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# **Allelochemicals: Role in Agriculture and Forestry**

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## Foreword

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation.

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**Symposium on**  
**Allelochemicals: Role in Agriculture, Forestry,**  
**and Ecology**

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## Preface

**M**OST PEOPLE ARE AWARE that skunks and porcupines have effective ways to repel their enemies. What they don't realize, and science is just now learning, is that plants also have defense systems.

Theophrastus (285 B.C.) and, later, Pliny recognized the existence of interference among plants and noted its significance in agriculture. However, involvement of plant-produced chemicals in plant-plant interactions was first suggested by the Swiss botanist M. A.-P. de Candolle in 1832. In 1937, Hans Molisch coined the term allelopathy to include both harmful and beneficial biochemical interactions between all types of plants and interactions involving microorganisms. This definition was later adopted by Rice in 1983 and is currently accepted. We would like to include the plant-insect and the plant-higher animal interactions in the terms allelopathy and allelochemicals for this book.

The nature of allelochemicals; the mechanisms and rates of their emission from the aggressive plant; their fate in the soil; and their uptake, translocation, and mode of action within the receptive plant are all processes that should be studied. These processes will be discussed in the plant-plant, plant-microorganism, plant-insect, and plant-animal sections of this book. In describing the allelopathic phenomenon, understanding how the aggressive plant (the donor) avoids autotoxicity is also essential.

In indigenous plant communities, allelopathy may determine the distribution patterns of plants in relation to their neighbors, whereas in agriculture and forestry allelopathy may affect yields. For example, weeds with allelopathic potential or crops that produce autotoxic aftereffects may reduce yields; conversely, using crops with the allelopathic potential to decimate weeds may improve yields.

### **Agricultural Implications**

Incorporating allelopathy into agricultural management may reduce the use of herbicides, fungicides, and insecticides; cause less pollution; diminish autotoxic hazards; etc. Plants and soil with their allelochemicals or those allelochemicals produced by associated microorganisms, insects, or higher animals could provide new strategies for maintaining and increasing forest and agricultural production in the future.

If the chemicals are allelopathic, they lend themselves to become starting materials for the synthesis of herbicides, pesticides, and fungicides

that are not based on petroleum compounds, which are a public health concern. We try to be responsive to the needs of the public.

There must be an interactive working group composed of foresters, entomologists, botanists, agronomists, biochemists, plant pathologists, and animal scientists to solve these research problems. Organizing this group would create coordination problems as well as funding problems.

In spite of these obstacles, the numbers of scientists working with allelopathy in its various forms are increasing.

### **Acknowledgments**

We thank each of the participants, both domestic and from abroad, for sharing with us the results obtained in their studies on allelochemicals. We also would like to thank the American Chemical Society for holding the symposium upon which this book is based because this is the third time they have honored this subject in the past year and a half.

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October 1985

# Introduction

ALLELOCHEMICALS have already been shown to impose numerous impacts in cultivated and natural ecosystems. Although their influences were observed at least as far back as the time of Theophrastus (285 B.C.), the major progress in this science has occurred during the past 25 years. Only during that period has the science gained credence among scientists of many disciplines. The phenomenon of allelopathy may be unique in that it probably involves more scientific disciplines than any other single phenomenon.

The diversity of disciplines and wide geographical area represented in this book are indicators of the worldwide importance of allelopathy. Allelopathy impacts virtually all the plant science and pestology disciplines as well as microbiology and natural products chemistry. One of my greatest pleasures has been to see the disciplines come together to create the critical mass necessary to study this science. Four recent international symposia have fostered this interdisciplinary effort. Numerous research teams have gelled as a result of these meetings. Elroy Rice's fine book has also served as an important focus for this science. The science has now clearly entered its logarithmic phase of growth.

## Challenge to All Disciplines

Allelopathic interactions are complex. I'm aware of no case where one chemical has been unequivocally proven to explain the entire situation. Almost all allelopathic interactions involve not only products of higher plants but also those of microbes, either as enhancers or detoxifiers. All cases require chemical characterization work followed by intensive studies by plant physiologists. Seldom can all the work be accomplished within one group. Simply, this means we must work together.

We should encourage that the term allelopathy be used in its broadest sense (as intended by Hans Molisch). Allelopathy would logically include the chemicals produced by microbial plants (*Actinomycetes*, algae, fungi, etc.) and those that enhance growth as well as inhibit growth.

We must refine our methods to prove allelopathy. Merely grinding up a plant and obtaining a phytotoxin is not proof of allelopathy. We urge that protocols similar to Koch's postulates be followed to develop proofs. Improved techniques are needed in many phases of the work. For example, we must be careful not to produce chemical artifacts during our extractions and separations. We must find better ways of collecting allelochemicals



from the rhizosphere. We have not yet identified probably 25% of the allelochemicals produced by higher plants and microbes.

Even if we prove that allelopathy is involved in plant interference, we should not forget that it is but one mechanism that influences the eventual outcome in plant communities.

### **Challenge to Crop and Forest Scientists**

The applied aspects of allelopathy should provide excitement for agricultural scientists. Allelochemicals pose both a problem and an opportunity. Allelochemicals undoubtedly cost world agriculture billions of dollars annually; however, by gaining an understanding of these natural mechanisms, we could put them to work to benefit agriculture. Seldom are plant rotations, tillage systems, cultivar selections, or planting configurations planned with the idea of reducing adverse allelopathic effects, much less to exploit beneficial impacts. We don't even know the best way to design our home vegetable gardens.

In recent years, American farmers have been challenged to produce crops with a profit margin. Although higher commodity prices and higher yields can enhance profitability, reduced input into production will also produce similar results. Wherever possible, we should gain an understanding of natural mechanisms and try to put them to work in agricultural production systems. This may allow us to reduce some costly fuel and chemical expenditures.

Little is known about the potential to exploit mutualism in agricultural systems. Almost all the work to date has concentrated on symbiotic nitrogen-fixers and mycorrhizal associations. Availability of soil nitrogen and phosphorus is a severe problem in many areas of the world. Allelochemicals may impact the availability of these nutrients through effects on the symbiotic microbes.

We have gained some understanding of autotoxicity and replant problems, which are common in perennial cropping systems, but much remains to be learned. These problems can cause serious economic losses, and many appear to involve allelopathy.

Plants and microbes will undoubtedly be a rich source of chemicals that are beneficial to plant growth, yield, or quality. Some interesting developments are now under way in this realm.

I've often wondered why we haven't done more to exploit allelopathic plants to manage vegetation on our right-of-way lands. Steve Horsley and others have shown that selected herbaceous species can virtually eliminate tree growth for as long as 80 years. We've also done little to exploit allelopathic turf grasses, although excellent weed-suppressing types have been reported in *Lolium* and *Festuca*. The work on dwarf spikerush gives hope that we might manage aggressive aquatic plants with nonweedy

allelopathic species. All these examples could greatly reduce chemical input into systems.

### **Challenge to Plant Physiologists**

We know very little about the fundamentals of allelochemical production and release. Manipulating allelochemicals by imposing the appropriate stress on the organism will probably be possible. Do plants produce or release chemicals as a result of exposure to an alien species? Much work has been done regarding defense to insect attack, but virtually no research on plant-plant responses has been done.

The limited work on mode of action of allelochemicals suggests that they affect a variety of sites and biochemical processes, many of which are similar to those affected by synthetic herbicides. Many novel sites and mechanisms probably remain to be discovered.

Proof of allelopathy could be strengthened if more research was done to document uptake and fate of allelochemicals in the recipient or susceptible plant. We need to determine relative toxicities (selectivity) of allelochemicals on the target species. What might be the outcome of a 30% growth reduction during only 2 weeks of a plant's life cycle?

Plant physiologists can contribute immensely in the technology of bioassay. Several *in vitro* systems could prove useful when more is known about the mode of action of the compounds. These types of assays may prove extremely useful in monitoring toxicity through fractionations of extracts or exudates.

### **Challenge to Plant Ecologists**

Although ecologists have recognized several possible mechanisms for plant interference, they have seldom determined the relative impacts of various mechanisms at different life stages or under different environmental conditions. Allelochemicals may be especially important for some species and at some stages of growth, but have little impact on others. They might be important under wet (anaerobic) environments and absent under dry conditions. Although some highly respected plant ecologists say it is virtually impossible to separate interference mechanisms in the field, I say instead that we must be more creative in our approach to that problem. Several years ago, C. H. Muller made important progress on this problem. His papers can teach all of us a lesson.

Plant ecologists and agricultural scientists have seldom agreed on anything. It is ironic that one general area of agreement involves the notion that when plants do not perform well together it is because of competition. I contend that this notion is false. In my view, interference is the outcome and competition for resources is but one mechanism. Allelopathy is another

important mechanism that produces interference. This has been stated before by others, but I feel it needs repeating.

More needs to be learned about plant succession and why it proceeds as it does in a number of different environments. Considerable evidence exists that allelochemicals may have impact in this area.

We also need a better understanding of mutualism. Undoubtedly, many mutualistic associations exist of which we are not aware. For example, might associated microbes produce chemicals that help plants defend their space?

### **Challenge to Microbiologists**

Some of the more important allelochemical interactions involve soil microbes. They may be either donors or recipients. Microbiologists should make an effort to identify soil organisms that produce phytotoxins because they may damage crops in the field or prove useful as biocontrol agents or sources of useful chemicals.

We should also learn which organisms and allelochemicals adversely affect the microbial symbionts. Some have already been shown to suppress growth of bacterial nitrogen fixers and nitrifiers as well as mycorrhizal fungi. Compounds that inhibit nitrification could prove to be important agriculturally.

Weed seed longevity is attributed, at least in part, to inhibitors that protect the seed from decay by microbes. One of the reasons weeds pose such a serious problem is because their seeds can persist for decades. This problem might be attacked by either destroying the inhibitors or by developing strains of microbes that can destroy the seeds.

### **Challenge to Pestologists**

We should determine which plant pests inflict their damage through production of phytotoxins. It now appears that several pathogenic fungi may do this. Numerous weed species may impose interference on crop growth, at least in part through allelochemicals. More than 70 species have now been alleged to have allelopathic potential.

Perhaps the most exciting concept is to use the natural product as a pest-regulating or pest-inhibiting compound. This approach might work on vertebrate pests as well as insects, nematodes, plant pathogens, and weeds. One of the most exciting new nematocidal and miticidal compounds is avermectin, a complex natural product produced by a *Streptomyces*. An exciting new herbicide with glyphosate-like activity has also recently been discovered in a *Streptomyces* culture. I am confident that the pesticide-producing factory of the future will be a biosynthetic unit. Many of our useful compounds will be produced by actinomycetes, bacteria, or fungi using plant products as substrates. This in itself could produce another

major market for agricultural products. The compounds produced in this manner might also pose less environmental hazard.

An obvious place for intensive work on allelopathy is in the weed science area. Here, plant interference is either our problem or our opportunity. We should be clever enough to exploit allelopathy as a weed-suppression strategy. This could be accomplished with crops that release allelochemicals through exudation or by crop residues placed into sequential cropping systems. My research team and others have already developed some promising leads in this area.

### **Challenge to Natural Product Chemists**

This aspect has been the rate-limiting step in many studies of allelopathy. Although all signs may point to allelopathy, proof requires positive identification of the allelochemicals. Not many plant scientists can accomplish this, and some simply go looking for the same old compounds because they can buy standards from the chemical supply house. What is needed is a cooperative effort with plant physiologists and chemists working side-by-side on fractionation and bioassay. We need to isolate and identify the more active compounds even if they are present in small quantities. We must be careful not to isolate chemicals that prove to be artifacts.

Our isolation techniques should begin with steps that might be expected to operate in nature. Usually, water will be the appropriate solvent. We should perform isolation in the absence and presence of microbes to see if they add new toxins.

Chemists should help develop better methodology for isolating compounds from the environment, particularly the soil environment. We need more breakthroughs along the line of the trapping resin developed by Tang and Young. Chemists should continually develop improved separation and spectral analyses systems. The instruments now available are extremely powerful but, because of cost, are available to only a few laboratories.

When novel chemicals are characterized, chemists should synthesize similar structures to search for useful analogs. Chemists should also consider more plant compounds as potential intermediates or even starting points in production of other useful products.

There appears to still be a shortage of natural product chemists. If more natural product chemists were available for postdoctoral positions, their appetites could be whetted for allelopathic research.

### **Acknowledgments**

I must thank several colleagues who have contributed a great deal to my success. I wish to first thank my department chairman, Jack Kelly, who has most importantly given me the freedom to pursue my research on

allelopathy. I wish to especially thank the allelopathy graduate students Jane Barnes, Joe DeFrank, Ron Lockerman, Tracy Sterling, Anne Hartung, and Leslie Weston; and postdoctorates Rod Heisey, Fred Lehle, and Saroj Mishra for all their contributions. I particularly appreciate the technical help of Bill Chase and Curt Whitenack and the fine secretarial assistance of Jackie Schartzner. In addition, I wish to thank Bill Duke of Cornell University and the entire Weed Science Staff at the University of California (Davis) for the invigoration they supplied during my sabbatical leaves. Finally, I must thank Stan Ries for teaching me some important lessons about intensity and creativity.

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# Chapter 1

## The Potential of Allelochemicals Opportunities for the Future

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How to deal with pests that attack cultivated plants is a continuing challenge to the food and agricultural production system. Natural resistance or tolerance to pests has proven to be one of the safest and least costly ways to protect plants. As we identify the specific plant components involved and their actions, it will be easier to incorporate into the plant the capacity to produce the desired chemical. There is the further possibility of identifying additional natural chemicals that may be useful as pest control materials, through the use of natural products or products of industrial synthesis patterned after the natural products. It is clear that allelochemicals are involved in these complex processes and they hold promise for even greater applications. Expanded research in the new biotechnologies offers great potential for further development of allelochemicals.

I appreciate the opportunity to make the opening presentation at this outstanding symposium on allelochemicals and their role in agriculture, forestry and ecology. It involves multiple disciplines, its recent advances have been facilitated by modern instrumentation, and it is moving in the full continuum from basic molecular biology through to practical applications. It is an important emerging area. I commend the American Chemical Society and the Division of Agricultural and Food Chemistry for putting together this impressive set of reviews, technical reports, and poster sessions. One cannot help but be impressed by reading through the program that research in allelochemicals is coming into its own.

I would first like to give my perception of the conceptual setting for food and fiber in the United States today. In the history of the Earth there has never been a species so successful as Homo sapiens in establishing and maintaining itself. Meeting the essential requirements of ever increasing numbers of human beings both in this country and now globally has been an unremitting challenge for agricultural research for decades. You are all

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familiar with how well the challenge has been met: fewer people on the land feeding more Americans than ever before, high export levels of agricultural products, and the provision of lifesaving food to less fortunate people around the world. It could not have been done without advances in knowledge through research. A successful, competitive industry is dependent upon sustained, high quality research and the development of new and innovative technology. In such a setting the transfer and the rate of adoption of new technology takes on great significance. This means that the private sector-university-Federal laboratory interface must be dynamic and responsive.

Now to some general comments on allelopathic chemicals. Interactions among plants and other organisms have long been recognized and described. For example, gardeners long ago observed that tomatoes do poorly under black walnut trees. Sorghum plant residues inhibit the growth of many weeds. Pyrethrum and neem plants have definite insecticidal properties. Certain varieties of crop plants exhibit resistance to insects and diseases. We now know that many such relationships involve chemicals, known as allelochemicals, produced by the plants or other organisms. On a chemical and molecular basis, we are now beginning to unravel why these interactions among various organisms occur. Balandrin *et al.* (1) have provided us with an impressive sampling of the wide array of chemical constituents in plants. Their main interest is in finding useful plant products, and one must be impressed by both the number and the complexity of constituents present. Some of these have identifiable functions in the plant or effects on other organisms. We are likely to learn that more of these constituents have as yet unrecognized and sophisticated interactions biochemically and physiologically in biological systems.

Dr. Putnam's review in *C&E News* two years ago (2) provided an excellent survey of where we were in this area and I am pleased to see that he will be giving the 1985 Sterling B. Hendricks Memorial Lecture later this week. From the abstracts for the presentations that follow this morning, I know that outstanding speakers will present an excellent review of the status of knowledge and of exciting developments in this emerging area.

How to deal with the pests that attack plants under cultivation is a continuing challenge to the food and agricultural production system. Natural resistance or tolerance to pests has proven to be one of the safest and least costly ways to protect plants. As we identify the specific plant components involved and their actions, it will be easier to incorporate the capacity to produce the desired chemical into the plant of interest. There is the further possibility to identify additional natural chemicals that may be useful as pest control materials. The latter might be through the use of natural products or it might be products of industrial synthesis patterned after the natural products. It is clear that allelochemicals are involved in these complex processes and they hold promise for even a greater role.

Better understanding of allelochemicals in plant population dynamics in ecosystems will provide a basis for improved management decisions for managers of rangelands and forests. This will be especially significant for ecosystems undergoing drastic changes such as a forest that has burned or has been harvested.

High performance levels for specific crops attained in some situations are often difficult to reproduce even though all recognized conditions are duplicated. There may be answers to some of the unknowns in allelochemicals. Previous crops and soil microorganisms are possible sources of such differences.

Another major benefit that will flow from the more basic research of allelochemical mechanisms is an improved basis for understanding and dealing with environmental issues that are also a concern of agriculture. As we have become more sophisticated in our view of the interactions occurring in the natural environment we have also become more sensitive to possible side effects from production practices in agriculture and forestry. This is particularly true for the use of agricultural chemicals. The study of allelochemicals is helpful both in providing new knowledge about plant and pest interactions and a basis for using specific chemicals that produce desired reactions with a minimum of undesirable side effects.

Improving knowledge of the functions of allelochemicals in biological systems contributes to development in an increasingly important area: risk assessment and risk management. The concepts of risk assessment and risk management are essential elements in developing sound environmental policy but in practice there has been frustration due to lack of adequate data and proven methodology. The concepts are still attractive and with more complete knowledge they will become less frustrating and more useful.

I think it is appropriate in this setting to discuss the exciting possibilities opened up by new developments in molecular biology and frequently referred to in terms of the new biotechnologies. The application of the science of genetics to agriculture is not new, but comparatively recent discoveries have catapulted our understanding of the hereditary apparatus of living organisms into a new era.

The transfer of genetic material from one organism to another that is not even closely related to the original donor is now commonplace. Recombinant DNA techniques now facilitate gene identification, characterization, splicing, replication, regulation, and transfer, all unknown "arts" a generation ago. The pursuit of promising leads may require the expertise of microbiologists, physiologists, and biochemists who may have to collaborate with plant pathologists, entomologists, agronomists, horticulturists, foresters, and geneticists.

Expanded research in the new biotechnologies is moving ahead with support from many sources. Private industry has shown a very significant interest through a number of new specialized companies as well as older established firms. Many States are putting up funds to establish biotechnology centers.

A part of the new support comes from an initiative by the Land-Grant Universities that was carried forward by USDA for a new program in the competitive research grants program for biotechnology. We are in the final award stages for the fiscal year 1985 program. Nearly \$20,000,000 of new funding was made available for fiscal year 85 in three broad program areas: molecular biology, molecular and cellular mechanisms of growth and development; and genetic and molecular mechanisms controlling responses to physical and biological stress. Approximately 890 proposals were submitted, of



which 165 can be funded from the funds available. Our 1986 budget is not yet final, but we anticipate a similar program for next year and plan to have an announcement published in the Federal Register in the near future.

The new techniques available in genetic engineering, molecular biology, tissue culture, and so forth offer great potential for identifying allelochemicals and their function. They also offer opportunities to more readily incorporate the capacity to produce specific allelochemicals into given plants for a desired effect.

One aspect of genetic engineering that is not yet resolved is the question of appropriate precautions and guidelines for recombinant DNA experiments. In particular, there is continuing dialog regarding the release of products of recombinant DNA into the environment. Discussions at the symposium on engineered organisms in the environment organized by the American Society for Microbiology last June and the recent exchange of letters between W. J. Brill (3) and R. K. Colwell (4) provide a good sense of the concerns and responses to them. The release issue has implications for agriculture broadly, and plans to use recombinant DNA techniques for application of allelochemical research results would be affected.

Because much of the release in question is of direct concern to agriculture, the agricultural research community has developed a proposed assessment system that would supplement existing regulatory and oversight programs. It would insure that adequate safety and environmental considerations are fully addressed before modified organisms are released into the open environment. Before I get into the details of the proposal, I want to review the background of how we got where we are today.

There has been for many years a very healthy, open debate on ethics and safety questions related to experiments involving recombinant DNA. Since its creation in 1974, the National Institutes of Health Recombinant DNA Advisory Committee (NIH-RAC) has been the primary forum for this debate. Guidelines for recombinant DNA research developed by the NIH-RAC became binding on all Federally supported research and in practice have guided all research in the United States. In the USDA, an Agricultural Recombinant DNA Research Committee (ARRC) has been in place for many years in close association with the NIH-RAC and has assisted the NIH-RAC in its handling of agriculturally related proposals.

The NIH-RAC guidelines have evolved as new knowledge has become available and they have served the country well. However, research and experimentation have progressed from contained facilities to the point that trials in the open environment are appropriate and products for commercial use are at hand. For a coordinated approach to the regulation and oversight of recombinant DNA products and activities, a government-wide Cabinet Council Working Group on Biotechnology was established through the President's Office of Science and Technology Policy. The Working Group prepared a proposal for a coordinated framework for regulation of biotechnology that was published in the Federal Register (5) for public comment. A revised draft taking into account the various reactions and comments is in preparation. I point out these activities to let you know that we are working within a coordinated overall framework.

Now I want to go back to the proposal that is part of our biotechnology initiative and give some details on the proposed

assessment system and what it would accomplish. The proposed system is referred to as the National Biological Impact Assessment Program. It will not assume a regulatory role, but is intended to complement existing regulatory and oversight activities. It is a research-based program, using the existing national network of expertise and experience in long-term assessments of agricultural biota. And the existing national network I am referring to is the State Agricultural Experiment Station System and the field locations of the Agricultural Research Service. The program will evaluate the impacts of biological changes occurring in our Nation, including the needs brought on by the new biotechnology research in agricultural systems. Currently, we assess the impact of plant, animal, and microbial biota in agriculture in a wide range of cropping and animal production systems. This is done through growth chambers, greenhouse studies, and research plots in the field. In the case of all major crops, a wide range of processes are used in which new varieties and species are assessed for their impact.

The need to know the impact of biological changes occurring in our Nation has increased very substantially since the development of recombinant DNA and closely related techniques in the early 1970's. It also has changed considerably in characteristics since those discoveries. On the one hand, recombinant DNA techniques require entirely new concepts of assessment, while on the other they provide wholly new tools for assessments. A research-based system is essential for successful involvement of the expertise, facilities, and instrumentation that are now available in the science and education system nationally. Fortunately, the emergence of this need can be supported by a rapidly growing system of new high-speed electronic assessment and processing techniques, which are essential to the development and application of biotechnology in agriculture and forestry. Again, I want to emphasize that the National Biological Impact Assessment Program will not assume a regulatory role. However, it will provide expertise and technical backup to the regulatory agencies.

For materials produced by the modern biotechnologies such as recombinant DNA, there will be a national system of projects for assessing the potential impact of these materials in a step-by-step manner prior to their release into the environment. We will draw heavily on the NIH-RAC guidelines and the local institutional biosafety committees. The program will operate as a highly participatory system that will appraise biological changes of importance to agriculture, forestry, and natural resources. There will be continuous cycling of data as assessments are made. The program will maintain a roster of knowledgeable scientists to assist in reviewing biotechnology proposals for safety and efficacy, information on safe test sites, and an inventory and ongoing assessment of materials under test.

The things I have been talking about are all part of the "big picture" for the food and agricultural sciences. In recent years we have devoted more attention to standing back from time to time to view the larger picture so that we can expect to do a better job of identifying the critical elements of the system and thereby "fit" them together into an effective comprehensive program. This involves better articulation among the Federal-State partners and

the private sector research. Much more needs to be done. The Congress of the United States encouraged us in this direction and in the 1977 Farm Bill called for establishment of a Joint Council on Food and Agricultural Sciences to foster coordination of the research, extension, and teaching activities of all public and private organizations and individuals. The 1981 Farm Bill called on the Joint Council to do a comprehensive needs assessment for food and agricultural sciences, which was published in January 1984.(6) The 1981 Farm Bill also called for an annual priorities report to promote coordination and joint planning. The edition for FY 1987 (7) lists the following broad priorities:

1. Increase agricultural profitability through management.
2. Improve water quality and management.
3. Expand biotechnology efforts on plants, animals, and microbes.
4. Develop necessary scientific and professional human capital.
5. Improve human nutrition and the understanding of diet/health relationships.

Bringing the entire system together in a set of priorities provides a framework for all of us and demonstrates to our supporters that we can make statements about what we think is important.

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## Chapter 2

# Allelopathy: An Overview

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Our increasing knowledge of allelopathy is aiding greatly in our understanding of many ecological phenomena. Our increasing awareness of conditions under which certain crop residues cause allelopathic effects on subsequent crops should enable us soon to guard against detrimental effects and to manage rotation to take advantage of stimulatory effects. Available evidence indicates that it will be possible, through breeding/or biotechnology, to develop crop cultivars that will inhibit growth of the chief weeds in a given area through allelopathic action and thus decrease the need for synthetic weed killers. We are already able to use allelopathic companion crops or residues of allelopathic crop plants and weeds to control weed growth in some crops and orchards. Our understanding of allelopathic interactions between various plant species has been used advantageously in reforestation, and future developments are encouraging. Considerable information is available concerning types of chemicals involved in allelopathy, and some information is available concerning movement of the chemicals from plants and factors determining their effectiveness after egression from plants. Nevertheless, these areas of allelopathy are probably the ones that merit the strongest research emphasis in the near future.

Theophrastus (1), about 300 B.C., stated that chick pea (Cicer arietinum) does not reinvigorate the ground as other related plants (legumes) do but "exhausts" it instead. He pointed out also that chick pea destroys weeds. Pliny (2) reported in the 1st century A.D. that chick pea, barley (Hordeum vulgare), fenugreek (Trigonella foenum-graecum), and bitter vetch (Vicia ervilia) all "scorch up" cornland.

In spite of the early suggestions concerning apparent allelopathic effects, no solid scientific evidence was obtained to support the suggestions until the present century. The term allelopathy was coined by Molisch in 1937 to refer to biochemical interactions between all types of plants, including microorganisms traditionally placed in the plant kingdom (3). His discussion indicated that he meant the term to cover both inhibitory and stimulatory biochemical interactions.

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A very important point concerning allelopathy is that its effect depends on a chemical compound being added to the environment. It is thus separated from competition which involves the removal or reduction of some factor from the environment that is required by some other plant or microorganism sharing the habitat. Muller (4) suggested the term interference to refer to the overall influence of one plant (or microorganism) on another. Interference would thus encompass both allelopathy and competition.

Evidence indicates that allelopathic compounds get out of plants by volatilization, exudation from roots, leaching from plants or residues by rain, or decomposition of residues (5).

The goals of this paper are to discuss some of the major generalizations that can be made about allelopathic interactions, provide some examples of suggested roles, and focus on desirable future research applications. Only a few of the pertinent investigations are cited in illustrating major principles.

### Allelopathy in Plant Pathology

Spores of most parasitic fungi remain ungerminated while located in their site of production (6). This can be due to several factors, one of which is production by the spores of fungistatic agents that are excreted into the water around the spores. These self-inhibitors generally assure dispersal of viable ungerminated spores. Endogenous germination stimulators that counteract inhibition by self-inhibitors occur in many spores. Nonanal and 6-methyl-5-hepten-2-one were isolated from uredospores of Uromyces and Puccinia (7). These compounds stimulate germination of stem rust spores that contain methyl ferulate as their inhibitor, but they do not stimulate germination of spores that contain a dimethoxycinnamate as inhibitor.

Most parasites have to survive prolonged periods of time apart from the host plant. Consequently, the formation of resting propagules, such as sclerotia, constitutes a critical part of the parasite's life cycle. Several observations indicate that formation of sclerotia may be stimulated by allelochemicals (8,9). Brandt and Reese (10) concluded that Verticillium dahliae produces a diffusible morphogenetic factor that stimulates production of microsclerotia. When low concentrations of the diffusible factor were added to cultures of the pathogen, the hyphae swelled and became constricted, septation was increased, and cell walls became thickened.

Kerr (11) grew sterile seedlings inside cellophane bags buried in soil inoculated with Pellicularia filamentosa and found an intense development of the pathogen on the cellophane opposite the roots of the two susceptible hosts, lettuce and radish, but no stimulation opposite tomato roots, which are not susceptible. Buxton (12) also demonstrated a definite specificity in relation to the germination of spores of Fusarium oxysporum f. pisi in the exudates of three pea varieties differing in susceptibility to this pathogen. Exudate from a wilt-resistant variety inhibited spore germination, whereas exudate from a susceptible plant stimulated such germination.

In soil that has not had recent additions of plant residue or other organic material, microbial respiration proceeds at a low rate (13). Moreover, fungi apparently exist mostly as spores in a state of fungistasis. This microflora usually responds to the addition of plant residue by spore germination, increased respiration, and growth. These responses were induced by volatile components from alfalfa tops, corn leaves, wheat straw, bluegrass clippings, tea leaves, and tobacco leaves, even when the residue

was separated from the soil by a 5-cm air gap. There was a rapid outgrowth of hyphae from the soil surface toward the residue before any growth of fungi could be seen in the plant material. Vapors from distillates of water extracts of the various plant residues mentioned had similar effects on growth of fungi and markedly increased numbers of bacteria and the respiratory rate of microorganisms in soil samples.

Witchweed (Striga asiatica) is an economically important root parasite affecting many warm-season grasses, including such important crop plants as corn, grain sorghum, and sugar cane. Viable witchweed seeds may remain dormant in the soil for many years (14). The seeds will usually not germinate unless pretreated in a warm, moist environment for several days before exposure to a chemical compound exuded from the roots of a host plant or some non-host plants. One such compound, strigol, was isolated from the root exudate of cotton and has proved to be a powerful stimulant of witchweed seed germination. Johnson, Rosebery and Parker (15) reported the synthesis and testing of several analogs of strigol, and some were powerful seed germination stimulants for species of both Striga and Orobanche. Cotton is not a host plant for Striga or Orobanche, and it is noteworthy that the structures of the germination stimulants exuded from the roots of the host plants remain unknown (16).

The haustoria of the parasites do not form when the plants are grown axenically, but are rapidly induced in the presence of the host roots or host root exudates (17). Several haustorial-inducing compounds have now been characterized. Xenognosin A and B were identified in gum tragacanth, an exudate of Astragalus gummifer, and soyasapogenol B was identified in roots of Lespedeza sericea.

#### Allelopathy in Natural Ecosystems

Patterning of vegetation. Curtis and Cottam (18) observed the fairy-ring pattern of the prairie sunflower Helianthus rigidus, which is due to a pronounced reduction in plant numbers, size, and inflorescences in the center of the clone. They subsequently demonstrated that the pattern was due to autotoxins produced by decay of dead parts of the sunflower.

Prostrate knotweed, Polygonum aviculare, rapidly encroaches into bermudagrass lawns and the grass dies in patches of prostrate knotweed while bermudagrass at the edges of the knotweed patches turns yellow. Soil minus litter was collected under a P. aviculare stand and under a bermudagrass stand and used to grow bermudagrass (19,20). Soil collected in March under knotweed markedly inhibited seed germination and seedling growth of bermudagrass compared with soil from under bermudagrass. Decaying roots and shoots of prostrate knotweed reduced seed germination and seedling growth of bermudagrass. Additionally, root exudates of knotweed reduced seedling growth of bermudagrass. Eleven allelochemicals inhibitory to growth of bermudagrass were isolated from soil under prostrate knotweed, whereas none of these occurred in soil under bermudagrass (20,21). Four were phenolics and seven were long-chain fatty acids.

Vegetation under the trees in Japanese red pine, Pinus densiflora, forests is sparse despite the fact that the interior of these forests is one of the brightest among forests (22,23). Many other forests have dense undergrowths of herbs in spite of much lower light intensities. Various parts of red pine and the soil under it contained chemicals toxic to many potential understory plants. Thus, it was concluded that allelopathy probably plays an important role in retarding understory growth.

Lycoris radiata is a dominant species along roadsides and slopes in Japan, and it appears to prevent some other plant species from emerging and growing near it (24). Ueki and Takahashi found that the bulbs of L. radiata exuded two allelochemicals that markedly reduced root growth of several weedy species usually occurring in the same general areas with Lycoris. Moreover, the same compounds were found in the soil adjacent to Lycoris bulbs.

Plant succession. In the tall grass prairie region of Oklahoma and Kansas, there are four main successional stages when fields that are infertile are abandoned from cultivation: a pioneer weed stage that persists for only 2-3 years, an annual-grass stage that lasts for 9 to 13 years, a perennial bunchgrass stage that remains for 30 years or longer after abandonment, and the climax prairie (25). The evidence is strong that the pioneer weed stage disappears rapidly because the species are eliminated through strong allelopathic interactions (5). Aristida oligantha, prairie threeawn, the dominant of the second stage, invades next apparently because it is not inhibited by the allelochemicals produced and is able to grow well in soil that is still too low in nitrogen to support species that invade later in succession (26).

A. oligantha and several pioneer species produce allelochemicals that inhibit growth of Rhizobium and free-living nitrogen-fixing organisms, and nodulation and hemoglobin formation in legumes (5). This indirect evidence suggested that biological nitrogen fixation was slowed in the first two stages of succession. Kapustka and Rice (27) measured nitrogen fixation rates in soils of the pioneer weed stage, the annual grass stage, and the climax prairie using the acetylene reduction technique. The rate was about four times as high in the climax soil as in the pioneer weed stage and about five times as high in the climax as in the annual grass stage, thus substantiating the indirect evidence. The slowing of nitrogen fixation in the first two successional stages probably gives Aristida oligantha a selective advantage in competition with species having higher nitrogen requirements and causes it to remain for a lengthy period.

There is a strong evidence that nitrification is slowed in the later stages of succession causing available nitrogen to be present chiefly as ammonium nitrogen (28,29,30). This should help to conserve nitrogen because the ammonium ion is adsorbed by the negatively charged micelles in the soil and is not readily leached below the depth of rooting or washed away into streams. There is evidence that tannins, phenolic acids, flavonoids, and coumarins may be important inhibitors of nitrification (30, 31).

The nitrogen concentration gradually increases to the point where some later species can invade. This apparently results in less inhibition of nitrogen fixation and more inhibition of nitrification. Thus, the rate of addition of nitrogen is increased and the rate of loss of nitrogen is decreased. Eventually the concentration of nitrogen is increased to the point where climax species can invade.

Urbanization around large cities in Japan has greatly changed the plant communities because of the creation of bare areas or serious disturbance of natural ecosystems (32-34). Weed succession on urban waste land is similar to that in old-fields in some parts of the U.S.A. with Ambrosia artemisiifolia being the first-year dominant followed by Solidago altissima and Erigeron spp. for a few years, and next by Miscanthus sinensis. Numata and his colleagues found that S. altissima and Erigeron annuus both produce polyacetylenic methyl esters (one in Solidago and three in E.

annuus) that inhibit seed germination of A. artemisiifolia, M. sinensis and a species of Tagetes, and growth of rice seedlings. The phytotoxin found in Solidago was also extracted from soil in a stand of the species, and the concentration present was sufficient to regulate germination and growth of associated species. A very dilute solution (5 ppm) of three of the phytotoxins inhibited growth of A. artemisiifolia in soil.

Kobayashi et al. (35) found that the roots of Solidago altissima contain 250-400 ppm of the C<sub>10</sub>-polyacetylene, cis-dehydromatricaria ester (cis-DME). They found that soil under a stand of the Solidago contained 6 ppm of cis-DME plus trans-DME. Both compounds were found to inhibit growth of rice seedlings. Three C<sub>10</sub>-polyacetylenes were identified from methanol extracts of Erigeron annuus, E. canadensis, E. floribundus, and E. philadelphicus. These were the cis- and trans-matricaria ester and the cis-lachnophyllum ester. All were inhibitory to seed germination of Ambrosia artemisiifolia and seedling growth of rice at a concentration of 5 ppm or above. Kobayashi et al. concluded that the dominance of Solidago altissima and Erigeron spp. in the second stage of secondary succession is probably due to their production of acetylenic compounds which strongly inhibit growth of many other plant species. They suggested also that the relatively short period of occupation by S. altissima and Erigeron spp. may be a consequence of the accumulation of such polyacetylenes in the soil to the point where they are toxic to these species also.

#### Allelopathy in Manipulated Ecosystems

Allelopathy in forestry. Walters and Gilmore (36) noted that height growth of sweetgum, Liquidambar styraciflua, was less in plots containing fescue, Festuca arundinacea, than in adjacent plots without fescue. Chemical and physical soil factors did not appear to explain the differences. Growth of sweetgum was correlated with residual phosphorus and magnesium, but this correlation was achieved across all experimental plots without respect to the presence or absence of fescue. Seeding of fescue into pots containing sweetgum seedlings resulted in a reduction in dry weight increment of sweetgum up to 95%. Elimination of competition through use of a stairstep apparatus suggested that an allelopathic mechanism was involved. Leachates from the rhizosphere of live fescue, dead fescue roots, and dead fescue leaves caused reductions in dry weight increments of sweetgum up to 60%. Chemical analysis of sweetgum seedlings from the stairstep experiment suggested that fescue leachates decreased absorption of phosphorus and nitrogen.

Tubbs (37) found that sugar maple seedlings inhibited growth of seedlings of yellow birch despite the apparent absence of competition in nursery experiments. Root elongation of birch was retarded by exudates of actively growing root tips of sugar maple. When seedlings of these species were grown together in aerated nutrient solution, the number of actively growing root tips of birch formed each day was inversely correlated with the activity of the allelochemical produced by maple, as indicated by the retardation of elongation of yellow birch roots.

Alder species are often important in forests because of the fixation of nitrogen by Frankia in nodules on their roots. Jobidon and Thibault (38) observed growth depression of alders near balsam poplar, Populus balsamifera, stands. Water extracts of leaf litter and buds, and fresh leaf leachates of balsam poplar inhibited seed germination and radicle and hypocotyl growth of green alder, Alnus crispa var. mollis, seedlings. There was marked inhibition of root hair development and necrosis of the radicle



meristems. The average number of nodules on alder plants treated with any one of the three balsam extracts described above was only 51% of that of control plants (39). Acetylene reduction (nitrogen fixation) was decreased 62% by green alder plants treated with the most concentrated bud and leaf litter extracts.

Certain tree species such as *Betula pendula* and *Picea abies* fail to develop in association with heather, *Calluna vulgaris* (40,41). This apparently results from the production by heather of an allelochemical toxic to growth of mycorrhizae of *Betula* and *Picea*. Fruticose soil lichens are often allelopathic to the growth of mycorrhizae and forest tree seedlings also (42). Removal of reindeer moss (a lichen) in field tests resulted in accelerated growth of pine and spruce.

Allelopathy in agriculture. Schreiner and his associates published several papers shortly after 1900 which indicated that certain crop plants produce compounds inhibitory to growth of the same and other crop plants (5). McCalla and Duley (43,44) reported the allelopathic effects of decaying wheat residues in 1948-1949, and many papers on allelopathic effects of crop plants have been published in the past three decades.

The unharvested parts of rice plants are generally mixed with the soil because this has been thought to be beneficial. It has been observed however, that productivity of the second crop of rice in a paddy is less than that of the first crop. Chou and Lin (45) found that aqueous extracts of decomposing rice residues in soil retarded radicle growth of rice seedlings and growth of rice plants. Maximum toxicity occurred in the first month of decomposition and declined thereafter. Some toxicity persisted for four months in the paddies. Five inhibitory phenolic acids were identified from decaying rice residues and several unidentified allelochemicals were isolated.

In the southern part of Taiwan, a crop of rice is often followed immediately by a legume crop. Yields of soybeans have been increased by several hundred kilograms per hectare by burning the rice straw prior to planting the soybeans. Rice *et al.* (46) hypothesized that the decreased yields in unburned fields may result from an inhibition of nitrogen fixation by *Rhizobium* in the nodules of the soybean plants. The five phenolic acids identified by Chou and Lin and sterile extracts of decaying rice straw in soil markedly inhibited growth of *Rhizobium*. The phenolics also reduced nodule numbers and hemoglobin content of the nodules of two bean varieties. Moreover, extracts of decomposing rice straw in soil reduced nitrogen fixation (acetylene reduction) in Bush Black Seeded beans.

It has been observed for some time in Senegal in west Africa that growth of sorghum is decreased markedly following sorghum in sandy soils but not in soils high in montmorillonite (47). Similar results occurred in the growth of sorghum seedlings when roots or tops of sorghum were added to sandy soils in laboratory experiments. No inhibition resulted, however, when the residues were added to soil high in montmorillonite. Water extracts of roots or tops retarded growth of sorghum seedlings in sandy soils similarly. Inoculation with *Trichoderma viride* or an unknown species of *Aspergillus* eliminated the inhibitory effects of aqueous extracts of sorghum roots in a short time. Several weeks were required, however, to detoxify nonsterile field soil after addition of root residues of sorghum. It was concluded that the microflora in the sandy soils of Senegal were not able to detoxify the soil fast enough to prevent inhibition of subsequent crops of sorghum.

Some crop residues and weeds appear to stimulate growth of other plants. Chopped alfalfa added to soil stimulated the growth of tomato,

cucumber, lettuce, and several other plants (48). The stimulatory allelochemical was identified as 1-triacontanol. Subsequent tests with this compound have given variable results, but addition of calcium or lanthanum salts to the triacontanol solution appears to make the stimulatory activity consistent (49). A steroid, brassinolide, has been isolated from rape (*Brassica napus*) and alder (*Alnus*) pollen (49). One nanogram applied to a bean plant causes significant growth increases.

Weeds versus crop plants. Velvetleaf, *Abutilon theophrasti*, is a serious weed of several crops in the United States and Canada. Average yield reductions of soybeans under a variety of velvetleaf densities, placements, and duration of interference ranged from 14 to 41% (50-52). Reductions in cotton yields ranged from 44 to 100% (53,54). All the cited researchers attributed the reductions in crop yields to competition although none performed experiments to determine whether allelopathy might be involved. Numerous other researchers have found velvetleaf to have marked allelopathic potential (55-58). Water extracts of velvetleaf residues were slightly allelopathic (5-24% inhibition) to radicle and coleoptile growth of corn and to hypocotyl growth of soybeans (57,58). Decaying residues were highly allelopathic (50% or more inhibition) to height growth and fresh weight increase of shoots of both corn and soybeans in double pot experiments.

Purple nutsedge, *Cyperus rotundus*, was listed by Holm (59) as one of the ten worst weeds in the world, and interference by this weed caused reductions in yields of various crops ranging from 23 to 89% (5). It is noteworthy therefore that numerous workers have found purple nutsedge to be strongly allelopathic. Soil previously infested with this weed for 9 to 12 weeks significantly reduced germination of mustard, barley, and cotton seeds; and soil infested for only 6 weeks significantly reduced germination of mustard and cotton seeds (60). Ethanol extracts of the previously infested soil inhibited radicle growth of barley also. Decomposing tubers of purple nutsedge reduced root and top growth of barley (61), sorghum (62), and soybeans (62).

Some polyphenols (63) and sesquiterpenes (64,65) were isolated from tubers and other parts of purple nutsedge. Seven sesquiterpenoids were identified in the steam distillate of soil in which purple nutsedge was growing (65) and the same compounds were isolated from essential oil in purple nutsedge (66). Several of the compounds identified were previously shown to inhibit elongation of wheat coleoptile segments in the presence of indoleacetic acid and second leaf sheath growth of rice seedlings in the presence of gibberellin A<sub>3</sub> (64).

Decaying ground-ivy (*Glechoma hederacea*) leaves (2 g per kg of soil) markedly stimulated both root and shoot growth of downy brome (*Bromus tectorum*) and radish (*Raphanus sativus*) (67). Radish root growth was stimulated 1354% in one experiment. Moreover, root exudates of ground-ivy significantly stimulated both root and shoot growth of radish. In fact, the roots attained table size in only 14 days.

Crop plants versus weeds. Both thin and dense field stands of Kentucky-31 fescue were observed by Peters (68) to be relatively free of weeds. Extracts of fescue, sand cultures, and split-root-system experiments demonstrated that fescue produced toxic chemicals which exuded from the roots and inhibited growth of wild mustard and birdsfoot trefoil.

Three thousand accessions of the USDA collection of oat, *Avena*, germplasm were screened for their ability to exude scopoletin, a compound

known to have root-growth-inhibiting properties (69). Twenty-five accessions exuded more blue-fluorescing material (characteristic of scopoletin) from their roots than a standard oat cultivar (Garry). Four accessions exuded up to three times as much scopoletin as Garry oats. When one of these was grown in sand culture for 16 days with a wild mustard, growth of the mustard was significantly less than that obtained when the weed was grown with Garry oats. Moreover, plants grown in close association with the toxic accession were chlorotic, stunted, and twisted indicative of chemical effects rather than competition. It appears possible therefore to breed allelopathic genes into standard cultivars to aid in weed control.

Allelopathic crop plants have already been used experimentally in weed control. Leather (70) found one of thirteen genotypes of the cultivated sunflower tested to be very allelopathic to several weeds. In a 5-year field study with oats and sunflower grown in rotation, the weed density was significantly less than in control plots with oats only.

Putnam and DeFrank (71) tested residues of several fall- and spring-planted crops for weed control in Michigan. The plants were desiccated by the herbicides glyphosate or paraquat, or by freezing. Tecumseh wheat and Balboa rye residues reduced weed growth by up to 88%. Mulches of sorghum or sudangrass applied to apple orchards in early spring reduced weed biomass by 90% and 85%, respectively. In a 3-year series of field trials, sorghum residues reduced populations of common purslane by 70% and of smooth crabgrass by 98% (72).

#### Chemical Nature of Allelopathic Compounds

Allelopathic compounds consist of a wide variety of chemical types which arise through either the acetate or the shikimic acid pathway (5). These compounds range from very simple gases and aliphatic compounds to complex multi-ringed aromatic compounds. Only a few examples are mentioned below.

Acetic and butyric acids were among the toxins produced during decomposition of rye residues (73), and salts of acetic, propionic, and butyric acids were the chief phytotoxins produced in decaying wheat straw (74).

A simple lactone, parasorbic acid, from the fruit of mountain ash, inhibits seed germination and also has antibacterial action (75). Another such compound, patulin, is produced by several fungi, including *Penicillium urticae*, which produced large amounts of the substance when growing on wheat straw (76).

Long-chain fatty acids have long been reported to be important allelochemicals produced by algae (77). These compounds were recently reported to be potent toxins in decaying residues of a higher plant, *Polygonum aviculare* (21). Polyacetylenes are apparently derived from long-chain fatty acids (78), and evidence is increasing that they are important allelopathic compounds (35,79).  $\alpha$ -Terthienyl produced by roots of marigold, *Tagetes erecta*, caused 50% mortality in seedlings of four test species in concentrations from 0.15 to 1.93 ppm (79).

Juglone is the only quinone identified as an allelopathic compound from higher plants (5). It is produced by walnut trees and is a potent inhibitor. Numerous antibiotics produced by microorganisms are quinones, including the tetracycline antibiotics such as aureomycin (80).

Simple phenols, phenolic acids derived from benzoic acid, and phenolic acids derived from cinnamic acid have been the most commonly identified

allelopathic compounds produced by higher plants. The most common allelopathic compounds identified in soil under allelopathic plants are *p*-hydroxybenzoic, vanillic, *p*-coumaric, and ferulic acids.

Coumarins are lactones of *o*-hydroxycinnamic acids in which side chains often are isoprenoid (78). Coumarin, esculin, and psoralen (a furanocoumarin) all strongly inhibit seed germination. Such inhibitors are produced by a variety of legumes and cereal grains.

Flavonoids are widespread in higher plants and a few have been implicated in allelopathy. Phlorizin in apple roots is toxic to young apple trees and often causes difficulty in replanting old apple orchards. Numerous flavonoids and their glycosides are produced by species from the tall grass prairie and post oak/blackjack oak forest and are inhibitory to nitrifying bacteria and to seed germination (31).

Several hydrolyzable and condensed tannins have been implicated in allelopathy (5). They have been identified as growth and germination inhibitors in dry fruits (81), as growth retarders of nitrogen-fixing and nitrifying bacteria in several plants, and as reducers of seedling growth in several plants (5).

Higher plants produce a great variety of terpenoids (78) but only a few of these have been implicated in allelopathy. The monoterpenoids are the major components of essential oils of plants and they are the predominant terpenoid inhibitors that have been identified from higher plants. Many fungi (82) and algae (83) produce terpenoid allelochemicals also.

There are only a few instances in which amino acids have been implicated in allelopathy and in most cases the specific amino acids have not been identified. Rhizobitoxine is produced by certain strains of *Rhizobium japonicum* and is a nonprotein amino acid (84). Several of the phytotoxins produced by pathogenic microorganisms are polypeptides and related glycopeptides (82).

Many alkaloids have been implicated in plant-animal chemical interactions but few have been associated with allelopathy (85). Several alkaloids were demonstrated by Evenari (75) to be strong inhibitors of seed germination. Little recent work has been done on alkaloids except for caffeine (78).  $\alpha$ -Picolinic acid is a microbial alkaloid with toxic action on plants (82). One of the more active synthetic herbicides on the market, picloram (Dow's Tordon), is a chlorinated picolinic acid derivative.

Cyanohydrins have been implicated in allelopathy in several instances. Dhurrin occurs in grain sorghum seedlings and the seedlings contain enzymes that hydrolyze dhurrin to glucose, HCN (hydrogen cyanide), and *p*-hydroxybenzaldehyde (86). The situation is similar in Johnsongrass, *Sorghum halepense*, a very allelopathic weed (87). Both the HCN and *p*-hydroxybenzaldehyde are potent allelochemicals. HCN and benzaldehyde are produced by the hydrolysis of amygdalin present in peach root residues (88). HCN and benzaldehyde are inhibitory to growth of peach seedlings and apparently cause the peach replant problem in old peach orchards.

Mustard oils, such as allylisothiocyanate, are products of the hydrolysis of mustard oil glycosides (78). Mustard oils are produced by all organs of plants belonging to the Cruciferae (mustard family) (75), and are strong inhibitors of seed germination and microbial growth.

Many antibiotics produced by various microorganisms are nucleosides (5). Among these are nebularine, cordycepin, and nucleocidin. The only known purines in higher plants shown to be involved in allelopathy are caffeine, theophylline, paraxanthine, and theobromine from the coffee tree (89).

### Factors Determining Effectiveness of Allelochemicals

Some allelochemicals have been shown to be bound by the humic material in the soil and presumably inactivated (90). When known amounts of tannic acid were added to a prairie soil that contained no tannic acid, a minimum of 400 ppm had to be added before any could be recovered immediately. It is noteworthy, therefore, that as small a concentration as 30 ppm added to the same soil reduced the nodule number of heavily inoculated legumes growing in the soil. Obviously, some of the bound tannic acid remained biologically active (91).

Some plants exert greater allelopathic effects in fine-textured than in coarse-textured soils and evidence indicates that the greater retention capacity of the fine textured soils for at least some allelochemicals may be important in the accumulation of physiologically active concentrations of these chemicals (92-94).

Many allelochemicals are decomposed in soil, either abiotically (37) or by microorganisms (95-100). Obviously, the attainment of active concentrations of allelochemicals in soil depends on the relative rates of addition and inactivation. It is important to understand also that microbial decomposition of allelochemicals does not necessarily result in a decrease in allelopathic activity. In fact, the reverse may be true. Hydrojuglone is oxidized in soil to juglone, a quinone that is inhibitory to some species at a  $10^{-6}$  M concentration (101). Isoflavonoids produced by red clover are decomposed to even more toxic phenolic compounds (95); and to repeat, amygdalin from peach roots is changed to hydrogen cyanide and benzaldehyde which cause the peach replant problem (88), and phlorizin from apple roots is decomposed to several phenolic compounds that appear to be responsible for the apple replant problem (100).

It is also important to understand that most allelopathic effects apparently result from the combined actions of several allelochemicals, often with each below a threshold concentration for impact. In allelopathic situations which implicate phenolic acids, soil concentrations have ranged from below 10 to above 1000 ppm for each compound. The lower end of the spectrum is below a concentration required for an effect in current bioassays. Additive and synergistic effects have been demonstrated, however, for combinations of cinnamic acids (102), benzoic acids (103), benzoic and cinnamic acids (104), and *p*-hydroxybenzaldehyde with coumarin (105). It appears that such combined interactions may be very important under field conditions.

It is revealing to consider microbial decomposition of allelopathic compounds in relation to synergism. As discussed above, partial decomposition of one compound may result in the presence of several active compounds, which may exert synergistic allelopathic effects. Thus, partial decomposition could increase allelopathic activity, rather than decrease it.

### The Direction of Future Research in Allelopathy

Most of our present knowledge concerning allelopathy has been obtained in the past three decades. Thus, it is a very young field of science and research should be continued in all areas of allelopathy investigated in the past. The point has been reached, however, where certain areas need special emphasis. Even though many types of chemical compounds have been implicated in allelopathy, there are probably many highly important ones that have been overlooked. Techniques are now available to identify allelochemicals much more rapidly and accurately than in the past, and

many more chemists are doing research in allelopathy. Therefore, there should be special emphasis in this area. There have been many demonstrated instances of allelopathic action on the part of many plant species where no allelochemicals were identified. Most species for which good evidence exists of allelopathic potential, should probably be examined again also with new techniques and expertise.

It is important to identify the allelopathic compounds in the substrate (soil or water) of the allelopathic plant and to determine whether these compounds have come from the plant, are produced by partial decomposition of other compounds, or are synthesized by microorganisms using carbon sources from the plant. It is important to keep in mind that the allelopathic compounds produced by bacteria, fungi, and algae are just as much a part of the science of allelopathy as are those produced directly by plants.

There is a large body of indirect evidence, but only a relatively small body of direct evidence, concerning the movement of allelopathic compounds from plants that produce them and the uptake and translocation of these compounds by neighboring plants. This is no doubt the weakest link in our chain of information concerning the phenomenon of allelopathy. There is an urgent need, therefore, for careful research in this area. Potential allelopathic compounds need to be tagged (with radioisotopes) in suspected allelopathic plants, and paths of the compounds should be traced out of the donor plant and into and through affected acceptor plants. Such investigations should include studies of the possible movement of allelopathic compounds from donor to acceptor through natural root or stem grafts, mycorrhizal fungi, and haustorial connections of parasitic plants (106-109).

After allelochemicals have been identified in the substrate, concentrations should be calculated, and threshold concentrations for activity should be determined against test plants using combinations of compounds present in the substrate, in addition to individual ones. Undoubtedly, many important allelopathic effects have been overlooked because of the use of single allelochemicals in determining threshold concentrations for activity.

A moderate amount of information is available concerning the factors affecting concentrations of phenolics in plants, and a little research has been completed concerning factors affecting concentrations of alkaloids and terpenoids. Little information is available concerning factors affecting concentrations of other types of allelopathic compounds; thus, research is urgently needed in this area.

There is a critical need for more study of factors affecting inactivation and effectiveness of allelochemicals after they move out of donor plants. Very little is known concerning the binding of these chemicals in soil and the effects of the binding on their activity. Virtually nothing is known concerning the role of texture in the accumulation of allelochemicals to physiologically active concentrations. Temperature stress markedly accentuates the allelopathic effects of ferulic acid on growth of sorghum and soybeans (110). There are obviously many stress factors which could affect response of a plant or microorganism to a given allelochemical or combination of allelochemicals, and such interactions should be investigated.

The surface has just been scratched in determining the mechanisms by which the different kinds of allelopathic compounds exert their actions. Therefore, it is important that much more research be done in this area of allelopathy.

I have emphasized to this point the need for research in all the basic areas of allelopathy. Such work could open up new horizons for applied research in the field of allelopathy; in fact, the results form the foundation of the entire field. This emphasis on basic research should in no way detract from the value and need for more progress in the various applied areas of allelopathy. In reality, only a relatively small amount of research has been carried out concerning the roles of allelopathy in natural or any of the man-made or man-altered ecosystems. Only a few of the more obvious areas in need of attention will be mentioned here.

Much research is needed on the quantitative effects on crop yields of interference by most of our serious weeds, and on the relative contributions of allelopathy and competition to the total interference by each weed species. Crop-crop relationships need to be investigated much more thoroughly to determine which crops can follow others with the least inhibitory or most stimulatory effects. More emphasis should be placed on investigations of stimulatory allelopathic effects, because these effects have been largely ignored in the past. Possible autotoxicity should be investigated also to determine if it is unwise to cultivate the same crop continuously without rotation.

Research in the use of allelopathy in biological weed control should be vigorously pursued. This should include the use of mulches of allelopathic plants; rotation of crops in which one or more of the crop plants is allelopathic to major weeds; use of allelopathic cover crops; underplanting of allelopathic companion crops in orchards, vineyards, etc.; and the development (through breeding or genetic engineering) of crop cultivars which can control major weeds in a given area through allelopathic activity. More research is needed on the possible use of certain allelochemicals as herbicides or as structural models for herbicide development.

The very broad area of allelopathic interactions between microorganisms and plants has been largely ignored by researchers. There has been some study of effects of selected weedy species on free-living and symbiotic nitrogen fixers and on nitrifiers in natural ecosystems but virtually nothing has been done on these relationships in other ecosystems. Much more research needs to be done also on the antagonistic effects of plants on soil-borne plant pathogens, and on the effects of allelochemicals in the predisposition of plants to infection by pathogens (111-113). There is a pressing need also for better understanding of the production by microorganisms of allelochemicals in soil or water that affect growth of plants. This extends also to the partial decomposition of allelochemicals from plants, which produces more active compounds or simply more compounds which can increase allelopathic effects through additive or synergistic action.

Obviously these suggestions for future research in allelopathy are only a few of the large numbers that could be given. Hopefully, however, they may give some impetus to progress in some vital areas of allelopathy.

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## Chapter 3

# Japanese Contributions to the Development of Allelochemicals

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The Japanese people live in a delicate microecosystem that can be easily polluted by industrial chemical accidents and the use of persistent agricultural chemicals. An intensive scientific effort has led to the isolation and identification of biodegradable natural products for potential use in agriculture. These include microbial metabolites that have activity against plants, microorganisms, nematodes, and insects. The chemical structures range from complex to simple and represent diverse classes of compounds. In addition, compounds isolated by non-Japanese researchers have been assigned specific uses by Japanese scientists for potential agricultural use.

If a scientist who knew nothing about the geography or demography of Japan visited that country he would begin to arrive at certain conclusions about the nature of the country quite quickly. Visits to restaurants in the cities and surrounding countryside would indicate that vegetables and fish are quite plentiful and relatively cheap; that the main grain for food and beverage is rice; that red meat is both expensive and difficult to obtain. Visits to shops and open-air market stalls would confirm these observations. Stacks upon stacks of fresh vegetables including some that are only just finding their way into western markets, such as daikon (Raphanus sativus longipinnatus), greet the eye. Plastic pans of assorted fish in all shapes and sizes, and an abundance of shellfish, are commonplace. While travelling along the highways our scientist would note that arable land is used to its maximum, even to the edge of the road, and to some extent one is reminded of the allotment gardens of World War II England, where all available land was used to produce vegetables to supplement the food rationing program. Even rice paddies extend to the road edge and one becomes suddenly quite aware of the vulnerability of this staple crop during the aquacultural stage of its life cycle. One chemical accident, or one malicious act, can spell disaster for the

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rice crop because of the rapid diffusion of agents in water. In addition, our scientist would see the backbone of mountains, the omnipresent sea that can be reached moderately easily, and one major conclusion would be that Japan is a country that cannot afford to have chemical pollution problems from either industry or agriculture. Furthermore, our observer would have been struck by the population density and the small, compact houses that are built quite closely together and have small, if any, gardens. Any effect on the food chain is immediately felt by the large population.

Our scientist would not be surprised if the map of Japan was shown to him. The country is bow-shaped, long, and consists of four main islands, Hokkaido, Honshu, Kyushu, and Shikoku, plus several smaller islands. The land mass is 377,748 square kilometers, or about 4% the size of the United States of America, and supports a population of 119.6 million people (1). Mountains comprise 71% of the land with 29% plains and basins of which approximately 15% is suitable for crop production. The mountains serve as a watershed and influencing factor on the environment, and as a source of timber. Compared with many other countries, Japan does have a dense population, especially when those figures are compared on an arable land ratio, but it is interesting to see the demographic figures for other countries. For example, the population per square kilometer in 1983 was 616 for Bangladesh, 388 for South Korea, 346 for the Netherlands, 323 for Belgium, 317 for Japan, 100 for China, and not surprisingly, 24 for the USA (1).

All the demographic figures and general observations lead one to the inevitable conclusion that Japan has a very delicate microecosystem that can be easily damaged by industrial or pesticide spills. And Japan is a highly competitive industrial nation while, at the same time, an intensely agricultural one. No one is more aware of the effects of pollution than the Japanese. In 1984, the Environment Agency of the Government of Japan published the "Illustrated White Paper on the Environment in Japan", in which the attitudes and law concerning pollution and waste control are discussed. The word "imperative" occurs throughout the text and it is quite obvious that the country intends to implement a rigorous set of standards for industry and the private citizen. The vulnerability of the environment and the population is vividly expressed in the examples of industrial spills. The first involved cadmium, which affects bones and kidney, that spilled into the Jintsu river in the Toyama Prefecture to produce the itai-itai disease. The second, which is better known because of a poignant set of pictures that were published of a mother bathing her son, was a methylmercury discharge that caused brain disorders and damage to the nervous system. This occurred along the Yatsushiro coast, Kumamoto Prefecture, and in the Agano River Basin in the Niigata Prefecture; the symptoms were described as the Minamata disease. Again, one notes the rain washing down the mountains, sweeping through the industrial and agrarian piedmont, carrying pollutants out into the bays and sea.

In spite of the population pressures, the shortage of arable land, the concentration of industry, and the relative isolation of the country, the Japanese have done a remarkable job in keeping their country in an orderly fashion. Parks abound and there is a respect for nature and art that is quite extraordinary. But aware

of what has happened in the past, and aware of what might happen in the future, Japanese scientists have asked themselves two important questions: "What sort of chemicals can we use on our crops as pesticides that have high specific activity against target organisms?" and "How biodegradable are these chemicals?" The answer to the first question may be microbial metabolites (allelochemicals) which are organic natural products and to the second, natural products are intrinsically biodegradable. In addition, a third question involves the nature of phytopathogenic microorganisms and the phytotoxins that they produce. For if one is to control these invaders, the biochemical pathways by which phytotoxins are produced by the pathogens must be elucidated. Besides, phytopathogens may produce toxins that can be used to control crop pests.

We shall see, in this brief review, that Japanese work has been intense in the isolation and identification of biologically active natural products from microorganisms. This intensity has been marked by the following steps: Extensive search for high-yielding strains of microorganisms, isolation and identification or proof of structure of a metabolite, and thorough testing on pests and crops that are of economic importance to Japan when sufficient quantities of the natural product are available, and synthesis (whenever possible) of the metabolite so that further screening may be carried out. In some instances metabolites have been rediscovered and new applications found for them that were overlooked by the primary discoverer. While the Japanese contributions to the area of biologically active natural products have been quite extensive, I have chosen a few examples to illustrate the wide diversity and activity of microbial metabolites against plants, microorganisms (including a self-inhibitor), nematodes, insects, and other zoological species.

Cylindrocladium scoparium is an ubiquitous phytopathogenic fungus that causes diseases in a wide variety of plants, especially ornamentals, and, more importantly, rice (Oryza sativa L.), where it induces sheath net-blotch. Hirota and coworkers first published on the nature of the toxins produced by this organism, in culture, in 1973 (2,3) and during the course of the next eleven years they carefully analyzed the structure of two metabolites possessing biological activity which they designated as cyl-1 and cyl-2. Finally, in 1984, the proposed structures for these compounds were published (4) and all the evidence pointed to two oligopeptides, specifically tetrapeptides arranged in the sequence of D-0-methyltyrosine, L-isoleucine, L-pipecolic acid (in cyl-2) or proline (in cyl-1), and 2-amino-8-oxo-9, 10-epoxydecanoic acid (Figure 1). Both metabolites were active against plant species but the more active of the two metabolites was cyl-1, which was isolated in rather small amounts. Cyl-1, for example, inhibited lettuce root elongation 50% at 0.5 ppm while cyl-2 inhibited extension 50% only at 1.0 ppm (2). In other tests, using Avena sativa L. cv. Russell coleoptiles, cyl-2 did not inhibit the growth of coleoptiles but when indole-3-acetic acid was added to the incubation medium at 1.0 ppm the extension normally induced by that substance did not occur. That is, cyl-2 at 10-100 ppm acted as an antagonistic agent to indole-3-acetic acid and while the mechanism of action was neither reported nor suggested the observation is, nevertheless, an interesting one.

The cyclic tetrapeptides are a fascinating group of compounds and the first observation that one makes on viewing the structures of the cyl group is the sequencing of the D and L amino acids. This leads to some thoughts about the synthetic permutations by substituting D and L species and, of course, the concomitant biological activity. But an even greater surprise is that while the cyl structures are unique they are part of a greater class of toxins, all of which possess the characteristics of having pipercolic acid (or proline) and the 2-amino-8-oxo-9, 10-epoxydecanoic acid residue. Among these are chlamydocin (*Diheterospora chlamydosporia*) (5), HC toxin (*Helminthosporium carbonum*) (6,7), and WF-3161 (*Petriella guttulata*) (8), the latter being another Japanese contribution. All these isolations and identifications are milestones in carefully constructed and meticulous work. It is estimated that approximately fifteen years were spent putting together the cyl-1 and 2 data.

Other cyclic tetrapeptides have also been isolated by Japanese workers and AM toxins I, II, and III, isolated from *Alternaria mali*, are extremely toxic to certain plant species (9,10). These are constructed of L- $\alpha$ -hydroxyisovaleric acid, L-alanine,  $\alpha$ -aminoacrylic acid and, in AM toxin I, L- $\alpha$ -amino- $\delta$ -(*p*-methoxyphenyl)-valeric acid. The phenyl residue in AM toxin II is L- $\alpha$ -amino- $\delta$ -phenylvaleric acid, while in AM toxin III, it is L- $\alpha$ -amino-(*p*-hydroxyphenyl)valeric acid (Figure 2). All the AM toxins produce leaf spot, or necrosis, in apple but as might be expected slight change in substitution (R-group) on the phenyl ring radically alters the specific activity of the molecule. Both AM toxin I and III induce interveinal necrosis in the "Indo" apple cultivar, which is also highly susceptible to *A. mali*, at concentrations as low as 0.1 ppb within 18 h after treatment. In contrast, the resistant apple cultivar "Jonathan" is only affected by 1 ppm of AM toxin I and 10 ppm of AM toxin III.

The fungal genus *Aspergillus* has been a rich source of allelochemicals for Japanese scientists. Among these are the piperazine, nigerazine B, from *Aspergillus niger* (11) (Figure 3). The compound tends to be unstable at room temperature and turns brown upon standing for any length of time. It inhibited root growth in *Lactuca sativa* L. cv. Great Lakes at concentrations above 50 ppm and, at 200 and 400 ppm, roots were inhibited over 50 and 80% respectively, relative to controls. However, leaves of *Oryza sativa* L., cv. Nihonbare seedlings were not affected by nigerazine B even at 400 ppm, though roots were slightly inhibited at 50 ppm only, but appeared to grow normally at 10, 100, 200 and 400 ppm. No explanation was given for these anomalous data. The LD<sub>50</sub> in mice (ip) is 75-150 mg/kg (11).

A structurally odd metabolite has been isolated from *Aspergillus nidulans*, 3-carboxy-2,4-diphenylbut-2-enoic anhydride, along with asperlin (12) (Figure 4). The anhydride was present in relatively small quantities in the malt extract medium and only 480  $\mu$ g were isolated per liter. Root growth of *Raphanus sativus* L., cv. Tokinashi was promoted 100% at 30 ppm and there was a decreasing linear response to 300 ppm, where promotion was approximately 75% greater than controls. At 1000 ppm roots were greatly inhibited. However, there was no response on hypocotyls of

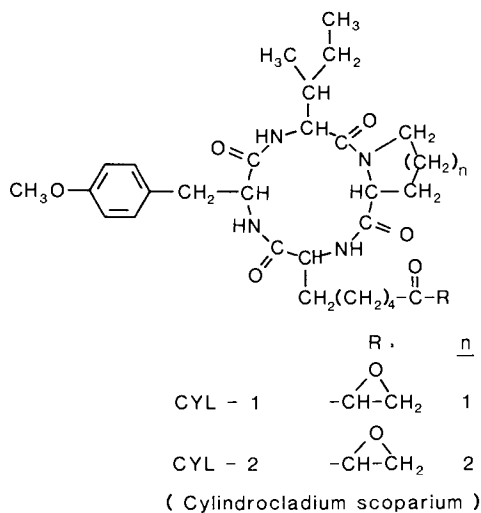


Figure 1. Cyl-1 and cyl 2.

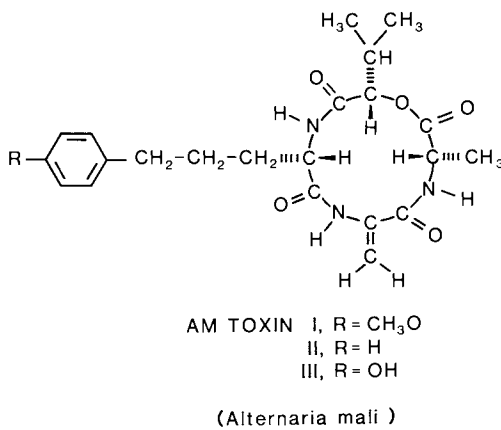
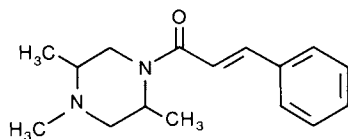


Figure 2. AM toxins I, II, and III.



NIGERAZINE B  
 ( *Aspergillus niger* )

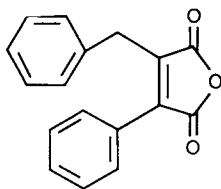
Figure 3. Nigerazine B.

the same species, except at 300 and 1000 ppm where inhibition occurred, of approximately 50 and 75%, respectively. The metabolite promoted root growth in Lactuca sativa L., cv. Great Lakes at 100 ppm only, by 50%, but hypocotyls were inhibited at the same concentration. At 300 and 1000 ppm, lettuce roots and hypocotyls were markedly inhibited. Thus, the metabolite appears to promote root growth in certain plant species at concentrations ranging from 30 to 300 ppm. Beyond that, inhibition of root tissue occurs. There is no effect on hypocotyl growth with the metabolite except at concentrations in excess of 100 ppm (this, too, is species dependent) when inhibition is effected. Later, 3-carboxy-2,4-diphenylbut-2-enoic anhydride was synthesized from phenylsuccinic acid and benzaldehyde (12,13).

One of the most interesting natural products to be discovered in recent years is hexylitaconic acid, from Aspergillus niger K-88 (14) (Figure 5). The molecule is relatively simple compared to other biologically active microbial metabolites and its discovery has an odd history. In 1978, McCorkindale and coworkers reported the biosynthetic pathway for the natural product canadensolide (15), a structurally interesting dilactone from Penicillium canadense which has been shown to have antifungal properties (16). In assessing the possible precursors to canadensolide, McCorkindale postulated that hexylitaconic acid might be a necessary intermediate. Hence, the compound was synthesized, <sup>14</sup>C radiolabelled, and introduced to cultures of P. canadense. The fermentation products contained [<sup>14</sup>C]canadensolide. But hexylitaconic acid was not bioassayed, which is interesting in light of the fact that canadensolide is a bioactive compound and most investigators would be curious about the anabolic and catabolic products thereof. Furthermore, the discovery of compounds structurally related to canadensolide in a number of fungi and lichens has not, apparently, led to an intensive search for active precursors in the lichens. The discovery of hexylitaconic acid in A. niger came six years after its reported synthesis and fairly extensive bioassays were carried out on Lactuca sativa L., cv. Great Lakes 54 and Oryza sativa, cv. Nihonbare. Lettuce roots were promoted approximately 25% relative to controls at 20 ppm. Rice shoots elongated 20-30% and roots were also promoted by hexylitaconic acid at 20 ppm, although shoots and roots were inhibited at 100, 200, and 500 ppm; the highest concentration induced total inhibition. The structure of hexylitaconic acid is similar to that of radiclonic acid (17,18), a root growth stimulator extracted from an unidentified fungus.

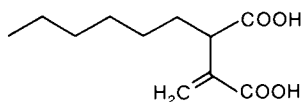
Colletotrichum nicotianae, a phytopathogenic fungus that causes tobacco anthracnose, has been the source of three phytotoxic substances (19,20) (Figure 6). These are colletotrichin and colletotrichins B and C. Insofar as structure and biological activity are concerned, the important features are the groups R1 and R2. Colletotrichin, which is by far the most inhibitory of the triplet, has OH groups in both positions and it inhibited Lactuca sativa L., cv. Great Lakes roots and hypocotyls at concentrations from 3 to 100 ppm. Colletotrichin B, which has a CHO function at R1 and an OH at R2, only inhibited roots and hypocotyls at 100 ppm. Colletotrichin C, where the R1 functional group is OH and R2 is CHO (the reverse of colletotrichin B), was slightly less active than





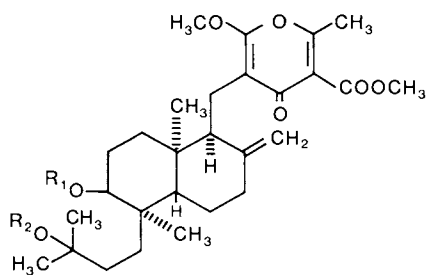
3-CARBOXY-2,4-DIPHENYLBUT-2-ENOIC ANHYDRIDE  
( *Aspergillus nidulans* )

**Figure 4.** 3-carboxy-2,4-diphenylbut-2-enoic anhydride.



(+) - HEXYLITACONIC ACID  
( *Aspergillus niger* K-88 )

**Figure 5.** Hexylitaconic acid.



	R <sub>1</sub>	R <sub>2</sub>
COLLETOTRICHIN	H	H
COLLETOTRICHIN B	CHO	H
COLLETOTRICHIN C	H	CHO

(*Colletotrichum nicotianae*)

**Figure 6.** Colletotrichin and colletotrichin B and C.

colletotrichin against hypocotyls and roots except at concentrations above 30 ppm, where in roots, it was more inhibitory. Both colletotrichins A and C inhibited lettuce seed germination at 30 to 1000 ppm, and B inhibited at 100 to 1000 ppm. So it would appear that colletotrichin and colletotrichin C are an order of magnitude greater in activity than colletotrichin B. This observation also holds true for induction of lesions in tobacco leaves. When 1  $\mu$ g of colletotrichin or colletotrichin C was added to the leaf surfaces and pricked into the tissues of Nicotiana tabacum L., cv. Bright yellow, or Xathi, lesions were formed that resembled those produced by C. nicotianae on field-grown tobacco. The chemical structures of the colletotrichins are interesting in that they are polysubstituted pyrones that have anomalous terpenyl side chains (19).

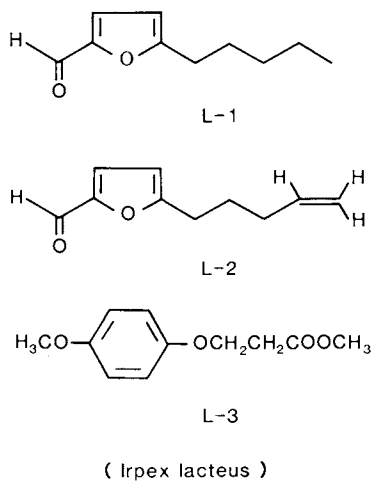
As was implied earlier, Japanese scientists are especially interested in directly controlling not only the growth and development of their national crops, but also in eradicating pests. One of these is the plant parasitic nematode Aphelencooides bessevi, which attacks soybean and also causes summer crimp of strawberry. The fungus Irrex lacteus has yielded three compounds that have specific activity against this nematode (21). These substances are designated L-1: (5-pentylfuraldehyde), L-2: [5-(4-pentyl)-2-furaldehyde], and L-3: (methyl 3-p-anisyloxypropionate) (Figure 7). Of these, L-1 and L-2 were equally active in nematode bioassays and each had an LD<sub>50</sub> of 50 ppm while concentrations of 100, 200, and 300 ppm killed all populations within 24 h. The L-3 metabolite LD<sub>50</sub> was 25 ppm and concentrations of 50 to 300 ppm killed nematodes within 24 h. Methyl 3-p-anisyloxypropionate was synthesized and shown to have the same nematocidal properties as the natural product L-3.

Another metabolite from Aspergillus niger is nigragillin, a piperazine closely resembling nigerazine B (from A. niger I-639), the significant difference between the two molecules being a terminal phenyl group in nigerazine B. Nigragillin, N-methyl-trans-2,5-dimethyl-N'-sorbylpiperazine, was originally isolated by Caesar et al. in 1969 from Aspergillus ustus but the compound was not tested in biological systems (22) (Figure 8). When silkworm, Bombyx mori L., were orally dosed with nigragillin incorporated into their diet, 40 ppm proved to be toxic within 48 h and 40% of the larvae, which had been treated after the third molt, died. At 72 h, 70% had died. Treatment with 80 ppm proved to be more lethal and 100% of the larvae died within 48 h. However, it should be pointed out that while the metabolite was incorporated into the media at these rates it is most probable that only small amounts were ingested by the larvae. A further dilution effect occurred because ten larvae were included in each diet assay. Other symptoms noted during the course of the experiments included vomiting, convulsions, and swooning (23). The effects of topical application of nigragillin in ethyl acetate to silkworm were noticeable almost instantaneously. Doses of 5  $\mu$ g/g caused immediate knockdown and poisoning, though death did not always follow. Synthetic dl-nigragillin was made after the method outlined by Caesar (22) and it had the same biological activity as the natural product.

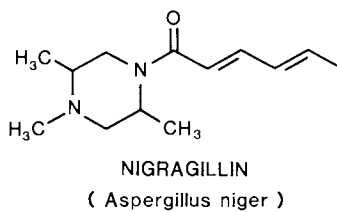
Another compound with marked insecticidal properties is

L-alanosine (Figure 9), isolated from a Streptomyces species, which inhibited larval ecdysis when administered to fourth-instar larvae of the common armyworm, Leucania separata, in artificial diet (24). The molecule is quite simple, L-2-amino-3-(hydroxynitrosamino)-propionic acid, and the reader quickly calls to mind the number of 'simple' amino compounds that have been discovered during recent years, that range from artificial sweeteners (aspartame) to the herbicide glyphosate. Doubtless, with all the possible permutations for amino acid derivatives, many more will find their way to the marketplace. The action of L-alanosine appears to be quite specific on ecdysis and rates as low as 5 ppm in diets caused inhibition of head capsule removal in 50% of larvae. With 40 ppm, not only was head capsule removal totally inhibited, but cuticle shed did not take place. If the metabolite was fed to the insect immediately following ecdysis, then larval growth was slightly delayed. The same effects were also observed in the cabbage armyworm, Mamestra brassicae. L-Alanosine was originally isolated in 1966 by Murthy and coworkers as an antiviral and antitumor metabolite from Streptomyces alanosinicus (25,26). It also inhibited reproduction in the housefly, Musca domestica (27). Its mechanism of action may involve blocking RNA adenine synthesis, thereby inhibiting production of the cuticular protein that is essential for sclerotization in the molting process (24). Again, L-alanosine is an excellent example of how previously discovered natural products have been re-isolated by Japanese researchers and tested in systems that have practical, national application.

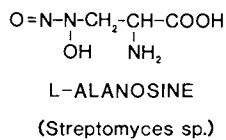
In addition to controlling plant growth and development and certain insect pests there is great interest in eliminating plant pathogens. One of these is Valsa ceratosperma, a fungus that produces apple canker and ranks among the most severe problems in apple cultivation. Mechanically injured parts of trees are readily attacked by the fungus, as are necrotic areas, and invasion proceeds methodically until the trunk is damaged. Infected parts become cankered by the fungus and eventually die. In an attempt to control V. ceratosperma several antibiotics were evaluated and microbial products tested. Micromonospora chalcone produced a novel antibiotic, designated propanosine (K-76), which had specific activity against V. ceratosperma (28) (Figure 10). The compound which was like L-alanosine in UV spectral properties, was of relatively simple structure. Disk assays in petri dishes were conducted against several microorganisms. Greater than 800 µg/mL were necessary to control Colletotrichum lagenarium, Fusarium oxysporum, f. lycopersici, Gibberella fujikuroi, Pellicularia filamentosa, and Saprolegnia parasitica. But 200 µg/mL controlled A. kikuchiana, Botrytis cinerea, Cochliobolus miyabeanus, Diaporthe citri, Gromerella cingulata, Pyricularia oryzae, and Rhizoctonia solani. In excess of 100 µg/mL were needed to control B. subtilis, ATCC 6633, B. stearothermophilus, Mycobacterium phlei 607, Staphylococcus aureus 209P, E. coli NIHJ, Pseudomonas aeruginosa M8152, Serratia marcescens, Vibrio percolens ATCC 8461, and Candida albicans M9001. Only 25 µg/mL were necessary to inhibit Saccharomyces cerevisiae Y21-1, but most importantly, only 1.0 µg/mL of the antibiotic was needed to control Valsa ceratosperma. Since only 45 mg of the Na salt were isolated initially, there was insufficient material for field trials. Synthetic material was



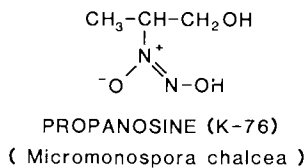
**Figure 7.** L-1 : 5-pentylfuraldehyde,  
L-2 : 5-(4-pentyl)-2-furaldehyde, and  
L-3 : methyl 3-p-anisoyloxypropionate.



**Figure 8.** Nigragillin.



**Figure 9.** L-Alanosine.



**Figure 10.** Propanosine (K-76).

obtained (28) but it was a racemic mixture and had only half the activity of the natural product.

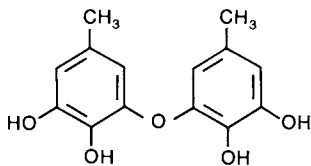
The catalog of natural products that control microorganisms is extensive. That is, after all, one of the early areas of medicinal research and really marks the beginning of antibiotics derived from microbes. But less common are natural products from microorganisms that are self-inhibitors at low levels of application. While higher plants contain apparent self-inhibitors and plant growth regulators, specifically abscisic acid, the role of these metabolites in the producer organism is not clearly understood. Aspermutarubrol, bis(5-methyl-2,3-dihydroxyphenyl) ether, from Aspergillus sydowi is such a compound (Figure 11). The presence of the organism on the surface of old shoe polish is something of a mystery but the curiosity of Satomura is to be commended! The first reaction on discovering a fungus on any household item is to discard it with alacrity. During the culturing of the organism it was observed that pigment production could be correlated with mycelial inhibition and that addition of sodium acetate increased both responses. Eventually, aspermutarubrol was isolated as colorless crystals from chloroform (1 mg/L) (29) but in aqueous solution the metabolite oxidized quite readily to give red products. The metabolite proved to be ineffective against Saccharomyces cerevisiae, Penicillium notatum, Aspergillus niger, A. oryzae, Mucor mucedo, Rhizopus japonicus, and very slightly active against E. coli, but it was very active at 50, 100, and 200 ppm against the gram-positive B. subtilis, Staphylococcus aureus, and Micrococcus lysodeikticus. At 12.5, 25, 50, and 100 ppm it moderately inhibited A. sydowi and completely inhibited at 200 ppm (29). The authors of this work were quick to point out that the secretion of antibiotic substances as a competitive mechanism for the survival and benefit of the producer organism is well established, but the puzzle posed by aspermutarubrol remains to be explained.

During the course of trying to isolate a self-inhibitor from benomyl-resistant strains of the cherry brown rot fungus, Monilinia fructicola, two new metabolites that had antimicrobial and phytotoxic properties, though not self-inhibitory characteristics, were isolated. These were monilidiol and dechloromonilidiol (30), "salicylaldehyde type octaketides (30)", which are structurally related to the phytotoxins pyriculariol (31) and pyriculariol (32) from Pyricularia oryzae (Figure 12). Monilidiol, when applied to cherry leaves at 2-5 µg, followed by pin pricks, induced dark necrotic spots. It also inhibited the growth of rice seedlings though hard data are not available (30). Dechloromonilidiol was not as active as the chlorinated compound in either cherry or rice plants. Other tests were carried out with monilidiol against selected organisms but greater than 100 ppm were necessary to inhibit plant pathogens (unnamed), including M. fructicola. The compounds were synthesized and the procedures were published in 1983 (33).

No system is perfect and sometimes events become quite tangled in research. What appears to be a placid area of endeavor is suddenly entered by several workers who are, unknowingly, independently pursuing common goals. Such was the case with the *p*-terphenyls, a curious group of compounds that are expected to be

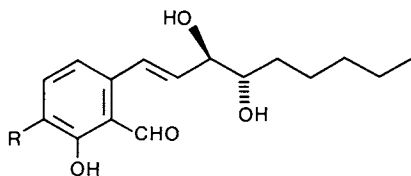
products of a chemical reaction in vitro rather than natural products. In 1975, Takahashi was in the process of isolating and identifying terphenyllin (Figure 13) from A. candidus when, as he states, "At this stage of the work Marchelli and Vining (34) reported the isolation of the terphenyl assumed to be identical with compound A..."(35). The compounds were identical. Takahashi had noted the effect of terphenyllin against HeLa cells, where 3.2 ppm produced slight cellular damage and higher concentrations induced greater changes so that at 100 ppm there was complete cytolysis. Cells also had varying degrees of R type changes, slightly enlarged cells, evenly distributed chromatin, and small nucleoli (35). In 1978, Cutler, et al., discovered hydroxyterphenyllin, also from A. candidus, using the etiolated wheat coleoptile bioassay and showed that it had plant growth regulatory activity (36) (Figure 14). Furthermore, terphenyllin significantly inhibited coleoptiles 35% at  $10^{-3}$ , while hydroxyterphenyllin inhibited 100, 42, and 8%, at  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  M. Peracetylation of the molecule rendered it biologically inactive and so the activity appeared to be related to the number of the OH groups. Four years later, Kobayashi et al., published the structures for candidusins A and B from A. candidus (Figures 15 and 16). Both were analogs of terphenyllin and hydroxyterphenyllin, respectively. Each blocked initial cleavage in sea urchin embryos when added 5 min after fertilization at  $1 \times 10^{-4}$  and  $5 \times 10^{-5}$  M, and inhibited B. subtilis at 50  $\mu\text{g/mL}$  (37). Further experiments were conducted with candidusin B to determine its effects on DNA, RNA and protein synthesis in the gastrula stage of sea urchin embryos. Uptake of [ $^3\text{H}$ ]-thymidine, [ $^3\text{H}$ ]-uridine, and [ $^3\text{H}$ ]-L-leucine was observed and percent inhibition noted. At 0  $\mu\text{g/mL}$  of candidusin B there was 0 inhibition of uptake of these radioisotopes, but at 1  $\mu\text{g/mL}$  there was 83, 58, and 0% inhibition of uptake of radiolabelled thymidine, uridine and L-leucine, respectively. At 10  $\mu\text{g/mL}$  these figures were 96, 86 and 0%, respectively. Therefore, candidusin B was not considered to be a respiratory inhibitor because it had been determined, in sea urchin embryos, that respiratory inhibitors suppress protein synthesis, and also DNA and RNA synthesis (37). In 1985, dihydroxyterphenyllin was isolated from A. candidus and was found to be approximately twice as active as hydroxyterphenyllin in blocking first cleavage and inducing irregularly shaped or odd-numbered cells in sea urchin embryos (38). The laboratory synthesis of these terphenyl derivatives is exceptionally difficult and has not yet been accomplished, but microorganisms appear to make them with relative ease.

These few examples of research indicate, at least in part, the intensity and perseverance with which Japanese scientists approach their work with microbial metabolites. We have seen that, in many instances, efforts are made to duplicate, by synthesis, these substances and it may only be a question of time before a marketable product is developed. Doubtless, what we are seeing in the literature is only a small portion of the energy being expended to successfully produce biodegradable agrochemicals based on natural product templates. Japan is committed to producing abundant crops on small parcels of land and to having an unpolluted environment.



ASPERMUTARUBROL  
( Aspergillus sydowi )

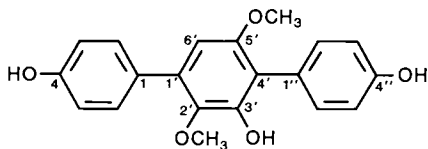
Figure 11. Aspermutarubrol.



	R
MONILIDIOL	Cl
DECHLOROMONILIDIOL	H

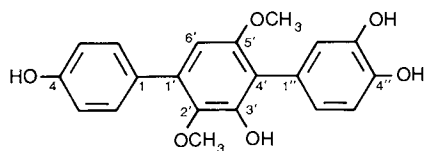
( Monilinia fructicola )

Figure 12. Monilidiol and dechloromonilidiol.

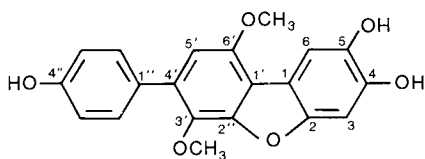


TERPHENYLLIN  
( Aspergillus candidus )

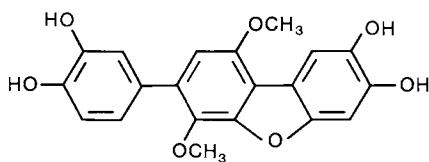
Figure 13. Terphenyllin.



HYDROXYTERPHENYLLIN

( *Aspergillus candidus* )**Figure 14. Hydroxyterphenyllin.**

CANDIDUSIN A

( *Aspergillus candidus* )**Figure 15. Candidusin A.**

CANDIDUSIN B

( *Aspergillus candidus* )**Figure 16. Candidusin B.**



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## Chapter 4

# Allelopathy in the Soviet Union

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Allelopathy has been developed in several main directions throughout Russia, which has a long and distinguished record in this research area. Soil sickness under wheat, oats, corn, rye, alfalfa, peas, sugar beets, clover, flax, woody plants and shrubs has received much attention. An understanding of recultivation-regulation of microbial activity and breeding of new plant varieties with less allelopathic activity are among the main objectives. Mechanisms of interactions between plants in various types of ecosystems have been intensively studied. Research on isolation and methods of evaluation of chemicals produced by plants and/or microorganisms and testing them are discussed.

Allelopathy is an old tradition in the Soviet Union. In the middle of the last century the botanist Levakovskii (1) from Kazan published his observations about interactions between forest trees and forest grasses. He suggested that forest litter chemically influences plant seedlings. At the beginning of the present century Periturin (2) investigated the causes of soil fatigue under common cereals -- oats, wheat, and barley. That found toxins accumulated in soil, which could be extracted with alcohol; after this the soil regained its fertility.

A special direction in research on chemical interaction among plants began in 1926, when Tokin (3) discovered the presence of volatile protective substances of plants, which he called phytoncides. Besides their influence on pathogenic organisms, which cause illnesses of men and animals, the most important function of phytoncides is the protection of plants against herbivorous animals and parasite damage and against fungal and bacterial infection. Independently of those researches Kholodnyi (4) at the end of the 1930s noted the ability of some microorganisms and the roots of higher plants to absorb volatile substances from air and to use them for growth. On the basis of these investigations he developed the idea of interaction among plants and between plants and microorganisms mediated by volatile compounds, such as terpenes and other hydrocarbons. In 1956 Chernobrivenko (5) summarized many such observations and field experimental data. Some interesting observations on the biological effects of volatile substances were also published by Sanadze (6).

At the end of the 1950s allelopathy was already well known among botanists and plant physiologists, but it was considered rather a minor and rare phenomenon that had no great ecological importance. We have screened many plant exudates, and reached the conclusion that many species are allelopathically active; indeed, practically any plant under certain conditions so affects other plants

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(7). Thus allelopathy is indeed very important ecologically, although skeptics remain.

Many investigations in chemical interactions in different types of phytocenoses (natural, artificial, forest, steppe, aquatic ecosystems) are currently conducted by specialists: botanists, plant physiologists, microbiologists, biochemists, soil scientists, agronomists, etc.

By drawing analogies with other sciences, we can mark several stages of allelopathy development and paradigms that change and add to each other. Originally, allelopathy was considered only as the harmful influence of certain active plant species on adjacent plants. This definition was extended by the author of the term allelopathy, Molisch (8). Molisch indicated that allelopathy included stimulatory as well as inhibitory growth effects. Many of the phenomena he discussed involved growth stimulation or a combination of stimulation and inhibition e.g., effects of ethylene. Most researchers now accept allelopathy. Active substances can be likened to herbicides and their high specificity is accepted without question.

For example, a very allelopathically active plant that we studied in 1959 is the crucifer Crambe tataria Sebeök (9). It is a biennial or perennial herbaceous plant with a well-developed root and large spherical bushlike stems with many dry fruits. After ripening, the stem tears loose from the root and is rolled throughout the steppe by wind. En route the fruits are lost in the grass. The fruits contain very strongly inhibiting substances, a mixture of many phenolic acids, amino acids, and some sulfur-organic substances. We still do not know the whole chemical constitution of fruit coats of Crambe tataria, but that plant first gave us the idea of chemical interaction between plants. The water-soluble inhibitors from the fruits go into the surroundings, suppress other plants, and make free places for germination and growth of Crambe.

Such plants as Crambe are not numerous -- they may constitute less than 7% of the general flora. However, allelochemicals can be not only harmful, but favorable, particularly at low concentration. Chernobrivenko (5) and other Soviet scientists assumed the possibility of positive chemical influence of adjacent plants. American authors, Rice (10) among them, took this position much later.

The notion of action by specific allelochemical compounds is also unjustified. Detailed study of some allelochemicals in active species has shown the presence of phenolic acid mixtures and other phenolic derivatives or terpenes. I think that we can never talk about the action of a single substance; everywhere many compounds having different biological activity act simultaneously, perhaps mutually increasing their activity. As a rule, such allelochemicals are the intermediate products of soil humus, synthesis, or the ground detritus in aquatic ecosystems (11). High concentrations of these substances are lethal, moderate ones inhibit growth processes, and low concentrations stimulate them.

Accordingly, the second paradigm of allelopathy was formed. In this pattern the chemical mutual influence is manifested as a cycling of physiologically active substances, which play the role of regulators of internal and external interrelations -- of initiation, development, and change of plant cover in biocenosis (ecosystem) (12). Allelopathy is part of the whole recycling of organic substances in the ecosystem; it involves low-molecular-weight carbon compounds, which are either mineralized or polymerized into large humic molecules. Such molecules do not penetrate into plants and thus have no allelopathic effect. This means that we are discussing the intermediate products in humus formation and decomposition.

This paradigm provided our basic notion of allelopathic soil fatigue (13). As a broad ecological notion it includes accumulation of toxic products of the vital activity of plants and heterotrophic organisms; this adversely affects the productivity of following plants. As a consequence of specialization and concentration of the agricultural industry, the soil fatigue problem became particularly serious and urgent. We managed to devise an isolation method for allelochemicals by using ion-exchange resins, which permitted us to obtain those substances without destruction of humus complexes that could not be absorbed by living plant roots. Our method simulates plant root absorption of allelochemicals. It was shown that cinnamic, *p*-coumaric, *p*-hydroxybenzoic, and other phenolic acids accumulate under monocultures of wheat, rye, and other cereals. The concentrations of these acids increase two- or threefold under permanent wheat culture, while that of neutral humus decreases. At the same time we observe changes in microflora; the diversity and number of bacteria decrease and the mass of soil fungi increases (14). To relieve allelopathic soil fatigue we can use either the old tested method of crop rotation or, if we know the chemical basis of the problem, the agrotechnical method, which accelerates mineralization/polymerization of allelochemicals into stable humus, or else use allelopathically inactive species and varieties that don't cause soil fatigue. We think that the last method is the most reliable. This second paradigm includes both negative and positive allelochemical effects on plant growth and physiological processes.

This new view of allelopathy, its new paradigm, conveys the notion of chemical information exchange among plants and other organisms. Current plant physiology makes it possible to suggest that plants are able to "perceive" a chemical environment and respond with appropriate reaction. This can be shown in changes of their life strategy and tactics. In this case an allelochemical plays the role of a signal; its effect does not depend on concentration, but releases a trigger connected with a genetic program. For example, seeds and bulbs of many herbal plants may rest in the soil many years under cover of a forest and germinate only after the trees are removed; humidity, temperature, and extent of aeration often changed and several times there were apparently perfect conditions for germination, except that the seeds were under living dominant plants. Obviously, the signal of mature trees holds the plant embryos in the resting stage. In other cases, root exudates, for example, those of oats, stimulate germination of weeds in the field. The research of Gajic' (15) from Yugoslavia found that the weed *Agrostemma githago* stimulates germination and growth of wheat. After many years of research she identified the allelochemicals, which is a mixture of amino acids, and includes allantoin and tryptophan. By analogy Gajic' created the biostimulator Agrostemin, commercially marketed, which accelerates growth and germination, and increases productivity of many cultivated plants and native meadows by 10-15%. Agrostemin is used at remarkably low concentrations -- about 10 g/ha.

For a long time phenomena of this kind have attracted Soviet researchers' attention. In the 1920s Gurvitch (16) suggested the existence of so-called mitogenetic rays, which were supposed to stimulate cell division of yeast, lower plants, protozoa, and so on. But no such physical rays could be found and in the 1950s Moiseeva (17) showed that the signal for mitosis is indeed chemical in nature. However, such signal substances have not been discovered yet.

In recent years in the agricultural high school in Kharkov, Naumov and his students (18) demonstrated that a water extract from 2 kg of grain (wheat, rye,

oats, barley) when added to 700 kg of the same or other cereal grain before sowing stimulates all vital functions of the growing plants and reliably increases the harvest. Moreover, reciprocally soaking the seeds of wheat in seed extract of rye, and of rye seeds in wheat extract a few times, is claimed to facilitate fructification by hybridization between the two genuses. The soaking of seeds in extracts from some weeds made the new plants more resistant to the weeds used.

These investigations are in progress and sometimes may be of doubtful validity, but suggest that allelochemicals of a plant can act on the expression of the heredity program in another plant. In other words, chemical signals coming from living plants causes recipient plants to follow a suitable life strategy -- for example, resting.

Perhaps the signal is other than chemical; in particular maybe it is slow (circadian) vibrations of electric fields near living plants (for example mature trees) and this is what prevents the germination of resting seeds and bulbs. Although concentration does not change essentially the character of the recipient reaction, the signal is operative only in a certain interval; at too high concentration the substance does not convey the message. This is related to a recent interesting observation on birch and pine trees by Marchenko (19) in Bryansk. When a coniferous tree and birch grow together, their needles and twigs deviate on opposite sides. Marchenko calculated that the forces required to cause such deviation amount to a few hundred or thousand horsepower.

To show the dimensions of the allelochemical effect with all three above-mentioned paradigms: before the era of mineral fertilizers, production of a crop required application of 20 tons of manure per hectare; later, with mineral fertilizers only several centner per hectare are required, with regulators such as herbicides, 2 to 20 kg, and regulators such as Agrostemin only a few grams per hectare.

Of course, the above-mentioned paradigms do not exclude or contradict each other; each of them concerns its own circle of phenomena, but all these phenomena apply to allelopathy.

I think that Soviet researchers contribute much to the development of this science, which is very important for understanding the nature of vegetation development and species evolution and especially for increasing production of consecutive crops.

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## Chapter 5

# Allelopathy Involving Microorganisms

## Case Histories from the United Kingdom

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Plant residues can provide substrates for the production of phytotoxic metabolites by soil microorganisms but they can also support the growth of pathogens and other deleterious microorganisms. This is illustrated by reference to the problems of establishing crops drilled in the presence of straw residues and of decaying weed and grass residues that have been previously killed with herbicides. Short-chain acids accumulate under anoxic conditions, which favor fermentative metabolism of bacteria. Such phytotoxins may damage the plant directly or predispose plants to infection by pathogens. However, plant residues may also be used as substrates for beneficial microorganisms to produce plant nutrients, soil conditioners, and plant protection chemicals. There is scope to promote the beneficial microbial effects against the harmful by soil management and by inoculation.

Microorganisms produce a vast range of metabolites that can potentially influence plant growth (1, 2, 3). This action can be positive or negative. Pathogens produce a negative effect by producing specific metabolites or enzymes. Beneficial organisms may act directly on the plant by producing chemicals that stimulate plant growth or enhance the uptake of nutrients. Indirect effects of beneficial organisms include the suppression of harmful organisms and the improvement of soil structure. This diverse range of microbial activities falls within the phenomenon of allelopathy as defined by Rice (4). The description of this phenomenon is useful, but the processes can also be described under the heading of plant/microbe interactions.

Whereas a wide range of potential allelopathic agents has been identified from soil microorganisms, it has seldom been proven that they are of true ecological significance. The minimum necessary criterion for this is that the product should occur in the form and

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concentration that influence plant growth. To demonstrate this usually involves very mild extraction procedures because, for example, even mild acids or alkalis can depolymerize lignin to yield phenols that would otherwise be inert because in the polymeric state.

Another factor rarely considered is the zone of the root system subjected to the microbial metabolite. It is likely that the metabolites will only be formed in particular regions of the soil where there are suitable substrates for producer micro-organisms and it is unlikely that the entire root system will come under the influence of a metabolite. For example, acetic acid is a common microbial fermentation product of cellulose and is phytotoxic (5, 6). However, in treating a single root tip with a small concentration ( $5 \text{ mol m}^{-3}$ ), root and shoot growth were stimulated. This was not observed when a greater number of tips were treated or greater concentrations of the acid were used (Table I). Lengths of roots were more sensitive than tips to inhibitory concentrations of the acid. Compensatory growth in non treated roots could occur in response to treatment of other parts of the root system.

Table I. Response of Barley Root Elongation to Treatment with  $10 \text{ mol m}^{-3}$  Acetic Acid

Region treated	No. of roots treated	Mean length of non-treated root + s.e.m.	Mean length of treated root + s.e.m.
2 cm tip	1	11.6 + 1.7	8.2 + 1.1
2 cm tip	3	15.8 + 0.2	9.1 + 0.6
2 cm section	1	16.5 + 0.5	12.9 + 2.6
2 cm section	3	17.5 + 1.3	7.4 + 0.8
Control*	-	11.2 + 0.7	12.4 + 0.7

\* Means of plants where either one or three tips or lengths were treated with plant culture solution or where no roots were treated.

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Whereas aliphatic acids can produce permanent shoot and tiller damage, when the acids are removed from the growth medium, root growth can be promoted (8), presumably by compensatory action.

Quite commonly micro-organisms and their products are bio-assayed together. When leaves of Anthoxanthum odoratum were decomposed aerobically the total suspension contained growth-inhibitory micro-organisms but no cell-free phytotoxic metabolites (Table II). By contrast wheat straw degraded anaerobically yielded phytotoxic metabolites but no growth-inhibiting micro-organisms.

Table II. Effect of Micro-organisms and Their Metabolites Formed During 14 Days Decomposition of Plant Residues on Longest Root Length (mm) of Barley Seedlings

Residue	Control (distilled water)	Total suspension	Filtrate	Micro- organisms
<u>Anthoxanthum</u> leaves, aerobic	75	79	74	59*
Wheat straw, anaerobic	84	35***	37***	76

Significantly different results indicated by \*\*\*  $\underline{P} \leq .001$ ; \*  $\underline{P} \leq .05$   
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 Martinus Nijhoff B. V.

### Straw Residues

In the direct drilling (no-till seeding) practice in the United Kingdom, straw residues from the preceding crop are usually burnt because poor crop establishment and yields can result, particularly on heavy soils in wet years (10). Similar problems can occur in the conservation tillage systems of the Pacific Northwest (L.F. Elliott and H.-H. Cheng, this volume).

The older agricultural textbooks indicate that this is due to the straw having a high C:N ratio (c. 100:1) compared with the decomposer micro-organisms (c. 5:1) and that N otherwise available to plants is immobilized into microbial biomass. It is quite easy to demonstrate this effect in pot experiments, where seedlings show obvious signs of N deficiency in the presence of straw. However, it is likely that in the full cropping season the immobilized N will subsequently become available to the crop, although little firm experimental evidence has been obtained to support this hypothesis. Indeed N immobilized in microbial biomass during winter could prevent winter leaching and therefore straw could even be beneficial to the N cycle. Certainly extensive trials in the UK show that application of seedbed N has little or no beneficial effect on crop productivity compared with normal application times.

There is a need for further studies on this topic. One model laboratory study has demonstrated that the N-immobilization potential is greatly reduced when fertilizer N is placed several centimetres below the soil surface (11). The N tie-up is much smaller when residues are left on the soil surface as opposed to being mixed in the soil.

In the USA some evidence (12) has been reported for Pythium spp. increasing as a consequence of direct-drilling into straw but in the UK little evidence has been reported as yet for pathogens building-up on straw, although this is likely to vary greatly between locations and conditions. Straw is a favorable substrate for pathogenic Fusarium spp. and Pythium spp. Growth-inhibitory bacteria may also be a part of the problem (L.F. Elliott and H.-H. Cheng, this volume).

All the present evidence points to phytotoxic steam-volatile fatty acids, particularly acetic, being a major microbiological factor responsible for the crop damage, and the conditions of ecological significance referred to earlier have been satisfied. However, the toxin is produced only in the straw tissue and its concentration declines exponentially with distance from the straw (13). A correlation of soil acetic acid content with phytotoxicity is therefore neither expected nor found.

### Weed Residues

When dense infestations of weeds are killed with herbicides, a situation analogous to the straw problem can occur because a large amount of readily degradable substrate becomes available to the saprophytic microbial population of soil. With herbicides that are translocated, such as glyphosate, there is a chance that the herbicide itself would be released to the soil, but this has not been found to be the case (14). Certainly phytotoxic acetic, propionic and butyric acids can be produced from the couch (quack) grass rhizome. However crop damage is usually observed in dry (50% water saturation) soils and this could be repeated in glasshouse trials (15). Thus it appeared unlikely that the necessary anaerobic conditions for bacterial fermentative metabolism would normally exist. Dry soils favor the development of the pathogen Fusarium culmorum (16) and large populations of this fungus have been found on decomposing rhizomes of the weed. However, even under dry conditions small concentrations of the organic acid can form and unless the pathogen population is very large the acid appears to provide a compounding stress on the host plant (Table III).

Table III. Effect of 5 mM Acetic Acid and Fusarium culmorum on the Growth of Barley Seedlings

Inoculum density (spores/ml)	Mean length of first three leaves (mm) 12 days after germination			
	0	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>
Seedlings treated with 5 mM acetic acid	120 <sup>b</sup>	98 <sup>c</sup>	89 <sup>cd</sup>	83 <sup>cd</sup>
No acid treatment	140 <sup>a</sup>	118 <sup>b</sup>	114 <sup>b</sup>	78 <sup>d</sup>

Results with different letters are significantly different  
( $P < 0.05$ )

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Permanent Neutral Grassland

It can be difficult to establish new grasses into permanent grassland. This may be an example of allelopathy and the reason why some species dominate old grassland. Newman (18) prepared a review on whether allelopathy is ecological adaptation or accident. His research team at Bristol University investigated allelopathy within permanent grassland using pot experiments with 'donor' or 'treatment' and 'receiver' or 'test' species of different grasses (19, 20, 21, 22, 23). These studies showed, for example, that the decomposing roots of *Rumex acetosa* had the greatest inhibiting effect on four species (Table IV). When the nutrient content of *Lolium perenne* as 'test' species was analyzed, *R. acetosa* residues gave rise to a similar P content in the test species as the P-deficient soil alone; there was no such effect on N content (Table V). They concluded that the allelopathy acted by the residues of the treatment species failing to make P available to the test species, an effect which was greatest in wet soil.

Table IV. Dry Weights (mg) of Shoots of 'Test' Plants of Grassland Species Grown on Soils Containing the Decomposing Roots of 'Treatment' Species

'Treatment' species	'Test' species			
	Ao	Lp	Pl	Ra
<i>Anthoxanthum odoratum</i> (Ao)	61b	122a	215a	174a
<i>Lolium perenne</i> (Lp)	155a	180a	456a	73ab
<i>Plantago lanceolata</i> (Pl)	199a	216a	326a	41c
<i>Rumex acetosa</i> (Ra)	47b	38b	18c	17c
Nil	44b	62b	77b	103ab

Values not sharing the same small letters in each column differ significantly ( $P < 0.05$ )

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Table V. Nitrogen and Phosphorus in Shoots of 'Test' Plants Grown on Soils Containing the Decomposing Roots of 'Treatment' Species

'Treatment' species	<u>Lolium perenne</u>	
	N%	P%
<u>Anthoxanthum odoratum</u>	2.95ab	0.217a
<u>Lolium perenne</u>	2.19c	0.195a
<u>Plantago lanceolata</u>	2.39bc	0.201a
<u>Rumex acetosa</u>	2.51bc	0.140b
Nil (wet)	3.22a	0.157b

Values not sharing the same small letters in each column differ significantly ( $P < 0.05$ )

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In parallel studies it was demonstrated that the rhizosphere populations of grassland species could affect each other, there being a large increase in fungal biomass (Table VI). The significance of this observation is still unclear. There have been relatively few quantitative studies of micro-organisms on plant roots in monoculture let alone in mixed stands. Such approaches should prove useful in assessing the potential magnitude of microbial metabolic processes in allelopathic interactions.

Table VI. Bacterial Cover and Fungal Mycelium Length on Root Surfaces of Lolium perenne (Lp) and Plantago lanceolata (Pl)

	Bacterial cover (%)		Fungi <sub>1-2</sub> (mm mm <sup>-2</sup> )		Mean plant weight (g)	
	<u>Lp</u>	<u>Pl</u>	<u>Lp</u>	<u>Pl</u>	<u>Lp</u>	<u>Pl</u>
Separate	4.3	5.6	0.7	1.8	0.71	0.69
Together	6.3	5.8	2.1	2.9	1.01	0.69

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### Reseeding Old Grassland

When grassland becomes unproductive because of poor species composition, the old sward can be killed off with herbicide and new grasses reseeded by direct-drilling into the treated sward. This

presents a situation of microbial decomposition which is analogous to the decomposition of straw and weed residues.

Shoots of a range of grass species were toxic to other grasses and clover when decomposed anaerobically (Table VII). The phytotoxicity seemed to be caused by organic acids and was less after 20 days of decomposition than after 10. Festuca rubra, Agrostis stolonifera, and Alopecurus pratensis residues were the most toxic; which to an extent is consistent with field observations that residues of the former two species are particularly difficult to seed into. When the shoots were decomposed aerobically, some were toxic after 10 days but this toxicity disappeared after 20 days when some residues could stimulate plant growth (Table VII).

Table VII. Effect of Solutions Produced after 10 Days Decomposition of Plant Residues on Root Extension

Residue	Root extension of test species (mm)						
	<u>Alopecurus</u> <u>myosuroides</u>	Fr	Hl	Lp	<u>Poa</u> <u>annua</u>	Pt	<u>Trifolium</u> <u>repens</u>
(a) Aerobic							
<u>Agrostis stolonifera</u>	13	7	11	28	8	4	38
<u>Alopecurus pratensis</u>	13	6	10	35	9	5	42
<u>Anthoxanthum odoratum</u>	13	7	10	25	10	6	34
<u>Festuca rubra</u> (Fr)	13	11	10	28	10	7	30
<u>Holcus lanatus</u> (Hl)	13	6	9	25	9	3	32
<u>Lolium perenne</u> (Lp)	11	6	10	24	10	6	24
<u>Poa trivialis</u> (Pt)	11	6	11	26	11	7	32
Control	11	6	11	30	8	5	46
(b) Anaerobic							
<u>Agrostis stolonifera</u>	0	0	3	9	5	0	2
<u>Alopecurus pratensis</u>	0	5	0	3	0	0	0
<u>Anthoxanthum odoratum</u>	15	7	14	25	10	6	33
<u>Festuca rubra</u> (Fr)	0	0	4	5	0	0	0
<u>Holcus lanatus</u> (Hl)	15	9	21	36	12	11	37
<u>Lolium perenne</u> (Lp)	6	8	10	26	10	7	22
<u>Poa trivialis</u> (Pt)	11	4	7	16	7	3	21
Control	10	7	10	32	10	5	48

Control contained soil and water only

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Even though toxins could at least in part be involved, Fusarium culmorum again seemed to be responsible for the damage (25). Whereas the fungicides carbendazim and drazoxolon were effective in controlling the disease, calcium peroxide was also effective (26). This compound had the added advantage of releasing alkali to

neutralise organic acid toxins and oxygen to minimize organic acid formation (27).

#### Conclusion: The Scope for Soil Biotechnology

The potential of manipulating soil micro-organisms, especially for the utilization of crop residues, has been outlined (28). For example, accelerating straw breakdown can reduce the time period in which organic acid toxins are produced (29). By inoculating straw with a consortium of a cellulolytic fungus and an anaerobic  $N_2$ -fixing bacterium in the laboratory, straw breakdown has been accelerated and the resulting residue is enriched in N (Table VIII). In other similar associations the cellulolytic fungus has biocontrol potential against root disease, and associated polysaccharide-producing bacteria can assist with the stabilization of soil structure (31). If such approaches could be carried to practice in the field, a new era of manipulative allelopathy would emerge. This presents a great challenge for soil biotechnologists but will certainly not be realised until the ecological aspects of allelopathy are clearly understood.

Table VIII. Decomposition of Non-Sterile Straw Contained in Glass Columns at 25°C for 8 Weeks

Treatment	Decomposition rate constant, $k$ ( $d^{-1}$ )	N gain (mg)	
		Per g straw lost	Per g original straw
Non-inoculated	0.0096	8.8	2.8
<u>Penicillium corylophilum</u> + <u>Clostridium butyricum</u>	0.0139	11.5	5.0

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## Chapter 6

# Allelopathy in Desert Ecosystems

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Allelopathy and autotoxicity in desert ecosystems in both hemispheres are reviewed. The following generalizations are made : (a) aggressive plants with allelopathic potential are often adult perennials, members of the Compositae or Labiatae, that are capable of reducing germination and/or growth of various annuals or of their own seedlings; (b) allelochemicals emanated from aggressive plants are either common secondary metabolites (terpenes, terpenoids, or phenolic compounds), or inorganic salts. Allelochemicals, although frequently nonspecific, do not constitute general phytocides. They may reach susceptible plants through the soil in different ways, either washed off from the fresh or dried shoots by rainfall, released as volatile substances later absorbed by the soil, or released in part from the mature or decomposed roots; (c) some ecological factors in the desert favor production of allelochemicals, e.g. water or mineral stresses, or grazing, whereas other factors improve the preservation of the allelochemicals, i.e. low rates of leaching, or reduced activity of the soil microflora. Wide desert areas are covered with sandy soils containing small amounts of organic material and this may account for a slow release of allelochemicals. Dependent upon such interactions, allelopathy may be manifested in a certain area and not at all in a similar one despite the presence of very similar plant populations in both areas. It is likely that further observations in desert areas that show a gradient of aridity will allow exploration of additional allelopathic effects.

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The sparse vegetation in arid environments provides an excellent model for studying germination, progressive growth, and mortality of individual plants. No wonder, then, that pioneering work on allelopathy was performed in various deserts of the world, and some of this work will be described herein. First, however, I raise the conjecture that arid conditions may favor plant species endowed with allelochemical potential, and this more often than currently realized. I believe the allelochemical effect interacts with other environmental factors and is diminished or enhanced in accordance with local changes in the aridity. Because of the spatial and temporal dependence of plant interactions, inconsistencies in the observations made at different localities are inevitable, and for those who struggle to prove allelopathy an element of uncertainty is thus introduced. It is to be hoped that an exacting combination of ecological observations with biochemical procedures will enable the tracing of pathways taken by phytotoxins from the producer plant to the susceptible one, and will eventually allow a quantification of the allelopathic effect. The purpose of the present paper is to review some of the work on allelopathy being undertaken in various desert ecosystems in the world, to point out factors that may modify the effect of aggressive plants or the response of susceptible ones, and to elaborate on the methodology employed to assess allelopathy or autotoxicity.

#### Allelopathy in Deserts of the Western Hemisphere

The best documentation of plant allelopathy derives from the United States. For instance, in the Borego Valley of the Mojave Desert, where the annual rainfall is 200-255 mm, Went (1) noted that only a few annuals were associated with living shrubs of Encelia farinosa (Compositae), whereas the density of annuals in the vicinity of dead Encelia shrubs, as well as near living shrubs other than Encelia, was much higher. Annuals not associated with living Encelia were: Malacothrix californica, Emmenanthe peduliflora, Rafinesquia neomexicana, and Hilaria rigida. It was first supposed that toxicity is induced by the roots of Encelia, but Gray and Bonner (2) were unable to show any inhibition by such roots, whereas either fresh or dried leaves of Encelia, when added to sand-grown cultures of tomato, inhibited growth of the seedlings. Aqueous extract of Encelia leaves (0.025% on dry weight basis) was extremely toxic to corn and pepper, but barley, oats and sunflower were only slightly affected. The phytotoxic principle, colorless needles with a pleasant odor, was crystallized from extracts of plants growing wild in the Colorado Desert, California and identified as 3-acetyl-6-methoxybenzaldehyde (Figure 1) (3). At a concentration of 1.4 mM, this compound killed, within 24 h, 100% of tomato seedlings grown in sand cultures. When test plants were grown in fertile garden soil, the effect was smaller, but Gray and Bonner have pointed out that, in nature, Encelia is common on sandy soils and the inhibitory effect therefore is to be expected. These authors, however, did not assess the response to the phytotoxin of such annuals as were absent from the vicinity of Encelia. Nor did they attempt to isolate the

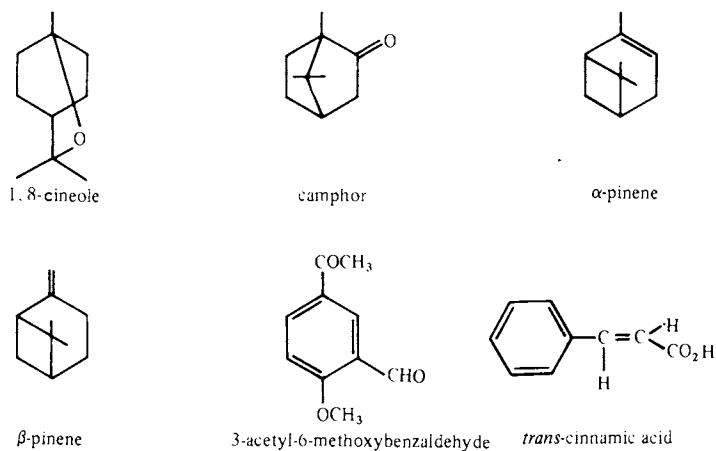


Figure 1. Allelochemicals from Various Desert Plants.

phytotoxin from soils around these shrubs. Interestingly, leaves of Encelia collected in Arizona yielded a different, as yet unidentified toxin (3).

Guayule (Parthenium argentatum, Compositae), a rubber-producing plant, is common in the Chihuahu Desert (Mexico and South Texas), where sparse populations of this shrub grow at an altitude of 700-3500 m above sea level, receiving 250 mm of annual rainfall (4). When this plant was grown under nursery conditions, marginal rows produced larger plants than rows in the center, and roots of one plant did not intermingle with those of neighboring plants. From gravel cultures with Hoagland solution, Bonner and Galston (5) isolated trans-cinnamic acid (Figure 1), which was found autotoxic to guayule seedlings grown in similar gravel culture. The autotoxic effect was still evident even at concentrations of  $6.7 \times 10^{-3}$  mM, and at 1 mM it caused an 80-90% reduction in growth of guayule seedlings. Subsequent attempts to isolate this or other phytotoxic compounds from various soils supporting guayule proved unsuccessful (6,7).

In a series of papers published during the 1960's, C. H. Muller (8-11) reported that in areas around shrubs of Salvia leucophylla, S. apiana, S. millifera (Labiatae), or Artemisia californica (Compositae), grasses and herbs are suppressed. The pertinent observations were made in the Santa Ynez Valley of Santa Barbara County, California, a region with an average annual rainfall of 200-250 mm. Zones entirely devoid of annual plants occurred within 60-90 cm from the canopy of each shrub, whereas further out, to about 6 m, various gradations of inhibition were observed. Volatile materials from the crushed leaves or twigs of the named shrubs inhibited root growth in seedlings of cucumber or oats, as well as of some annuals common in the area. Highest inhibition was exerted by Artemisia californica, while no inhibition was obtained with macerated young or mature roots of Salvia leucophylla or their leachates (10). Several terpenes and terpenoids, e.g.  $\alpha$ -pinene,  $\beta$ -pinene, camphor, and cineole, released from the shrubs canopy were identified (Figure 1). Of these, camphor displayed the highest toxicity. The agent transporting the phytotoxins into the soil was first assumed to be dew (9) but subsequently it was shown that dry, rather than wet, soils absorbed more of the volatile phytotoxins and these proved to be toxic for the seedlings of annuals common in the regions studied (11). This led to the conjecture that volatile compounds accumulate in the soil during the long dry summer and in the winter, when germination commences, to be released by rainfall into the soil microsphere, where they inhibit growth of the annuals (12).

#### Allelopathy in Deserts of the Eastern Hemisphere

In the Negev Desert of Israel, near Sede Boquer, within a region boasting up to 100 mm of rainfall per year (Figure 2), Friedman et al. (13) observed that on south-facing slopes the yield of annuals was 6-8 times that on adjacent north-facing slopes (Figure 3). This was confirmed both by the annual-plants density and by the dry matter yield ( $\text{g/m}^2$ ), and proved true during 4 years, despite the

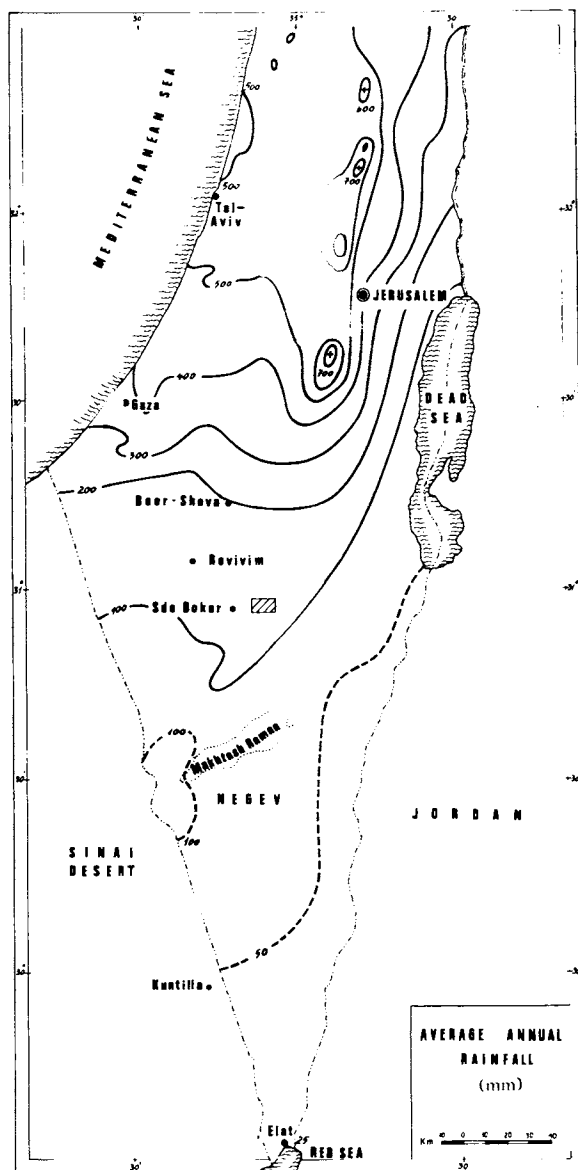


Figure 2. Map showing Location of the Study Area Including Isohyetal Lines.

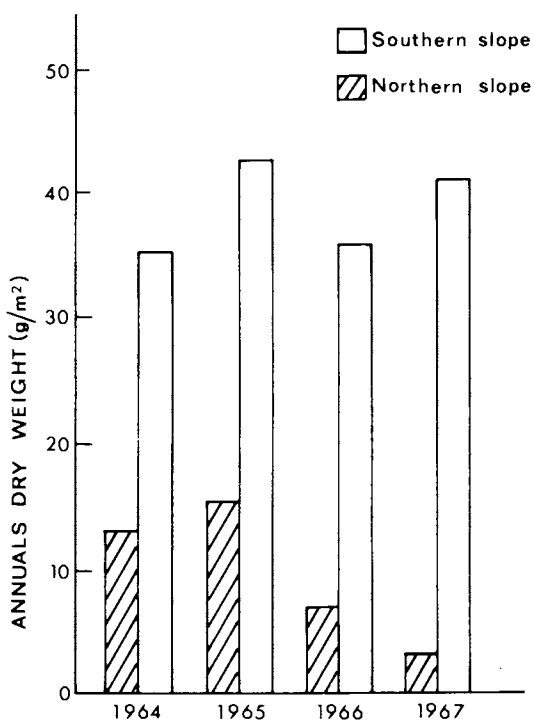


Figure 3. Annual Yield of Annuals on Southern and Northern Slopes in Sede Boquer, in the Years 1964-1967.

fact that the south-facing slopes are much more arid than the north-facing ones (due to high solar radiation, higher salinity, and higher water run-off). It was noted, however, that the north-facing slopes are dominated by the aromatic semi-dwarf shrub Artemisia herba-alba (Compositae) (Figures 4a & b), whereas the south-facing ones are dominated by a nonaromatic shrub (Zygophyllum dumosum, Zygophyllaceae) (Figure 5). One year following removal of perennials from both slopes, the yield of annuals on the northern slope increased significantly (Figures 6a & b), albeit it did not match that on the perennial-free southern slope. Counts made during germination time showed that density of the seedlings of annuals in the vicinity of Artemisia herba-alba was only half that observed 100 cm from the canopy. On the suspicion that volatile allelochemicals were responsible for the decimation of annuals on the north-facing slopes, laboratory tests were undertaken using small plastic beakers (Figure 7). These tests confirmed that Artemisia herba-alba, like A. californica, produces volatile phytotoxins. Thus, one gram of fresh shoots of A. herba-alba in a sealed 50-ml flask arrested the germination of various annual species common in the studied area, whereas no such inhibition was induced by the leaves of Zygophyllum used as a control (13). Of the species examined for germination inhibition, Stipa capensis and Helianthemum ledifolium were strongly inhibited, Zygophyllum dumosum less so, and the two varieties of Medicago laciniata not at all. Major volatile inhibitors turned out to be terpenes and terpenoids, such as  $\alpha$ -pinene, camphor, and cineole (Figure 1). We then postulated that chemical inhibition is mainly responsible for the absence or scarcity of sensitive species in the vicinity of Artemisia and that the yield of annuals on plots free of perennials on the less arid north-facing slopes does not exceed the yield on the southern slopes owing to persistence of residual phytotoxins in the soil. All our attempts to demonstrate soil toxicity by sampling of soils in pots failed, probably because of the volatile nature of the phytotoxins. When shoots of Artemisia herba-alba collected in the desert were placed near seeds of various annual plants, germination was inhibited and such inhibition was highly reproducible. However, when plants of A. herba-alba were transplanted in a more humid region in Tel Aviv, similar inhibitory effects were obtained only when 3-4 times as many shoots were applied. Water stress, high sun radiation, and high temperatures are believed sometimes to favor higher production and release of volatile terpenes and terpenoids. This is a common, ancient belief among mint growers which gained support from the work of Clark and Manary (14). Interestingly, our observations on populations of Artemisia herba-alba in less arid regions in Israel (albeit with no more than 350 mm of annual rainfall) not only failed to show any reduction in the number of nearby annuals, but actually revealed that annuals may aggregate around the Artemisia plants. In general, one would expect the degree of inhibition to depend both on the susceptibility of different plant species as well as on the reactivity of the inhibitors, their concentration and their proximity to the susceptible plant. Populations of A. herba-alba are relatively dense (about 3 plants/m<sup>2</sup>) and the shrubs are extremely aromatic, particularly during the long summer



Figure 4. Artemisia herba-alba , Branch (a) and Inflorescences (b).





Figure 5. Artemisia herba-alba on a North-facing Slope (bottom) and Zygophyllum dumosum on a South-facing Slope (top).



Figure 6. a. A flash of annuals on an Artemisia-free plot (left) compared with those of an undisturbed plot used as a control (right), 8 months after removal of the Artemisia shrubs. (Reproduced with permission from reference 13. Copyright 1977 Blackwell Scientific Publications Ltd.)

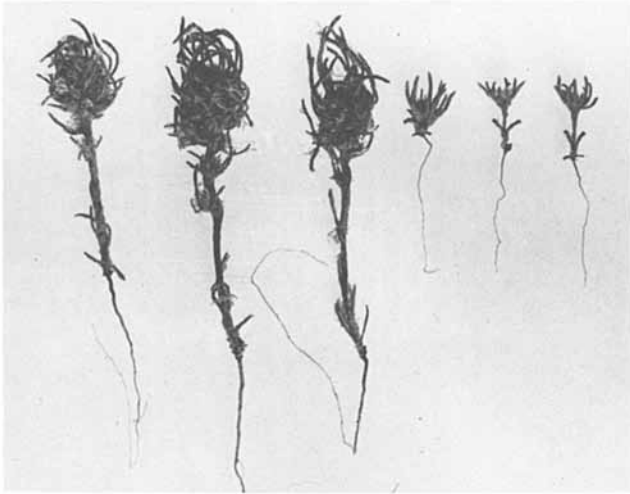


Figure 6. b. Mature Plants of *Ifloga spicata* Sampled from the Artemisia -free Plot (left) and from the Undisturbed Plot (right).

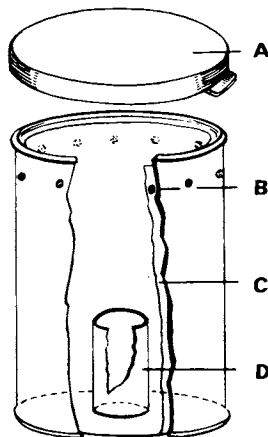


Figure 7. Polythene Beaker Used in Germination Experiments : A, Plastic cover; B, Seeds; C, Filter Paper; D, Open beaker employed in the "volatiles method" (13) (by permission of Blackwell Scientific Publications).

(May-October), when the upper soil layer may adsorb inhibitors to the point of saturation. In such a case, a rainfall of 100 mm per annum, such as occurs on the slopes and part of which (10-30%) disappears quickly as run-off, would be unlikely to deplete the soil of inhibitors by leaching. Variable ecological conditions may trigger allelopathy of A. herba-alba in one habitat and abrogate it in the next one. The wide global distribution of A. herba-alba from North Africa to the Iranian deserts suggests that the allelopathic phenomenon associated with this species may manifest also in geographic regions other than in Israel.

In the region studied, near Sede Boquer, dwarf shrubs of Artemisia herba-alba disperse 85% of their diaspores (achenes) under the canopy and yet most of the germination takes place outside the shrubs' canopy (15). This is true even though the canopy of A. herba-alba provides both shade and litter during the germination period, so that humidity under the shrubs is believed to be higher than outside it. Population regulation by autotoxicity is thus suggested. Our field observations further suggest the occurrence of similar interactions between the parent plant and its seeds and seedlings (probably by the shrubs' litter) in the case of the sand-dune-located non-aromatic Artemisia monosperma.

Tamarix aphylla (Tamaricaceae) is a tree of moderate height (8-11 m). In Israel it is prevalent in the coastal plain and in the Negev desert. It normally grows in xeric areas with 100 mm of annual rainfall (16) and is recognized as a salt-excreting tree (17). Litwak in 1957 (18) studied the influence of this tree on soil salinization. At that time he noticed that in arid localities with less than 200 mm of annual rainfall no plants of any kind grew under the canopies of the large trees, even in rainy years. At the periphery and still under the partial influence of the litter and water dripping from the canopy, some halophytic and ruderal species appeared, e.g. Bassia muricata, Mesembryanthemum nodiflorum, or Chenopodium opulifolium (Figures 8a, b). Compared with soil samples from the open area, those from under the canopy contained twice as many soluble salts (Table I).

TABLE I. Total Soluble Salts under the Canopy of Old Trees of Tamarix aphylla and out in the Open (average values, ppm)

Depth (cm)	Under the canopy (a)	In the open (b)	a/b
0	1201 + 345	480 + 120	2.5
40	1198 + 216	512 + 102	2.3
80	859 + 187	490 + 78	1.7

Source: Reproduced with permission from Ref. 18. Copyright 1957 The Weizmann Science Press of Israel.

Under small trees of T. aphylla (3-5 m in height), annuals were not entirely absent.

To evaluate the effect of the trees on annuals, along a distance gradient from the stem, we determined both the density of

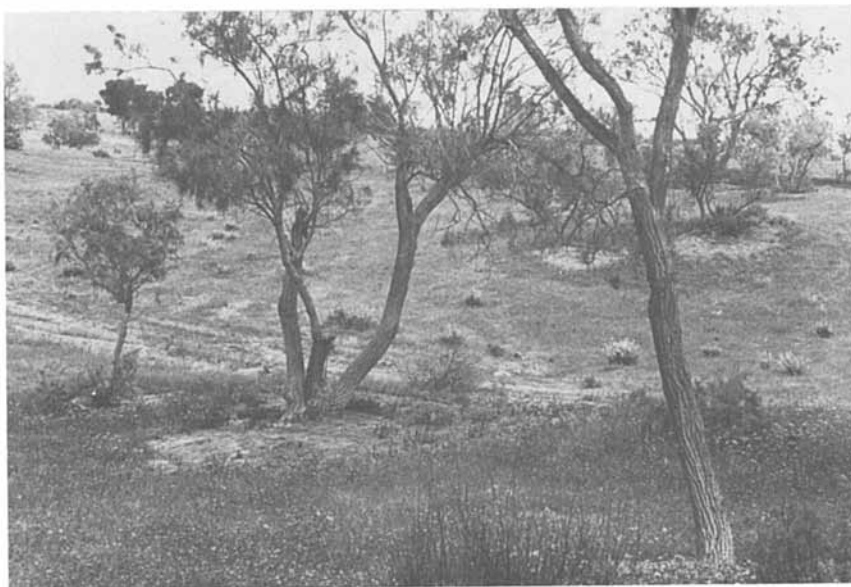


Figure 8. a. Annuals-free Areas around Trees of Tamarix aphylla, 15 km South of Beer Sheva (cf. Fig. 2).

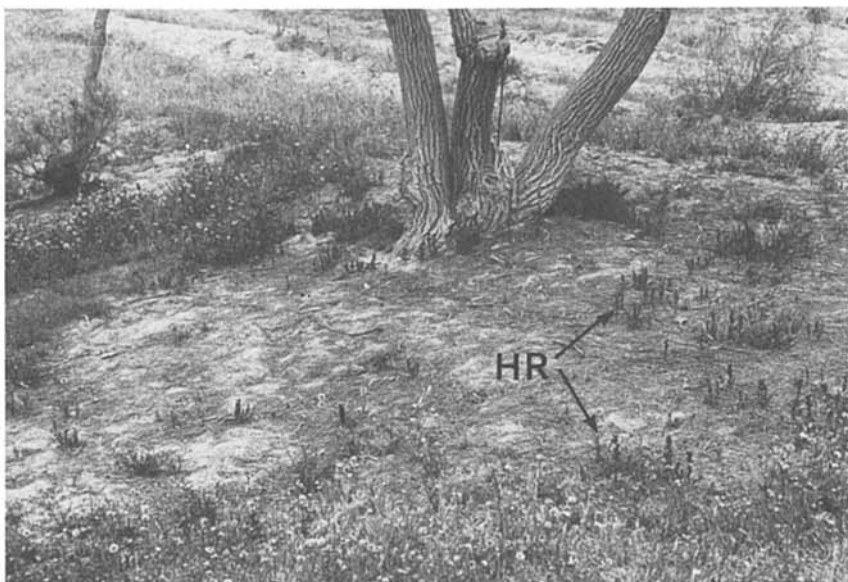


Figure 8. b. Halophytes and Ruderals (HR) in the Periphery of an Annuals-free Area around Tamarix aphylla.

annuals and the chloride ( $\text{Cl}^-$ ) and sodium ( $\text{Na}^+$ ) concentrations in the upper soil layer (0.5 cm), proceeding seriatim from the stem towards the periphery of the tree. The data presented in Figure 9 show that close to the stem the  $\text{Cl}^-$  concentration is highest, and the annuals density lowest, but the situation reverses towards the tree's periphery. A similar pattern was observed also for the  $\text{Na}^+$  concentration in the upper soil layer vis-a-vis the annuals density. Sodium chloride is the major salt excreted by Tamarix aphylla. However, the composition of the excreted salts is to a certain extent influenced by the composition of the salts encountered by the roots (19). The rate of excretion of sodium chloride, when measured in relation to its concentration around the root system, shows an optimum pattern, i.e. highest at a 0.2 M concentration and diminishing when the roots are irrigated with either lower or higher concentrations of sodium chloride (19). The marked allelopathic effect in the more arid regions is probably due to a combination of the following: the ecological conditions, the fact that the soils in these areas are sufficiently saline to enhance the rate of salt excretion by the trees, and the annual rainfall is insufficient to wash the excreted salts into lower salt layers. It seems that this allelopathic effect is a secondary event resulting from the excretion mechanism of tamarisks which removes the salts from the roots, subsequently eliminating them by the salt glands and finally concentrating them in the upper soil layer around the canopies. The contribution of this allelopathic effect to the survival of the species is doubtful. Trees of T. aphylla were found to be susceptible to sodium chloride even when irrigated with as little as 0.1 M NaCl (19). It is not surprising, therefore, that in the annual-free concentric areas under the canopies, we could not find any roots of tamarisk capable of exploiting the non-utilized water. Nevertheless, it is clear that inorganic minerals of plant origin can induce allelopathy. Indeed, in the case of Ceratophyllum demersum elemental sulfur was recently found accountable for the fact that very few epiphytes associate with this water plant (20). It is possible that other trees or shrubs active in the salinization process of soils or affect soil pH, e. g. Sarcobatus vermiculatus (Chenopodiaceae) in Escalante Desert, Utah (21) may under specific ecological conditions induce allelopathy.

#### Discussion and Conclusions

From the accumulated information on allelopathy or autotoxicity in arid regions of both hemispheres, some generalizations may be drawn:

- (a) aggressive plants with allelopathic potential are adult perennials or young seedlings of such perennials;
- (b) the allelochemicals are either common secondary metabolites or inorganic salts. They are often nonspecific, not general phytocides. They reach the susceptible plant through the soil in various ways - by being washed off during rainfall from the fresh or dried leaves and stems, by escaping as volatile substances from the shoots to be adsorbed later by the soil, or by liberation from the mature or decomposed roots to reach the rootlets of the young plant;

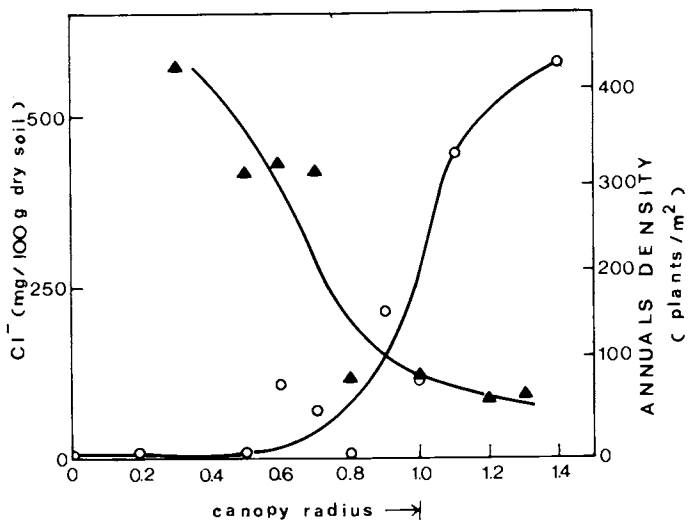


Figure 9. Annuals Density (○) and Chloride Concentration (▲) at Various Distances from Stem to Periphery of Tamarix aphylla (mean values of 5 trees). Distances expressed as fraction of the trees' radii.

- (c) the allelochemical effect is subject to local small-scale changes as well as to large geographical ones. Very few annuals, for instance, are associated with shrubs of Artemisia herba-alba on the hillslopes of the Negev Desert, but this is not so in the runnels or in less arid northern regions, for transplantation of the plants from the dry desert climate to the wetter Mediterranean, one reduces the production of volatile phytotoxins.

It is therefore suggested that arid regions that manifest an aridity gradient are fitted for the exploration of new allelopathic effect. Clearly, allelopathy is more common under desert conditions than in humid environments. This is not because the wide spacings between the plants in the desert allow an easier detection of the allelopathic effect, but because there are ecological conditions that favor allelopathy, such as those affecting the rate of production of allelochemicals or determining the effectiveness of the allelochemicals already in the soil. Among further findings of the present study are the following :

1. Water and nutrient stress may often increase allelochemical production. In a recent review, Gershenzon (22) has pointed out that numerous allelochemicals of various chemical groups are often produced and stored in much higher concentrations by plants under water or nutrient stress than by plants growing under optimal conditions.
2. There is some evidence that grazing can also trigger the production of allelochemicals (23-25) , albeit this was reported for only a few plants and was not studied at all in desert plants. Future investigation may perhaps reveal that the grazing effect importantly aggravates allelopathy in the deserts.
3. Allelochemicals in desert soils are predictably less prone to leaching and rapid biodegradation than are soils in humid environments.
4. Sandy soils, with low amounts of organic matter, are most common in deserts. As such, they may release the adsorbed chemicals more readily than will heavy soils rich in organic matter.

As in other environments, so also in arid regions, allelopathy is associated with plant-plant competition, but here the paucity of resources may lead to considerable mutual interference resulting not only in diminution in size or number of the plants, but also in total extinction of a vulnerable species.

This does not mean that analysis of allelopathy in an arid environment should be done differently from that which is customary in a humid environment; yet it is important to estimate the extent to which inorganic salts (excreted by the plant or released from its litter) are involved in the allelopathic effect. So far as secondary metabolites are concerned, it should be of interest to compare their production under humid and stressed conditions. It is suggested that for the evaluation of the allelochemical effect, species suppressed in their natural habitat should be preferred over any other standard seeds commonly used for evaluating germination inhibitors. Also, efforts to isolate allelochemicals from soils will assist in the establishment of allelopathy on a more concrete basis than is available at present.

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## Chapter 7

# Improving Crop Productivity in India: Role of Allelochemicals

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The possible role of allelochemicals has been explored for pest control, crop rotation, and agroforestry. Various allelochemicals were screened for herbicidal and fungicidal activities against Amaranthus spinosus and Alternaria solani. Caffeine and geraniol completely inhibited germination of the test weed at 6.0 and 0.3 mM, respectively, and similarly inhibited mycelial growth of the test fungus at 10.3 and 2.5 mM. In a tobacco-maize rotation system, nicotine at 5 mM significantly increased the growth of maize. Leucaena leucocephala is widely recommended for agroforestry, so the effect of its most important allelochemical, mimosine, on several crops was studied. It appreciably inhibited growth of rice and wheat at 1 to 5 mM concentrations. These observations establish in principle that allelochemicals can be important in improving pest control, crop rotation, and agroforestry programs and thereby increase crop production.

Both the harmful and the beneficial effects of plant-plant, plant-microorganism, and plant-insect interaction must be considered aspects of allelopathy, as advocated by Rice (1). The role of allelopathy in natural and manipulated ecosystems is poorly recognized. In the past decade we have been concerned mainly with exploring the frontiers of applied allelopathy that may lead to increased crop production. There are two ways of this kind by which crop production may be improved: allelopathy can be exploited by developing new crop management systems and improving existing ones, and purified allelochemicals may be used commercially as agrochemicals.

### Management Systems

Different types of management may be developed for weed, insect, and disease control, for crop rotation, and for agroforestry employing allelopathy. In the following paragraphs these are discussed separately.

In weed management systems involving allelopathy, crop varieties may be screened or new varieties developed for their potential for controlling weeds. Such varieties may be left as residues in the field, or be incorporated in every rotation system, and/or used as a companion crop. Similarly, if crop varieties allelopathic to pathogens can be found, their residues can be used similarly for disease control. Research groups of Putnam at Michigan State University and of Gliessman at the University of California are two of many that are involved in research of this kind.

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Crop rotation is a "cropping system in which two or more crops are grown in a fixed sequence" (2). Any crop may release allelochemicals either directly in the form of exudate or vapors, or its residue/litter can produce them afterward. Irrespective of their source, allelochemicals may affect the next crop in the rotational sequence either positively or negatively. In India, many farmers grow two or three crops/year on the same field, and there are definite recommendations on the sequence of crops. The rotational sequences are based on maintenance of soil fertility, soil structure, and plant nutrients, etc., but they have been formulated with little or no consideration for allelopathy, although it is unlikely that when crops are continuously supplying the soil with allelochemicals, these would not affect the following crop. This thought persuaded us to test our views experimentally, with two locally practiced rotational sequences: tobacco and maize, and tobacco and rice. On a very conservative estimate the leaf litter from a chewing tobacco crop adds about 133 kg/ha of organic matter to the soil (3), and the average nicotine content in leaves of tobacco is approximately 4%. Thus every harvested crop to tobacco adds about 5.32 kg nicotine/ha merely through its leaf litter. Of course, roots also contribute some nicotine. Therefore we studied the effect of nicotine on germination and growth of maize and rice *in vitro*, using the technique of Rizvi et al. (4). Nicotine significantly increased the radical and plumule length of maize, but decreased all the parameters studied in rice; the radical length was most affected at 5 mM concentration (Table I). These results demonstrated the differential effect of nicotine, and suggest that careful consideration of allelopathic interactions of crops of a rotation management can improve its productivity, either by eliminating detrimental interactions or by exploiting beneficial ones.

Agroforestry. Agroforestry may be defined simply as intercropping of woody plants with food or forage crops in order to maintain or increase total yields (5). While agroforestry has a potential to increase yield, it has its own limitations: competition of trees with food crops, damage to food crops during tree harvesting, etc. The literature remains silent on allelopathic interactions between trees recommended for agroforestry and food crops, and no suggestion has been made to consider such effects before recommending food/forage crops for agroforestry programs (6). Hence we worked with mimosine, and allelochemical produced by leguminous trees -- *Leucaena* sp. The concentrations of mimosine in air-dried leaves of such species range from 2.35% to 6.37% (7). Our studies showed that mimosine is toxic for rice and wheat. Seed germination and radicle and plumule length of both rice and wheat were adversely affected by mimosine (Table II). Earlier reports of Smith and Fowden (8) and Kuo et al. (7) on the injurious effect of mimosine on mung bean and rice, respectively, support our results. Therefore, possible allelopathic interactions between trees and food/fiber crops should be studied to improve agroforestry management.

#### Allelochemicals as Sources of Agrochemicals

Now we come to the other potential application of allelochemicals: the development of new, safer and effective agrochemicals as pesticides and growth promoters. However, we restrict the discussion of pesticides to herbicides, fungicides, and broad-spectrum pesticides.

It is obvious that synthetic pesticides are important in controlling weeds and plant-pathogenic fungi, but in judging the efficacy of a method, it is essential to assess its nontarget toxicity, including health and environmental hazards. The literature abounds with reports that synthetic pesticides affect nontarget plants, their consumers and the environment (9). Thus, plant protection needs

Table I. Change Caused by Nicotine in Seed Germination, Radicle Length and Plumule Length over Control in Two Crops Plants

Concentration (mM)	Change (%)		
	Germination	Radicle	Plumule
For <u>Zea mays</u> (var. Diarra composite): increase			
2.5	2.73 ± 0.98	43.56* ± 1.10	25.82* ± 1.38
5.0	2.63 ± 0.88	46.20* ± 0.98	33.11* ± 1.20
For <u>Oryza sativa</u> (var. Pusa 221): decrease			
2.5	4.72 ± 0.21	14.99* ± 0.98	3.10* ± 0.96
5.0	11.02 ± 0.82	57.64* ± 1.08	26.54* ± 1.01

\* Significant at 5% level.

Table II. Reduction of Seed Germination, Radicle Length and Plumule Length over Control in Two Crop Plants by Mimosine

Concentration (mM)	% Reduction		
	Germination	Radicle	Plumule
<u>Oryza sativa</u> (var. Saket)			
1.0	2.44 ± 0.32	5.48 ± 0.18	4.21 ± 0.68
2.0	9.76 ± 0.91	11.39 ± 0.25	15.72 ± 1.01
3.0	13.42* ± 1.01	19.40* ± 0.86	32.01* ± 1.20
4.0	21.96* ± 1.32	84.38* ± 1.20	83.15* ± 1.82
5.0	25.62* ± 1.42	100.00* ± 0.63	85.08* ± 1.82
<u>Triticum vulgare</u> (var. Sonalika)			
1.0	12.72 ± 0.36	2.27 ± 0.12	11.33 ± 1.10
2.0	14.84 ± 0.96	30.60* ± 0.98	20.20* ± 1.02
3.0	15.90* ± 0.93	57.12* ± 1.11	43.84* ± 0.98
4.0	19.88* ± 1.01	65.51* ± 1.01	70.44* ± 0.68
5.0	23.32* ± 1.32	80.93* ± 1.20	63.05* ± 1.20

\* Significant at 5% level.

improvement. It is important to note that many allelochemicals are known to inhibit the germination and growth of weeds and fungi (1), which is an essential feature of all herbicides and fungicides, respectively. Therefore, allelochemicals can be an alternative to synthetic pesticides, and, being natural products, are safer to use and easily biodegradable (9-11).

**Herbicides.** The idea of using allelopathy in weed control was conceived in the late seventies (12), and several workers have considered this possibility, as already mentioned (13-16). However, characterization and possible use of allelochemicals as selective herbicides received attention only recently, and our demonstrations of the selective herbicidal activity of caffeine are among the pioneer ones (17-21).

To explore the potential of allelochemicals as herbicides, we tested some monoterpenes and terpenoids for suppression of germination and growth of the test weed *Amarathus spinosus*, using the bioassay of Rizvi et al. (4). Among the monoterpenes tested, geraniol was most potent. It inhibited radicle growth by 100% and seed germination by 90% at a concentration of 2 mM, and completely inhibited plumule growth at 3 mM concentration (Table III). The phytotoxicity of geraniol indicates that it and other allelochemicals may serve as alternatives to synthetic herbicides.

**Fungicides.** Since most commercial fungicides are synthetic products, almost all demerits of synthetic pesticides in general are associated with them. To look for an alternative to such synthetics, some allelochemicals were tested in vitro by a technique of ours (17). Surprisingly, geraniol again proved the best. It inhibited mycelial growth of *Alternaria solani*, the test fungus, by 92% at a concentration of 2 mM, and proved fungicidal above 2 mM (Table IV). Thus geraniol in particular and allelochemicals in general may be of use as fungicides.

**Multipurpose pesticides.** Occurrence of more than one pest-controlling property in a single allelochemical has persuaded us to propose using allelochemicals as multipurpose pesticides (18). Such use would be beneficial in several ways:

(i) Reduction in cost of pesticides. Use of a single compound for the control of several pests may reduce the total expenditure on crop protection.

(ii) Reduction in cost of production and research. The cost of production of single multipurpose compound would probably be less than the total cost of product of several pesticides.

(iii) Improved quality of life. Being plant products, most allelochemicals should affect fewer nontarget organisms, and improve quality of farm produce.

Further, multipurpose pesticides can be very important in developing integrated pest management systems (IPMS). Any IPMS is designed to minimize losses of crop yield and quality due to pests, through integration of various approaches to pest control so as to get maximum benefits with minimum disadvantages. In spite of their hazardous nature, synthetic pesticides are often among the dominating components of an IPMS. Moreover, an IPMS often requires simultaneous use of several synthetic pesticides, which, harmless singly, may become poisonous through interaction among themselves or their metabolites (22,23). Therefore, the number of chemicals used in any IPMS should be minimized. A viable approach to this may be to find multipurpose pesticides. A multipurpose allelochemical pesticide for IPMS would not only reduce the chances of synergistic toxicity but also, being plant-derived, have all the merits of natural products.

Our studies with geraniol indicate its ability to control more than one agriculturally important pest, a weed and a pathogenic fungus. Moreover, up to

Table III. *Amaranthus spinosus*: Reduction of Seed Germination, Radicle Length, and Plumule Length over Control by Geraniol

Concentration (mM)	% Reduction		
	Germination	Radicle	Plumule
1.0	2.86 ± 0.64	9.16 ± 0.98	9.09 ± 1.6
2.0	90.90* ± 1.60	100.00* ± 1.10	35.00* ± 1.2
3.0	91.60* ± 0.98	100.00* ± 0.52	100.00* ± 1.4

\* Significant at 5% level.

Table IV. *Alternaria solani*: Inhibition of Mycelial Growth over Control by Geraniol

Concentration (mM)	Inhibition of Mycelial Growth %
1.0	53.20* ± 1.10
2.0	92.80* ± 1.68
3.0	100.00* ± 0.09

\* Significant at 5% level.

2.5 mM geraniol exerted no visible adverse effect on a test crop, tomato (*Lycopersicon esculentum*), in which both the test pests are problems. Thus, the possibility of exploiting allelochemicals to develop multipurpose pesticides has promise.

Growth promoters. The beneficial effects of allelochemicals have been recognized only recently (1). Our studies with nicotine (Table I) suggest that nicotine and other allelochemicals with such potential may be used as growth promoters.

### Conclusions

Any improvement or new development in crop management based on allelopathic studies could not only increase production, but reduce expenditures on farm labor and agrochemicals, and reduction in use of synthetic agrochemicals would lead to an improved quality of life. If allelochemicals can be developed as botanical pesticides, they would be better than synthetic ones owing to their smaller nontarget toxicity, easy biodegradability, and nonpollutive nature. It is even possible that allelochemical production can be induced by genetic manipulation into cultivars, to provide an inexpensive, safe and permanent means of biological pest control.

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## Chapter 8

# Variation of Root and Microflora Rhizosphere Exudates in Genotypes of Barley

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The potential of crop plants in controlling their rhizosphere is important in agricultural systems. Variation between cultivars in rhizosphere exudates can be observed, provided that the right analytical techniques are used. Rhizospheres from two barley cultivars with different adaptations to acidic soils were investigated. Plants of the two species were cultivated under identical conditions in soil with a high peat concentration. Volatile components in the soil were sampled after three weeks by sucking air from the pots through adsorption tubes. All samples were taken at the same time in a greenhouse and then analyzed by capillary gas chromatography. The large amount of data from the GC analyses was then transferred to a computer for calibration and multiple component analyses (SIMCA). The results show differences in the occurrence of volatiles in the rhizosphere between the two cultivars. This is discussed in relation to possibilities of the genetic capacity of plants to control their own environment.

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It is of great interest to know more about the capacity of crop plants in controlling their root environment in order to increase crop production and adapt plants to new agricultural systems. Besides, crop improvement by plant breeding needs genetic variation of the character of interest and one such character is the potential of the plant to control the soil microflora, especially the rhizosphere flora. One possibility to record differences between plant genotypes in their root environment and microflora is to analyze the pattern of chemical compounds present in the root zone. The advantage of such an analysis is the possibility to carry out the measurements in an intact root-soil system with unstressed plant material. If volatile compounds are chosen for investigation, sampling can even be made in a undisturbed root zone.

The aim of the present study was to find whether differences could be detected in the compositions of mixture of volatile compounds sampled from the root zone of two different cultivars of barley. To have a reasonable base for a relevant genetic variation in the plant material, two cultivars with different adaptation to acid soils were selected. The sampling was done from young plants, as the establishment of the rhizosphere microflora is of importance in early stages of plant development.

#### Plant Material and Soil System

Seeds of two barley (*Hordeum vulgare* L.) cultivars, Tellus (not tolerant to acid soils) and Etu (tolerant to acid soils), were sown in standard plastic (PVC) pots (height 15 cm; upper diameter 14 cm) containing 800 g of a soil mixture. Before use the pots were purified by heating in boiling water for 10 minutes to avoid leaking of compounds from the plastic material.

The soil had a high concentration of peat and was mixed with Perlite (aluminum-silicon material) and Leca (burned clay) particles to make the mixture more porous. The soil had a pH of about 6, contained 45 mg/l of nitrate nitrogen, 119 mg/l of phosphorus and 284 mg/l of potassium, and was mixed carefully before transfer to the pots.

The plants were thinned to four per pot and no additional fertilization was given. Each pot was watered with the same volume of tap water. The plants were grown in a greenhouse in natural light supplemented with light from metal halide lamps (HQI-E 400W/DW) to a daylength of 18 h. The temperature was 20-25°C in the day and 5-12 °C in the night.

The experiments were carried out 21 days after sowing. Two days before the experiments started the plants were transferred to a small greenhouse for sampling. They were cultivated in four parallel pots for each cultivar and four pots without plants were included as a control and were treated in the same way as the other pots.

### Sampling Method

At the Department for Chemical Ecology we have developed adsorption techniques for sampling and analyzing of volatile components in air. These gas chromatographic (GC) methods have been used in allelochemical research, i.a. for analyses of volatiles emitted from plant leaves (1,2). We believed that such an adsorption method could be adapted for sampling volatiles in the soil by allowing for the high humidity in the soil and its effect on the adsorbent.

Because of its low affinity for water we have used TENAX TA, poly-(2,6-diphenyl-p-phenylene oxide). This very porous polymer has a high thermostability and may be used with a thermal desorption technique. It is also possible to dry it to avoid plugging the GC column, with only minimum losses of other collected components.

The sampling tube is made of heat-resistant glass and one end is drawn out to form a capillary injection needle (Figure 1). The other end of the tube is a glass cone (Quickfit 7/16) which allows a clean connection, without any gasket, to the vacuum pump or gas supply. The adsorbent, 0.3 g of TENAX TA, 35-60 mesh, is kept between plugs of glass wool.

Adapters (Figure 1) were designed to plug into the drain holes at the bottom of the cultivation pot. The adapters were made of acetal plastic and had caps with silicone septa of the same type as used in gas chromatography. The needle of the sampling tube was inserted through each septum and the other end connected to a flow regulator and vacuum pump.

To make sure of identical background and other conditions all sampling were done at the same time in the greenhouse. Twelve pots, four with plants of the barley cultivar Etu, four with barley cultivar Tellus, and four with soil only, were placed on a table with holes for the pots. The bottom of the pot was then easily accessible for all the necessary connections. Two samples from each pot and in all twenty-four samples in each set were obtained. After sampling, all tubes were stored in screw-capped glass tubes under inert conditions (low temperature and helium atmosphere) until gas chromatographic analysis.

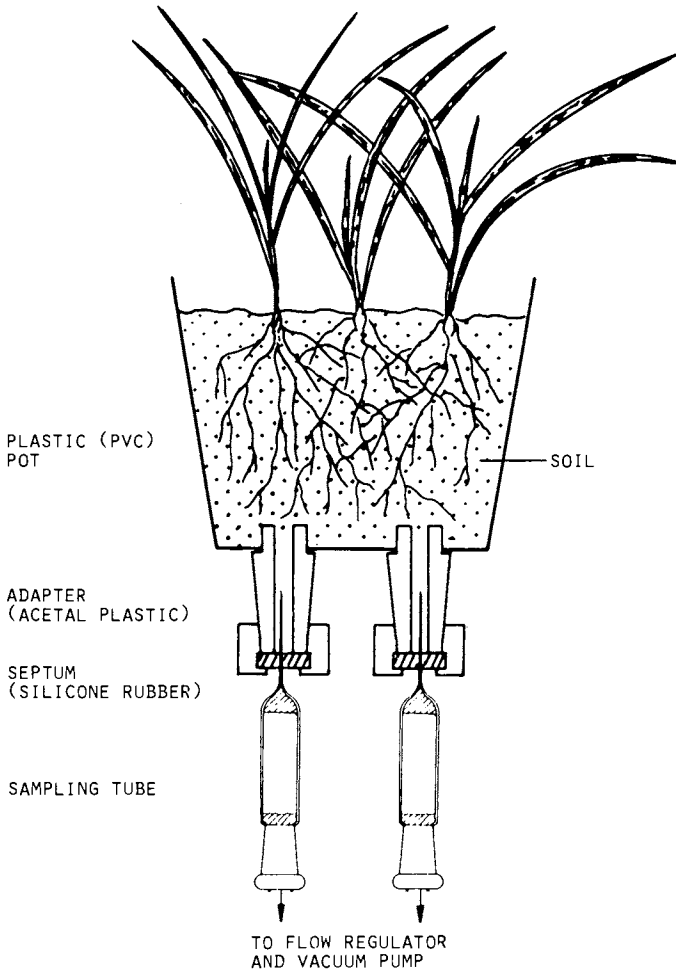


Figure 1. Assembly for Sampling of Volatiles

### Sample Injection System

Gas chromatographic analysis of the adsorption samples were made on a capillary gas chromatograph (Carlo Erba 2900) equipped with a modified injection system constructed at our department (3).

It consists of three main parts (Figure 2):

1. desorption oven
2. ordinary split/splitless injector (Grob type)
3. heatable cold trap.

The sampling tube is put into the desorption oven and the gas supply is connected. If the sampling tube is very wet, drying is performed by flushing dry helium gas through the tube at low (ambient) temperature for ten min. The tube is then heated to 250°C and the desorbed substances are carried off and condensed in the cold trap cooled with liquid nitrogen. The sample is trapped in the first part of the capillary column which is located inside the glass-lined steel tube (GLT tube). When the desorption step is finished (it takes about ten min), the trap is heated rapidly to 250°C in 30 s by applying an electric current (10 A) through the GLT tube. This is made automatic by the use of a temperature regulator.

The starting time of heating of the cold trap is also the starting time of the chromatographic run. The integrator (Hewlett Packard 3385) is automatically started and all retention times are referred to this time. The integrator is also used for time programming of various events.

With this injection technique a very rapid and distinctive start of the separation is obtained which is particularly important for the most volatile components. The most significant property of the inlet system is its ability to yield reproducible retention time values.

### Data Pre-Processing

Figure 3 shows a gas chromatogram obtained from soil with plants of one barley cultivar, Etu. It is possible to count over 400 peaks, and the figure illustrates the very complex pattern of volatiles in the rhizosphere. If we compare chromatograms obtained from samples of Etu, Tellus and soil only, no specific difference can be seen. Most of the peaks seem to be present in all three chromatograms, although the intensities of the peaks vary. The necessity of computer help with the pattern recognition is obvious.

Before we can use any computerized method for pattern recognition the considerable amount of data from the GC analysis must be preprocessed.

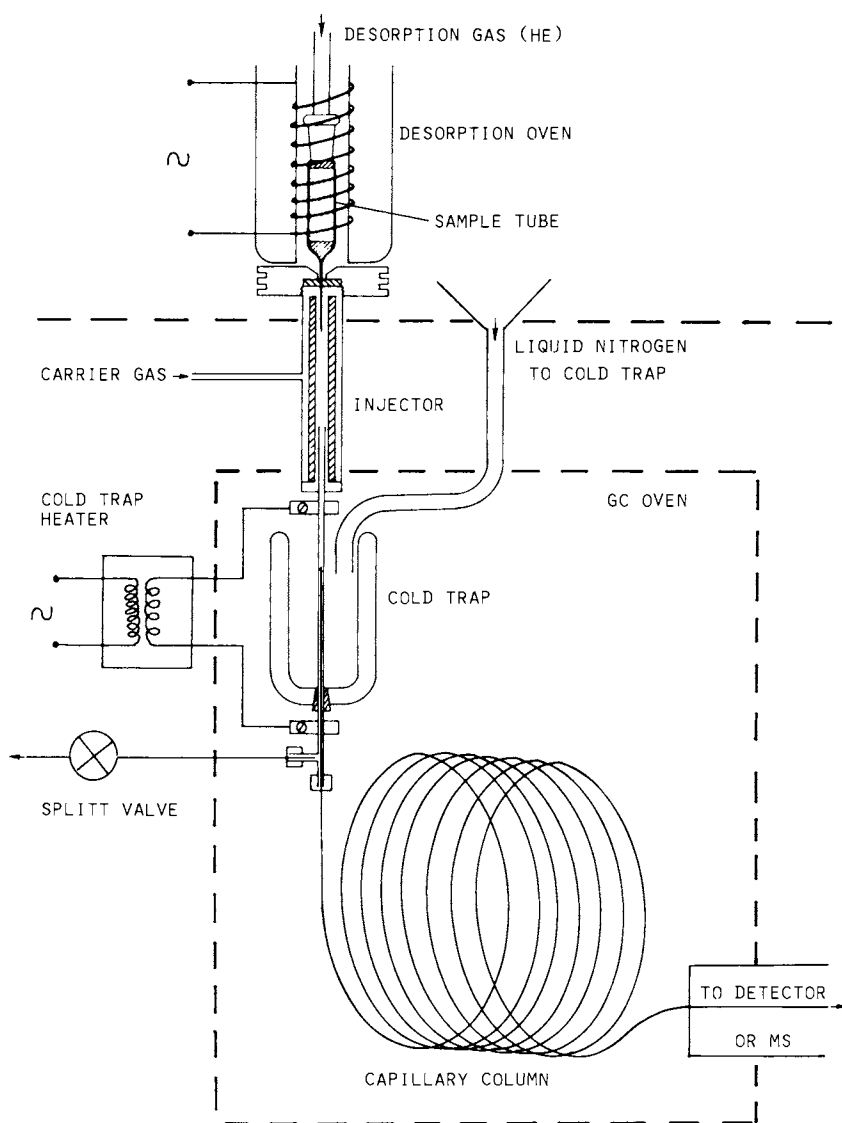


Figure 2. Gas Chromatographic Inlet System

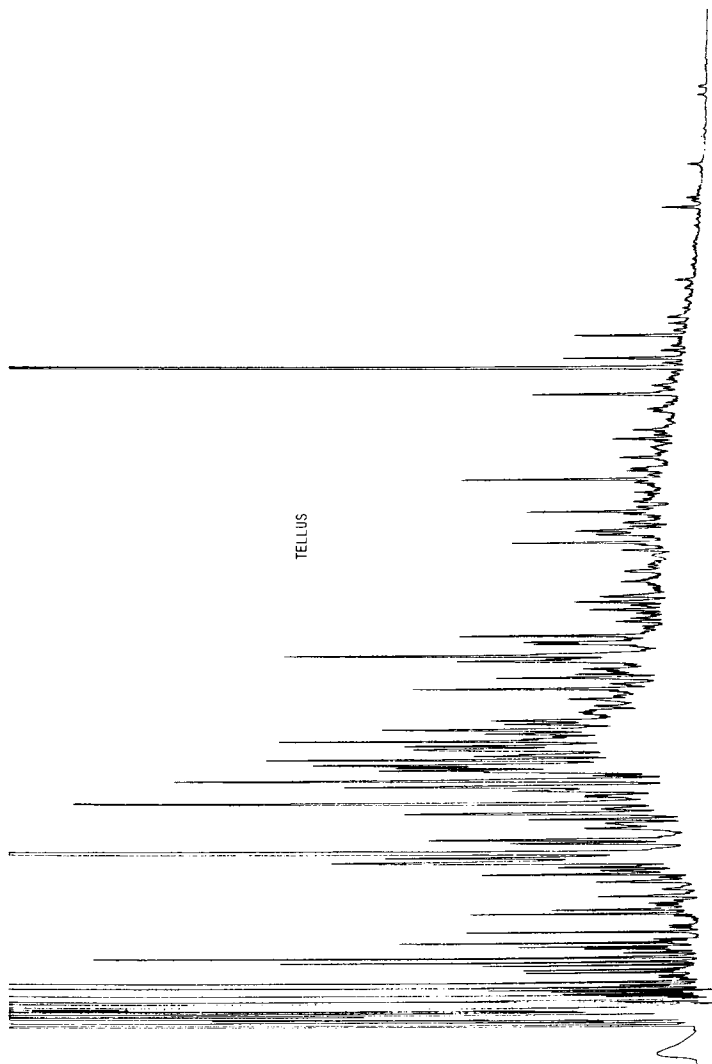


Figure 3. Capillary Gas Chromatogram of Volatiles from Soil. Chromatographic data: adsorption: 4 h on Tenax TA; desorption: 8 min at 200°C; column: fused silica 0.3 mm, 20 m, SE33; temperature programming: 40 to 230°C at 5°C/min; carrier gas: helium (50 cm/s).

The reports from the integrator consist of retention time and sample amount for each integrated peak. These are transmitted to a small computer. The integrator has the capacity to process up to 250 peaks in a run. However, because of the limited memory space of the computer, we had to decrease the number of peaks processed. Chromatographic runs with more than 150 peaks were reduced to 150 peaks by elimination of those with the smallest area. The reduced reports were then stored on tape.

Retention time calibration. In spite of all effort to obtain reproducible retention time values these varied for the same component between different chromatographic runs, mainly because of different sample amounts. To solve this problem all retention time values were calibrated. Values for a limited number of peaks, that could easily be found in all the chromatograms in a set, were manually entered into the computer. For each of these reference peaks the mean value  $M(j)$  over all the runs was calculated. New retention times,  $Rtcal(i)$ , for the peaks in the data set were then calculated by the straight-line expression:

$$Rtcal(i) = (Rt(i)-A(i))/B(i)$$

The coefficients  $A(i)$  and  $B(i)$  are based on the mean values  $M(j)$  of the nearest reference peaks on both sides of the peak to be calibrated.

Reference vector. After calibration a reference vector is created. This vector is used to match all the same fractions together in a data matrix and is created by ordering of all existing calibrated retention time values. The number of the values is reduced by replacing each value that falls into a small retention time "window" with its mean value. The "window" was set wide enough to reduce the number of values to 250.

Matching to form a data matrix. Each value of the reference vector is compared with the retention time values for the fractions in every chromatographic run. Only a small variation in the values is tolerated; thus no doublet matching should occur.

The result of the pre-processing is a data matrix with information about the amount of each fraction, for all runs, on a line. This matrix is the base for the multivariate data analysis (Figure 4).

		OBJECT											
		1	2	3	.	.	.	.	.	.	.	N	
VARIABLE	1	Y11	Y12	Y13	.	.	.	.	Y1K	.	.	.	Y1N
	2	Y21	Y22	Y23	.	.	.	.	Y2K	.	.	.	Y2N
	3	Y31	Y32	Y32	.	.	.	.	Y3K	.	.	.	Y3N
	4	Y41	Y42	Y43	.	.	.	.	Y4K	.	.	.	Y4N
	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.
	M	YM1	YM2	YM3	.	.	.	.	YMK	.	.	.	YMN
	CLASS ETU				CLASS TELLUS					NON CLASSIFIED SOIL			
TRAINING SET								TEST SET					

Figure 4. Available Data in the Pattern Recognition Problem Form a Matrix of Dimensions M Times N.



### Pattern Recognition

In the nineteen-seventies, new methods for pattern recognition have been developed by means of quantitative analogy models. The analogy analysis implies looking for regularities in the observations made. The individual items that are analyzed are called objects. The objects are alike that will be brought into the same class. In our case the number of classes would be two, the two barley cultivars (Etu, Tellus). The samples obtained from soil are used as test objects.

One question we want an answer to is: is there any difference between the gas chromatographic separation pattern of the three objects?

A method successfully used for chromatographic data and capable to answer this and related questions is the SIMCA method (Statistical Isolinear Multiple Component Analysis). It has been constructed and developed by Svante Wold and his group at the University of Umeå, Sweden.

The SIMCA method and the principal components (PC) analysis, a common method for obtaining a view of multivariate data, have been described in detail elsewhere (4,5); thus only a short presentation will be given here.

Principal vector plot (PC plot). The principle of the PC plot is shown in Figure 5. The plane that best approximates the data set (in the sense of least squares) is calculated. The coordinates in the plane of the projection of each point are calculated and the point plotted in the diagram. Already in this simple plot we see that the three classes are separated from each other and that objects 1-3 (soil) lie far from the others.

SIMCA (each class described by a PC model). The basic idea of the SIMCA method is that multivariate data measured on a group of similar objects, a proper class are well approximated by a simple PC model.

The dimensionality of the model,  $a$ , is estimated so as to give the model as good predictive properties as possible. Geometrically, this corresponds to the fitting of an  $a$ -dimensional hyperplane to the object points in the measurement space. The fitting is made using the least squares criterion, i.e. the sum of squared residuals is minimized for the class data set.

The class belonging can be determined when the distance of an object to the class model is compared with the typical distance of the class objects to the same model.

In Figure 6 models are fitted separately to each class and the distances for each object to the two classes are plotted. The two classes are well separated and the objects corresponding to the soil samples are located close to the dashed line, indicating equal class distance.

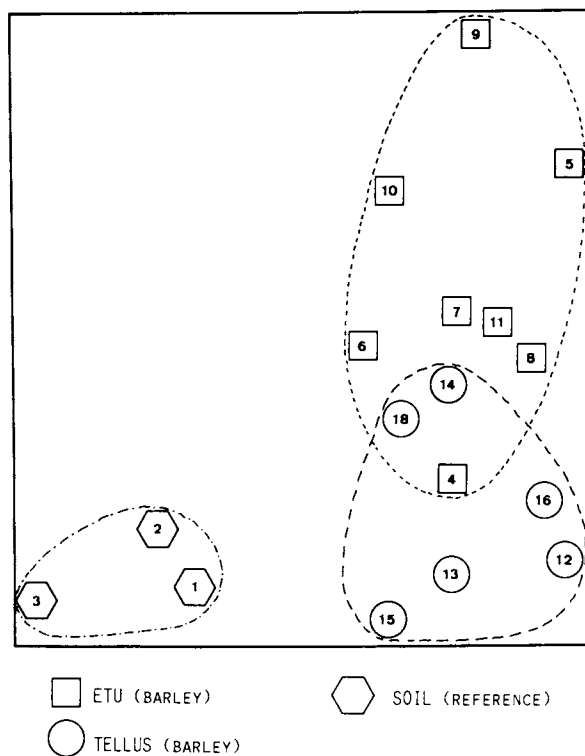


Figure 5. Eigenvector Projection (Principal Vector Plot). A plane is least squares fitted to all the data. This plane constitutes a two-dimensional window into the multi-dimensional measurement space. The projections of the object points down to the plane are visualized in this plot.

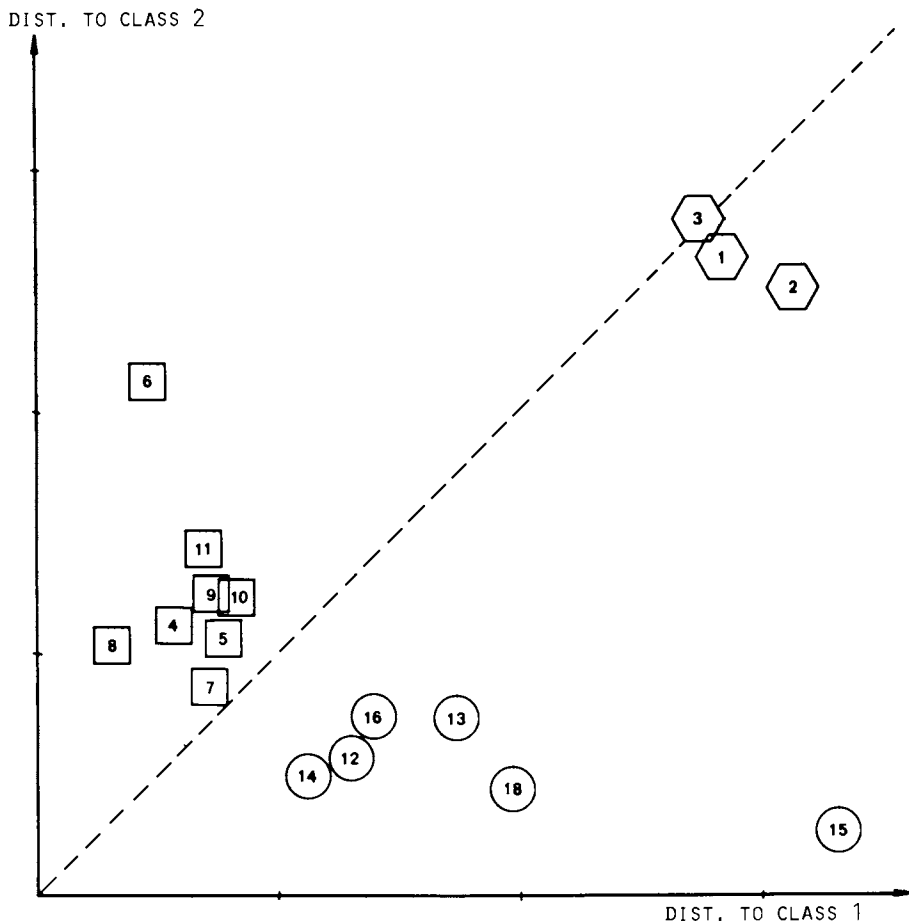


Figure 6. Class Distance Plot (Coomans Plot). Models are fitted separately to each class (Etu resp. Tellus). The distances for each object to the two classes are plotted. The dashed line indicates equal class distance. The soil samples (object 1-3) are located close to this line.

### Discussion

The results show a significant difference in composition of the volatiles from the root zones of the two barley cultivars. The volatile samples include several hundreds of different compounds and the distinction between the cultivars was possible only by using the technique presented.

The present analyses did not reveal which compounds varied, or if the variation was predominantly qualitative or quantitative. However, further SIMCA analysis of the present data can give information of this kind.

The origin of the volatile compounds that differ between the two barley cultivars is not known. Three sources for the differing compounds are possible. The first one is the original root exudate (6,7). In this case the results reflects cultivar variation in plant metabolism and thus the potential of the plant in controlling its environment.

A second source might be the products of the soil microflora, which then directly indicate a variation in the composition of the microflora in the root zone for the two cultivars. A third possible source for the observed variation is degradation of the components in the whole soil system. In this case different levels or kinds of microbiological activity are recorded. Probably we have a combination of the three suggested sources.

In conclusion, we have found that even young unstressed plants of different cultivars show a variation in the chemical composition of their undisturbed root zones. We suggest that this variation shows a genetic potential of the plant in its control of the root zone environment and especially of its rhizosphere microflora. We suspect that this genetic potential will be of importance in selection and adaptation of agricultural crop plants in the future.

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## Chapter 9

# Allelopathy in Mexico

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Studies of allelopathy in Mexico were initiated in 1970 within the project entitled Recovery of Tropical Rain Forests. This paper summarizes the main results obtained from: research about the allelopathic potential of some tropical secondary plants in Veracruz; studies in coffee plantations (herbs, coffee shrubs, and shade trees); the discovery of the allelopathic potential of water hyacinth, and the use of this plant as fertilizer in the "chinampas"; the relationships of crops and weeds in "chinampas" and the allelopathic properties of corn pollen. Likewise studies on *Helietta parvifolia* and *Piqueria trinervia* are mentioned and finally research that is in progress in temperate and tropical agroecosystems in order to permit more efficient agricultural and forest management of the agroecosystems in Mexico is mentioned, mainly the biological control of weeds and pests, the use of green manures and composts, and the management of water and biological diversity.

The studies on allelopathy in Mexico were initiated in 1970 within the project entitled Recovery of Tropical Rain Forests, as a suggestion of its director, Dr. Arturo Gómez-Pompa, at the Institute of Biology of the National Autonomous University of Mexico.

### Studies of Secondary Vegetation

The main objective of this project was to study some of the ecological processes that occur during secondary succession in warm and humid tropics. This process is triggered after a perturbation in the tropical rain forest or the abandonment of crop land (1,2).

The quantity and quality of leached plant metabolites, in warm and humid regions, suggest that there exists a great variety of complex interactions among plants and microorganisms.

In 1970, studies on allelopathy in the tropical zones were scarce, particularly in Mexico. The contributions of McPherson (3), Frei and Dodson (4), Quarterman (5), Webb, Tracey and Haydock (6), Marinero (7), and Gliessman (8) are some important antecedents for the study

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that was carried out concerning the allelopathic potential of the secondary vegetation in Veracruz, Mexico (9) at the biological station of the National Autonomous University of Mexico located at Los Tuxtlas. In this place we selected the most abundant species in order to detect their allelopathic potential. The species were:

Piper auritum, Piper hispidum, Croton pyramidalis, Siparuna nicaraguensis, and Cecropia obtusifolia.

The aqueous extracts of roots and leaves, the leachates of aerial parts, aqueous extracts of soils and in some cases organic extracts of leaves, the essential oils, and isolated pure compounds were tested for their effects upon germination and growth of several test species from the same area. Likewise, bioassays of simultaneous germination were carried out with some of the available seeds.

The test seeds used for the bioassays were: Mimosa pudica, Achyranthes aspera, Bidens pilosa, and Crusea calocephala (herbaceous species); Ochroma lagopus and Helicarpus donell-smithii (arboreal species).

First the tolerance of these species to osmotic pressure was determined in order to avoid interference of this factor with the results (10). Then we tested the following aqueous extracts of leaves, made with 1 and 4 g of dried leaves at 30°C, and 100 mL of distilled water in a blender. Root extracts were made with 15 g of plant in 100 mL of distilled water.

The aerial parts were leached by soaking 100 g of fresh plants in 100 mL of distilled water. Soil extracts were prepared in a 2:1 proportion. The organic extracts of leaves were obtained with the following solvents: hexane, ethyl acetate, chloroform, benzene, acetone, and methanol. The essential oils were obtained by steam distillation and the pure substances with several extraction techniques (11, 12, 13, 14).

All materials were tested upon seeds in Petri dishes with agar (1%) or filter paper as substrate, at 27°C and a 12-h photoperiod.

Lengths of roots and stems were measured and the percent of germination was calculated. All results were statistically analyzed with an F test.

The bioassays showed the wide allelopathic potential of these plants as well as the phytotoxicity of some soils extracts and essential oils (Tables I, II, and III).

All species studied inhibited the growth of certain test species. This confirms the selectivity of the allelopathic compounds (15).

The species with the higher allelopathic potentials were: Piper auritum, Piper hispidum, Croton pyramidalis, and Siparuna nicaraguensis. The essential oils of the Piperaceae were highly inhibitory while that of Croton pyramidalis was less inhibitory and even produced stimulations (Table III).

A very interesting result was the isolation of safrole from the essential oil of Piper auritum. This compound is abundant in the Monimiaceae and Lauraceae families. It was found to constitute 60 to 70% of the essential oil of P.auritum (16).

The benzenic extract from leaves of Croton pyramidalis was highly inhibitory. From this extract we isolated a flavone and a diterpene (Figure 1) but there are other compounds not yet identified in this extract that are much more toxic (11).

The tests of simultaneous germination with seeds of Siparuna nica-

Table I. Effects of the Extracts of Leaves and Roots of *Piper auritum*, *P. hispidum*, *Croton pyramidalis*, *Cecropia obtusifolia*, and *Siparuna nicaraguensis* on Radicle Growth of Some Secondary Species

Treatments	Inhibition/Stimulation <sup>1</sup> (%)									
	<i>Piper auritum</i>		<i>Piper hispidum</i>		<i>Croton pyramidalis</i>		<i>Cecropia obtusifolia</i>		<i>Siparuna nicaraguensis</i>	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
<i>Mimosa pudica</i>	59 <sup>2</sup>	27 <sup>2</sup>	56 <sup>2</sup>	17 <sup>3</sup>	67 <sup>2</sup>	21 <sup>2</sup>	37 <sup>2</sup>	(13) <sup>3</sup>	54 <sup>2</sup>	8
<i>A. aspera</i>	42 <sup>2</sup>	(44) <sup>2</sup>	79 <sup>2</sup>	(67) <sup>2</sup>	49 <sup>2</sup>	(22) <sup>2</sup>	51 <sup>2</sup>	(29) <sup>2</sup>	52 <sup>2</sup>	(7)
<i>B. pilosa</i>	68 <sup>2</sup>	28 <sup>2</sup>	76 <sup>2</sup>	(11)	56 <sup>2</sup>	15 <sup>3</sup>	(3)	0	64 <sup>2</sup>	11
<i>Crusea calcephala</i>	35 <sup>2</sup>	(75) <sup>2</sup>	100 <sup>2</sup>	(42) <sup>2</sup>	25 <sup>2</sup>	(49) <sup>2</sup>	(11)	5	4	(9)
<i>H. donnell-smithii</i>	47 <sup>2</sup>	-	86 <sup>2</sup>	-	66 <sup>2</sup>	-	(8)	-	51 <sup>2</sup>	-
<i>O. lagopus</i>	11	21 <sup>2</sup>	24 <sup>2</sup>	18 <sup>3</sup>	56 <sup>2</sup>	31 <sup>2</sup>	41 <sup>2</sup>	0	(3)	8

<sup>1</sup> mean of five repetitions.

<sup>2</sup> significant to the 1% level.

<sup>3</sup> significant to the 5% level.

Numbers in parenthesis are stimulations.

Table II. Effects of the Aqueous Extracts of Soils Associated with Piper auritum, P. hispidum, Croton pyramidalis, Cecropia obtusifolia, and Siparuna nicaraguensis on the Radicle Growth of Some Secondary Species

Treatments	Inhibition/Stimulation <sup>1</sup> (%)				
	<u>Piper auritum</u>	<u>Piper hispidum</u>	<u>Croton pyramidalis</u>	<u>Cecropia obtusifolia</u>	<u>Siparuna nicaraguensis</u>
Species:					
<u>Mimosa pudica</u>	12.4 <sup>3</sup>	10.7	11	0	(7)
<u>A. aspera</u>	(6)	24 <sup>2</sup>	(16) <sup>3</sup>	(4)	10
<u>B. pilosa</u>	9.4	21 <sup>2</sup>	18 <sup>3</sup>	15 <sup>3</sup>	11
<u>Crusea calocephala</u>	19.2 <sup>3</sup>	25 <sup>2</sup>	(3)	10	(20) <sup>2</sup>
<u>H. donnell-smithii</u>	20 <sup>2</sup>	24 <sup>2</sup>	34 <sup>2</sup>	14 <sup>3</sup>	25 <sup>2</sup>
<u>O. lagopus</u>	2	10	6	(77) <sup>2</sup>	(87) <sup>2</sup>

<sup>1</sup> Mean of five repetitions.

<sup>2</sup> significant to the 1% level.

<sup>3</sup> significant to the 5% level.

Numbers in parenthesis are stimulations.



Table III. Effects of the Essential Oils of *Piper auritum*, *Piper hispidum*, and *Croton pyramidalis* (100 ppm), on the Growth (Radicle and Stem) of Some Secondary Species

Species	Treatments					
	inhibition/stimulation <sup>1</sup> (%)					
	<i>Piper auritum</i>		<i>Piper hispidum</i>		<i>Croton pyramidalis</i>	
	Root	Stem	Root	Stem	Root	Stem
<i>Mimosa pudica</i>	87 <sup>2</sup>	91 <sup>2</sup>	90 <sup>2</sup>	100 <sup>2</sup>	19 <sup>3</sup>	(55) <sup>2</sup>
<i>Achyranthes aspera</i>	81 <sup>2</sup>	100 <sup>2</sup>	100 <sup>2</sup>	100 <sup>2</sup>	84 <sup>2</sup>	34 <sup>2</sup>
<i>Bidens pilosa</i>	100 <sup>2</sup>	100 <sup>2</sup>	100 <sup>2</sup>	100 <sup>2</sup>	49 <sup>2</sup>	27 <sup>2</sup>
<i>Crusea calocephala</i>	100 <sup>2</sup>	100 <sup>2</sup>	-	-	(5)	15 <sup>3</sup>
<i>Heliocarpus donnell-smithii</i>	91 <sup>2</sup>	100 <sup>2</sup>	100 <sup>2</sup>	100 <sup>2</sup>	(22) <sup>2</sup>	16 <sup>3</sup>
<i>Ochroma lagopus</i>	74 <sup>2</sup>	100 <sup>2</sup>	-	-	(26) <sup>2</sup>	(35) <sup>2</sup>
<i>Solanum nitens</i>	100 <sup>2</sup>	100 <sup>2</sup>	100 <sup>2</sup>	100 <sup>2</sup>	-	-

<sup>1</sup> Mean from five repetitions.

<sup>2</sup> significant to the 1% level.

<sup>3</sup> significant to the 5% level.

Numbers in parenthesis are stimulations.

raguensis showed that they are inhibitory to the germination and growth of other test seeds. Rodriguez-Hahu (personal communication) mentioned that this effect is due to a terpene, a rhamnoside and a flavonol, among other substances that are not yet identified.

As part of the same project a study of one of the most common weeds in some disturbed habitats from many regions in Los Tuxtlas (Veracruz) was carried out. Ambrosia cumanensis is found as an important species of the ruderal vegetation. It grows vigorously and in almost pure stands. We decided to assess its allelopathic potential in order to evaluate this phenomenon as a determining factor for the structure of the community as well as in the secondary succession process.

Root and leaf aqueous leachates of Ambrosia cumanensis did indeed produce a strong inhibition on the growth of weed species. Aqueous extracts of soil collected under A. cumanensis in July (during its flowering) were strongly allelopathic to weed growth. Decomposition of leaves and roots in pots caused inhibition of some weeds also. Micro-organisms have a major role in this process, as shown by results from sterile and nonsterile soils (17).

Bioassays with several sesquiterpenic lactones from A. cumanensis showed that these compounds produce different effects (stimulatory and inhibitory) on the germination and growth of several species of the secondary vegetation (18). Therefore, it is possible that the allelopathic potential of A. cumanensis contributes to the autocontrol of its population by preventing the growth of seedlings of its own species (Figure 2).

The information obtained at Los Tuxtlas shows that the studied species from the secondary vegetation produce one or more allelopathic substances, mainly in leaves or through the decomposition of their organic matter, that can inhibit growth or have deleterious effects on plants and may cause parallel effects that are related to the role of auxins and to tropisms and other metabolic processes.

The production of allelopathic compounds in tropical zones, particularly if they are continuously released into the environment, may contribute to the elimination of secondary species already established and to the selection of those that are beginning to establish in the habitat.

### Studies in Coffee Plantations

In 1979, we decided to extend our studies to one of the agroecosystems of greater importance in Mexico: the coffee plantations. These studies were realized within the Program of Agroecosystems at the Instituto Nacional de Investigaciones sobre Recursos Bioticos. We worked at the coffee plantations in Coatepec, Veracruz, which are characterized by the presence of shade trees which resemble the structure of the deciduous temperate forests, with three well defined strata: the herbaceous layer, the shrub layer represented by coffee plants and the tree layer. The main objective of this study was to assess the allelopathic interactions among the species that constitute this community, in particular the coffee plants (19).

Figure 3 shows the effect of the soil extracts from the coffee plantation. Waller et al. mention that these effects might be explained by the accumulation of caffeine and other alkaloids in soil in old coffee plantations (20).

The greatest allelopathic effects were produced by plants from the

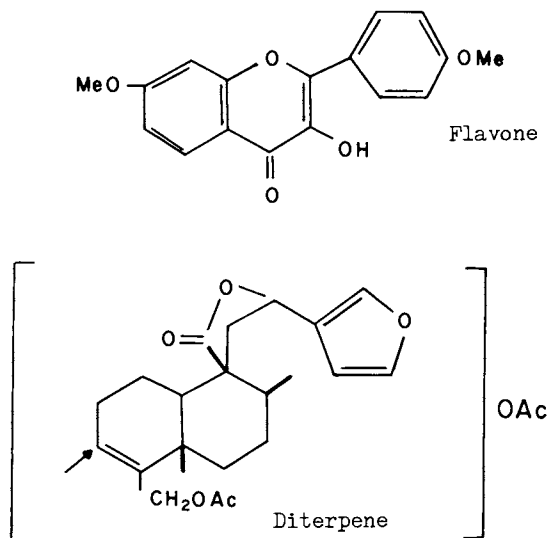


Figure 1. Structures of Pyramidolactone (Furolactone Diterpene) of the Norclerodane group (A), and 3,5-dihydroxy-7,4'-dimethoxyflavone (B) isolated from *Croton pyramidalis*.

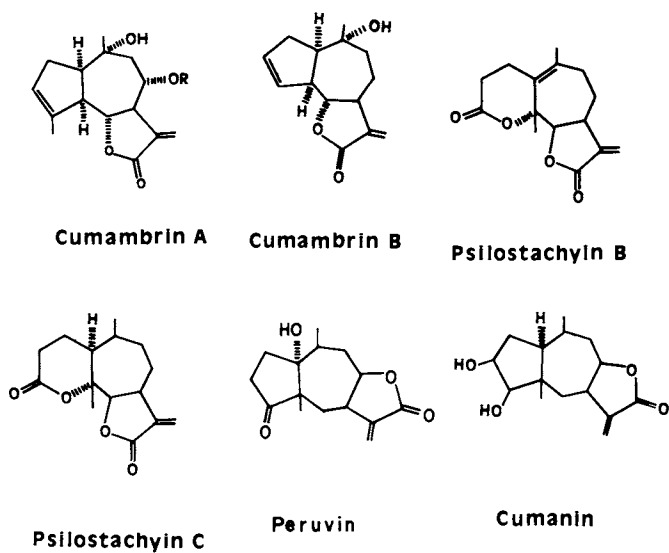


Figure 2. Sesquiterpene lactones isolated from the *Ambrosia cumanensis-psilostachya* complex.

herbaceous layer (Figure 4), particularly from various species of Commelinaceae. These results led us to the study of three of the most abundant species in the coffee orchards: Commelina diffusa, Tripogandra serrulata, and Zebrina sp. All species, fresh, dried, and chopped, as well as their litter, exerted a significant inhibition in the growth of Bidens pilosa (21).

#### Studies on Water Hyacinth

Simultaneous to the studies at the coffee plantation was the study of the allelopathic potential of the water hyacinth (Eichornia crassipes). This aquatic plant, introduced in Mexico at the beginning of this century, invades many of the water reservoirs and streams and is considered as one of the worst aquatic weeds in our country. Its capacity to establish itself in several water habitats and its extensive vegetative growth suggested a strong mechanism of invasion, perhaps of allelopathic nature. Several bioassays were used for testing aqueous extracts from leaves, roots, and flowers upon weeds and cultivated plants. Results showed a strong inhibitory effect, especially from leaves and flower leachates (Figure 5). Water hyacinth is widely used as a green fertilizer in the Valley of Mexico, mainly in the ancestral traditional agroecosystems known as "chinampas". These are long narrow strips of land surrounded on at least three sides by water.

Once the allelopathic potential of water hyacinth was demonstrated in laboratory assays, we decided to study the effect of this plant upon the agricultural production and growth of weeds in a chinampa where turnip, radish, lettuce, and cabbage were cultivated.

The soil in the chinampa was prepared in the traditional way of peasants at Xochimilco, by making a seed bed with mud from the bottom of the channels that surround the chinampa. When the mud was dry, it was cut in small cubes where the seeds were planted. The seedbed was then covered with soil and twigs. Once the seedlings reached 10-15 cm they were transplanted to a plot previously weeded and plowed. Treatments were placed randomly and covered with mud. These were: 1. control (without fertilizer); 2. inorganic fertilizer (10:10:15), 250 g/m<sup>2</sup>; 3. cow manure; 4. water hyacinth (whole plant); 5. water hyacinth (roots); 6. water hyacinth (aerial part). Treatments 3,4,5, & 6 were added in a proportion of approximately 2kg/m<sup>2</sup>.

Figure 6 shows that all plots with water hyacinth exhibited a higher crop yield than the other treatments. This was mainly due to: 1) improvement of the soil texture; 2) a decreased in the salinity of the soil due to a reduced water evaporation and salt deposition on the soil surface.

Leaves and whole plants added to the soil caused the greatest inhibitions to weeds in turnip, radish, and cabbage plots (Figure 7). This effect might be due to: 1) a selective allelopathic effect of water hyacinth and vegetables upon weeds, and/or 2) competition with crops. Water hyacinth has been widely used as a fertilizer in the chinampas because it improves the physical and chemical properties of soil and it exerts a certain control of weeds through its decomposition in the soil.

#### Studies on Crop-Weed Relationships

As part of the Program of Agroecosystems from INIREB (Instituto

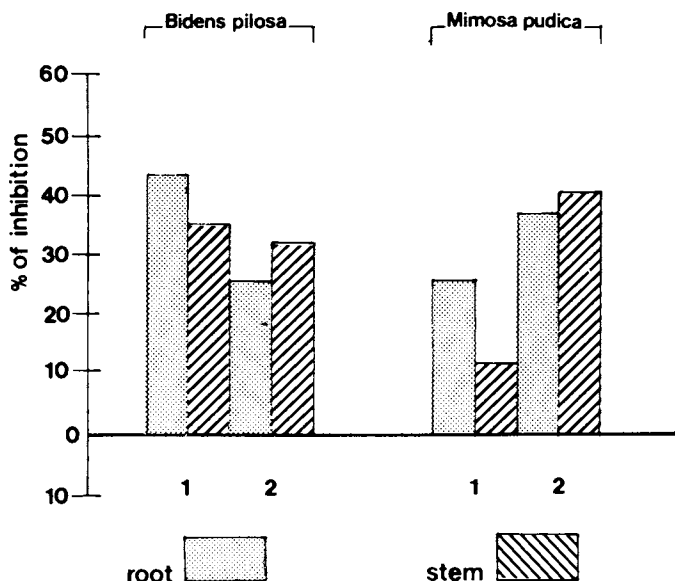


Figure 3. Effect of the aqueous extracts of soils of the shrubs stratum (coffee) on weed growth. (1) Soil of *Typica* coffee. (2) Soil of *Bourbon* coffee.

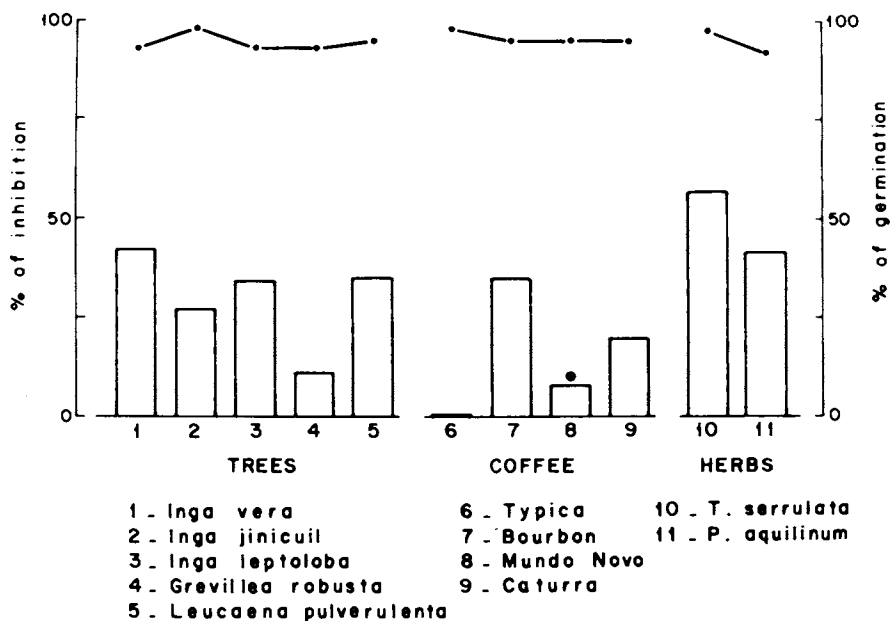


Figure 4. Effect of the aqueous extracts of dry leaves of trees, coffee and herbs on the germination and growth of *Rumex* sp. (● non significant).

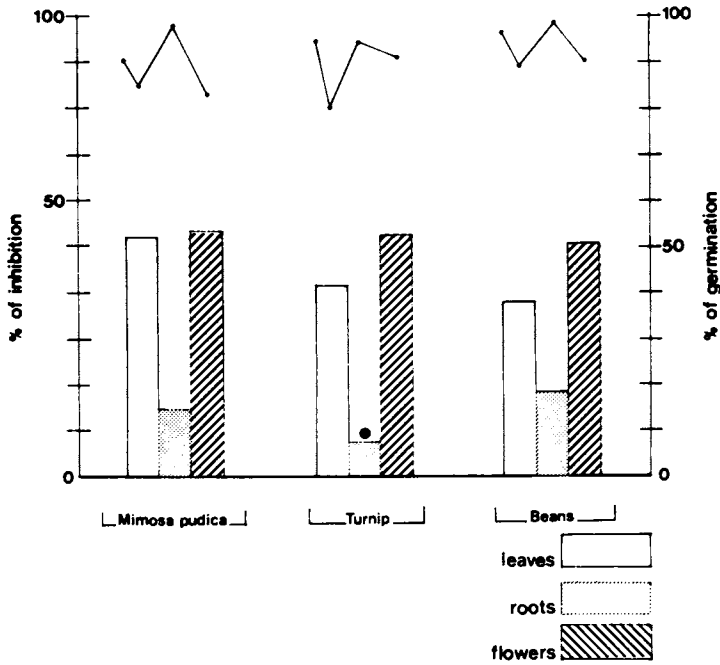


Figure 5. Effect of aqueous leachates of *Eichhornia crassipes* (leaves, roots and flowers) on the germination and growth of three species (● non significant).

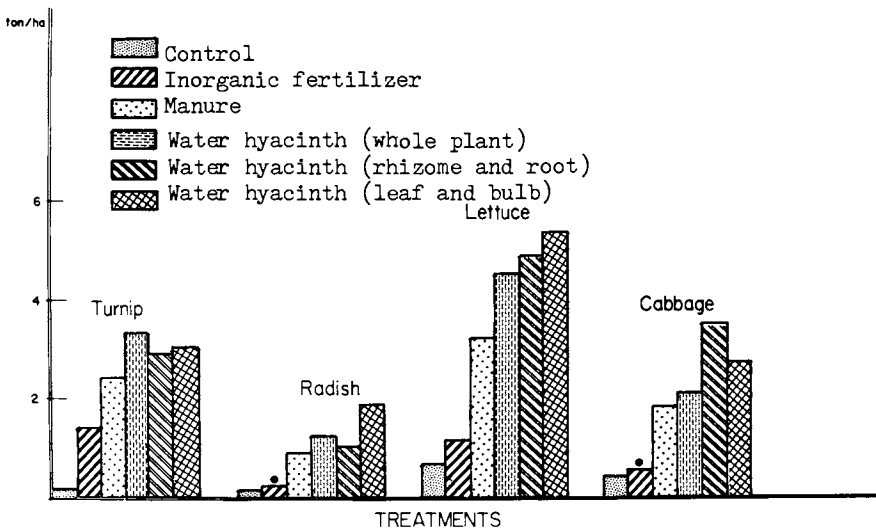


Figure 6. Yield of turnip, radish, lettuce and cabbage with the six treatments (● non significant).

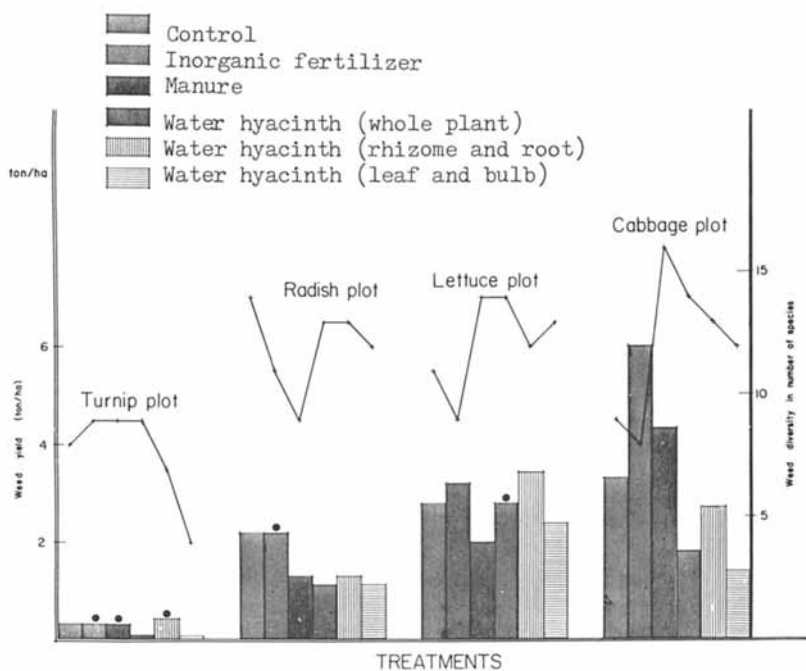


Figure 7. Yield and diversity of weeds with the six treatments (● non significant).

Nacional de Investigaciones sobre Recursos Bioticos), we conducted a study of the relationships between cultivated plants and weeds in a "chinampa" at San Andres Mixquic, D.F., in the southeastern Valley of Mexico. In this study it was found that leachates from cultivated plants (corn, squash, and beans) stimulated the growth of their own seedlings and inhibited that of weeds. Also, we found that corn production is less affected by the presence of weeds when it is associated with Cucurbita ficifolia (Jimenez et al., in preparation). Finally, it was demonstrated that corn pollen has a strong allelopathic potential. These results led us to the study of several organic extracts of such pollen tested upon Cassia jalapensis. The hexanic and methanolic fraction proved to be very inhibitory to Cassia jalapensis seedlings(22) and the ethanolic extract was found to act as an inhibitor of electron transport in isolated mitochondria from watermelon seedlings (Cruz, R., in preparation).

Studies on Helietta parvifolia. In the northern arid region of Mexico, Rovalo et al. (23) carried out a study of the potential uses of Helietta parvifolia. They demonstrated that the essential oil of Helietta acts as a fungicide upon Penicillium, Rhizopus, Fusarium, and Aspergillus and also acts as an insecticide upon Anastrepha ludens (fruit fly). The allelopathic potential of Helietta leaves was demonstrated upon a common weed: Convolvulus arvensis.

Studies on Piqueria trinervia. A very interesting study is that of the allelopathic potential of Piqueria trinervia and its piquerols A and B, by González de la Parra et al. (14). It was found that this widely distributed weed in the Valley of Mexico has a wide biological activity upon other plants.

Present studies in warm and temperate region. At present, we are assessing the allelopathic potential of weeds from a tropical region of the country (Uxpanapa, Veracruz), as a complement to the project entitled Recovery of Tropical Rain Forests from INIREB. This information is necessary to permit more efficient agricultural and forest management of the secondary vegetation in the tropics.

At the same time we are studying the allelopathic interactions among crops, weeds, and microorganisms and some aspects of the traditional agroecosystems known as "camellones" in Tlaxcala, a central state in Mexico. Those are distinguished by their biological diversity, the presence of water channels along the border of the crop lands, and the traditional management system, which involves biological control of pests and the use of green manure in mono cultures of corn and in mixed cultivation with beans and squash. Our final goal is to help generate a multiple model of production and to maintain our natural resources.

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## Chapter 10

# Allelopathy in Subtropical Vegetation and Soils in Taiwan

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Allelopathy plays an important role in subtropical vegetation and soils, regulating the formation of plant dominance, succession, population dynamics of understory plants, and the productivity of many crops in Taiwan. This paper describes research findings on autointoxication phenomena of rice plants, sugar cane plantation, asparagus plants, and pangola grass (*Digitaria decumbens*), and on allelopathy in relation to agricultural practice, forest plantation, and environmental stresses. Allelopathy even plays an appreciable role in plant adaptation in many natural vegetation and plantations. The responsible phytotoxins reported here are phenolics, flavonoids, alkaloids, and other unidentified compounds.

Since the 1960s allelopathy has been increasingly recognized as one of the important ecological factors in plant interactions and has been regarded as impossible to single out from an environmental complex (1). Koeppe et al. (2, 3) also reported several allelopathic studies, in which the tested plants were placed under conditions of environmental stresses. Duke and Putnam (4) introduced the concept into agricultural practice to select a crop variety with high phytotoxic potential in order to avoid using herbicides. In the last decade, a tremendous growth of publication on allelopathy has occurred in the world (5, 6, 7, 8, 9). Wang and his associates described methods of extraction and identification of phytotoxins in soil (10, 11, 12), and subsequently studied the behaviors of phytotoxic phenolics in soil (13, 14, 15). Since 1972, Chou and his co-workers have conducted such research in a subtropical humid zone of Taiwan and accumulated substantial information concerning allelopathic interactions in vegetation and soils (5). These findings of allelopathic studies are of great significance to understand the role of allelopathy in the natural and agricultural ecosystems in Taiwan.

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Autointoxication And Agricultural Productivity

Autointoxication, in which an organism releases a toxic chemical that suppresses its own growth, is one phase of allelopathy. Autointoxication can also be important in intraspecific interactions, such as the regulation of population size by self-thinning. Several case studies conducted in Taiwan are described below.

Autointoxication as the cause of low yield of the second crop of rice. Rice (*Oryza sativa*), the most important crop in Taiwan, is planted twice a year by a continuous monoculture system. For nearly a century, the yield of the second crop there has been generally lower by 25% than that of the first crop (a reduction of about 1000 kg/ha). This reduction of rice productivity has been particularly pronounced in areas of poor water drainage. The cropping system of rice in Taiwan is different from that of other countries. For example, the fallowing period between the first crop and the second crop is only 3 weeks, as compared with a 10-week period elsewhere. In growth of the first crop (from March to July) the temperature increases gradually from 15 °C to 30 °C but for the second crop (August to December) it decreases from 30 °C to 15 °C. Between these two crops, the farmers always leave rice stubble in the field after harvesting, and submerge these residues in the soil for decomposition during the fallowing time. During the second crop season, the typhoon (or monsoon) brings a great amount of rainfall, leading to a high water table in some areas where water drainage is rather poor. Chou and his associates therefore conducted a series of experiments to elucidate the reason for the low yield of rice in the second crop season. Aqueous extracts of paddy soil collected in Nankang were bioassayed and found to be phytotoxic. In pot experiments, a rice straw-soil mixture (100 g: 3 kg) was saturated with distilled water and allowed to decompose for 1, 2, and 4 weeks under greenhouse conditions. Soil alone was treated in the same manner, as a control. At the end of each decomposition time, 5 rice seedlings (3 weeks old) were transplanted into a pot containing straw-soil mixture or into the control soil. After one month, rice seedlings grown under control conditions were normal and usually over 66 cm tall, while the seedlings grew poorly (about 36 cm tall) in the straw-soil mixture. The roots of retarded plants were dark brown and the root cells were abnormal and enlarged. Further experimental results showed that when the amount of rice straw mixed was increased to 100 g/3 kg soil, the phytotoxicity increased with the increase of straw added. The toxicity was still persistent after 16 weeks of decomposition. The rice straw-soil mixture with different intervals of decomposition was extracted with ethanol, the ethanol evaporated, and the residue reextracted with ethyl ether; then the phytotoxins present in the ether extract were identified by chromatography. The compounds identified were *p*-coumaric, *p*-hydroxybenzoic, syringic, vanillic, *o*-hydroxyphenylacetic, and ferulic acids (16), and propionic, acetic, and butyric acids (17). Particularly, *o*-hydroxyphenylacetic acid, first reported to be a phytotoxin by us, was toxic to

rice growth at a concentration of  $1.64 \times 10^{-4}M$ . We found that the concentration of *o*-hydroxyphenylacetic acid reached about  $10^{-4}M$  in the soil containing decomposing rice residues. The additional evidences of phytotoxic effects arising from the study will be described later in this paper.

Inter- and intra-specific interactions between *Oryza perennis* and *Leersia hexandra*. The wild rice, *Oryza perennis* Moench, distributed throughout the humid tropics, is considered to be a progenitor of cultivated *O. sativa* (18). The Asian race shows a perennial-annual continuum, varying greatly in various life-history traits among its varieties (19, 20). *Leersia hexandra* Sw. is a perennial grass with short rhizomes, commonly found in marshy habitats in Taiwan and other tropical Asian countries. It is a companion of *O. perennis* in about 40% of the habitats observed in India and Thailand (21). In Taiwan, three small populations of *O. perennis*, hybrid with *O. sativa* (18), had existed in marshes along natural streams at Patu, Taoyuan Hsien, but they became extinct around 1975, displaced by *L. hexandra*. An experimental introduction of *O. perennis* populations into different habitats indicated that *L. hexandra* was a key determining the biotic environment of the former. To look into the interaction mechanisms of the two species, their allelopathic interrelations were examined by several methods, such as bioassay of the effects of aqueous leachates and extracts of the two species on the radicle growth of rice and lettuce and on the growth of adventitious roots from nodes of cuttings of the two species, and the effects of powdered plant material added to soils on the root development of cuttings. Both grass species showed phytotoxic effects on the radicle growth of rice and lettuce, intra- and inter-specifically. *L. hexandra* showed in many cases higher phytotoxicity than *Oryza* although the pattern of variations was complex. The concentrations of many phytotoxins identified were higher in *Leersia* extract than in that from *Oryza*. Observation of plants growing from buried seed pool in soils to which powdered plant materials were added also showed higher phytotoxicity of *Leersia* than *Oryza*. Probably, allelopathy plays an appreciable role in the successional replacement of the two species (22).

Autointoxication of sugar cane plantation. Inadequate germination and growth of ratoon cane have been found to be the two major problems in the farms of Taiwan Sugarcane Corporation (TSC). The yield of monoculture sugar cane has declined in many sugar cane fields. The causes of this yield reduction have been investigated, but no single factor causing the reduction can be found. Wang et al. (23) demonstrated by field and laboratory experiments that phytotoxic effects are one of the important factors involved. Five phenolic acids (*p*-hydroxybenzoic, ferulic, *p*-coumaric, syringic, and vanillic) and formic, acetic, oxalic, malonic, tartaric, and malic acids were identified in the decomposing sugar cane leaves in water-logged soil. At  $3 \times 10^{-4}M$  solution of these phenolic acids in water culture, the growth of young sugar cane root was inhibited. The aliphatic acids were also found to inhibit the growth of ratoon sugar cane at  $10^{-3}M$ . Furthermore, Wu et

al.(17) found that the population of Fusarium oxysporum associated with the rhizosphere soil of poor ratoon cane roots was much greater than that of good growing ratoon or of newly planted sugar cane roots. They found that fusaric acid, a secondary metabolite of the organism, was toxic to the growth of young sugar cane plants in vitro (17).

Autointoxication of Asparagus officinalis L. Asparagus officinalis is a perennial ratoon crop widely planted in many plantations of Taiwan. A significant reduction of yield and quality of asparagus often occurs in old plantation soil. The wilting of asparagus plants has been found to be due to monoculture of the crop. Young (24) indicated that there was about 40% of asparagus seedlings missing from the plantation. Young further showed that the root exudates of asparagus retarded the seedling growth of asparagus cultivars, namely Mary Washington, California 309 and California 711 (24). Exudate collected by use of the circular trapping with a XAD-4 resin significantly retarded radicle and shoot growth of asparagus seedlings (25). Six phytotoxic phenolics, namely 3,4-dihydroxybenzoic, 3,4-dimethoxybenzoic, 2,5-dihydroxybenzoic, 3,4-dihydroxyphenylacetic, and  $\beta$ -(*m*-hydroxyphenyl)propionic acid, and 3,4-dimethoxyacetophenone were found in the extracts and exudates of asparagus plant parts. The amount of phytotoxins identified was significantly higher in the stem than in the root, and was well correlated to phytotoxicity (25). It is concluded that the reduction of asparagus productivity in old asparagus fields is due primarily to phytotoxins released from the plant parts and those produced from the decomposition of residues remaining in soil.

#### Allelopathy And Agricultural Practice

Allelopathy of native and pasture grasses. Miscanthus floridulus, widely distributed in Taiwan, is a native and predominant grass and often occurs in poor soil on hillsides and/or channels. A field experiment conducted at a Nankang hillside showed that the botanical composition in Miscanthus stands is about 65% for M. floridulus, 17% for Lactuca indica, and less than 4% for Eupatorium formosanum, Brachiaria distachys, Sporobolus fertilis, Pouderia scandens, Cyperus pilosus, Digitaria violascens, and three unknown grasses (26). A successional trend of botanical composition was caused by the aggressive nature of Miscanthus floridulus. For example, in an experiment in which M. floridulus was cleared, the dominance of Miscanthus recurred after three years. The associated species found in the Miscanthus stands were again found to be suppressed by the Miscanthus (5). Furthermore, aqueous extracts and leachate of Miscanthus leaves caused a significant reduction of radicle growth of tested species (26). Additionally, the extracts of soils collected from the Miscanthus rhizosphere, between stands, under the canopy of Miscanthus, and in open ground control area adjacent to the Miscanthus stands were also bioassayed for their phytotoxicity. Of these, the extract of root soil of Miscanthus exhibited the highest inhibition of the tested plants (26).

Some 12 subtropical introduced species of forage grasses gave aqueous leaf extracts evaluated for their phytotoxicity on tested species. Acroceras macrum, Cynodon dactylon, Chloris gayana, Digitaria decumbens, Eragrostis curvula, Panicum repens, and P. maximum always caused significant inhibition of radicle growth of test plants. Of them, Digitaria decumbens had the highest phytotoxicity upon the tested species at 10 milliosmols, in which the osmotic inhibition is zero (27, 28). Chou furthermore found that D. decumbens was also an autotoxic species, and the productivity was significantly depressed after several years of planting (Chou, unpublished data). The aqueous leachate and exudates of Digitaria plants showed a significant reduction of growth of this species.

Selection of weed control grass for pasture. An increased amount of allelopathic research on grassland species has been conducted in many parts of the world during recent decades (5, 9). Most of the studies have been concerned with the interpretation of allelopathic phenomena in the field. Only a few studies have employed the allelopathic effect as a practical means of directly controlling weeds. In Taiwan, many grasses have been introduced into pasture but only a few varieties can be established as forage pasture. As already mentioned, among 12 species studied (28), pangola (Digitaria decumbens) exhibited the highest toxic effect on test species. Under sufficient nitrogen fertilizer application, pangola grass forms a pure stand where almost no other weeds can grow. We also found that different varieties of pangola had different growth performance and competitive ability. Liang et al. (29) thus selected eight varieties of pangola for field trials and laboratory assays. These showed that the invasion ability of cultivars A65, A255, and A254 were highest in Hsinhwa, Hengchun, and Hwalien station, respectively; while cultivars A79 and A80 were inferior in all stations. Cultivars A84, A254, and A255 possessed the highest toxicity, which was due to phytotoxins, of which nine phytotoxic phenolics were identified. The interference of grasses in the field is very complicated, and allelopathy alone cannot account for the complicated phenomena. Further field and laboratory experiments thus need to be performed in order to clarify the role of allelopathy in grassland ecosystems.

Phytotoxic effect of cover crops on orchard plants. Wu et al. (30) compared the phytotoxic effects of some cover crops, namely Centrocema sp., Indigofera sp., and Paspalum notatum (Bahia grass), on the growth of pea, mustard, cucumber, cauliflower, rape, Chinese cabbage, mungbean, watermelon, tomato, and rice. They found that rape was most sensitive to the extracts of these cover crops. Among them, Centrocema and Indigofera exhibited the greater phytotoxic effect; moreover, the leachate of Centrocema inhibited the growth of banana. More recently, several cover crops including Bromus catharticus, Pennisetum cladestinum, Lolium multiflorum (both chromosome 4X and 2X cultivars), Paspalum notatum, and white clover are now under investigation for allelopathic effects on the productivity of apple and peach plantations

in the Lishan area of central Taiwan. A vast area of apple plantations has been situated on the hillsides of the Central mountain since the 1960s. The productivity of these plantations was exceedingly high in the first decade after planting but has gradually decreased in recent years. In fact, this problem has been encountered in many European countries and Northern America as well.

Forest-pasture intercropping system. Taiwan is an island, with two thirds of the land occupied by mountains, and its forests are extremely important for water conservation. The limited amount of agricultural land for crops and pasture forces farming activities to move upward to hillsides and higher elevations. A forest-pasture intercropping system has been thought to be a possible way to increase livestock production. Recently we have conducted several experiments in the forest area of Hoshe Experiment Station of National Taiwan University located at an elevation of about 1200 meters. An area of about one hectare was deforested, part was cleaned by removing the leaf litter of the conifer tree (*Cunninghamia lanceolata*), and part was left unchanged to serve as control. The cleaned and unchanged plots were planted with kikuyu grass (*Pennisetum cladestinum*) or left open. The experiment was designed to determine the reciprocal interaction of fir litter and kikuyu grass, and to evaluate the allelopathic potential of the two plants on weed growth under natural condition. Results indicated that the biomass of kikuyu grass in the cleaned plot was significantly higher than that in the control plot. In addition, the number of weeds that grew in the plot planted with kikuyu grass was lower than that in the control plot, indicating that the kikuyu grass may compete with and suppress weeds. The seedlings of fir regenerated in the deforested area grew well and seemed to not be affected by the neighboring newly planted kikuyu grass. However, the growth of kikuyu grass was inhibited by the fir litter left on the unchanged plot in the first three months after deforestation. Furthermore, bioassay of aqueous extracts showed that the fir litter extract exhibited higher phytotoxicity than the kikuyu grass. Nevertheless, four months after deforestation the kikuyu grass growth in the field was luxuriant, indicating that the phytotoxicity of fir litter disappeared (Chou et al., 1985 unpublished data).

Forest intercropping system. On the hillsides of mountainous district in Taiwan, there is an increasing area of deforestation. Forest regeneration of the area is very important to ecological conservation. Many highly valuable forest species have been planted in a forest intercropping system, such as bamboo, conifers, *Acacia confusa*, *Leucaena leucocephala*, *Liquidambar formosana*, *Casuarina glauca*, *Alnus formosana*, and *Pinus taiwanensis*. We have evaluated the suitability of intercropping systems among the aforementioned species. The first experiment was conducted with *L. leucocephala*, an allelopathic plant, intercropped with other species mentioned above. *Pinus taiwanensis* grew very well and could tolerate the leachate of *L. leucocephala*, but the remaining species were damaged by the leachate to some

extent. As mentioned earlier, we found several phytotoxic phenolics and mimosine produced by L. leucocephala. It is notable that the growth of Mimosa pudica was suppressed by Leucaena leaf leachate, even though the leaf juice of M. pudica contains a relatively high amount of mimosine. Among 84 seedlings of M. pudica tested only 2 seedlings survived, showing that mimosine can be practically useful to control a notorious weed such as M. pudica in the field.

#### Allelopathy And Forestry Plantation

Allelopathic nature of some bamboos. On many hillsides of mountainous districts in Taiwan, there is a vast area of bamboo plantations, and in the Chitou area we often found Cryptomeria japonica (conifer) and Phyllostachys edulis (bamboo) growing adjacent to one another. However, the P. edulis often encroaches on the C. japonica area, resulting in the gradual decline of productivity and ultimately the death of the latter trees. Chou and Yang (31) found that the litter of Phyllostachys edulis possesses phytotoxic phenolics, which suppress the growth of its understory. The floristic composition of the two vegetations showed that the understory species are different. For example, five predominant species of the understory in the P. edulis community are Ageratum conyzoides, Commelina undulata, Pilea funkikensis, Pratia nummularia, and Tetrastigma formosana; while in the C. japonica community, they are Ficus pumila, Pellionia scabra, Pilea funkikensis, Piper arboriola and Urtica thunbergiana. These species respond differently either to light intensity or to the phytotoxic leachates, so that there is a different distribution of species density and biomass under the canopy of the two tree species. The total number and dry weight of seedlings per square meter were much higher in the conifer community than in the bamboo forest, although the light intensity, soil moisture, and nutrient contents were significantly higher in the bamboo habitat than in the conifer. Further experimental results indicated that the aqueous extracts and leachates of bamboo leaves were more phytotoxic than those of conifer leaves, reflecting that allelopathy plays a significant role in the regulation of species diversity and production under the canopy of at least these two forests. Nevertheless, the difference in potential for species exclusion between the two forests may be due partly to an anatomic factor, such as the rhizome. There are two types of rhizomes, sympodial rhizocauls and horizontal rhizomes with lateral culms. P. edulis has the latter type, which grow rapidly. Thus, the invasion of P. edulis to territory of C. japonica may be due to (a) the fast-growing rhizomes, which may possibly release phytotoxic root exudates, and (b) allelopathic substances produced by the bamboo leaves and decomposing litter. The continuous release of water-soluble phytotoxins from P. edulis and accumulation of these in the soil may result in suppression of the growth of understory or in elimination of neighboring plants. In addition, the aqueous leaf extracts of 14 bamboo species were evaluated for allelopathic potential. The bioassay results showed that Sinocalamus latiflorus possessed the highest phytotoxicity



for lettuce, rye grass, and rice plants, but Bambusa oldhami, B. pachinensis, B. ventricosa, Phyllostachys edulis, and P. makinoi also showed significant phytotoxicity. Aqueous extracts obtained from the associated bamboo soils also exhibited some inhibition, which in most extracts was correlated to that of leaf extracts (32).

Allelopathic effect of Leucaena leucocephala. Leucaena leucocephala trees have been widely planted in Taiwan because of its high economic value for producing nutritious forage, firewood, and timber. Generally, after a few years of growth, the floors of these plantations are relatively bare of understory plants, except Leucaena seedlings. This pattern of weed exclusion beneath Leucaena trees is particularly pronounced in areas having a drought season. Chou and Kuo (33) therefore undertook a series of experiments conducted in fields, greenhouse, and laboratory. Field data showed that the phenomenon was not due primarily to physical competition, involving light, soil moisture, pH, and nutrients. Instead, aqueous extracts of Leucaena fresh leaves, litter, soil, and seed exudate showed significantly phytotoxic effects on many test species, including rice, lettuce, Acacia confusa, Alnus formosana, Casuarina glauca, Liquidambar formosana, and Mimosa pudica. However, the extracts were not toxic to Leucaena seedlings. Decomposing leaves of Leucaena also suppressed the growth of the aforementioned plants grown in pots but did not inhibit that of Leucaena plants. By means of paper and thin-layer chromatography, UV-visible spectrophotometry, and high performance liquid chromatography, 10 phytotoxins were identified. They included mimosine, quercetin, and gallic, protocatechuic, p-hydroxybenzoic, p-hydroxyphenylacetic, vanillic, ferulic, caffeic, and p-coumaric acids. The mature leaves of Leucaena contain about 5% (dry weight) of mimosine, the amount varying with varieties. Seed germination and radicle growth of lettuce, rice, and rye grass were significantly inhibited by aqueous mimosine solutions at a concentration of 20 ppm while that of the forest species mentioned was suppressed by mimosine solution at 50 ppm or above. However, the growth of Miscanthus floridulus and Pinus taiwanensis was not suppressed by a mimosine solution at 200 ppm. Seedlings of Ageratum conyzoides died in mimosine solution at 50 ppm within 7 days and wilted at 300 ppm within 3 days. It is concluded that the exclusion of understory plants is due to the allelopathic effect of compounds produced by Leucaena. The allelopathic pattern was most clearly shown in the area with a heavy accumulation of Leucaena leaf litter, which was a result of drought and heavy winds.

Allelopathic potential of Vitex negundo. Vitex negundo is a dominant component of coastal vegetation and widely distributed in the southern parts of Taiwan. Chou and Yao (34) found that the biomass and density of its associated understories are relatively lower than in adjacent pasture. Field results showed that the natural leachate of V. negundo significantly retarded the growth of Digitaria decumbens but stimulated the growth of Andropogon nodosus as compared to the rainfall control. The growth of D.

decumbens grown in pots under greenhouse conditions was significantly retarded by watering with a 1% aqueous extract of V. negundo, but the growth of Andropogon nodosus and Mimosa pudica was stimulated. The aqueous extract was phytotoxic to lettuce and rye grass seeds. The aqueous effluents obtained from a polyamide column chromatograph were also bioassayed. Some fractions inhibited radicle growth of lettuce and rice seedlings, whereas other fractions had a stimulatory effect. The responsible substances were isolated and identified. These included phenolic acids, *p*-hydroxybenzoic, ferulic, *p*-coumaric, vanillic, and syringic acids, and 10 flavonoids. One flavonoid, 3'-hydroxyvitexin, and nine other flavonoids were identified (34).

#### Allelopathy And Environment Relationship

The actions of many allelopathic compounds produced by plants are often affected by environmental factors, such as water potential of the environment, temperature, light intensity, soil moisture, nutrient, and soil microorganisms. The compounds are released to the environment by means of volatilization, leaching, decomposition of residues, and root exudation (1, 5, 7, 9). Firstly, the terpenoids, such as  $\alpha$ -pinene,  $\beta$ -pinene, cineole and camphor, are released to the environment by volatilization, which is noticeable under drought conditions. Secondly, the water-born phenolics and alkaloids are washed out by rainfall through leaching. Thirdly, phytotoxic aglycones, such as phenolics and others are produced during the decomposition of plant residues in soil. Fourthly, many secondary metabolites, such as scopoletin, hydroquinone and others, may be released to the surrounding soil through root exudation. The following paragraphs describe the phytotoxins produced and interacted with factors under conditions of environmental stresses in the subtropics.

Plant under drought stress. A study site of Leucaena leucocephala plantation was selected at the Kaoshu village of Pintung county, situated in the southern part of Taiwan. After 3 to 4 years of growth, there was an almost total lack of understorey except Leucaena seedlings during the winter season. The absence of weeds is due to a heavy accumulation of Leucaena plant residues, such as leaf litter, branches, etc. Chou and Kuo (33) found that the biomass of ground cover beneath Leucaena plantations was relatively low as compared to its adjacent grassland control area, being 9.3 g/m<sup>2</sup> on the Leucaena floor and 330 g/m<sup>2</sup> in the grassland area. There is about 80% bare ground on the Leucaena floor. However, the amount of litter accumulated on the Leucaena floor was remarkably high (1027 g/m<sup>2</sup>), which was primarily due to the drought winter season and heavy wind. Chou and Kuo (33) reported that the aqueous extracts of Leucaena leaves and litter were phytotoxic to many test plants, except Leucaena itself. The responsible phytotoxins were found to be mimosine and nine other phenolics. It is generally believed that the phenomenon would be more pronounced when the plantation goes for several years and even under severe drought season.

Plant growth under water-logged and oxygen-deficient conditions. Many aquatic plants grow very well in a water-logged and oxygen deficient environment because of their adaptation mechanism. Although rice plants are not aquatic plants, they grow very well in the paddy soil. Patrick and Mikkelsen (35) indicated that the level of oxygen reached almost zero when it was measured at 25 cm below the soil surface in the paddy field. We obtained similar results in Taiwan paddy soils. In many areas of Taiwan, namely Tsingshui (the central part of Taiwan), Chiatung and Yuanlin (the southern part), and Tungshan (the east coast), where the paddy fields are either poor in water drainage or have a higher water table, leading to oxygen deficiency. This is even pronounced in the second crop season when the monsoon comes. In addition, the farmers in Taiwan have always submerged rice straw into soil and allowed them to decompose. During the decomposition of rice residues in soil, a significant amount of phytotoxic substances, such as short-chain aliphatic acids and phenolic acids were produced. The amounts of these compounds produced reached its maximum the first month after rice residues are submerged into soil, resulting in the suppression of root growth and panicle initiation; thus, the rice yields decrease (16, 36, 37).

Allelopathy in relation to soil redox potential (Eh) of paddy soil. As mentioned earlier, the oxygen level is nearly zero at a depth of 25 cm below the soil surface of paddy fields, resulting in the reduction of soil redox potential (Eh) (35). Chou and his co-workers found that the soil Eh ranged from -100 to 200 mV during the first crop season and from -200 to 100 mV during the second crop season in the Nankang paddy field. At the farm of the National Chungshing University of Taichung, the Eh was remarkably low, ranging from -500 to 100 mV during the second crop season. In pot experiments, we found that the soil Eh was below -300 mV in the treatment of rice straw mixed with soil and was above 100 mV in the treatment of soil alone. Thus, the reduction of soil Eh was apparently related to the decomposition of rice residues in soil. The reduced soil Eh was remarkable at the tillering stage (30-45 days after transplanting) and at the panicle stage (80-90 days after transplanting) (36). During this period, the growth of rice roots was retarded, the root cells swelled, and many adventitious roots developed. Wu et al. (17) postulated that the swelling of root cells could be a kind of adaptive mechanism in order to obtain more oxygen.

Allelopathy in relation to microbial activity. During the decomposition of plant residues in soil, microbial activities are involved. Wu et al. (38) found that the denitrified bacterium Pseudomonas putida became dominant in the rhizosphere of the rice paddy, and the population of P. putida was positively correlated to phytotoxin production when rice residues were submerged in soil and to the poor water drainage. They pointed out that in the well-drained area of Tsautune soil the number of P. putida was  $701 \times 10^5$  /g dry soil, while in the poor water drainage area of Lotung, Taan, and Taichung, the number of P. putida ranged from  $347$  to  $3412 \times 10^5$  /g dry soil. It was evident that the number of P.

putida was exceedingly high in the poor water drainage soil, indicating that the organism might use the residues as its carbon source. Wu et al. (30) furthermore indicated that the phytotoxic phenolics did not come from the metabolites of this microorganism but were released from the decomposing rice residues. Chou et al. (36) pointed out that ammonium sulfate mixed with rice residues enhanced phytotoxicity, reflecting that the addition of nitrogen fertilizer might favor the growth of decomposing microorganisms and thus expedite the decomposition rate of rice residues in soil. Wu et al. (36) also indicated that the application of ammonium sulfate fertilizer to paddy soil was beneficial to the growth of P. putida, but that may expedite the formation of  $H_2S$ , which is toxic to rice growth. Similarly, the cause of yield decline of sugar cane in Taiwan has been investigated by Wang and his associates (23) and Wu et al. (38). They found that the reduction is partly due to the phytotoxic effect of decomposing cane residues in soil and the microbial activity of Fusarium oxysporum, which produced a phytotoxin, fusaric acid, in addition to phytotoxic phenolics. They found that the F. oxysporum population was much greater in the rhizosphere of ratoon sugar cane soil than in the soil without planting. At 10 ppm of fusaric acid mixed in Murashige and Skoog's medium, leaves of sugar cane wilted and became chlorotic (17).

Phytotoxins in relation to nitrogen availability. Chou et al. (39) concluded that the more rice stubble left in the paddy soil, the higher would be the phytotoxic phenolics and the less amount of leachable nitrogen, reflecting that the phytotoxins produced may interact with nitrogen available in soil. They also found that the amount of leachable  $NH_4^+$ -N was about ten times as great as that of  $NO_3^-$ -N (36). Chou and his co-workers (31) used  $^{15}N$ -isotope tracer techniques to study the distribution of nitrogen in soil or soil-rice residues mixture under different temperature regimes and sequences. In the absence of straw, most of the fertilizer N remained in the mineral form. Straw enhanced N immobilization only moderately. The gradual decrease in the proportion of fertilizer N in the mineral form was accompanied by a steady increase of fertilizer N in the amino acid fraction of organic N. Little accumulation of fertilizer N in the amino sugar or the insoluble humin fraction was found (37). Although the experimental results did not show a distinct trend in relation to temperature variations, the temperature range of 25-30 °C tended to favor N transformation activities.

Interaction of decomposing rice residues with soil leachable cations. During the decomposition of rice residues in soil, the amount of available minerals might be affected and consequently alter plant growth. Chou and Chiou (36) studied the effect of rice straw incorporated into soil on the dynamics of some cations in pot soil. The results revealed that the concentrations of cations, K, Cu, and Mn, were higher in the first crop season whereas those of Na, Ca, Mg, and Zn were higher in the second crop season in Nankang paddy soil regardless of nitrogen fertilizer application. Most of our findings agree with those of

Patrick and Mikkelsen (35). In flooded soil, the concentrations of reducible iron and manganese were relatively low. When the pot soil was mixed with rice straw and allowed to decompose, the amount of K was significantly higher than that in soil alone, but the concentrations of Cu, Fe, Mn, and Zn were, on the average, significantly lower in the soil in terms of the ratio of soil to straw. It is interesting to note that in several poor water-drainage areas in Taiwan, such as Changhwa, Taitung, and Pingtung, Zn deficiency is particularly pronounced during the second crop season.

#### Ways of Eliminating Phytotoxins to Improve Crop Productivity

Improvement of water drainage. As mentioned earlier, the reduced rice yields in the second crop season in Taiwan is partly due to the phytotoxic substances produced during the decomposition of rice residues in soil in areas of poor water drainage. In addition, it was mentioned that the denitrified organism, *Pseudomonas putida*, was the dominant microbe during the decomposition of rice residues in soil. To eliminate the phytotoxins in paddy soil, a large scale experiment of improving drainage system has been conducted in Chiatung, where the water table is relatively high and water-drainage is poor. The results showed that the rice yield has been increased by at least 30% since the system was improved. We have analyzed the poor water-drainage and improved water-drainage soils for the phytotoxicity and phytotoxins present. Significantly greater phytotoxicity and higher amounts of phytotoxins were present in the poor water drainage soil than in improved water drainage soil, indicating that the phytotoxins were leached from the poor water drainage paddy soil; thus the productivity of rice was greatly increased. Furthermore, Wu et al.(38) found that the total amount of phytotoxic phenolics in the well-drained Tsao-tun soil ( $0.52 \times 10^{-2}$  mole/ 100 g soil) was significantly lower than that in the poorly drained Lotung soil ( $1.92 \times 10^{-2}$  mole /100 g soil). They also reported that the amounts of non-volatile and volatile fatty acids also were significantly lower in the well-drained soil (7.61  $\mu$ mole/ 100 g soil) than that in the poorly drained soil (37.16  $\mu$ mole /100 g soil). Rice yield in these poorly drained soils was lower. The amounts of phytotoxic phenolics were also correlated to that of denitrifying bacteria as described in the earlier session.

Removal of soil phytotoxins by water flooding. It has mentioned that after several years of growth of sugar cane, a significant reduction of yield usually occurred. This was thought to be due to the phytotoxins produced during the decomposition of sugar cane residues left in the soil and the imbalance of the microbial population. In order to eliminate the soil phytotoxins and to improve the soil condition of the microbial balance, the effects of water flooding on the sugar cane soil were studied. The population of *Fusarium oxysporum* was exceedingly high in the low-yield sugar cane soils before water flooding, but the population decreased after flooding. In addition, the amount of phytotoxin,

fusaric acid, produced by F. oxysporum, was significantly lower after water-flooding, indicating that phytotoxins had been leached out.

Crop rotation. Many monoculture fields often produce a soil-sickness problem, which is assumed to be due to the imbalance of soil microorganisms, accumulation of soil toxins, mineral deficiency, or abnormal soil pH, resulting in a decrease in crop productivity. Rotation of crops will avoid or eliminate the cause of the problem. Although many successful examples of crop rotation have been reported, only a few studies concerned allelopathic effects. In Taiwan, pangola grass (Digitaria decumbens) produces a highly productive pasture and constitutes a dominant species; however, after several years of growth, productivity declines. Chou and his associates found that the pangola grass produced phytotoxins, which suppressed its own growth (5). The decline of productivity of this grass has been particularly pronounced in the farm of Hengchun Experiment Station of Taiwan Livestock Research Institute. Thus, a crop rotation system of pangola grass-watermelon-pangola grass was established. The watermelon was planted in the drought winter season and pangola grass in the spring season following the harvest of watermelon. The yield of pangola grass after watermelon was significantly increased to about 40% as compared to that without the rotation. It is assumed that the increase of grass production could be a result of the disappearance of phytotoxins produced by pangola grass, and the effect is apparently lessened by the crop rotation.

Detoxification of soil phytotoxins by nutrient dressing. Wu and her co-workers (38) conducted field experiments in the area of poor-drainage paddy soil of Lotung by applying ammonium sulfate, lime, and green manure in order to compare the phytotoxic effect of rice residues decomposing in soil. The results indicated that the paddy soil supplied with lime exhibited significantly higher yield than that with other treatments (38). They concluded that the increase of rice yield was simply due to a detoxification of phytotoxins, and calcium may bind with some toxins, these being converted into nontoxic substances. In addition, Chou and Chiou (36) reported that different nitrogen fertilizer dressings gave different yields of rice. Ammonium sulfate fertilizer produced higher yields than nitrate nitrogen fertilizer. Apparently, ammonium sulfate fertilizer may overcome the phytotoxic effect of decomposing rice residues in soil. This finding agreed with that of Chandrasekaran and Yoshida (40), who concluded that ammonium sulfate effectively eliminated the injury caused by phytotoxins. We also found that the root system of rice plants was healthy and well-developed under the ammonium sulfate dressing as compared to those under the nitrate fertilizer dressing. It is concluded that some nutrients may play a chelating role in detoxifying the phytotoxins in soil, and consequently gives a better yield of crops.

Detoxification of phytotoxins by polymerization of humic substance. Many phytotoxic substances bind to clay minerals or other organic compounds, with a resulting decrease in phytotoxicity (9, 12). Wang et al. (13) found that protocatechuic acid, one of the phytotoxins related to trans *p*-coumaric acid, can be polymerized with humic acid by using clay minerals as heterogeneous catalysts. In fact, humic acid can polymerize many kinds of substances, such as amino acids, flavonoids, terpenoids, aliphatic acids, and other nitrogen containing compounds, thus keeping the soil in a fertile state. However, it is also possible that the polymerization of phytotoxic phenolics fixed in humic substances can be reversed under certain environmental conditions, with subsequent release of free phenolic compounds that will exert a phytotoxic effect on nearby susceptible plants. If this is the case, the natural device of organomineral complexing of humic acid would actually be a pool of detoxification of toxic substances produced by plants.

#### Allelopathy And Plant Adaptation

Allelopathy and autointoxication are involved in adaptation and evolution processes. Newman (41) doubts that allelopathy is an adaptation strategy; it may not be of benefit to the producer, simply because toxins can be both harmful to the producer and to other plants growing nearby. At this point, I can not fully agree with Newman's viewpoint. Some autointoxicating species can survive and adapt for many generations because the organism possesses adaptive autoinhibitors that limit the population to certain numbers, but do not destroy the species. We have evidence to show that rice plants produce phytotoxic substances that can reduce the productivity of the second crop yield in Taiwan; nevertheless, the rice plants are not killed by autoinhibitors. The autoinhibition of rice plants can have adaptive significance and is a good way to favor natural selection. For example, we found that both wild rice, *Oryza perennis*, and the associated community of *Leersia hexandra* have both autointoxication and allelopathic properties, and these species interact in the field. It is difficult to demonstrate which one exhibits the higher allelopathic potential and causes the death of the other, although *L. hexandra* sometimes showed higher phytotoxicity than *O. perennis* (22). The autointoxication of *O. sativa* as well as wild rice, *O. perennis*, and *L. hexandra* does not imply a harmful effect on the cell and tissue producing and containing toxic metabolites, but it will suppress sprouting of juveniles, the tillering stage, from the mother plant, seed germination, and seedling growth. The self-regulation of population density by self-thinning and phenotypic plasticity is an important adaptive mechanism of plants. There may be conditions in which natural selection works in the direction of reducing autointoxication and increasing allelopathic potential. The selective advantage of allelopathy would change according to nature of the coexisting plants and community structure, rendering the mode of selection diffuse and disruptive. This might have resulted in the complexity of response patterns observed in many studies (22). Other evidence also indicates that

allelopathic phenomena are more pronounced in the areas in which plants are under environmental stresses. This is also of particular importance to plant adaptation. Koeppel et al. (3) found that Helianthus annuus produces significantly higher amounts of phytotoxic substances, such as chlorogenic and neochlorogenic acids, under conditions of phosphorus deficiency than under normal conditions. The production of phytotoxins can be interpreted as an adaptation strategy to suppress the growth of competitors for nutrients. In addition, the plant produces high amounts of toxic alkaloids in low-nitrogen soils. This may be an adaptive strategy, i.e., to suppress the growth of adjacent plants and reduce the competition for nitrogen. Autointoxication, by itself, limits the population for survival. Many examples of autointoxication or allelopathy as described above demonstrate the fact that allelopathy is beneficial to the producer and has an adaptive significance. Allelopathy may also play an appreciable role in natural selection; thus many crops or natural vegetation can survive under different environmental regimes in time and space.

#### Acknowledgments

The author thanks Professors G. R. Waller and Otis C. Dermer for critically reviewing and editing this manuscript. This research was supported in part by the Academia Sinica, the National Science Council, and the Council of Agriculture of the Republic of China. The author is grateful to his former research assistants and graduate students for their diligent assistances.

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## Chapter 11

# Germination and Growth Inhibitors as Allelochemicals

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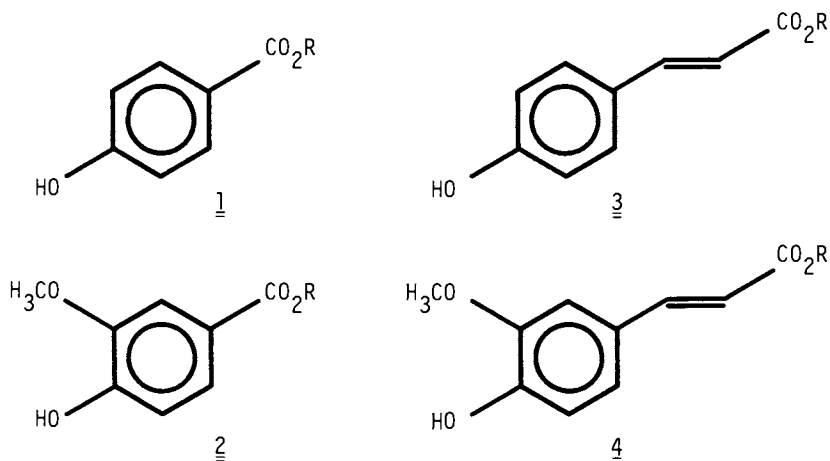
Germination inhibitors have been discussed for a long time as one of the reasons for dormancy of seeds. Some observations, however, point to the possibility that such inhibitors may also act as allelochemicals. When we tried to germinate oat caryopses we observed a decreasing rate of germination with increasing density of caryopses. Inhibition of germination could also be achieved with aqueous extracts from the caryopses (1). Interestingly, the inhibitory activity of oat caryopses is by no means restricted to germination of oats. We found that the seeds of several other plants (e.g. of *Raphanus* sp. or of *Amaranthus caudatus*) are much more sensitive against inhibitors from oat caryopses than oats itself. This means that germination of such seeds is effectively inhibited if they are in the immediate neighborhood of oat caryopses even if germination of oats itself can take place. Oats are more active in allelopathy than other cereals (2).

Such allelopathic activity is developed during maturation of oat plants. Whereas only little activity is found in roots, somewhat more is found in stem and leaves. The highest activity, however, is found in the caryopses during maturation, especially in the husks (3). In the first part of this paper, we shall describe isolation and structural elucidation of the main inhibitor from the husks of *Avena sativa*, which can be considered as an allelochemical against seeds of several plants. The second part of the paper deals with allelochemicals from rose seeds which, although of different chemical structure, bear some relationship to the allelochemical from oats.

The basis for this type of investigation is a quantitative bio-test as described in detail by Karl and Rüdiger (1). The crude aqueous extract from oat husks inhibits germination of seeds from several plants, e.g. *Avena sativa*, *Sorghum* sp., *Phalleris* sp., *Raphanus* sp., *Amaranthus caudatus*, *Lepidium sativum* L.. The inhibitory activity increases with increasing concentration of the extracts. Figure 1 shows the concentration dependency of inhibition of root growth after fractionation of the extract by ether extraction. Whereas less activity is found in the neutral fraction (pH 7), at pH 1 about equal activity is present in the ether layer and the water phase. The latter contains bioactive conjugates of organic

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acids; after alkaline saponification, the bioactivity is also extractable into ether after adjustment to pH 1. For further fractionation and search for active compounds, it is important that the bio-test be made quantitative as indicated in Figure 1. The main compounds of the acid fraction are phenolic acids. After methylation, the methyl esters were separated by gas chromatography and identified by comparison with authentic compounds, some of which are shown here (structures 1 - 4). Since these compounds (and the following ones) occur naturally as free carboxylic acids but are investigated as the methyl esters, the letter "R" is used in the structures for either "H" (natural compound) or "CH<sub>3</sub>" (investigated methyl ester).

Phenolic acids have already been discussed as inhibitory compounds. But inhibition of germination requires a concentration of  $10^{-3}$  to  $10^{-2}$ M phenolic acids. (1). Such high concentration may locally be achieved if oat straw is accumulated in the fields (4). This could sometimes be a reason for poor plant growth on fields after harvest of oats without removal of straw. We did not find such concentrations in our husk extracts, however; they were 100 - 1000 fold smaller than expected from bioactivity. Phenolic acids are therefore not the active compounds in our inhibitory extracts.

Consequently, we undertook to remove the phenolic compounds and retain the bioactivity as shown in Figure 2. The acid fraction was applied to a Sephadex LH-20 column and eluted with water. The bioactivity was eluted very early (Fraction A<sub>1</sub> and A<sub>2</sub>) whereas most phenolic compounds were eluted much later. The compounds of fractions A<sub>1</sub> (and A<sub>2</sub>, not shown here) were again investigated by gas chromatography/mass spectrometry. Their structures (5 - 13) are given here. The compounds are aliphatic carboxylic acids of the tri-carboxylic acid cycle and structurally related compounds. Their concentration in the original extract is at least one order of magnitude smaller than that of the phenolic acids. The aliphatic acids 5 - 13 do not inhibit germination in concentrations up to  $10^{-3}$  -  $10^{-2}$ M. The true inhibitor(s) must therefore be found amongst the many minor compounds of fraction A<sub>1</sub>. In another connection, we

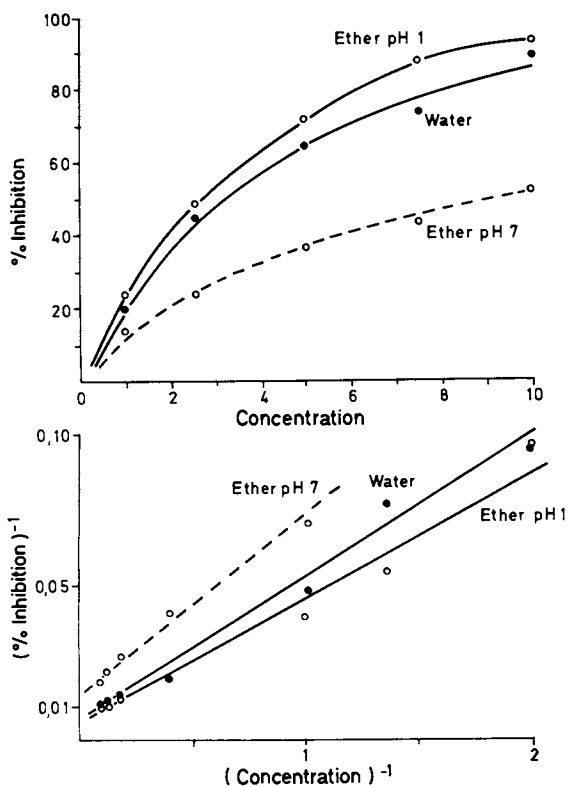


Figure 1. Inhibition of root growth of *Avena sativa* by the aqueous extract from oat husks after fractionation. The aqueous extract was extracted with ether first at pH 7 (o---o) and then at pH 1 (o-o); (●-●) the remaining water phase. Lower part: doubly reciprocal plot. (Reproduced with permission from reference 1. Copyright 1982 Verlag der Zeitschrift fur Naturforschung.)

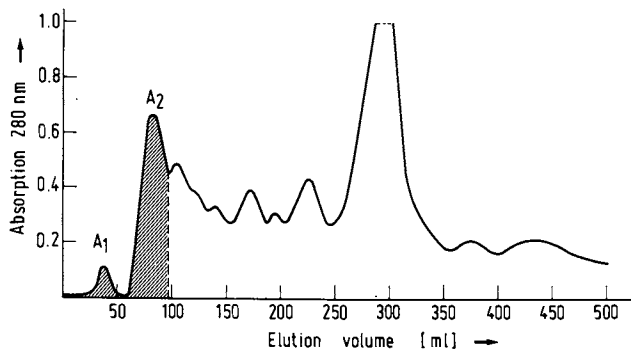
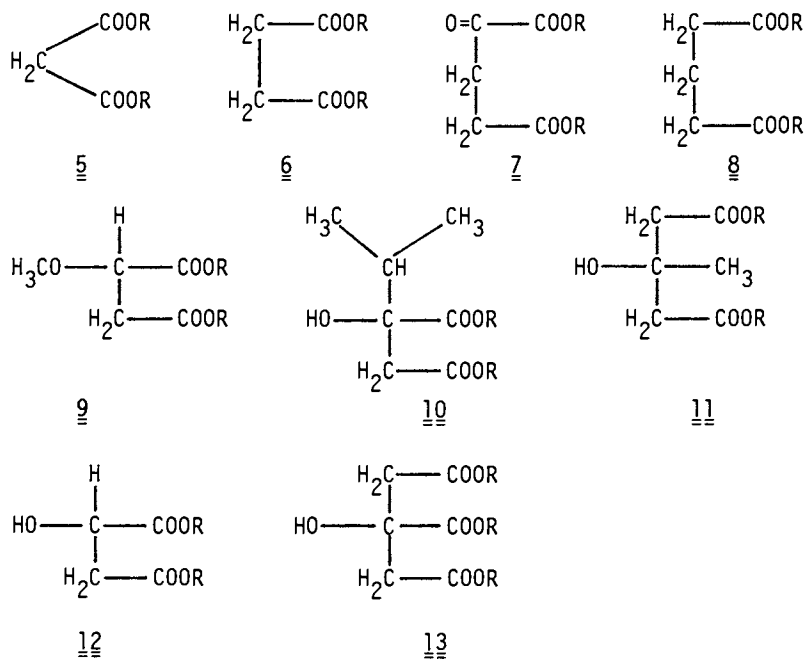


Figure 2. Column chromatography of the fraction ether pH 1 (see Figure 1) on Sephadex LH-20. Fractions with bioactivity (A<sub>1</sub> and A<sub>2</sub>) are hatched. (Reproduced with permission from reference 6. Copyright 1982 Verlag der Zeitschrift fur Naturforschung.)



studied minor compounds from the acid fraction of several cereals (5). We were able to identify 83 such compounds, but many remained unidentified. This analysis did not help in the identification of the inhibitor. Total analysis is not the best way to find bioactive compounds. The inhibitor was identified by a combination of further separation by column chromatography and quantitative biotest (6). Figure 3 shows the result of analysis of fractions 62-75 from the column chromatography by gas chromatography. The x axis is the retention time, the y axis is the fraction number of column chromatography. The vertical bars indicate the occurrence of peaks with a given retention time in the single fractions of column chromatography. The inhibitory activity was found only in fractions 68-72. The only peak that occurs exclusively in these fractions is the one with retention time 29.5 min (indicated as black bar). In Figure 4 the relationship between peak size (crosses) and magnitude of inhibitory activity (open circles) in the fractions 68 to 72 is given. The good correlation proved the identity of the compound causing this peak with the inhibitor even before the isolation of the compound. The approach is described here in some detail because it may be relevant for the solution of similar problems. Subsequently, the compound was isolated and investigated by mass spectrometry. The structure of the compound was derived from the fragmentation pattern of the protonated and the deuterated ester (6): it is pentane-1,3,4-tricarboxylic acid, or dihydrohematinic acid (14), which had not been known before as a natural compound. It had been obtained by chemical degradation of chlorophylls (7, 8) and by total synthesis (9). Total synthesis

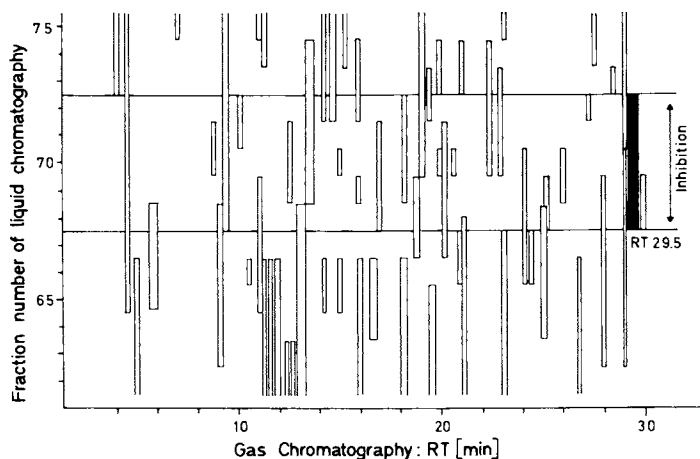


Figure 3. Analysis by gas chromatography (GC) of fractions 62-75 of the liquid chromatography. The only GC peak which occurs in all inhibitory fractions is that with retention time 29.5 min. (Reproduced with permission from reference 6. Copyright 1982 Verlag der Zeitschrift für Naturforschung.)

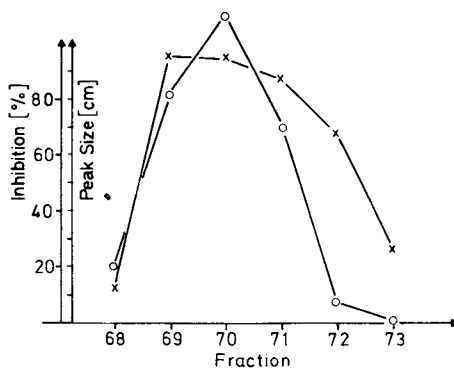


Figure 4. Correlation of seed germination inhibition (x-x) with GC peak size of compound RT = 29.5 min (o-o) of fractions 68-73 of the liquid chromatography (see Figure 3). (Reproduced with permission from reference 6. Copyright 1982 Verlag der Zeitschrift für Naturforschung.)

yields two pairs of diastereomers (14a, 14b). The natural compound from oats is the three isomer. We have not yet elucidated the absolute configuration of the natural compound. The synthetic racemate has a somewhat smaller activity than the natural compound.

During our synthetic approach, we found that several tricarboxylic acids have bioactivity similar to that of dihydroematinic acid. Some of the structures (15 - 17) and the corresponding activities (Table I) are given here. Essential is the presence of three carboxylic acid groups near each other and a certain chain length of the aliphatic residue. Recent investigations showed some inhibition of respiration by these tricarboxylic acids. Germination is possibly inhibited via this inhibition. Although these synthetic compounds are not natural allelochemicals they could be used for the same purpose, namely for inhibition of seed germination.

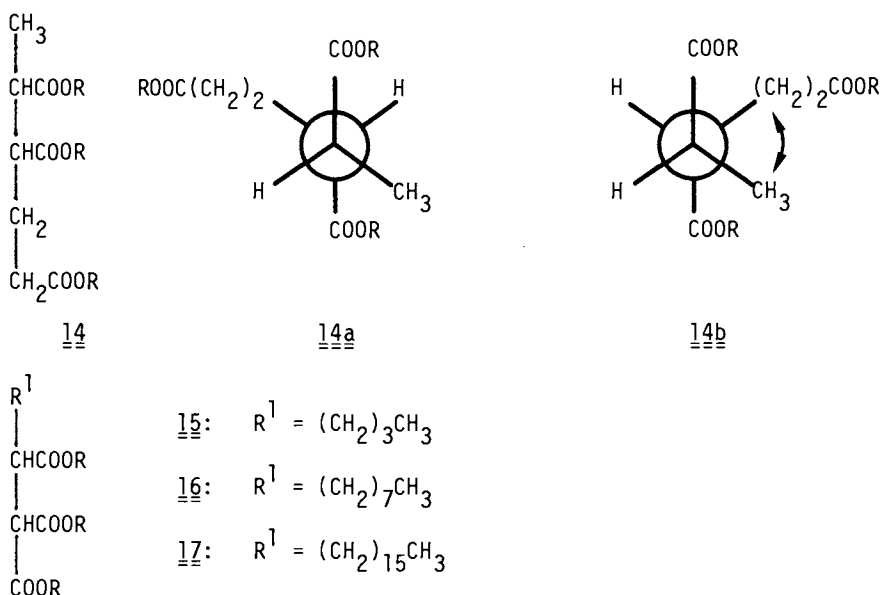
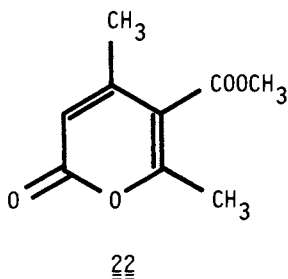
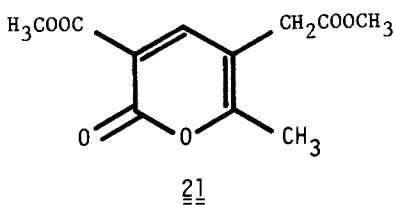
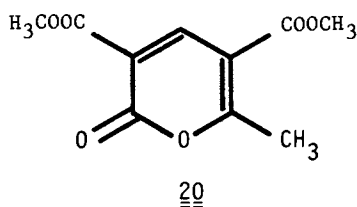
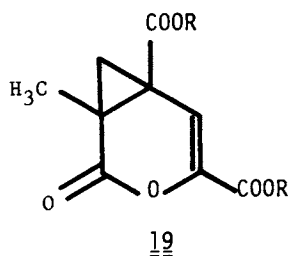
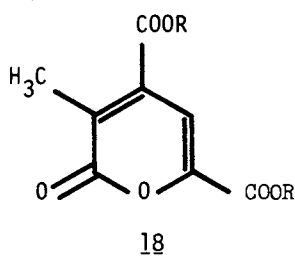


Table I. Inhibition of Germination of *Amaranthus caudatus* Seeds by Synthetic Tricarboxylic Acid Trimethyl Esters

Compound	% Inhibition of Germination	
	$10^{-4}$ M	$10^{-5}$ M
<u>14</u> a, b	100	54
<u>15</u>	100	20
<u>16</u>	100	50
<u>17</u>	5	0

The second example described here is dormant seeds from *Rosa canina*. Extracts of these seeds also inhibit germination of seeds of several plants (10). In Figure 5 a scheme is given for extraction and separation of three different inhibitor compounds. All these are present in the acid fraction. The first essential step is chromatography on Sephadex LH-20, which separates inhibitor I from inhibitor II and III. Inhibitor I was identified as abscisic acid. The other two inhibitors were separated by methylation with diazomethane, fractional distillation, and column chromatography. The second inhibitor is the  $\alpha$ -pyrone 18. Reaction with diazomethane transforms it into the bicyclic compound 19. This bicyclic compound is even more active than the parent  $\alpha$ -pyrone 18. Since we sought structural requirements for bioactivity here also, we tested several synthetic  $\alpha$ -pyrones (20 - 22) for bioactivity. These compounds had no inhibitory activity. We also tested the cyclopropane derivatives 23 and 24. In Table II, the bioactivity of the bicyclic compound 19 and two such derivatives is compared. The presence of several carboxylic acid groups seems to be essential (or at least helpful) for bioactivity in this case also.





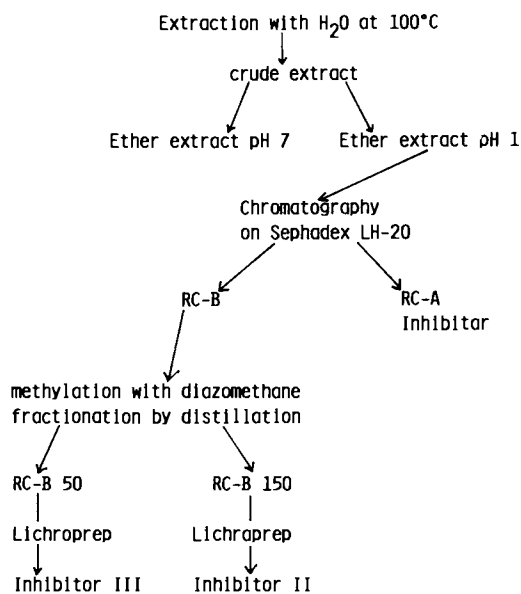
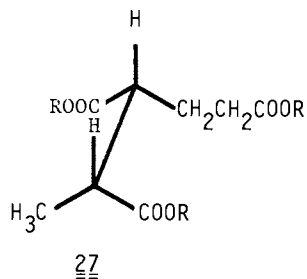
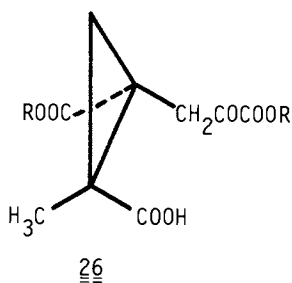
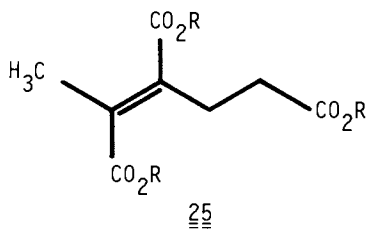
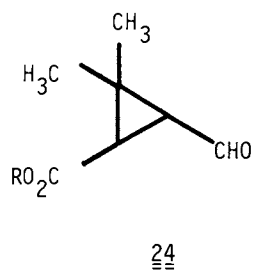
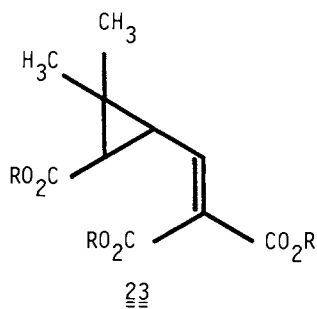


Figure 5. Scheme of isolation of germination inhibitors from hips of Rosa canina.

Table II. Inhibition of Germination of *Amaranthus caudatus* Seeds by Carboxylic Acid Methyl Esters

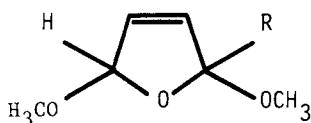
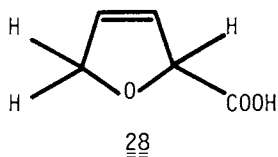
Compound	% Inhibition of Germination		
	$10^{-3}M$	$10^{-4}M$	$10^{-5}M$
<u>19</u>	100	100	30
<u>23</u>	100	90	10
<u>24</u>	90	40	10
<u>25</u>	100	90	50



(Product of ring opening of 19) (unfavored conformation of 14b)

The bioactivity is similar to that of isohematinic acid (25) (see Table II). We therefore raised the question whether there could be some closer relationship between the investigated compounds from roses and oats. Such relationship is indicated above. Opening of the lactone ring of 9 would lead to the tricarboxylic acid derivative 26. This has a structure like a certain conformation of dihydrohematinic acid (27). This conformation is certainly disfavored because of steric hindrance, but could exist at a binding site of an enzyme or a receptor. The presence of a cyclopropane ring as in 26 could stabilize such an otherwise unfavorable conformation that is eventually needed for high bioactivity.

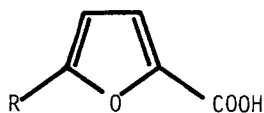
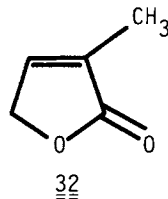
The last inhibitor from rose seeds was identified as 2,5-dihydrofuran-2-carboxylic acid (28). The specific activity of this compound is not very high, but rose seeds contain enough of it to exert measurable inhibition of seed germination. The bioactivity seems to depend on this particular structure because a number of similar compounds (29 - 37) have no activity or much lower activity than 28.



29: R = H

30: R = CH<sub>2</sub>NH<sub>2</sub>

31: R = CH(CH<sub>3</sub>)<sub>2</sub>

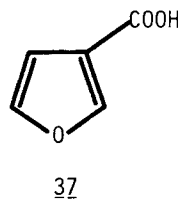


33: R = H

34: R = Br

35: R = NO<sub>2</sub>

36: R = CH<sub>3</sub>



The identification of natural germination inhibitors enables us to study their effect as allelochemicals in detail. Because most of these compounds are natural products not previously known, they have to be synthesized at first in order to obtain sufficient material for such investigations. This work is in progress.

#### Acknowledgments

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## Chapter 12

# Resistance of Cereal Crops to Aphids: Role of Allelochemicals

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Aphids cause important economic losses in cereals. Thus, development of resistant varieties is desirable. This paper describes the possible role of various natural chemical compounds in the resistance of barley, wheat, triticale and maize to the aphids *Schizaphis graminum*, *Rhopalosiphum padi* and *Metopolophium dirhodum*. Resistance of barley to aphids may be affected by the concentration of the alkaloid gramine. Conversely, increased susceptibility of barley seedlings grown under water stress is partially caused by accumulation of glycine-betaine in their leaves. Resistance of wheat, triticale and maize to these insects is mainly determined by the presence of hydroxamic acids.

Resistance of plants to herbivorous animals is determined in many cases by the presence in plant tissues of secondary metabolites that may cause feeding deterrence or toxicity to the animal. Insects cause damage to cereals, decreasing grain production. Development of varieties resistant to insects is then desirable for improving cereal productivity.

Resistance of barley to aphids has been reported to be influenced by the presence of several phenolic and flavonoid compounds (1). Gramine and related indole alkaloids cause toxicity in ruminants feeding on various Gramineae and Leguminosae (2,3). These alkaloids have also been reported to cause toxicity and feeding deterrence to aphids (4,5). Since these compounds also decrease palatability of fodder plants, it has been proposed to reduce the alkaloid content of various Gramineae by plant breeding (6). Resistance of Sorghum to the greenbug *Schizaphis graminum* appears to be affected by the degree of methylation of the intercellular pectin (7). Sorghum (8), barley (9), and other cereals have cyanogenic glucosides that may be important in plant protection. Dhurrin was identified as a feeding deterrent towards the greenbug (10). Dhurrin is located entirely in epidermal cells of Sorghum leaves (11). Hydroxamic acids may be important in determining the resistance of maize to the European corn borer *Ostrinia nubilalis* (12) and of wheat and maize to aphids (13 - 15). These compounds are found more concentrated in the vascular tissues than in other parts of the leaf (16).

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In this paper we summarize our work on the role of indole alkaloids and hydroxamic acids on the resistance of cereals to aphids. In addition, we describe the effects of water stress on susceptibility of barley to aphids.

### Results

#### Susceptibility to aphids and gramine and hydroxamic acid content.

Seedlings of several species of cereals were infested with aphids. At this time, the content of gramine and hydroxamic acids in the leaves was measured. Six days later aphid population growth rate was measured. Gramine was found only in barley while hydroxamic acids were present in wheat, triticale, and maize (Table I). Correlations were observed between resistance of barley to aphids and gramine content. Correlation coefficients between gramine content and population growth rate of *Rhopalosiphum padi* and *Metopolophium dirhodum* were -0.99, and -0.96, and -0.99, respectively. *R. padi* was more affected than *S. graminum* and *M. dirhodum* by the gramine content of leaves. Similarly, hydroxamic acid content of leaves of wheat, triticale and maize correlated with resistance to *S. graminum* and *M. dirhodum*.

Distribution of compounds in barley and wheat tissues. Tissues of barley and wheat leaves were mechanically separated under the microscope. It was observed that in barley gramine was more concentrated in the epidermis than in the entire leaf (Table II). Hydroxamic acids in wheat were absent in epidermic tissues and were more concentrated in the vascular tissues than in the entire leaf. Neither compound was detected in xylem exudates nor in guttation drops.

Biological activity of gramine and hydroxamic acids. Gramine decreased survival, feeding, and reproduction of aphids in artificial diets (Table III) at a concentration similar to those found in leaves of several barley cultivars. DIMBOA, 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (the main hydroxamic acid from wheat and maize), and its glucoside also showed deleterious effects on the aphids at concentration levels found in leaves of wheat and maize.

Effect of water stress on susceptibility of barley to aphids. Barley seedlings were subjected to water stress. These plants accumulated among other compounds proline and glycine-betaine (Table IV). Also, the more stressed plants were the most susceptible to aphids. The cultivar used in this experiment lacked gramine. However, when a gramine-containing cultivar was used gramine concentration was not affected by water stress.

To test if some stress compounds had beneficial effects on aphids proline, choline and glycine-betaine were incorporated into artificial diets. Proline and choline appeared to decrease survival of aphids, while glycine-betaine did not (Table V). Moreover, glycine-betaine caused a drastic increase in aphid reproduction. Thus, the increased susceptibility to aphids of water stressed plants may be partially due to the higher content of glycine-betaine.

Table I. Gramine and Hydroxamic Acid Content and Susceptibility of Various Cereals to Aphids

	Compounds (mmoles/kg fr wt)		Population Growth Rate (day <sup>-1</sup> )		
	Gramine	Hydroxamic Acid	Sg	Rp	Md
<i>Hordeum distichum</i> <sup>+</sup>					
cv F. Union	ND	ND	0.38	0.32	0.43
Brea "S" Celaya	1.2	ND	0.35	0.24	0.40
79 AN MN	1.7	ND	0.33	0.15	0.38
Abyssinian 5	2.9	ND	0.30	0.12	0.34
<i>Triticum aestivum</i> <sup>+</sup>					
cv Sonka	ND	0.6	0.44	-	-
Likay	ND	0.9	0.43	-	-
Cajeme	ND	1.5	0.24	-	-
Naofen	ND	1.5	0.24	-	-
<i>T. durum</i> <sup>+</sup>					
cv SNA-1	ND	1.8	0.21	-	-
<i>T. aestivum</i> <sup>++</sup>					
cv Huenufen	ND	0.5	-	-	0.35
Naofen	ND	1.2	-	-	0.31
<i>T. durum</i> <sup>++</sup>					
cv SNA-3	ND	3.1	-	-	0.23
<i>Triticale</i> <sup>++</sup>					
	ND	4.5	-	-	0.14
<i>Zea mays</i> <sup>++</sup>					
cv T125 L22	ND	7.3	-	-	all dead

The infestation was carried out on 10-day-old (+) or 7-day-old (++) seedlings in greenhouse-grown plants. After six days population growth rate was determined (growth rate =  $\ln(N_f/N_i)/\Delta t$ ). Sg, *Schizaphis graminum*; Rp, *Rhopalosiphum padi*; Md, *Metopolophium dirhodum*; ND, not detected.

Table II. Distribution of Gramine and Hydroxamic Acids in Barley and Wheat Tissues

Leaf Part	Compound (mmoles/kg fr wt)	
	Gramine	Hydroxamic Acids
Complete leaf	0.28	4.2
Veins	ND	7.0
Guttation drops	ND	ND
Xylem exudate	ND	ND
Epidermis	0.72	ND

Gramine was determined in barley (cv X81-T-1031) and hydroxamic acids in wheat (cv SNA-3) tissues. Plants were grown for 10 days in a greenhouse with a day-time temperature of 25°C and a night-time temperature of 16°C. Tissues were mechanically separated under a microscope.

Table III. Effects of Gramine, DIMBOA and DIMBOA-Glucoside on *Schizaphis graminum* feeding on artificial diets

Compound	Survival (%)	Aphids feeding (%)	Reproductive Index
None	100	60	4.0
Gramine	10	25	1.8
DIMBOA	15	0	3.0
DIMBOA-Glucoside	50	-	3.4

Survival after 24 h and the number of aphids feeding were determined at 4 mM compound in the diet. Reproductive index (number of nymphs/average number of adults) was determined at 0.15 mM compound in the diet. Values are the average of three samples of ten aphids each. For reproduction studies five samples were used. Standard errors were always less than 10%. DIMBOA: 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one.



Table IV. Effect of Water Stress in Barley Plants on Proline and Glycine-Betaine Content and Susceptibility of Barley to *Schizaphis graminum*

Water Potential (-bars)	Proline (mmoles/kg dry weight)	Betaine	Population Growth Rate (day <sup>-1</sup> )
6.3	5	57	0.36
10.6	11	79	0.39
15.5	25	126	0.43

Barley seeds (cv F. Union) were germinated at 25°C and after 6 days were subjected to water stress. Four days later, plants were analyzed for proline and betaine content and the water potential of the leaves was measured. At this time, plants were infested with aphids and the insects were counted six days later.

Table V. Effects of Proline, Choline and Glycine-Betaine on *Schizaphis graminum* Feeding on Artificial Diets

Compound	Survival (%)	Aphids Feeding (%)	Reproductive Index
None	93 ± 3	90 ± 4	2.45 ± 0.10
Proline	96 ± 6	57 ± 3	2.13 ± 0.13
Choline	72 ± 5	92 ± 3	2.00 ± 0.29
Glycine-Betaine	98 ± 3	74 ± 5	3.63 ± 0.11

Biological assays were performed with 6 mM compound in the diet. The reproductive index of aphids fed with 12 mM of glycine-betaine was 5.1 after 72 h of feeding. Values shown are the average (± standard error) of three samples of ten aphids each. Reproduction studies were performed with five samples.

### Discussion

Correlations between content of various compounds in cereals and population growth rate of aphids on the plants were found. In addition, these compounds cause deleterious effects on aphids on artificial diets at concentrations similar to those found in plants. Thus, it is possible that gramine and hydroxamic acids play a role in protecting barley and wheat, respectively, against aphids.

*S. graminum* feeds preferentially from phloem (17). Hydroxamic acids are more concentrated in the vascular bundles of wheat leaves than in other tissues. Moreover, these compounds are not present in

xylem exudates. Although the exact location of hydroxamic acids within the veins is unknown, it is likely that these compounds protect phloem against aphid feeding. Gramine was found preferentially in the epidermis of barley and was not detected in the veins. Thus, gramine is not found in the main feeding site (phloem) of the greenbug. Nonetheless, the feeding deterrent properties of gramine may be relevant to plant protection.

A variety of compounds may accumulate in plants under water stress (18, 19). One of these compounds, glycine-betaine, increased reproduction rates of aphids in artificial diets. It is likely that the observed increased susceptibility of barley to aphids may be due to glycine-betaine accumulation in barley leaves. It is possible that cultivars that accumulate preferentially proline and other amino acids instead of glycine-betaine under stress conditions may be more resistant to aphids.

### Experimental

Analyses of compounds. Plant tissues were homogenized in water, adjusted to pH 3, and centrifuged at 3500 g for 15 min. The supernatant fluid was extracted with diethyl ether (2:1 v/v, 2x) and the extract evaporated to dryness. This extract was used for quantitation of hydroxamic acids as previously described (14).

The samples for gramine analyses were frozen and macerated with 20 ml of MeOH: NH<sub>4</sub>OH (100:1 v/v). The extract was filtered through glass wool. Solvent and endogenous water were evaporated to dryness. The chlorophyllous residue was dissolved in 5 ml 0.1 M HCl, and the solution filtered (Whatman N°1 paper). The aqueous filtrate was adjusted to pH 9 with concentrated NH<sub>4</sub>OH and shaken twice with chloroform (1:2 v/v). The organic phase was evaporated to dryness. Gramine was quantified in these extracts by using Ehmann's reagent for indoles (20), as described previously (5).

Proline was quantified by the method of Bates *et al.* (21). Glycine-betaine was quantified by the method of Grieve and Grattan (22).

Feeding assays: Assays were performed with diets placed between two layers of Parafilm M (23). The diet was as described (24). When young aphids were used (survival and feeding deterrence assays) they were 3rd and 4th-instar nymphs.

Water stress treatment. Four-day-old seedlings were kept at 28°C (without irrigation for 48 h). Three groups of plants were then watered daily with different amounts of water for six days. Water potential of leaves was measured at this stage (25).

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## Chapter 13

# Exploring Allelochemistry in Aquatic Systems

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Allelochemistry in aquatic systems has been little studied. A secondary, opportunistic, form of activity resulting from the natural capacity of water as a carrier complicates studies in aquatic systems. Reflecting this complexity, experimental manipulations of apparent allelochemical relationships require unusual parameters of control, including control of inorganics to ppb levels and absolute partitioning of all organisms (including bacteria) in test systems. Comparisons of more than 200 pairs of algal species, all isolated from Linsley Lake, Connecticut, indicate that two-thirds of these pairs exhibit allelochemical activities. Since only autoclave-labile activity in cell-free filtrates was considered, and since much additional activity (not based solely on metabolites with these characteristics) was also noted, an ubiquitous presence and a pervasive influence of allelochemical activity on aquatic communities is postulated.

During the last several decades, as its potential for generating natural product-based pesticides emerged, the study of allelochemistry in terrestrial ecosystems has intensified. Yet, during the same period little effort has been invested in the pursuit of allelochemistry in aquatic ecosystems. In spite of this apparent neglect many field and laboratory examples have accumulated. A number of reviews are available; among the more extensive are those by Hartman, Lucas, Schwimmer and Schwimmer, Pourriot, Ruggieri, Keating, Maestrini and Bonin, and Provasoli and Carlucci (1-8).

This lack of development in aquatic allelochemistry appears an anachronism. It is not. Specifically reflecting the pervasive and peculiar effects of water, analysis of allelochemical events in aquatic systems has presented unique problems. Among the most significant are (a) the widespread and unpredictable occurrence of secondary activity, and (b) the difficulties of distinguishing between ultra-trace nutrient requirements and allelochemical effects. Both generate a need for unusually rigid experimental control.

The state of the art of aquatic organism culture has been inadequate to this challenge. Only recently (9) has the level of control been sufficient

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to insure that organisms, other than relatively few selected algae, (a) could be isolated and kept, while isolated, in good health, and (b) kept on a long-term basis in this state in defined, controlled, and repeatable experimental circumstances. Thus, only recently has it been possible to partition the dimensions of an aquatic niche to suit the demands of analysis of allelochemical events.

Primary allelochemical activity has essentially the same genesis and role in terrestrial and aquatic systems—offering an advantage to the producing organism which justifies the investment of resources in some active metabolite. In the aquatic environment, however, the water carrying the active substance circulates, randomly bathing the surfaces of many organisms other than the target. Some of these nontarget organisms respond to the active metabolite. This secondary response occurs only because the active substance happens to be there. It requires neither a competitive advantage for the producer, nor an association between the producer and target organisms. In fact a secondary target organism may benefit from an interaction that offers nothing to the producer.

The absence of a special relationship that generates some competitive or symbiotic advantage for the producer makes the possibilities for such secondary actions much more numerous, and much less likely to be recognized, than would be the possibilities for primary reactions. Thus, to assure the needed partitioning of, and the correct identification of, producer and target organisms, exertion of exceptionally tight control over experimental details becomes imperative.

Sometimes the active metabolic product of a planktonic organism is surprisingly familiar. One algal species, *Chlorella vulgaris*, manages to produce both hydrogen cyanide (10) and chlorellin (11, 12). Chlorellin, a toxin peculiar to this species, is a mixture of chlorophyllide derivatives (13). Undoubtedly, such toxins offer *Chlorella* an advantage in its competition for a niche. They also affect many organisms which in no sense compete with *Chlorella* for a niche or for any single dimension (e.g. nutrients, space) of a niche. Thus, this single organism offers at least two distinct types of allelochemically active metabolic products and each, in turn, offers a capacity for both primary and secondary actions.

This dichotomy of allelochemical activity is clearly evident among algal species that dominate the waters of Linsley Lake, North Branford, Connecticut. The natural programming of the series of algal species which dominate in the lake is greatly influenced by allelochemistry. In our laboratory tests the products of bloom-dominant blue-green algae (Cyanophyta) were either negative or neutral in their effects on predecessors and/or positive or neutral in their effects on successors (14). That is, they inhibited the algae they were replacing and left behind selective fertilizers when they, themselves, ceased to dominate. Comparisons of algal growth in waters collected before, during, and after blooms indicated that these waters produced positive, negative, and "neutral" effects in a pattern similar to those of the bloom-dominant blue-greens that had been prevalent. The "neutral" effects probably indicate that we were not testing enough parameters, or were not properly handling the metabolic materials.

In Linsley Lake this pattern persisted for at least 5 years. In a sense dominants selected their successors. This, however, was a secondary action. No value to the producer is required to justify its existence. The producer would no longer be present when the allelochemistry is in action. As a dominant weakened, it would make little difference to its survival as a species, or to its reoccur-

rence a year later, during the next annual bloom sequence, what organism was favored sufficiently to dominate immediately after it in the current year. If some dominant left behind a product damaging to the organism that dominated next, that "next" organism would cease to be next. Some other of the hundreds of candidates would replace it in the annual bloom sequence. Thus, it would be most unlikely that a producer would negatively affect its successor. On the other hand, promoting its own replacement is also not a reasonable evolutionary basis in an organism for the selection of a trait that results in production of metabolically active extracellular materials. When a producer releases metabolites that selectively favor successor-dominants, this benefit results from a successor's opportunistic use of some metabolite produced for another purpose. It is likely that the general, justifying, basis for the production of such active metabolites is in the widespread metabolite-based interference with those organisms with that producers do actively compete for dominance. Once this variety of metabolic material is present in the water, any possible successor-dominant which could use the material to its advantage would be favored.

### Methods and Materials

Complete methodology for algal experiments (15) and for all zooplankton/algal experiments (9) are available elsewhere.

### Results and Discussion

Several hundred algal pairs were tested for allelochemical activity and fully two-thirds (Table I) showed activity. In most tests algae were isolates from Linsley Lake. Except as noted in Table I tests were limited to autoclave-labile allelopathic materials carried in the cell-free filtrates of bacteria-free cultures of producer-algae. This usually translated into lability to a sudden increase in temperature, pH, or both. Most of our algae produced effects on most of our algae. Yet, no effects were inappropriate to the natural sequence of bloom dominance in the lake. In addition to the effects tallied in Table I, a variety of additional effects of the same cell-free filtrates was observed. Because so many instances of allelopathy were readily demonstrated, detailed study was restricted. Heat/pH-labile materials were selected for in-depth study including concentration, isolation, and partial identification of substances produced by dominant algal species (15). Study of materials related to remaining allelochemical events was limited to confirmation that the activity in the filtrate was tied to the presence of dissolved organic material that could be removed by activated charcoal. Since algal cultures were bacteria-free and unialgal, and were always in inorganic media, all organics in filtrates of those cultures could be identified as having been produced by the algae.

It is, specifically, the sheer number of instances of allelochemical activity in these studies which militates against drawing too close a parallel between the roles this phenomenon plays in terrestrial and aquatic systems. In contrasting the significance of allelochemistry to aquatic systems with its significance to terrestrial systems, it is especially constructive to take into account the absence of the need for protection against desiccation. This permits an exposure of action sites on the cell surfaces of aquatic organisms that would be destructive to organisms exposed to the drying effects of even excep-

**Table I. Allelopathic Effects of Cell-Free Filtrates of Axenic Cultures of Dominant Algae on Nondominant Algae**

(Number of species with +, -, o responses)

	DIATOMS			BLUE-GRNS			GREENS			MOTILE			TOTAL SPECIES TESTED
	+	-	o	+	-	o	+	-	o	+	-	o	
<b>PRODUCER</b>													
<u>Oscillatoria aghardi*</u>	0	5	3	5	0	3	2	0	1	6	1	3	29
<u>Oscillatoria rubescens*</u>	0	6	0	3	3	2	2	1	0	3	1	6	27
<u>Anabaena</u> sp.	0	29	0	3	3	2	3	2	8	-	-	-	50
<u>Pseudanabaena galeata</u>	0	7	1	1	4	3	7	2	0	0	2	2	29
<u>Oscillatoria</u> sp.	0	5	1	1	4	3	0	5	1	1	2	3	26
<u>Synechococcus</u> sp.	0	0	9	4	4	0	3	2	2	3	2	0	29
<u>Aphanizomenon flos-aquae</u>	0	4	3	3	2	3	3	0	3	1	0	4	26
<u>Anabaena</u> sp.	1	0	2	3	5	0	-	-	-	-	-	-	11
<u>Nostoc muscorum**</u>	0	4	4	4	1	3	0	1	2	0	1	2	22
<u>Nostoc</u> sp.**	0	3	5	-	-	-	-	-	-	-	-	-	9
<b>TOTALS</b>	1	63	28	27	26	19	20	13	17	14	9	20	257

\*Not axenic

\*\*Not isolated from Linsley Lake

tionally humid ambient air. The capacity of water as a solute/carrier and the common cell surface characteristics of aquatic organisms offer far more opportunity for a form of allelochemical activity which, due to its arbitrary pairing of producer and target organisms, is more appropriately characterized as secondary than as primary.

There are reports of algae releasing as much as 90% of their total photosynthetic product into the water (3-8). A great deal of energy is invested in those complex molecules. If these were of no value, such incredibly inefficient organisms could not dominate a community. Since they do, there is a value and the trait is fixed in evolutionary terms. This is the basis for primary allelochemical activity.

While direct competitions with other algae for niche allocation must be included as explanations for some primary allelochemical activity, there are other benefits to be considered. Algae are the prey, the food, of zooplankters of the second trophic level. If a particular algal species could produce a metabolite that made the zooplankters sick (indications: sluggish movement of feeding and/or swimming appendages; gut passage of nondigested algal material; loss of fecundity; loss of color; assumption of irregular swimming position), it would certainly provide a survival advantage--primary allelochemistry. This would also assist other phytoplankters, direct competitors. Thus, it would carry a price, but the *net* result would be beneficial to the producer. The potential interactive pattern is enormously complex. Coevolution, planktonic forms (both phytoplankton and zooplankton) constantly responding to the metabolites of other planktonic forms, must be considered as one of the basic themes of aquatic systems. The ties that impose that coevolution would be allelochemical.

After observing how intricately the pattern of allelochemical actions was woven, and taking into account the amount and variety of bioactive material dissolved in ambient waters, we decided to develop an *in vitro* demonstration of the action of phytoplankton metabolites on zooplankters.

Some activities were obvious. Since the algae are the basic food of the animals, there were positive effects. Any trace organic-based nutritional value could be an example--vitamins, coenzymes, etc. Even the provision of ordinary calories would be a legitimate "positive activity" although in no sense an allelochemical one.

Also, several algae have been shown to be extremely toxic to just about any animal on which they were tested (4). The most thoroughly studied of these are the toxins of blue-greens (16), some of which (17) can kill a cow in minutes.

We sought something less dramatic. The algal producer need not be a usual prey of the zooplankter. Some algal species always seem to prosper when the zooplankters are in trouble. The blue-greens, in particular, have often been observed to dominate waters in which there are few, or no, zooplankters. They have been consistently shown to be poor foods not only for zooplankters, but also for a variety of other eukaryotic organisms (1-8).

Algal extracellular products, present in great quantities during blooms, are of interest. To study such interactions highly controlled zooplankton cultures in which all the possible producers could be identified would be essential. That is, random microbial infections in cultures would be unacceptable.



Complex exogenous organics could also not be allowed. These requirements almost ended our studies. This was because, although there are hundreds, perhaps thousands, of published papers concerning zooplankton culture, there were no prior examples of controlled zooplankton culture from which both all bacteria and all exogenous complex organics were excluded. The latter guaranteed that there were none in which inorganic trace inclusions were controlled, because even a diet comprised of carefully selected pure proteins and lipids suffers from the inevitable contamination that accompanies commercial proteins and lipids. All that is needed is a few sulfhydryl groups and substitutions will occur.

In fact, with the single exception of some very special work by Provasoli and several students (18-21) in extremely high-organic media, the only successful long-term cultures of zooplankters were in lake, well, or tap water with mixed microbes. All were useless for our studies of allelochemistry.

It took five years to develop the cultures we needed. We refer to the final product of our efforts as the "MS" Cladoceran maintenance system (9). All media, both food and animal versions, are essentially the same, Table II. The inevitable contaminants carried into animal cultures by organic nutritional components (plant, animal, protein, lipid, peptone, etc.), which have plagued nutritionists for years, were by-passed by producing food algae in 100% inorganic media. The algae produce everything organic that the animals need, excepting vitamin B<sub>12</sub>. Since the organics made by those algae cannot be contaminated with inorganics that are not present in algal growth media, inorganic contamination can be limited to the level of the purest available inorganic salts.

The MS system supports an extensive variety not only of Cladocera, but also of other aquatic animals from several trophic levels. It is also, literally, the only extant system for maintaining permanent, healthy, cultures of zooplankters in *defined* circumstances. Unfortunately for its ultimate purpose (the study of allelochemistry between the first and second trophic levels), the system has two less than ideal characteristics. The first concerns the concentrations of several inorganics, especially copper, molybdenum, and calcium, which we consider higher than desirable. The second is the possibility that the algae which serve as food for our animals might also produce allelochemicals, not only directly, but also indirectly by stimulating animals or other algae into production.

Our recent efforts have been directed at refining the system and, as these problems are addressed, allelochemical interference repeatedly imposes itself. Initially, our extreme control of inorganics produced a disaster. Animals were falling apart, losing their major swimming appendages. In some ways they were quite healthy, producing larger broods than had been reported in the literature; however, they showed the deteriorated and ill-formed cuticle (thus the appearance) of old age in quite young adults. The problem could have been an infection—bacteria or fungus feeding on components of the cuticle, weakening its structure. It could have been a deficiency that interfered with the biosynthesis of critical structural components of the cuticle. It could have been allelochemicals generating either damage to the finished structure of the cuticle or interference with its formation in the first place. The more diatom (a food alga) added, the

Table II. Composition of MS Media

	A-MS Algal Medium	MS Animal Medium
<b>*M* COMPONENTS*</b>		
Disodium EDTA	5 ppm	5 ppm
B ( $H_3BO_3$ )	1000 ppb	1000 ppb
Fe ( $FeCl_3$ )	400 ppb	400 ppb
Mn ( $MnCl_2 \cdot 4H_2O$ )	200 ppb	200 ppb
Li (LiCl)	100 ppb	100 ppb
Rb (RbCl)	100 ppb	100 ppb
Sr ( $SrCl_2 \cdot 6H_2O$ )	100 ppb	100 ppb
Br (NaBr)	50 ppb	50 ppb
Mo ( $Na_2MoO_4 \cdot 2H_2O$ )	50 ppb	50 ppb
Cu ( $CuCl_2 \cdot 2H_2O$ )	25 ppb	25 ppb
Zn ( $ZnCl_2$ )	25 ppb	25 ppb
Co ( $CoCl_2 \cdot 6H_2O$ )	5 ppb	5 ppb
I (KI)	5 ppb	5 ppb
Se ( $SeO_2$ )	2 ppb	2 ppb
V ( $NH_4VO_3$ )	0.5 ppb	0.5 ppb
<b>*S* COMPONENTS**</b>		
Glycylglycine	250 ppm	250 ppm
$NaNO_3$	120 ppm	50 ppm
$CaCl_2 \cdot 2H_2O$	38 ppm	38 ppm
$MgSO_4 \cdot 7H_2O$	20 ppm	20 ppm
$Na_2SiO_3 \cdot 9H_2O$	145 ppm	10 ppm
KCl	10 ppm	10 ppm
$K_2HPO_4 \cdot 3H_2O$	10 ppm	10 ppm
$KH_2PO_4$	25 ppm	10 ppm
<b>VITAMINS***</b>		
Thiamine (HCl)	75 ppb	
Biotin	0.75 ppb	
$B_{12}$	0.75 ppb	1 ppb

\*Target ion or element is listed first. Compound employed in solution is in parentheses. Concentration is for ion or element.

\*\*Compound employed in solution is listed. Concentration is for whole compound.

\*\*\*Vitamin  $B_{12}$  should not be included in *Chlamydomonas reinhardtii* cultures. All vitamins can be omitted from such cultures.

more trouble introduced. This was *prima facie* indication of allelochemistry. If a metabolite were involved, it should have been possible to locate it in either the cells or the cell-free filtrates of food cultures.

Damage appeared proportional to the amount of diatom present in cultures. Yet, when the diatom was withdrawn from the animal's diet, the damage, though less pronounced, persisted. Thus, it could not have been the diatom alone that generated the problem. It was clearly demonstrable (22) that the diatom did cause part of the trouble. This was initially interpreted as indicating that more than one of the food algae was producing an undesirable material. We sought to isolate that material, but were unsuccessful.

The basis for this problem proved to be a selenium deficiency (23). The addition of  $1 \times 10^{-4}$  mg/L eliminated overt cuticle deterioration in the first generation. Ultimately, it was determined that a minimum of  $2 \times 10^{-3}$  mg/L of selenium in culture media (9) is necessary to avoid selenium deficiency in the animals tested in the MS system with exogenous organics excluded. Prior to this time no certain information had been published concerning specific requirements for inorganic materials essential to zooplankters. Therefore, when media were originally formulated, reasonable suppositions concerning nutritional requirements, based on nonspecific nutritional information relating directly to Cladocera, or on specific information relating to other organisms, including mammals, were made. Since a requirement for selenium had never been suggested for organisms even remotely related to the Cladocera; it had not been included in the original formulations. It was, therefore, available at critically low concentrations. That damage observed to be proportional to the amount of diatom present in cultures was the result of the food diatom taking up the last traces of selenium that had been carried into the culture system as contamination. More diatom simply meant a greater depletion of the already deficient trace element. There was no allelochemistry involved. The selenium was apparently being sequestered in a part of the alga that was not digested. In fact, its presence or absence did not initially affect diatom reproduction as measured by cell number although color and longevity of algal cultures were both diminished.

It is important that this problem be recognized as an ultra-trace inorganic nutritional problem masquerading as an allelochemical one because this reinforces the demand for exceptional control of both inorganic and organic incorporations into test systems. There are natural waters, for instance those of Lake Superior, which are sufficiently low in selenium to restrict zooplankton reproduction (24). Thus, the absence of secure information concerning minimum requirements for inorganic trace nutrients can interfere with accurate interpretation not only of laboratory results, but also of events in natural settings.

In short, a good food, one that is a source of desirable organics and useful calories (25), limits growth and shortens life span in proportion to its presence--this presents the appearance of allelochemistry; however, it is the exaggeration of a deficiency which proportionally increases. It is not the production of some allelochemical substance that proportionally increases. Like it or not, in aquatic systems the interplay of allelochemistry and nutrition can *not* be ignored.

Diatoms also have their nutritional problems. Many blue-greens interfere with diatom growth simply by interfering with their uptake of silica, which

happens to be singularly critical to diatoms (26). Since blue-greens give no indication of a nutritional requirement for silica, this does not represent direct competition for a nutrient. However, sequestering so critical a nutrient in a manner that makes it difficult for diatoms to satisfy their needs is a useful way to inhibit diatom populations (14, 27)--with which blue-greens do compete for just about every other nutrient. This is a relatively uncomplicated example of primary allelochemistry. When the blue-greens produce a bloom population, diatom numbers are greatly reduced. This does not help the zooplankton of the second trophic level. Although they might benefit by eliminating diatoms in that uncommon natural system in which selenium were somewhat deficient, generally, the animals lose the food value of the diatoms, and do very poorly on the blue-greens as food.

Blue-greens have also long been suspected to produce allelochemical substances that interfere with zooplankton physiology (4,5,7,8,15). When blue-greens bloom, they release a variety of allelochemical substances into the water. Some sequester nutrients (25), some act as toxins (4,15,16). All must serve some purpose for their producers. These metabolic products of blue-greens interfere with the normal growth and development of many other species. It is not surprising that once entrenched, blue-greens are difficult to eliminate.

Diatoms and blue-greens are not the only algae that cause trouble under the right circumstances. Even our most desirable food algae produce negative effects. We usually feed three algae (*Nitzschia frustulum*, *Chlamydomonas reinhardtii*, and *Ankistrodesmus convolutus*) to our zooplankters, being careful to feed from the log phase which offers high protein (28) and the likelihood of low allelochemical complications.

*Chlamydomonas reinhardtii* has been included in our standard diet for several years because it is generally regarded as a desirable food (18,29-32). Two years ago, when the supply of food algae was less than adequate, cells were harvested from cultures that were about a week and a half older than usual. It was soon clear that, as the *C. reinhardtii* cultures got older, the animals fed from these cultures got sicker. Animals fared better if they went hungry. In some cases, the *C. reinhardtii* actually killed them.

The same "desirable" food species, *C. reinhardtii*, introduces another problem in animal cultures. When *C. reinhardtii* is reared in algal A-MS medium containing vitamin B<sub>12</sub>, a diet containing relatively young such cells produces a mild negative effect on animals. Progeny of older mothers fed this diet show a pronounced Lansing effect--a loss of fecundity of progeny which is directly proportional to the mother's age at the time progeny are born (33, 34). Since there is no Lansing effect whatsoever when animals are fed a diet that includes the same clone of *C. reinhardtii* reared in the same A-MS medium excepting that the A-MS contains no B<sub>12</sub>, it appears to be the algal response to the presence of ambient B<sub>12</sub> that changes this desirable food organism into a self-protecting, allelochemical-producing, "prey" of the animals.

At this time we speculate that *C. reinhardtii* (a facultative producer/user of B<sub>12</sub>), when reared in the presence of exogenous B<sub>12</sub>, would produce a B<sub>12</sub>-binder (35). To date effort has not been invested in isolation of the binder; however, in controlled cultures in which both *C. reinhardtii* and any of several other food algal species are reared, *C. reinhardtii* produces the same growth

curve while the second species is grossly inhibited. Direct competition for available nutrients can not satisfactorily explain the absence of growth (reproduction) for the second alga. For example, simultaneous inoculation of *C. reinhardtii* and the diatom, *Nitzschia frustulum*, into a dual-algal culture results in *C. reinhardtii* growth quite similar to that of an uni-algal culture, but little perceivable growth for the diatom initially and elimination (death) of the diatom within two to three weeks.

When these algae are transferred into animal cultures (as food), it is suggested that the binder would interfere with the animal's utilization of ambient  $B_{12}$  in a manner that inhibits the development of reproductively viable progeny. If correct, this could be interpreted in natural circumstances as either primary or secondary allelochemical activity (or both). It is primary in that the production of a  $B_{12}$ -binder would offer a variety of advantages in terms of *C. reinhardtii*'s competition with other algae for occupancy of its niche and secondary in that the alga's capacity to thwart predators is incidentally enhanced. It is, however, important to consider that the speculated interference with the predator would also offer protection to other, competing, algal species.

To emphasize the complexity of this phenomenon, it must be noted that while additional exogenous  $B_{12}$  might help some of *C. reinhardtii*'s algal competitors, and it appears in preliminary tests to improve the animal's condition, as little as 0.5  $\mu\text{g}$  of additional ambient  $B_{12}$  (150% of "optimum") introduces a consistent drop in animal reproduction in some circumstances (35). This complexity of allelochemical interferences that desirable food algae introduce underwrites our belief that exceptional levels of control must be established prior to study of allelochemistry at the interface of the first and second trophic levels.

What it all means is hard to say. It is certain that, without exception, a community in a freshwater system offers an incredible number of interacting allelochemical phenomena all at once, all of the time. Every organism is affected, not just by predators and foods, but by every other organism which releases metabolites in some form into the water in which they all dwell. When we take the concepts of terrestrial ecology and impose them on aquatic systems, we lose a lot of this allelochemical local color.

Algae release just about any substance they make, whether useful to the producer or not, into the water. They have a storage problem, so they dump. With that array of plausibly active metabolic material in the water, allelochemical events of the primary sort are easy to find simply by looking for useful effects and seeking out the metabolites involved. Examples of secondary allelochemistry, while plentiful, are a bit more difficult to recognize since they need not tie the producer and target organism together by a logical association.

Allelochemistry is so pervasive in aquatic systems that in our laboratory, even when we specifically try to avoid it, we find it wherever we look. Our greatest problem is sorting it out.

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## Chapter 14

# Allelopathy: A Biotechnological-Agrochemical Approach

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In recent years, the biological activity of allelochemicals in plants has been demonstrated mainly by horticulturists, ecologists and biologists. ARCO Plant Cell Research Institute views these allelochemicals as a potential source of new types of natural biological agents. With the special emphasis on plant-plant, plant-fungi and plant-bacteria interactions we have initiated an in-house program with substantive dependence on our chemists now, and our biotechnology group later. In this report we will emphasize the scientific methods and resources utilized, and our interactions with the university community.

Allelopathy has caught the imagination of scientists throughout the world. Large industries are now contributing to its progress by supporting not only research in this area, but also the forum for exchanging the results. In this report I will offer insights on the involvement of our Plant Cell Research Institute - as an industry - in allelopathy, and our approach to this emerging science.

### The biotechnological-agrochemical approach

At ARCO's Plant Cell Research Institute our approach to allelopathy may be outlined as follows: Firstly, a survey is made of the biological and chemical properties of plants from which is selected those which might be usefully modified by addition of a foreign gene. The approach then requires us to develop scientific evidence that the gene carries the desired property. This gene from the source organism is then cloned and characterized to permit construction of a transferable recombinant form, which will be expressed at a satisfactory level in response to an appropriate signal, and direct the protein to the correct subcellular compartment. If the preceding is successful it is then necessary to construct a recombinant form and insert it into a transfer vehicle. Finally, one would expose the plant or plant cells to the transferable form in the transfer vehicle, select cells of plants with the transferred gene, and then, if necessary, regenerate the plants.

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In brief, it begins with establishing the biochemical basis for the interaction and climaxes when the desired trait is inserted in the genetic system of a selected plant, which formerly helpless is now equipped with a means of providing allelochemicals for its own defense. The project is undoubtedly very ambitious. To be fruitful it will depend on the ability of the molecular biologists to successfully engineer the appropriate gene into a selected crop plant. More immediately, however, our plan involves the combined efforts in ecology and natural products chemistry.

The survey by scientists in these latter fields must identify allelochemicals which effect a desired change in an agricultural system. In addition, the biochemistry of the compounds must be explored to provide suggestions for a causal relationship between increased amounts of the compound and increased levels of the allelopathic effect. The next phase of investigating the biosynthetic route to the target molecule to assess the feasibility of increasing the target by gene transfer is very critical and may be complicated by various factors.

- a. The target molecule may be the product of a single gene. This is the simplest and the dream of the molecular biologists.
- b. The target molecule may be the product of an endogenous pathway, where one enzyme represents the rate-limiting step in the pathway leading to the target molecule. Here the objective would be to transfer the gene controlling the level of that rate-limiting enzyme.
- c. A proximate precursor could be produced by an endogenous pathway. Here the target plant lacks a key enzyme which has been identified and which upon incorporation by the appropriate gene transfer should convert the useful precursor into desired allelochemicals.
- d. The most difficult case exists when the target molecule is the product of multiple genes, all of which would need to be transferred to the recipient plant to achieve production of the end product. This is the least feasible case, unless the genes are physically linked on one large piece of DNA and may be transferred simultaneously.

Further stages in the bioengineering feat require the tools of molecular biology, genetics, and tissue culture. Problems in cloning, much of which are due to technical barriers involving low levels of mRNA and complex ploidy which defies standard genetic analysis, have to be studied and overcome. Developing the gene transfer and plant regeneration system remains an art, in which each recipient plant system requires individual attention in attempts to regenerate it. In fact, we can predict that in some cases the inability to regenerate a plant, from say protoplasts, may be the single obstacle to achieving our goals. Nevertheless, until our long-term goals of appropriate gene transfer are realized, the allelochemicals isolated could yield new agrochemicals which may provide intermediate solutions to our agricultural problems.

PCRI's present organizational structure includes groups of Natural Products Chemistry, Genetics and Tissue Culture, and Molecular Biology. Together with a flux of visiting scientists, postdoctoral associates, and summer interns, it affords a completely integrated approach to solving our problems. Much of the results of our efforts will find outlets through ARCO Seed, our sister facility.



### Our concept of allelopathy

ARCO's concept of allelopathy has been reflected many times, from Molisch (1) through Rice (2), Putnam (3), and Thompson (4). It is probably most graphically summarized by the representation of Putnam (3) (Figure 1). Our ultimate goal is to engineer plants to produce their own defense chemicals. We believe this is a natural pathway to achieve our goals and to hasten the passage from discovery to development (sales) of an agrochemical (5) or agrochemical commodity (crops, resistant seeds, shoots, etc.).

The use of allelochemicals may overcome one of many long-term drawbacks of synthetic agrochemicals, namely the unnatural excessive application of these chemicals. Such an application can be counterproductive at times, in that the process of exogenously applying a high selection pressure of chemicals can cause rapid selection for organisms resistant to these agrochemicals, and thereby defeat the objective. However, such interim measures are, at times, necessary.

Over the long term, the resources of ecologists, natural products chemists, biochemists, biologists, and molecular biologists must be coordinated to meet the individual goals of:

- a. characterizing novel structures leading to new herbicides, pesticides, etc.,
- b. identifying old structures with novel applications,
- c. developing and enhancing allelopathic properties of agronomic species, and
- d. developing a genetic engineering approach for moving a controlling gene into agronomic crops for their own protection.

The commercial benefits. This biotechnological approach to use allelopathy is natural and has ingredients for success. Two targets for this approach are velvet leaf (*Arbutilon theophrasti*, Medic) and *Bacillus thuringiensis* (6). Velvet leaf caused a half billion dollars in economic loss in 1982 (7,8). The basis of activity and the allelopathic potential of this plant must be identified as chemical or biological and harnessed.

The second example, the selective effects of BT toxin on lepidopteran larvae and its use as a commercial product, is well known (9). However, the endogenous incorporation of the genes for expressing this toxin (10) into a crop plant would obviate the wasteful topical application of the commercial product. This bioengineering feat of transferring the allelopathic defense mechanism of one type of organism (bacteria) to another (plants) would be a great scientific achievement and commercial success.

Despite this preferred approach, one method being developed by large agrochemical manufacturers extends the tools of biotechnology to augment their trade in synthetic herbicides and pesticides. In the case of synthetic agrochemicals that affect fundamental biopathways of plant metabolites, the direction is to establish, through molecular biology and tissue culture, crop plants that are resistant to these commercially successful herbicides. The idea is that once the users (the farmers) of these agrochemicals have access to these resistant crop plants they can apply the agrochemicals with less reserve, thereby enhancing the attractiveness and sale of the resistant crops and the agrochemicals. The commercial future of these commodities are thereby insured.

At ARCO, we wish not only to employ this manner of protecting plants against artificially obtained chemicals, but also to provide such plants with their own defense against living organisms. We also hope that our plant-microorganism studies will supply us with effective controlling agents by way of mycotoxins or phytotoxins, which can be commercialized as effective agrochemicals.

### PCRI's efforts

Our studies at PCRI are preliminary, but are supplemented through our relationships with Dr. Alan Putnam of Michigan State University, and with former colleagues at the University of the West Indies and other universities. These relationships expand PCRI's efforts, and establish an interface between university and industry, which allows reciprocal movement of personal and technological resources through a process that hastens the achieving of our combined goals.

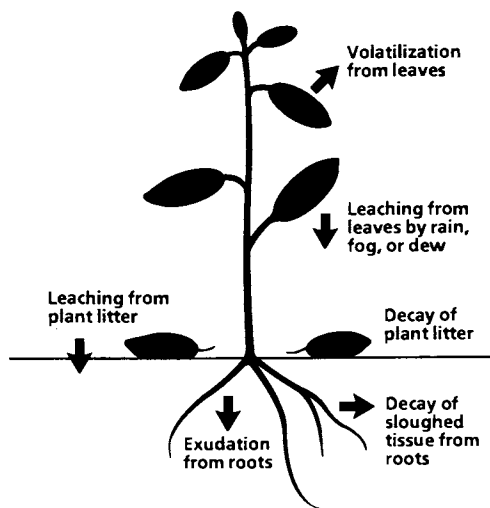
Of the several plants with reported allelopathic activity that we at PCRI have examined, none has yet yielded dramatic results. A bioassay-directed approach has yielded modest biological activity which seems to lessen or disappear in pursuing a purified product. This latter accentuates the difficulties of pursuing allelopathic chemicals, the activity of which are due to additive and or synergistic effects of the compounds involved. They must be meticulously studied.

One plant investigated was *Kalanchoë daigremontiana*. This has been repeatedly investigated by others, primarily in search of answers to its asexual reproduction on the leaves (11), but also because of the observed allelopathic activity associated with the inhibition to growth of plantlets in soil close to the parent plant (12). We have investigated greenhouse-grown plants in pots, concentrating on the possibility of epicuticular leachates from the leaves and possible exudates from the roots into the soil.

Chloroform washings from leaves of greenhouse-grown plants have yielded 1-triacontanol (A) as a major component, together with a few known sterols and other unidentified components. Acetone extracts of the roots gave ferulate esters of normal C<sub>22</sub> to C<sub>30</sub> alcohols (B-F). In addition, isolates from the vermiculite used as soil on which these plants are grown gave phenolics from which ferulic acid (G) has been identified (Figure 2). No ferulic acid or other phenolics were extracted from vermiculite that was not exposed to plants. Despite the marginal growth inhibition activity shown by these extracts, the detection of triacontanol and ferulic acid early in the chemical analysis prompted further study.

Phenolics in general and ferulic acid in particular have ample precedence as allelopathic agents (2,3,13). On the other hand, 1-triacontanol has been reported to be a plant growth regulator (14). However, because these compounds are well studied, experiments to confirm or reject these reports have not been our main focus. Furthermore, we have not been able to duplicate the growth-promoting activities of 1-triacontanol, either with the whole plants or with callus or suspension cultures. Yet, the discovery in the roots of *K. daigremontiana* of ferulic acid and triacontanol linked together as a conjugate ester is interesting. Despite a healthy skepticism, it is conceivable that this conjugate ester could, through enzymatic hydrolysis, give rise to 1-triacontanol which, as a putative plant hormone, would travel to the leaf and promotes the well being of the plant. Ferulic acid would

Leaves, roots, and litter are  
allelochemical sources



Allelopathic chemicals from plants may be released from living leaves as volatiles or leachates or from roots through exudation or sloughing off of dead tissues. They also may be leached from leaf litter on the soil surface.

Figure 1. Production of Allelochemicals

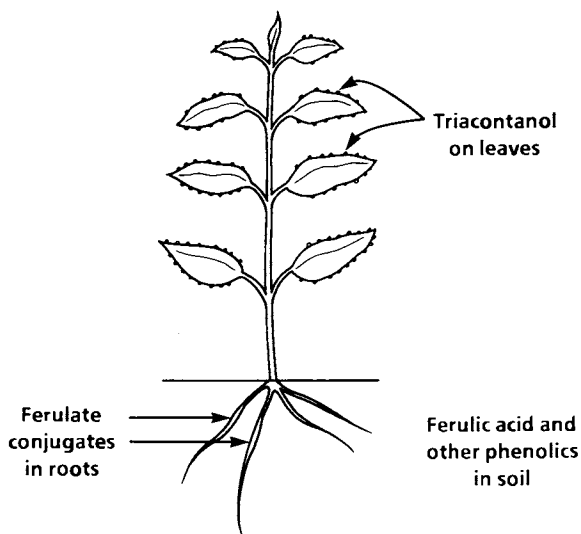
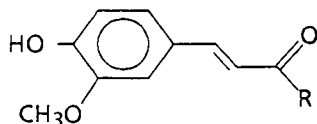


Figure 2. Allelopathy of *K. daigremontiana*

**A.**  $n\text{-C}_{30}\text{H}_{61}\text{OH}$  triacontanol



**B.**  $\text{R} = \text{O-}n\text{-C}_{22}\text{H}_{45}$

**C.**  $\text{R} = \text{O-}n\text{-C}_{24}\text{H}_{49}$

**D.**  $\text{R} = \text{O-}n\text{-C}_{26}\text{H}_{53}$

**E.**  $\text{R} = \text{O-}n\text{-C}_{28}\text{H}_{57}$

**F.**  $\text{R} = \text{O-}n\text{-C}_{30}\text{H}_{61}$

**G.**  $\text{R} = \text{OH}$

simultaneously be extruded into the soil and thereby inhibit the growth of other plants in the surrounding areas. In our study, triacontanol and ferulic acid were identified by comparison with authentic samples, and the ferulate esters characterized by NMR, HRMS, UV, and synthesis of the ferulate (**B**) of behenyl ( $\text{C}_{22}$ ) alcohol. The mass spectrum (Figure 3) shows the molecular ions (502, 530, 558, 586, 614) of the mixture of ferulate esters (**B** to **F**, respectively), each a homologue differing from its next highest neighbor by two methylene groups (28 u). The ferulic acid fragment ( $m/z$  194) is a product of McLafferty-type elimination. Figure 4 displays the mass spectrum of the synthetic ferulate ester (**B**).

Although the compounds are well known and their effects well studied this finding gives rise to a new concept in agrochemical design. Such a design would include, in a single molecule, a plant growth regulator and a herbicide or pesticide - somewhat like having one's vitamins and antibiotics in a single capsule. This is an example where one reward of pursuing allelochemicals is the novelty of the idea that accompanies trivial chemical products.

From our cooperative efforts with Dr. Putnam of Michigan State University, the benzoxazinone and benzoxazolinone derivatives DIBOA (**H**) and BOA (**I**) were isolated and identified from rye, a plant used in no-till agrosystems (15,16). Both DIBOA (**H**) and BOA (**I**) inhibit seed germination. For example, DIBOA completely inhibits germination of seeds of velvet leaf at 2 mM (17). These results amplify those of Worsham (18) and help to explain the allelochemical effects of rye residues in suppressing weeds.

Although the compounds are known (19), this biological activity is new. The biosynthesis (20,21) of these heterocyclic derivatives from anthranilic acid and ribose has been studied, and further study may reveal information on the possible enzymes and the controlling gene. There is a plethora of activities associated with these compounds (22-24). At least one has been quantitatively correlated with the presence of benzoxazinones in corn (25). This activity may soon be incorporated into other plants.

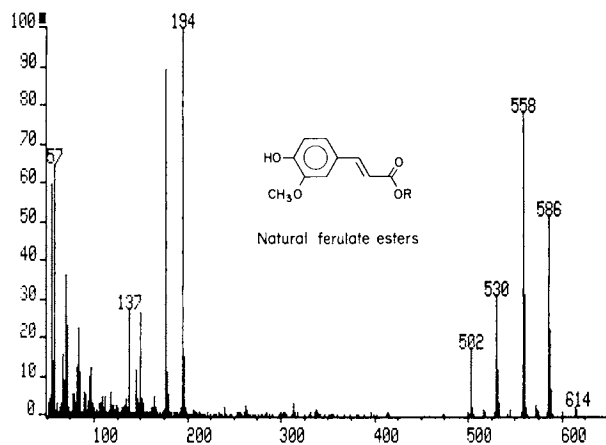


Figure 3. Mass Spectrum of Mixture of Natural Ferulate Esters

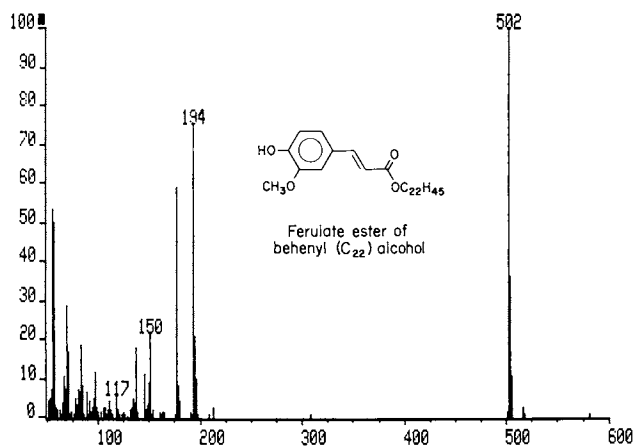
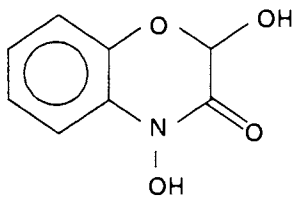
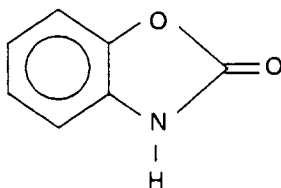


Figure 4. Mass Spectrum of Ferulate Ester of Behenyl Alcohol



H DIBOA



I BOA

### Success and regulation

This scientific approach to allelopathy is necessary for progress in this area. Although challenging, ARCO feels it has great potential to solve agrochemical problems. Natural adaptations to the carbamates, organophosphates, and synthetic pyrethroids demand a look into the untapped resources of lesser developed regions of the world where undiscovered and undescribed plant species and other organisms exist in a struggle for survival. Using the techniques of micropropagation, tissue culture, and molecular biology we must overcome many obstacles if our goals are to be met. However, not all problems are technological. Some are associated with regulation, legislation, and fear of genetically engineered plants. We must demonstrate that genetically engineered plants are safe and hope that the regulations for such engineered systems will be measured by the same yardstick as traditionally bred systems.

PCRI is equipped and resolved to evaluate this agrochemical-biotechnological approach to allelopathy. We have excellent facilities and resources in chemistry, genetics, tissue culture and molecular biology. We are probing this young science of allelopathy. Our hope is that through this combined approach our understanding will grow and enable productive adaptations to be made in agriculture.

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## Chapter 15

# Allelopathy in Australia: Bacterial Mediation

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Australian workers have reported allelopathic phenomena in native plant communities, both undisturbed and managed. In agriculture, allelochemicals have been identified with plant interference during life and, from their residues, after death. Bacteria are involved in examples of allelopathy from these several milieux.

Allelopathy is currently gaining acceptance as a factor having ecological significance in Australia, although reports of phenomena which contemporary workers would regard as allelopathic may be found in the literature over the past 30 years. Such examples represent several broad areas of current interest. Rovira (1) found that 27 amino acids, and various sugars, were excreted by roots of peas and oats and concluded that such excretions were important in stimulating the growth of microorganisms in the rhizosphere. Florence and Crocker (2) implicated antagonistic microbial activity in autotoxicity of Eucalyptus pilularis Sm., and Kimber (3) discussed phytotoxicity that may occur during decomposition of crop residues in the field. So far as can be determined there are no early reports of allelopathy manifested by weed species. However, all 18 of the world's worst weeds, as identified by Holm et al. (4) (Table I), are present in Australia and many of them have been associated with allelopathy.

More recent work can, for convenience, be divided into that dealing with plant species native to Australia (Table II) and plants which have, accidentally or deliberately, been introduced to the continent (Table III). Lovett (31) has reviewed these studies, and examples in both categories will be briefly discussed.

### Allelopathy and Native Species

Rabotnov (32) considers that allelopathy contributes to maintaining homeostasis in natural plant communities. Economically important losses may occur when the ecological balance in such communities is disturbed. For example, Booth and Barker (33) recorded lost grazing capacity due to invasion of pastoral lands by shrub species such as

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Table I  
The World's Worst Weeds (4)

Botanical Name	Common Name	
	Australia	U.S.A.
1. <u>Cyperus rotundus</u>	nutgrass	purple nutsedge
2. <u>Cynodon dactylon</u>	couch grass	Bermuda grass
3. <u>Echinochloa crus-galli</u>	barnyard grass	barnyard grass
4. <u>Echinochloa colonum</u>	awnless barnyard grass	jungle rice
5. <u>Eleusine indica</u>	crowsfoot grass	goosegrass
6. <u>Sorghum halepense</u>	Johnson grass	Johnson grass
7. <u>Imperata cylindrica</u>	blady grass	cogon grass
8. <u>Eichhornia crassipes</u>	water hyacinth	water hyacinth
9. <u>Portulaca oleracea</u>	pigweed	common purslane
10. <u>Chenopodium album</u>	fat hen	lamb's-quarters
11. <u>Digitaria sanguinalis</u>	summer grass	large crab grass
12. <u>Convolvulus arvensis</u>	bindweed	field bindweed
13. <u>Avena fatua</u> , et al.	wild oats	wild oats
14. <u>Amaranthus hybridus</u>	slim amaranth	smooth amaranth
15. <u>Amaranthus spinosus</u>	prickly amaranth	spiny amaranthus
16. <u>Cyperus esculentus</u>	nutgrass	yellow nutsedge
17. <u>Paspalum conjugatum</u>	sour grass	sour paspalum
18. <u>Rottboellia exaltata</u>	kokoma grass	Raoul grass

Eremophila sturtii R.Br. and Dodonaea attenuata A.Cunn. in western New South Wales (Table IV). Overgrazing by introduced domestic livestock and the relative absence of fire since European settlement are implicated in the development of this situation which, in several respects, resembles that of the Californian chaparral communities (34). Allelopathy is a possible contributing factor.

Many Eucalyptus species are economically important for their timber, their essential oil content, or their contribution to reafforestation in tropical and subtropical countries. Monoterpenoids, which have been linked with allelopathy by several workers, including Muller (34) and Lovett (24), are common constituents of Eucalyptus oils. These and other compounds may contribute to allelopathic manifestations by Eucalyptus baxteri ssp. (9), E. regnans F.Muell. (13) and E. globulus ssp. (8).

#### Allelopathy and Introduced Species

Examples of allelopathy between weeds and crop or pasture species in Australia have been documented within the past decade. Lovett and Lynch (17) discussed the association of Salvia reflexa Hornem. (mintweed), an annual member of the family Lamiaceae introduced to Australia from North America (35). This species produces aromatics as well as water-soluble compounds that may be inhibitory to species such as wheat and sorghum. Kloot and Boyce (21) documented inhibitory effects of allelochemicals produced by Polygonum aviculare L. (wireweed) on the annual Medicago species, important leguminous components of annually regenerating pastures in southern Australia.

Table II. Reports of Allelopathic Phenomena in Australia-- Native Species

Author(s)	Year	Allelopathic species	Species affected	Allelochemicals present	Effects	"Prima facie" evidence	Comments
Florence and Crocker (2)	1962	<u>Eucalyptus pilularis</u>	Seedlings of <u>E. pilularis</u>	?	Affects root and root hair development and shoot growth	-	Microorganism antagonism implicated
Story (5)	1967	<u>Eucalyptus molluccana</u> <u>Acacia pendula</u> <u>Casuarina leuhamii</u> <u>Callitris calcarata</u> <u>Eucalyptus crebra</u> <u>E. dawsonii</u> <u>E. melliodora</u> <u>Norclacu microcarpa</u>	<u>Bothriochloa ambigua</u> <u>Eragrostis leptostachya</u> <u>Sporobolus elongatus</u>	?	-	Grasses statistically sparser beneath tree canopies	-
Bevee (6)	1968	<u>Pinus sp.</u> , <u>Araucaria sp.</u> , <u>Plindersia sp.</u>	<u>Araucaria cunninghamii</u>	?	Seedling tip necrosis, chlorosis	-	'Halo' effect visible on aerial photographs
Trenbath and Silander (7)	1976	<u>Eucalyptus bicostata</u>	<u>Trifolium repens</u>		Blackening of radicle tips	Exclusion from zone around <u>E. bicostata</u>	Fraas from Propagulae resulted in growth inhibition
Silander et al. (8)	1983		<u>Festuca rubra</u> var. <u>fallax</u>	?			
Del Moral et al. (9)	1978	<u>Eucalyptus Baxterii</u>	<u>Leptospermum myrsinoides</u> <u>Casuarina pusilla</u>	Gentisic, ellagic, gallic, sinapic, caffeic acids Phenolic aglycones Glycosides Terpenoids	Inhibition of growth	Suppression of L. myrsinoides and C. pusilla beneath canopy of <u>E. Baxterii</u>	Chemicals from foliar and litter leachates inhibitory in bioassays
Ellis et al. (10)	1980	<u>Nothofagus cunninghamii</u> and other rainforest species	Seedlings of <u>E. regnans</u>	Lipids	Inhibition of growth	Dieback of mature trees; inhibition of regeneration by seedlings	
Lange and Reynolds (11)	1981	<u>Eucalyptus microcarpa</u>	<u>Gonocarpus elatus</u>	?	-	Suppression of <u>G. elatus</u> under <u>E. microcarpa</u>	
Willis (12)	1980	<u>Eucalyptus regnans</u>	Seedlings of <u>E. regnans</u>	Lipids	Difference in abundance of rhizosphere fungus <u>Colindrocarron destructans</u> between healthy and unhealthy roots of seedlings	Failure of seedlings to regenerate in absence of fire	NH <sub>4</sub> /NO <sub>3</sub> balance critical for seedling survival. Mature trees strongly mycorrhizal - competition with microorganisms for NH <sub>4</sub> . Antagonistic soil factors may be affected by root exudates
Ashton and Willis (13)	1982						

Table III. Reports of Allelopathic Phenomena in Australia--Introduced Species

Author	Year	Allelochemicals present		Effects	"Prima facie" evidence	Comments
		Year Allelopathic species affected	Species affected			
Kimber (3)	1967	Triticum aestivum	Avena sativa	Root growth reduced		Phytotoxic effect diminished with time, depended on previous weathering and variety of wheat
		Triticum aestivum (residues)	Avena sativa			
Kimber (14)	1973a	Hordeum vulgare	Triticum aestivum	Inhibition of root growth	Germination, plant growth and ultimate yield affected	Toxic effect is of an ephemeral nature
		Avena sativa				
		Medicago sativa				
		Secale cereale				
Kimber (15)	1973b	Pisum sativum	Triticum aestivum			Effect diminished with time. N immobilization may be involved
		Triticum aestivum (residues)	Triticum aestivum	Reduction of germination, growth and ultimate yield	Proximity of new wheat straw residues to sown wheat seed	
Bendall (16)	1975	Cirsium arvense	Carduus pycnocephalus	Inhibition of germination and seedling growth	Failure of C. arvense seedlings	C. arvense is auto-toxic - both roots and foliage inhibit growth of seeds and seedlings
			Cirsium vulgare			
			C. arvense			
			Helianthus scaberrimus			
			Lolium perenne			
			Trifolium subterraneum			
Lovett and Lynch (17)	1979	Salvia reflexa	Triticum aestivum	Inhibition of germination and early growth	Observed competitive ability in the field	Soil type shown to affect expression of allelopathy
		Salvia reflexa	Triticum aestivum	Inhibition of germination and early growth	Trichomes implicated as reservoirs for allelochemicals	
Lovett and Speak (18)	1979	Salvia reflexa	Triticum aestivum	Inhibition of germination and early growth	Field effects noted by earlier workers	Phyllospheeria - Pseudomonas fluorescens and Enterobacter cloacae convert benzyl isothiocyanate to benzylamine and hydrogen sulphide
		Salvia reflexa	Triticum aestivum			
Lovett and Burfield (19)	1981	Camelina sativa	Linum usitatissimum	High concentrations of benzylamine inhibited radicle elongation		A noxious weed, especially of summer crops
		Camelina sativa	Linum usitatissimum			
Lovett et al. (20)	1981	Stramonium	Linum usitatissimum	Reduced germination and inhibition of radicle growth		Water soluble allelochemicals present in green leaves and stems
		Stramonium	Linum usitatissimum			
Kloot and Boyce (21)	1982	Polygonum aviculare	Medicago truncatula	Causes morphological deformities in germinating medic seedlings by interfering with cell division and early growth of meristems	Failure of medic pastures to self-regenerate in paddocks dominated by Polygonum	Failure of medic pastures to self-regenerate in paddocks dominated by Polygonum
		Polygonum aviculare	Medicago truncatula			

Continued on next page

Table III. Continued

Author	Year	Allelopathic species	Species affected	Allelochemicals present	Effects	"Prima facie" evidence	Comments
Lovett (22)	1982	<i>Camelina sativa</i>	<i>Linum usitatissimum</i>	Benzylamine ?	Inhibition of early growth		Indirect effect of benzylamine through creation of hydrophobic conditions in soil
Lovett and Jessop (23)	1982	<i>Pisum sativum</i> <i>Vicia faba</i> Glycine max Lupinus max Lupinus angustifolius Cicer arietinum Cicer arietinum Carthamus kinctorius Helianthus annuus Brassica napus Sorghum bicolor Avena sativa Hordeum vulgare Triticum aestivum	<i>Triticum aestivum</i> L. cv. Songlen	?	Crop residues affect early growth of wheat by affecting germination emergence, coleoptile height and length of longest seminal root		Phytotoxic effect increased when crop residues were incorporated into the soil
Lovett (24)	1983	<i>Salvia reflexa</i>	<i>Triticum aestivum</i>	Monoterpenes	Germination and seedling growth adversely affected		Unidentified but active water-soluble compound(s) also present
Cheam (25)	1984	<i>Cenchrus ciliaris</i>	<i>Calotropis procera</i>	?	Suppression of root, stem and leaf growth of Calotropis seedlings	Calotropis seedlings fail to establish in well groomed buffel grass areas	
Levitt and Lovett (26)	1984	<i>Datura stramonium</i>	<i>Helianthus annuus</i>	Tropane alkaloids	Inhibition of radicle growth		Primary effect of allelochemicals on metabolism of food reserves. Effect documented in the field
Levitt et al. (27)	1984	<i>Datura stramonium</i>	<i>Helianthus annuus</i>	Tropane alkaloids	Inhibition of radicle growth		Soil type shown to affect expression of allelopathy
Rajan (28)	1984	<i>Imperata cylindrica</i> (L.) Beauv.	Trifolium subterraneum Calopogonium mucunoides	p-hydroxy-benzoic acid vanillic acid p-coumaric acid ferulic acid	Delay of germination and inhibition of radicle growth	A noxious weed which colonizes large areas to the virtual exclusion of other species	
Purvis et al. (29)	1985	<i>Brassica napus</i> <i>Sisymbrium officinalis</i> <i>Pisum sativum</i> <i>Helianthus annuus</i> <i>Triticum aestivum</i> (residues)	<i>Avena fatua</i> <i>Avena ludoviciana</i>	?	Germination and growth of wild oats differentially affected by crop residues		Implications for crop rotation development
Mason-Sedun et al. (30)	1986	<i>Brassica campestris</i> <i>B. juncea</i> <i>B. napus</i> <i>B. nigra</i>	<i>Triticum aestivum</i>	?	Variable inhibition of growth	Effects persisted through to final grain yield	Effects documented in the field

Table IV  
Grazing Capacity Loss Due to Shrub Invasion on Four Properties  
Located West of Wanaaring (33)

Property	Lost grazing capacity (1968-70)(%) <sup>a</sup>	Lost grazing capacity (1978)(%)
A	1	8
B	4	10
C	6	22
D	9	15
Mean	5	14

<sup>a</sup>Properties A, B and C were inspected in 1968 and property D in 1970.

An example of allelopathy by a useful, introduced pasture grass against a weed is provided by Cheam (25, 36) who has studied the effects of *Cenchrus ciliaris* L. (buffel grass) on *Calotropis procera* (Ait.) W.T. Ait (calotrope), also an import to Australia [Meadly (37)]. Six- and 9-week old buffel grass plants significantly suppressed the growth and development of calotrope seedlings, which were also inhibited when grown in soil which previously supported a buffel grass stand (Table V). Cheam (36, 38) considers that planting of buffel grass will prevent ingress by calotrope in areas that are now free of the weed, that introduction of buffel grass on land already infested should lead to a steady decline in the calotrope population, and that the bioactive compound may prove useful as a 'natural herbicide' for *C. procera* and other species.

Table V  
Germination and Growth of Calotrope in Soil in which Buffel Grass  
Had Grown for 6 Weeks Previously  
[The treated soil was free from buffel grass roots.] (25)

Treatment	Cumulative germination(%)		Plant height(mm)	Growth response	
	1 week	2 weeks		First pair of true leaves Length(mm)	Breadth(mm)
Control soil	80.0	86.7	49.6	30.6	18.5
Treated soil	83.3	90.0	32.8	21.7	12.8
Level of significance	N.S.	N.S.	P = 0.001	P = 0.001	P = 0.001

This example illustrates the benefits and costs of introducing plants to new localities. Over the mere two hundred years of European settlement in Australia many of the World's most important crop and pasture plants and all of the World's worst weeds (Table I) have been introduced to the continent. The latter have attained problem status and several other introductions, such as *Echium plantagineum* L. (Paterson's Curse) from the Mediterranean region, freed from those organisms which maintain them in balance in their native communities, have posed threats to agriculture.

Parthenium hysterophorus L. (parthenium weed), native to North and Central America and introduced into Queensland as recently as 1960 (39), is a contemporary example. It is aggressive, persistent and lowers crop yield through interference, a component of which is allelopathic (40).

#### Allelopathy and Microorganisms

The production of compounds which may act as allelochemicals is not restricted to higher plants. For example, Heisey, DeFrank and Putnam (41) have discussed substances produced by soil microorganisms which may have herbicidal activity. Bacteria may also mediate allelopathic activity in economically important situations such as forest regeneration (Line, personal communication); crop/weed associations (42), and reduced cultivation systems where plant residues are retained (43).

Cruciferous species, in which the glucosinolates are biologically active compounds (44), have been studied in both of the latter categories. In Australia, allelopathy has been associated with introduced crucifers such as Brassica tournefortii Gouan (wild turnip) and a more complete study has been made of Camelina sativa (L.) Crantz (false flax).

Grümmer and Beyer (45) reported allelopathy between Linum usitatissimum L. and Camelina species in the field, providing that rain fell during a critical (unspecified) period of growth. Lovett and Sagar (42), working in the United Kingdom, established that the presence of bacteria in the phyllosphere of C. sativa was necessary for allelopathy to be manifested. The organisms were free-living, motile, Gram-negative rods representative of the bacteria which tend to predominate in the phyllosphere (46) and were identified as Enterobacter cloacae (Jordan) Hormaeche and Edwards. In subsequent work, carried out in Australia, Pseudomonas fluorescens (Trevisan) Migula has been identified as playing a similar role (47).

Typically, bacteria are recovered from aqueous washings of fresh foliage of C. sativa. If foliage washings are incubated at +23°C for 24 h the washings become strong smelling and cloudy in appearance. If bacteria are removed from the washings after collection, by filtration, no change is noted. GC/MS analyses have shown that organic acids of the citric acid cycle are present in fresh washings but are much depleted after 24 h incubation with bacteria (Figure 1, Figure 2). Similar results are obtained by inoculation of leaf washings, freed of bacteria by Millipore filtration, with either of the two bacteria identified (19).

Lovett and Jackson (47) observed that C. sativa leaf washings exhibited allelopathic activity in bioassay after incubation for as little as 12 h and demonstrated that during this period of time there was exponential growth of the bacterial population (Figure 3).

Benzyl isothiocyanate was identified in aqueous extracts of C. sativa foliage by Lovett and Duffield (19). Tang, Bhothipaksa and Frank (48) showed that E. cloacae was capable of degrading benzyl isothiocyanate to hydrogen sulfide and benzylamine. Tests were, accordingly, carried out with incubated leaf washings of Camelina and showed the presence of hydrogen sulfide and

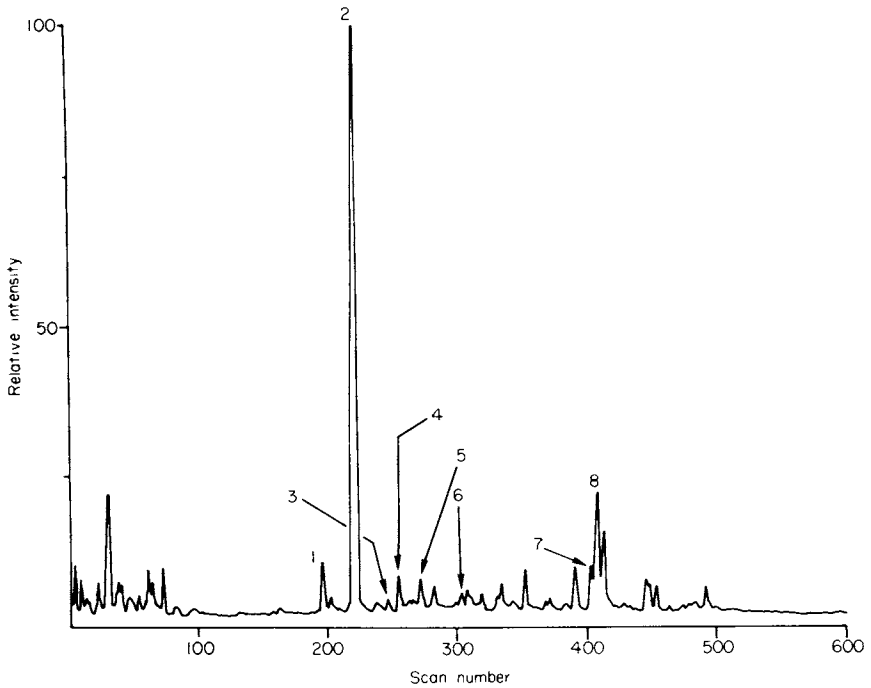


Figure 1. Total ion current of n-butyl esters of the acidic fraction from non-sterile leaf washings of *C. sativa*. Key: 1 = oxalic acid; 2 = malonic acid; 3 = maleic acid; 4 = succinic acid; 5 = fumaric acid; 6 = alpha-ketoglutaric acid; 7 = cis-aconitic acid; 8 = citric acid (19).

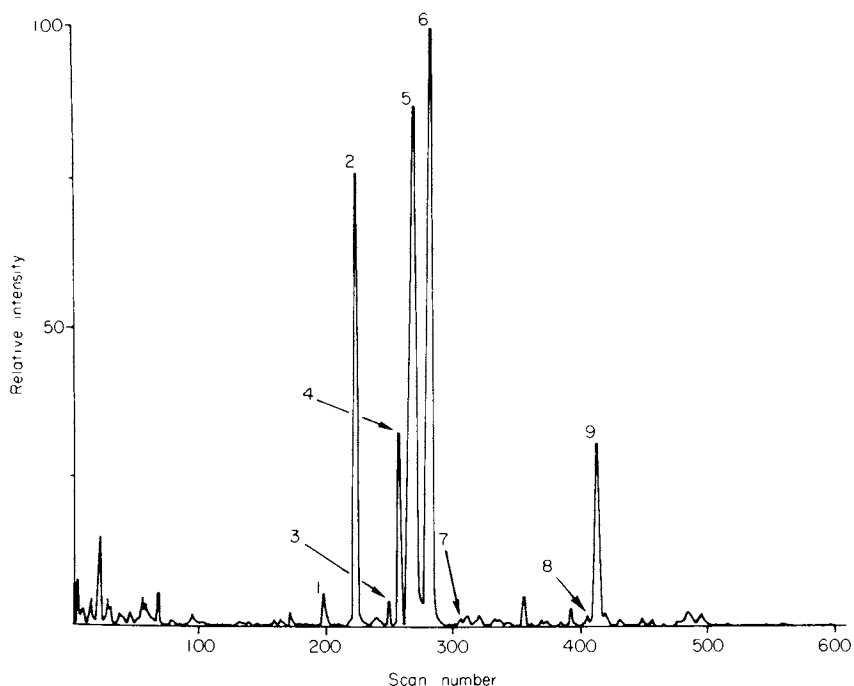


Figure 2. Total ion current of *n*-butyl esters of the acidic fraction from sterile leaf washings of *C. sativa*. Key: 1 = oxalic acid; 2 = malonic acid; 3 = maleic acid; 4 = succinic acid; 5 = fumaric acid; 6 = malic acid; 7 =  $\alpha$ -ketoglutaric acid; 8 = *cis*-aconitic acid; 9 = citric acid. (Reproduced with permission from reference 19. Copyright 1981 Blackwell Scientific Publications Ltd.)



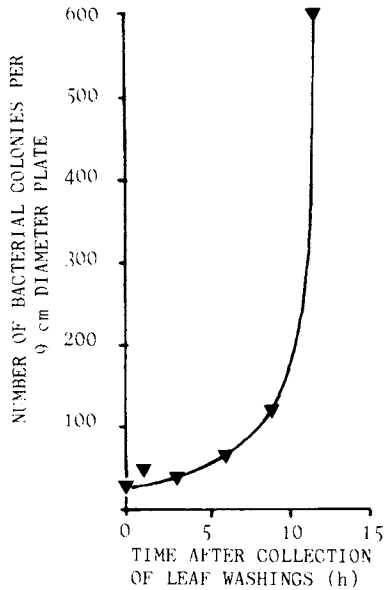


Figure 3. Increase in bacterial colony numbers during a 24-h incubation period. (Reproduced with permission from reference 47. Copyright 1980 The New Phytologist.)

benzylamine (19). The level of the latter component, in particular, is variable depending on age of the plant and season of the year. This observation accords with the report of Grümmer and Beyer (45) that allelopathy in the field occurred only at a particular time of the year. Our data (43) have demonstrated that bacteria are most prolific on senescent leaves, a finding which agrees with that of Ettlenger and Kjaer (49), namely that injury to plants containing glucosinolates results in the liberation of isothiocyanates from those substances. Thus, the presence of available quantities of isothiocyanate and large numbers of bacteria during senescence of the weed could explain field observations of allelopathy.

Two effects of benzylamine as an allelochemical have been documented. When *L. usitatissimum* is used in bioassay, germination is impaired but only at relatively high concentrations of the allelochemical (in excess of 500 ppm). Radicle length of germinating *L. usitatissimum* in bioassay is increased at low concentrations of benzylamine (less than 200 ppm) but is increasingly inhibited as concentration increases. In soil, however, inhibition may occur even at low concentrations (Figure 4), probably as a result of improved contact between germinating seedlings and substrate.

In addition to direct effects on the plant, benzylamine may induce hydrophobic (water repellent) conditions in soil (Figure 4). These data indicate a linear increase in moisture content as benzylamine content increases, attributable to the development of a lower unsaturated hydraulic conductivity in the surface soil, which thus became less able to transfer water from depth in response to evaporative demand. McGhie (50) suggests that poor germination of crop and pasture plants may be related to the development of hydrophobic conditions, the affected soil being unable to supply water to the germinating seed.

Strains of *E. cloacae* are known to fix nitrogen (51). An additional component of the complex *Camelina/Linum*/bacteria association was the finding by Lovett and Sagar (42) that *E. cloacae* cultured from *C. sativa* foliage washings gave indications (through acetylene-ethylene assays) of a nitrogen-fixing capability. It is not known whether such nitrogen contributes to the nitrogen economy of the plant, although Jones (52) suggests several means by which nitrogen fixed in the phyllosphere of conifers may aid the growth of these trees.

Both *E. cloacae* and *P. fluorescens* are capable of activity in the phyllosphere and in soil. This may be of significance in relation to the findings of Elliott and Lynch (53) and Lynch and Clarke (54) that pseudomonads, in large numbers, are a dominant feature of the microflora of the rhizosphere and straw of some temperate cereals. Among the plant growth-promoting rhizobacteria that are being tested for commercial applications, Burr and Caesar (55) have found the most effective strains to be fluorescent *Pseudomonas* species. Lynch and Clarke (54) suggested that some strains of pseudomonads stimulate root and shoot extension and dry matter production of barley, a species that shows variable sensitivity to benzylamine (Figure 5). However, Elliott and Lynch (53) indicated that pseudomonads antagonistic towards seedling growth colonize roots of wheat, their inhibitory effects also

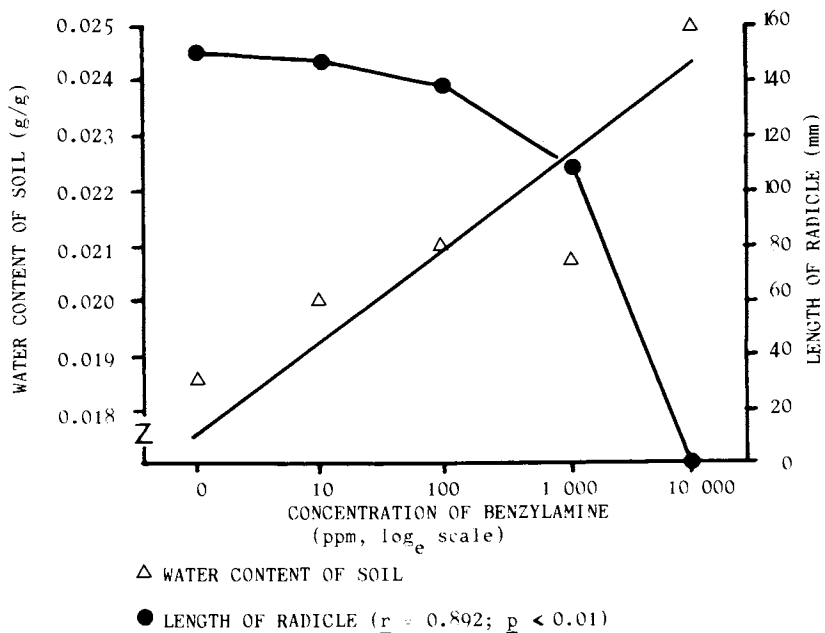


Figure 4. Effect of benzylamine on soil water content and radicle length of *Linum usitatissimum*.

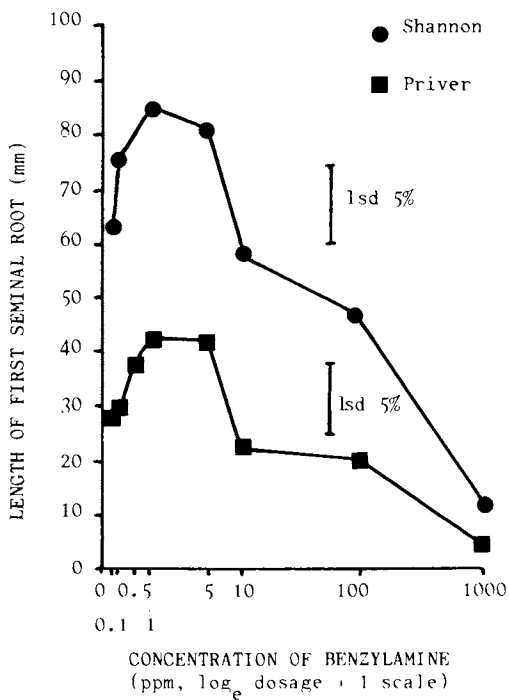


Figure 5. Effect of benzylamine on length of the first seminal root of two cultivars of Hordeum vulgare.

varying with cultivar. Although considerable advances in knowledge of this complex area have been made, clearly there remains a need for more precise understanding.

Lynch and colleagues have been concerned with cereal production under reduced tillage where impaired germination of crop plants has been attributed to nitrogen immobilization (56). From the observations of Lovett and Sagar (42) a *prima facie* case for possible enhancement of nitrogen status as a consequence of the presence of commonly occurring Gram-negative bacteria in the phyllosphere/rhizosphere could be made. Work of Roper (57), in two areas of the wheat belt of New South Wales, supports this contention. Using the acetylene reduction technique in the field, she obtained data that indicate the presence of free-living bacteria in the soil which are capable of utilizing some of the energy provided by decomposition of straw residues for nitrogen fixation. The data suggest that during the course of each experiment, a period of 10 days, nitrogen fixation was at the rate of 0.3-1.23 kg/ha/day. Roper (57) concludes that there is considerable potential for nitrogen fixation in soils where straw and other crop residues are retained. Fertilizer nitrogen input into dryland cropping in Australia is limited; for example, 7.8 million ha of wheat, the most important Australian crop, received a typical input of 23 kg/ha N in the season 1977/78 (58), so that nitrogen fixation by free-living organisms could lead to a further reduction in fertilizer nitrogen inputs.

The work of Kimber (3, 14, 15), on phytotoxicity of plant residues, received comparatively little attention prior to the widespread adoption of reduced cultivation/plant residue retention in Australia during the past decade. Reports by farmers of reduced emergence of crops, often accompanied by chlorosis, were commonly attributed to nitrogen immobilization. Such data as were available related to phytotoxicity from residues of cereal crops.

In much of eastern Australia it is possible to grow summer and winter crops in the same ground in the same year. Since rainfall is subject to great variability, dryland farmers seeking to capitalize on a rainfall event may rapidly seed a second crop into the residue of its predecessor at a time when microbial activity and the products of decomposition are at a peak. In order to assess the likely effect of crops grown sequentially, experiments with a range of species have been carried out (23). With wheat as the test species in glasshouse experiments it was found that all twelve crop residues tested affected height of the shoot and length of the longest seminal root five days after sowing. In the field, residues of the same crops reduced emergence relative to control whilst at final harvest residues of sorghum and pea significantly reduced the 1000 grain weight of the harvested wheat crop (Table VI).

Work with cereals, performed by a number of researchers in diverse locations, has shown that the short-chain aliphatic or volatile fatty acids (VFA), especially acetic acid, are the only chemicals produced in sufficient quantities from decaying residues to be toxic in the field (59, 60, 61, 62). VFA have also been

Table VI  
Effects of Crop Residues on Emergence and 1000-grain Weight of  
Wheat Grown in the Field (23)

Species	Emergence (plants per 1-m length of row)	1000-grain weight (g)
Control	28.0a	30.3a
Sorghum	9.3b	26.8b
Rape	17.7ab	28.7ab
Lupin	19.7ab	29.1ab
Pea	6.3b	25.7c
Wheat	11.3b	28.9ab

Means identified by the same letter are not significantly different at the 5% level, Studentized Range Test.

identified from residues of rape (63) and pasture grass species (64). In our experiments, field pea provided one of the most phytotoxic residues. Clarke and Humphries (65) reported the presence of VFA in pea silage effluent but there are no data to confirm the presence of VFA in residues similar to those of other crops. As pea residues were most phytotoxic when incorporated into soil, that is, under anaerobic conditions, 10 g air-dry residues were digested in 140 ml sterile water in closed glass jars at 24°C for 25 days. Acetic acid was the major VFA produced, which corresponds with findings from cereal residues (60, 63) and pasture grasses (64). The highest acetic acid concentration recorded was  $6.5 \times 10^{-4}$  M/g residue (Mehaffey, unpublished data), the concentration produced from wheat straw being generally about  $4 \times 10^{-4}$  M/g residue (63, 66, 61, 62, 67). The greater toxicity of pea as compared with wheat residues (23) may, therefore, be a result of the production of higher acid concentrations.

Work of Cochrane (68) suggests that most of the acetic acid produced resulted from bacterial rather than fungal activity. In our experiments, a large range of fungi was observed at the commencement of digestion but the range decreased with time. In contrast, the population and range of bacterial species increased with duration of digestion. Positive identification to species level was not attempted but representatives of the genera Bacillus, Micrococcus, Erwinia, and Flavobacterium were relatively abundant (Mehaffey, unpublished data).

As reported by other workers, for example, Putnam and DeFrank (69), crop residues may selectively toxify weed species. In our field experiments dry weights of grass weeds excluding wild oats were reduced by residues of rapeseed, sorghum, pea, sunflower and wheat. Germination, growth, and grain yield of wild oats tended to be slightly stimulated by most residues but significantly so by residues of wheat, a crop with which it is particularly associated (29). This finding confirms that, although some products of

decomposition, and their effects, are common to many species, there remains the possibility that a specific component will exert a singular effect.

Bevege (6) canvassed the possibility that microorganisms may be involved in the production of bioactive compounds in forest soils in Queensland, and microorganisms have also been implicated in allelopathic phenomena related to forest regeneration in north east Tasmania. Of particular interest is Eucalyptus delegatensis R.T.Bak. a commercially important species which often represents a subclimax stage of succession, maintained by burning. Two problems have emerged in recent times (a) dieback of E. delegatensis and invasion by rainforest species, and (b) lack of vigor of E. delegatensis seedlings in grassland dominated by Poa spp. Ellis, Mount and Mattay (10) suggest that allelopathy may be involved in the first case and work in progress has demonstrated the presence of factors inhibitory to E. delegatensis seedling growth in two soils where invasion by rainforest is taking place. Monoterpenes, identified by Fisher (70) as allelochemicals in forest systems, are among compounds monitored to date (Potts, personal communication). Allelopathy appears to be part of an invasion complex in which soil temperatures decrease, precipitation and organic matter increase (71, 72), and ingress by rainforest proceeds. The hypothesis that, once established, cold, wet conditions beneath the rainforest canopy are unfavorable to the breakdown by microorganisms of organic matter and accumulated phytotoxins is currently being tested.

Characteristically, seedlings of E. delegatensis taken from grassland dominated by Poa spp. show a lack of vigor. Mycorrhizal development on these seedlings is slight. Two microorganisms are presently being tested for pathogenicity to seedlings, a fungus (Cladosporium sp.) and a bacterium (?Xanthomonas sp.). Both have been isolated from the root surfaces of inhibited E. delegatensis plants taken from Poa-dominated grassland and formed the predominant microflora isolated from root surfaces of these plants (Line, personal communication).

In other experiments, where the grassland soil has been steamed at 70°C for 30 min before potting, 10- to 25-fold increases in dry weight of seedlings have been obtained over results obtained with untreated soil. Fumigation with methyl bromide for 24 h before potting resulted in a 45-fold increase in seedling growth. With both of these treatments, subsequent mycorrhizal formations resembled those from a eucalypt-dominated soil. This evidence, together with a lack of response by seedlings to major plant nutrients in other experiments, suggests that microorganisms are important in both communities where E. delegatensis loses vigor and that the importance of burning in these communities is to reduce concentrations of allelochemicals and to contribute to the maintenance of a nonpathogenic microflora in which bacteria are important.

### Conclusion

In the several examples discussed, where problems of biological and economic significance can be attributed to allelopathy, bacteria

play an important but often ill-defined role. The genera so far identified are cosmopolitan and capable of activity in both the phyllosphere and rhizosphere. Chatterjee and Nandi (73) determined that, under favorable conditions, a succession of microorganisms successively degrades complex organic compounds to simple forms which become decreasingly toxic in effect. It seems likely that allelopathy occurs when environmental conditions retard the sequence of breakdown and permit accumulation of concentrations of phytotoxic compounds to occur.

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## Chapter 16

# Allelopathy: A Potential Cause of Forest Regeneration Failure

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The failure of forest tree regeneration is a major problem in many areas. Although such failures are often climatically induced, there is a growing body of data that suggests allelopathy is an equally likely cause of failure. Complete elucidation of the exact role of allelopathy in regeneration problems awaits a better understanding of the pathway and fate of allelochemicals in the environment. A schematic approach to this problem is presented.

Failures of newly established forest tree plantations or abnormal delays in tree seedling growth seldom have clear-cut causes. In the absence of knowledge of cause, foresters often attempt remedies that are unnecessarily costly or environmentally damaging, even when they succeed. Allelopathy, the direct or indirect deleterious effect of one plant upon another through the production of chemical inhibitors released into the environment, is likely a common cause of such failures or delays.

Foresters generally think of interactions among plants in terms of competition for light, water, nutrients, or space. It has become increasingly clear, though, that many species influence others through chemical inhibition or interference.

Although the phenomenon of allelopathy was first described by De Candolle (1), Pliny the Elder writing in the first century A.D. in his Naturalis Historia described the failure of certain plants to grow in the shade of Juglans regia. In addition nearly every society has had folklore about the effects of one plant upon another.

In the early days of plant pathology, viruses were mysterious and poorly understood. Nearly every malady of unknown cause was

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ascribed to a virus and for a time the virus as a legitimate cause of plant disease was held in great question by many. Currently allelopathy enjoys a similar reputation among foresters and tree physiologists.

Much of the early experimental work on allelopathy employed questionable methods and failed to produce unequivocal results. Now there is a substantial body of data obtained by sound techniques, that verifies the role of allelopathy in many plant communities.

Table 1 lists some allelopathic plants of interest to foresters, together with the classes of toxic compounds produced and examples of species they are reported to suppress. The list is not exhaustive; many species that may be allelopathic have not been studied in depth. One easily observed effect--though sometimes difficult to distinguish from effects of competition--is the exclusion of shrubs, herbs, and other trees from beneath the crowns of particular tree species.

Walnut (*Juglans nigra*) is the most notorious of allelopathic trees, although *Eucalyptus* can have equally dramatic effects. Jameson (2) found that several species of *Juniperus* and *Pinus* in the pinyon-juniper type of the Southwest inhibit grasses and forbs to a greater degree than would be expected from simple competition. His work, however, like much of that on the subject, stopped short of conclusively demonstrating allelopathic activity.

Sycamore (*Platanus occidentalis*), sugarberry (*Celtis laevigata*), and sassafras (*Sassafras albidum*) produce phenolics and terpenoids that reduce the growth of some species of herbs, grasses, and even trees (3-5). Tubbs (6) demonstrated that sugar maple (*Acer saccharum*) root exudates could inhibit the early growth of yellow birch (*Betula alleghaniensis*). Cherrybark oak (*Quercus falcata* var. *pagodaefolia*) as well as other oaks preclude or retard the growth of some trees, shrubs, herbs, and grasses beneath their crowns (7-9). This natural herbicidal effect is not only ecologically interesting, but it constitutes both a blessing and a curse to the forester trying to manage such trees.

The allelopathic effects of shrubs, herbs, grasses, ferns, and lichens upon trees is of particular concern when considering regeneration failure or seedling growth suppression. Poor survival of hardwood seedlings in weedy abandoned fields has troubled foresters for decades. Recent work has shown that several common plants inhibit tree establishment in such habitats. Walters and Gilmore (10) reported that fescue grass (*Festuca*) inhibited the growth of sweetgum (*Liquidambar styraciflua*) planted in old fields in Illinois, and Fisher et al. (11) found that goldenrod (*Solidago*) and *Aster* interfered with the establishment and growth of sugar maple in old fields in Ontario. Horsley (12, 13) has shown that black cherry (*Prunus serotina*) is inhibited by old-field plants such as shorthusk grass (*Brachyelytrum erectum*) and *Aster* and by forest plants such as New York fern (*Dryopteris noveboracensis*) and clubmoss (*Lycopodium*). Certainly poor nutrition and microclimate are the major barriers to the establishment of late successional

**Table 1. Some Allelopathic Plants Important in Forestry, the Chemicals They Produce, and the Plants They are Reported to Affect**

Allelopathic Species	Class of Chemical Produced	Example of Affected Species
<b>Trees</b>		
Sugar maple	Phenolics	Yellow birch
Hackberry	Coumarins	Herbs, grasses
Eucalyptus	Phenolics, terpenes	Shrubs, herbs, grasses
Walnut	Quinone (juglone)	Trees, shrubs, herbs
Juniper	Phenolics	Grasses
Sycamore	Coumarins	Herbs, grasses
Black cherry	Cyanogenic glycosides	Red maple
Oaks	Coumarins, other phenolics	Herbs, grasses
Sassafras	Terpenoids	Elm, maple
Poplar	Phenolics	Shrub mycorrhizae
<b>Shrubs</b>		
Laurel	Phenolics	Black spruce
Manzanita	Coumarins, other phenolics	Herbs, grasses
Bearberry	Phenolics	Pine, spruce
Sumac	Phenolics, terpenoids	Douglas-fir
Rhododendron	Phenolics	Douglas-fir
Elderberry	Phenolics	Douglas-fir
Lyonia	Phenolics	Slash pine
<b>Other</b>		
Aster	Phenolics, terpenoids	Sugar maple, black cherry
Goldenrod	Phenolics, terpenoids	Sugar maple, black cherry
New York fern	Phenolics	Black cherry
Bracken fern	Phenolics	Douglas-fir
Fescue	Phenolics	Sweetgum
Shorthusk grass	Phenolics	Black cherry
Clubmoss	Phenolics	Black cherry
Reindeer lichen	Phenolics	Jack pine and white spruce mycorrhizae
Bahiagrass	Phenolics	Slash pine

tree species on old fields, but just as certainly allelopathy is often an additional adversity that must be overcome.

Numerous other allelopathic interferences to tree establishment have been discovered, although their importance is yet to be demonstrated in the field. For example del Moral and Cates (14), using an elaborate bioassay technique, found that litter extracts of Pacific madrone (Arbutus menziesii), vine maple (Acer circinatum), elderberry (Sambucus racemosa), Rhododendron, sumac (Rhus ursinus), and several other common shrubs inhibited radical elongation in germinating Douglas-fir (Pseudotsuga menziesii) seeds. Also, Stewart (15) found that bracken fern (Pteridium aquilinum) inhibited Douglas-fir seedling growth in greenhouse cultures.

Reitveld (16) demonstrated that grass (Festuca arizonica, Muhlenbergia montana) residues reduced the germination and early growth of ponderosa pine (Pinus ponderosa) while Priester and Pennington (17) have reported that broomsedge (Andropogon virginicus) has inhibitory effects on loblolly pine (Pinus taeda) seedlings. Fisher and Adrian (18) found that Bahiagrass (Paspalum notatum) was a strong inhibitor of the growth of slash pine (Pinus elliottii) seedlings.

Peterson (19) found that sheep laurel (Kalmia angustifolia) was toxic to black spruce (Picea mariana) seedlings, and Fisher (20) reported that reindeer moss (Cladonia) restricted the growth of jack pine (Pinus banksiana) and white spruce (Picea glauca) by reducing root formation. Fisher also observed that leachates from common forest plants such as bog laurel (Kalmia polifolia) and bigleaf aster (Aster macrophyllus) inhibited germination and early growth of white and black spruce in the laboratory. Such inhibition may explain why regeneration success on organic soils in northern Ontario is more closely related to the species than to the density of interfering plants.

Several authors have obtained circumstantial evidence that allelopathic compounds reduce mycorrhizae formation (20-23). Kovacic and associates (24) have shown that understory plants in a live ponderosa pine stand are largely nonmycorrhiza-forming species. They hypothesized that this was due to inhibition of the vesicular-arbuscular mycorrhiza necessary for the growth of herbaceous mycorrhizal plants, under living pines. They demonstrated that more mycorrhizal plants occurred under dead pines, bioassay plants formed mycorrhizae in soils beneath dead pines but not in soil beneath live pines, and mycorrhizal inoculum appeared to be absent from the live pine stand.

Tobiessen and Werner (25) found that the hardwood understory in Scotch and red pine plantations differed considerably in its ability to survive. Those seedlings in the Scotch pine plantation grew well and became mycorrhizal. However, hardwood seedlings growing under red pine were shown to be nonmycorrhizal, deficient in P, and unable to grow beyond the seedling stage. Read and Jalal (26) attempted to correlate suppression of conifers by Calluna vulgaris with inhibition of the ectomycorrhizal fungi needed for their growth. In

contrast to the other studies mentioned, they found that in pure culture studies there was no evidence of antagonism between the fungal symbiont of Calluna and ectomycorrhizal fungi. However, they did not examine the effect of Calluna extracts on ectomycorrhizal fungi.

Hollis and associates (27) investigated the allelopathic effect of nine of the most abundant herbaceous and shrubby understory associates in Lower Coastal Plain flatwoods pine stands on germination, radicle extension, and shoot growth of slash and loblolly pine. They identified lyonia (Lyonia lucida) as a strong inhibitor of both pine species. Subsequent field studies confirmed that lyonia reduced the growth of planted slash pine. Few studies have followed lab or greenhouse results with corroboration from field studies. Fewer yet have adequately traced the path of allelochemicals in the environment.

The potential fate of an allelopathic chemical in the environment is outlined schematically in Figure 1. Donor plants produce toxins or their precursors, and these toxins are released into the environment. Whether there is "feedback control" or any other form of control over this process is unknown (28).

Whatever quantity of toxin is released generally enters the soil, where several things may happen to it. The order of the second, third, and fourth steps in Figure 1 is not necessarily the order in which the reactions actually occur. In fact, if the toxin is a volatile substance, these steps may be skipped entirely. In most cases, however, it appears that allelopathic chemicals enter the soil.

Soil colloids are capable of adsorbing most allelopathic chemicals. Such adsorption would result in temporary loss of toxin activity. Chemical changes could occur during adsorption that would permanently deactivate the toxin. The adsorption reactions are usually reversible, however, so that some or all of the toxin would still be available for uptake by a receiver plant.

The toxin is also likely to be adsorbed or complexed by soil humic acids. If the reaction is a simple adsorption reaction, all or part of the toxin might later become available for absorption by a receiver plant. If the toxin is complexed or precipitated by its reaction with soil humic substances, then it would be deactivated.

The toxin may undergo microbial degradation either while it is free in soil solution or while it is adsorbed. This could destroy all or part of the toxin, and there is evidence that most of the natural organic chemical groups that contain allelopathic compounds can be metabolized by some microorganism. The possibility always exists, however, that the microbial degradation product from the metabolism of an active toxin will itself be an allelopathic chemical.

The reactions that a toxin undergoes in the soil are largely controlled by edaphic factors such as moisture regime, nutrient

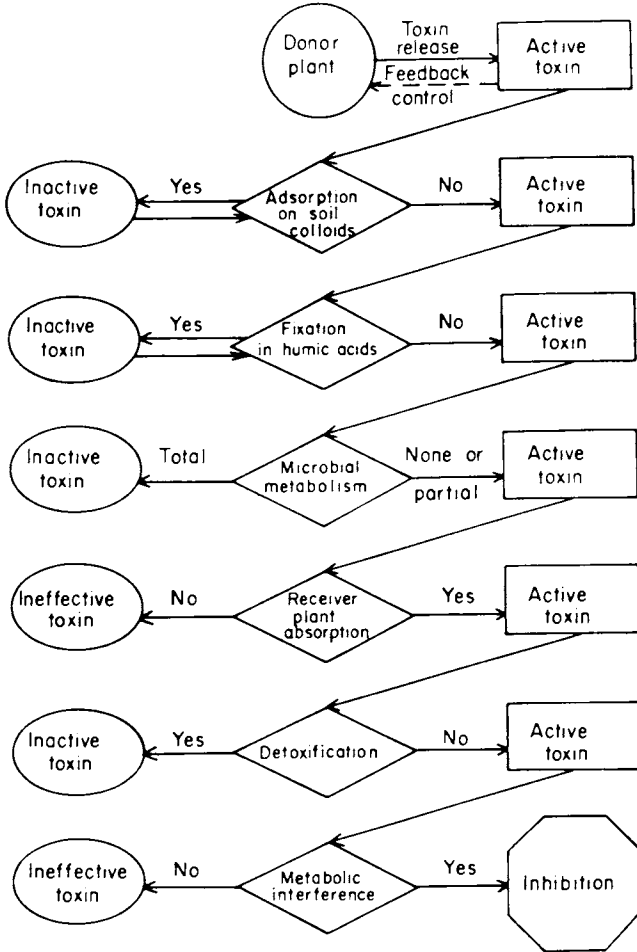


Figure 1. The potential fate of an allelopathic chemical in the environment. (Reproduced with permission from reference 28. Copyright 1979 Academic Press.)



status, or organic matter content. Soil moisture regime helps to determine whether aerobic or anaerobic decomposition takes place, which, in turn, helps to fix the quantity of toxin metabolized and the nature of the decomposition products. Soil nutrient status and soil temperature help to determine the rate of microbial activity. The nature and amount of soil organic matter determine whether simple adsorption or complexing by humic substances take place. Microbial degradation is also controlled by the spectrum of microorganisms present in the soil. These edaphic effects mean that different things will happen to the same toxin introduced into different soils or even into the same soil at different times.

It seems unlikely that the allelopathic chemicals that may be extracted from plant material are actually those that reach the host plant, yet nearly all our information on allelopathic compounds is derived from extracts that have never been exposed to the soil. Some compounds, such as juglone, may remain unchanged in the soil under some circumstances (29), but many compounds, such as ferulic or salicylic acid, are converted to other chemicals in the soil.

Toxin that is free in the soil solution is available for uptake by the receiver plant. Most, if not all, allelopathic chemicals are taken up by plants, but plants may discriminate against certain toxins on the basis of size (molecular weight) or some other factor. However, we do not know exactly which plants absorb which chemicals. It is only poorly understood why and how plants are able to discriminate against some chemicals. If the plant does not absorb the toxin, the toxin becomes ineffective.

Once the toxin is absorbed, it must be translocated to the site where it is capable of interfering with metabolism. If translocation is blocked, the toxin will be ineffective. Some plants may be capable of detoxifying an allelopathic chemical that is absorbed. The evidence for such capability is largely indirect, but this is certainly an area deserving of considerable research. If the toxin is absorbed and translocated but not detoxified within the plant, the toxin interferes with the host plant's ontogeny or its metabolism.

If we are to move forward to a clear understanding of the role of allelopathy in regeneration we will need integrated studies. These must contain an element of field corroboration, and they must elucidate the pathway and fate of the specific allelopathic chemicals involved. Such studies will eventually both convince skeptics and lead to techniques for the avoidance of allelopathic interference.

Allelopathic interactions may occur throughout the life of a stand, but are most commonly observed during reforestation or regeneration. Allelopathy prevents some tree species from regenerating, but most regenerate in spite of it. The allelopathic plants of abandoned fields are not common forest species. In contrast, however, Douglas-fir, jack pine, black and white spruce, wild cherry, and slash and loblolly pine seedlings appear to be inhibited by species common in the forest. In such cases

regeneration appears to be accomplished in a number of ways. For one thing, the distribution of the allelopathic plants is seldom continuous and the phytotoxic effect usually does not extend far from the source. Trees can grow in the empty spaces and shade the phytotoxic plants, thus reducing their vigor and activity; eventually a stand of trees can become established.

If the forester knows which trees are particularly susceptible, which plants are most likely to produce toxic effects, and which site conditions contribute to interactions, most allelopathic problems can be avoided or easily dealt with by site preparation and weed control. Indeed, by these and other practices foresters often control allelopathy inadvertently.

If current techniques become too costly or otherwise impractical silviculturists will be forced to rely upon the natural resistance of some species, or to select alternative silvicultural systems. This will require improved understanding of the allelopathic phenomenon and some alteration in the selection of species.

Whatever the cause, allelopathy is not a problem for all plants nor at all locations where allelopathic plants occur. It should not be used as an easy explanation for any mysterious regeneration failure or case of poor stand growth. Rather it should be considered as a potential cause and analyzed as an explanation just as other possible causes are analyzed.

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## Chapter 17

# Allelopathic Effects on Mycorrhizae Influence on Structure and Dynamics of Forest Ecosystems

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Ectomycorrhizal species differ in their sensitivity to allelochemicals originating in litter and soil organic material. This phenomenon produces a successional change in mycorrhizal species as ecosystems rebuild litter layers following disturbance, and possibly acts to structure rooting zones in such a way that competition among higher plants is decreased. Mycorrhizae alter foliage chemistry, and thus potentially form a closed loop in which they both act on, and are acted upon by, system biochemistry.

A mycorrhiza (literally, fungus-root) is a symbiotic association between a fungus and a plant. Mycorrhizae occur most frequently on plant roots, but may be found on any tissue involved in uptake of elements from soil. Mycorrhizae, formed by numerous fungi in the orders Phycomycetes, Basidiomycetes, and Ascomycetes, can be divided into two broad groups: those that penetrate host cells (endomycorrhizae) and those that do not (ectomycorrhizae). A few fungal species defy this neat classification, penetrating the cells of one host but not those of another.

Among the endomycorrhizae, the most common are those that, because of distinctive structures produced by the fungus, are called vesicular-arbuscular mycorrhizae (VAM). VAM are Phycomycetes of the family Endogonaceae, and may occur on trees, shrubs, or herbs, and in any plant phyla (1). Ectomycorrhizae (EM), formed by numerous species of Basidiomycetes, Ascomycetes, and Phycomycetes, are distributed less widely in the Plant Kingdom than VAM. Although EM have been reported on herbs, principal hosts are trees and woody shrubs, particularly Gymnosperms and Angiosperms of the families Betulaceae and Fagaceae (1). Most of the important commercial tree species of the temperate zone are ectomycorrhizal. Despite their relatively narrow host range, EM fungi are highly diverse. At least 5000 species of fungi form EM, compared to fewer than 30 forming VAM (2).

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Over 90 percent of the roughly 200 plant families so far investigated form mycorrhizae and for many the relationship is obligate (3). Mycorrhizae directly benefit host plants by increasing water and nutrient (particularly phosphorus) uptake, increasing the lifetime of feeder roots, and protecting against root pathogens. The ecological role of mycorrhizae is in general poorly understood, but they are likely to mediate competitive relations between higher plants (4-6), alter plant palatability to herbivores (5, 7), and influence the physical structure of soils (8). The latter point is particularly important in soils with low clay content, in which virtually all aggregation and consequently water-holding capacity and porosity may result from the activity of mycorrhizae and associated rhizosphere organisms (e.g., 9, 10).

For most of the world's plants mycorrhizae are the primary interface between physiologically active areas of the root and the external environment. Hence it is likely that many, perhaps most, allelochemical interactions involving higher plant roots are mediated by the fungal symbiont. Herein we briefly review past research dealing with allelopathic effects on mycorrhizae, and discuss how interaction between mycorrhizae and chemicals may influence structural and functional aspects of ecosystems. Most studies to date have dealt with EM in forest ecosystems, and this is where our discussion will focus.

#### Allelopathic Effects on Mycorrhizae

Allelopathic effects on mycorrhizae have been known for 40 years. Melin (11) demonstrated that EM were inhibited by ethanol-soluble, heat-stable, substances contained in litter of Scots pine (Pinus sylvestris) and various deciduous tree species. Since then various workers have shown inhibition of EM by organic matter or water-soluble extracts from litter or roots (12-19). EM may also be inhibited by some Streptomyces species (20). Mikola (21) found that the degree of inhibition of EM by leaf and litter leachates varied with concentration: low concentrations actually stimulated growth of Cenococcum geophilum and Lactarius tommentosus. Allelopathic effects on EM vary not only with concentration, but are highly specific to fungal species and nature of the allelochemical as well. Tan and Nopamornbodi (22) found that several fulvic acids and soil extracts stimulated growth of Pisolithus tinctorius, a fungus that is also inhibited by leachates from a number of litters. Rose et al (23), studying the influence of water-soluble leachates from various types of litter on growth of four EM fungi, found a highly significant interaction between EM species, litter type, and concentration.

Allelopathic effects on EM may produce striking changes in plant communities. Widespread failure of Sitka spruce (Picea sitchensis) plantations in Scotland was attributed to inhibition of spruce EM by substances leaching from heather (Calluna vulgaris) roots and/or raw humus (12). In Finland, unidentified substances leached from reindeer lichen (Cladina sp.) inhibit EM formation on various tree species (24). Trees growing in the absence of lichen cover may be up to 20 times as large as trees growing with lichen (25). In other cases effects are more subtle, with allelopaths

altering EM composition rather than causing outright failure of EM formation. Schoeneberger and Perry (19) found that leachates from the litter of an Oregon forest reduced formation of a single EM type on Douglas-fir (*Pseudotsuga menziesii*), while EM of Western hemlock (*Tsuga heterophylla*) were unaffected. Litter from a nearby, very similar, forest had no effect. This disparity in results is not unusual; while most studies have found that organic material inhibits EM, some find just the opposite--stimulation of EM by organic material (26, 27). Such differences are probably due largely to the highly specific nature of the allelopath-EM relationship, although physiological vigor of the allelopath-producing plant also plays a role (12).

Our work has consistently shown that trees of a given species form different proportions of EM types depending on whether they are grown in soils from undisturbed forest or from clearcuts, and that the relative frequency of types formed in the latter depends on whether logging slash was burned or not. More research is needed, but the most likely explanation for these observations is that soils from plant communities in different stages of succession, or that have experienced different types of disturbance, vary in their characteristic chemical signature, and this in turn influences the type of EM forming on seedlings.

Apparently a variety of chemical compounds inhibit EM. Olsen et. al. (15) identified benzoic acid and catechol as the EM inhibitors present in aspen leaves (*Populus tremula*). They also showed that fungi decomposing wood and litter were less sensitive to these compounds than EM, and speculated that this may be due to greater production of extracellular phenol oxidases by the decomposer groups. Harley and Smith (1) suggest that the differential susceptibility to allelochemicals that occur among EM species may be related to production of phenol oxidases. Melin and Krupa (28) found inhibition of EM by several mono- and sesquiterpenes present in Scots pine (*Pinus sylvestris*) roots, although the two fungi they tested differed in their response to individual compounds.

Relatively little work has been done on allelopathic effects on VAM. Tobiessen and Werner (29) found reduced VAM formation in hardwood tree seedlings growing under pines, and spores of VAM fungi are absent from soil beneath living ponderosa pines, although they are abundant under dead trees (30). Members of the nonmycorrhizal family Cruciferae sometimes inhibit VAM formation in associated plants though this doesn't always happen (31-34).

#### Influence on Succession

Temperate forests are characterized by periodic catastrophic disturbance. Often this is due to fire, but various other agents such as wind and volcanic eruption also are responsible. Disturbance may influence the allelochemical environment in various ways. Living biomass is reduced, and fire reduces or eliminates litter and humus layers as well. Hence we should expect the total production of allelochemicals to be lowered following disturbance. As succession proceeds and total biomass and litter layers rebuild,

allelochemicals should become an increasingly important factor in the ecosystem.

There are likely to be qualitative as well as quantitative changes in allelochemicals during succession. Incomplete combustion of organic matter produces various polynuclear aromatic hydrocarbons, many of which are mutagens, carcinogens, or prototocarcinogens (35). Fire dramatically alters the microflora of forest soils, increasing the ratio of bacteria to fungi and altering the proportion of Streptomyces spp. that inhibit root pathogens or mycorrhizal fungi (20). Concentrations of hydroxymate siderophores (HS), an important class of iron chelators, are reduced by disturbance, particularly fire (36). Because iron oxides play a role in the breakdown of phenolic compounds (H. H. Chang, personal communication), lower levels of HS may well influence allelochemical interactions. Finally the change in plant species that defines a successional sequence undoubtedly produces temporal differences in the nature of allelochemicals.

The diverse nature of mycorrhizal response to allelochemicals suggests that the changing biochemical environment during succession may drive a successional sequence of mycorrhizal fungi. It is well established that a sequence of mycorrhizal species occurs on a given tree as it matures (37, 38), and in one case this has been linked to the buildup of litter around trees (39). Sensitivity of mycorrhizal species to litter leachates correlates well with what we know about their ecological role. For example, Rose et al (23) found that growth of Pisolithus tinctorius in pure culture was inhibited by a wide range of litter types, while that of Cenococcum geophilum was stimulated. The former species is a rapid grower that greatly aids survival and growth of trees on highly disturbed sites such as mine spoils while the latter has characteristics, such as an ability to infect roots of plants grown under low light, that make it particularly suited for mature forest conditions. It is difficult to avoid concluding that P. tinctorius is an early successional fungus, adapted to the low allelochemical environment of recently disturbed sites, while C. geophilum is a late successional species that thrives, and perhaps even depends, on allelochemicals.

In our studies we find that, where more than one mycorrhizal fungus occurs in the system, disturbance invariably results in a shift in the proportion of types that are formed on tree seedlings (5, 19, 40). For example, in high-elevation forests growing on granitic soils in southern Oregon, two mycorrhizal types predominate on Douglas-fir seedlings, an unidentified species that we call "brown," and Rhizopogon vinicolor. Ninety-five percent of the mycorrhizae on seedlings grown in soils from mature forests are "brown". When seedlings are grown under the same conditions, but with soil from an adjacent clearcut in which logging slash had been burned (standard practice), the proportion changes to 22% "brown" and 68% R. vinicolor. Evidence suggests that this change is at least partially related to destruction of the upper soil layers, which contain a large proportion of organic matter. When Rose et. al. (23) added litter leachates to Douglas-fir seedlings grown in soil containing both R. vinicolor and "brown," colonization of roots by R. vinicolor was inhibited while "brown" was either

unaffected or stimulated. Comparison of soils between mature forest and disturbed sites suggests at least one mechanism by which this change in fungus occurs. The former is characterized by a heavily organic surface layer 20 to 30 cm deep, overlying a sandy mineral zone. The boundary between the two is quite distinct, and is emphasized by a predominance of roots and hyphae in the upper zone. Bioassays show that "brown" is the predominant mycorrhizal fungus in this organic layer. The clearcut soil, although its total carbon content differs little from the forest, has no coherent surface organic layer; texturally its surface is very similar to the soil that is found at depth in the forest. *R. vinicolor* is its predominant mycorrhizal fungus (at least for Douglas-fir). Apparently, the buildup of the organic layer over time in these forests results in a partitioning of the two fungal species, the allelochemical-insensitive "brown" occupying the organic layers and the sensitive *R. vinicolor* the lower mineral layers. Logging and fire destroys the organic layer, leaving *R. vinicolor* in the mineral soil to serve as the primary fungus colonizing roots of invading tree seedlings. As litter and humus layers rebuild, "brown" becomes the dominant mycorrhizae-former, at least in the top 20 to 30 cm of soil.

Empirical evidence suggests that, in at least some cases, allelochemical-insensitive mycorrhizal fungi preferentially colonize late-successional trees. In pure culture experiments *Cenococcum geophilum*, which is stimulated by litter leachates, readily forms mycorrhizae both with early-successional Douglas-fir and late-successional western hemlock (41). However when these tree species are grown in soil from either disturbed areas or mature forest, *C. geophilum* forms a much higher proportion of the total mycorrhizae of western hemlock than of Douglas-fir (19). This raises the intriguing possibility that the shift in mycorrhizal fungi that occurs during succession facilitates change in higher plants.

#### Effects on Rooting Structure and Interactions Among Plants

Studies discussed earlier clearly show that allelopathic influences on mycorrhizae may alter the structure of communities by inhibiting one or more plant species. The diversity of response to allelochemicals among mycorrhizal species suggests that rooting pattern may also be influenced by the biochemical background of soils. Such structuring, rather than increasing the level of interference among individual plants, could produce the opposite effect, distributing roots throughout the soil volume and hence decreasing competition among individuals for water and nutrients. Such patterning would be strongest where plant species grown together tend to form different mycorrhizal types. For example, in a mixture of Douglas-fir and Western hemlock the former, with a relatively high percentage of allelochemical-sensitive mycorrhizae such as *Rhizopogon vinicolor*, would tend to root in lower soil layers, while hemlock, with its allelochemical-insensitive symbiont *Cenococcum geophilum*, would root in or close to surface organic layers. Because a single tree species may form several types of mycorrhizae that differ in sensitivity to allelochemicals, this



mechanism of root dispersion and subsequent decrease in belowground competition could occur in monospecific stands as well.

In order to test these ideas, we are growing Douglas-fir and ponderosa pine (*Pinus ponderosa*) in a Replacement Series. In this standard design the total number of individuals in a pot remains constant (12 in our case) while the proportion of each species varies. Our experiment actually involves four Replacement Series, one in unpasteurized soil, one in pasteurized soil in which no mycorrhizae were added, one in pasteurized soil with a species-specific mycorrhiza synthesized on each tree species (*Rhizopogon vinicolor* on Douglas-fir and *Rhizopogon oechorubens* on ponderosa pine), and one with four mycorrhizae in the system, the two *Rhizopogons* plus the two generalists *Laccaria laccata* and *Hebeloma crustiforme*. Soils are from a mixed Douglas-fir - ponderosa pine forest in southwestern Oregon. A and B soil layers, plus litter, were lifted separately in the field and reconstructed in the pots to simulate natural conditions as closely as possible. Five replications are being grown in a greenhouse with pots rotated weekly so that all treatments cycle periodically through all bench locations.

This experiment is in progress; however initial results clearly show that interference between seedlings of the two tree species is altered by addition of mycorrhizae to the system. Six months after being transplanted into the same pots, height growth of ponderosa pine is suppressed by Douglas-fir in the absence of mycorrhizae (Figure 1a). When each tree has its own mycorrhizal symbiont, however, this trend is reversed, ponderosa pine increasing in height with increasing proportions of Douglas-fir in the mix (Figure 1b).

Growth of Douglas-fir is lower in unpasteurized than in pasteurized soil (Figure 1d); In previous experiments we have found that Douglas-fir growth may be either inhibited or stimulated by sterilization, depending on soil type and disturbance history, and that this may vary from one year to the next in a single soil (20, 42). Reinoculation of pasteurized soil indicates that this is not an artifact of pasteurization. In contrast to Douglas-fir, ponderosa pine, when grown only with other ponderosa pine, performs at least as well in unpasteurized as in pasteurized soil. However, the presence of Douglas-fir has a much more depressing effect on pine height growth in unpasteurized than in pasteurized soil. Reduced height growth of Douglas-fir may indicate greater root growth, and this could create more competition between the two tree species, however this is pure speculation--at this point we cannot explain why responses are so different between pasteurized and nonpasteurized soil.

Initial results of the experiment support the hypothesis that mycorrhizal diversity influences interactions between plants. Isotope labeling now in progress will tell us whether this is due to different rooting patterns, and future experiments will specifically address the role of allelochemical diversity on rooting pattern. Our results in nonpasteurized soils simply emphasize what every soil biologist knows -- patterns occurring in nature are complex and include interactions among numerous organisms. There is little question that the belowground dynamic

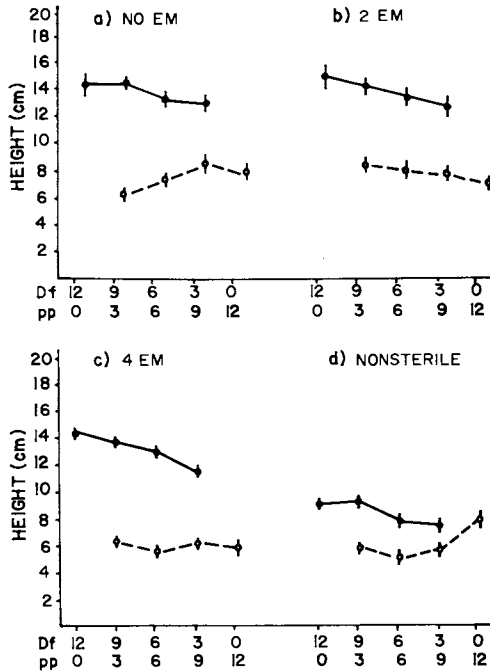


Figure 1. Height of Douglas-fir and ponderosa pine seedlings as a function of species mix and mycorrhizal infection.

Solid line = Douglas-fir  
 Dashed line = Ponderosa pine  
 Vertical bar = Standard error

- a) Pasteurized soil with no added mycorrhizae  
 b) Pasteurized soil with two mycorrhizal species added  
 c) Pasteurized soil with four mycorrhizal species added  
 d) Unpasteurized soil

is strongly influenced by allelochemicals, although the task of unraveling mechanisms is likely to be a long one.

### The Ecological Demon

The diversity of response to allelochemicals that exists within the community of mycorrhizal fungi has considerable implication for structure and functioning of terrestrial ecosystems. The repertoire of behaviors available to a given plant is greatly enhanced if it forms different mycorrhizal types, as is that of plant populations and communities forming a variety of mycorrhizae. The fungi translate information contained in allelochemicals into pattern within the ecosystem, and in this sense are reminiscent of James Clerk Maxwell's imaginary Demon, which could sort fast molecules from slow and thus impose structure on a gas. This allelochemically driven information processing ability suggests that mycorrhizae, like Maxwell's Demon, may play a far more important role in the ecosystem than indicated by their biomass or energy use.

Among the many questions remaining is the degree to which mycorrhizal fungi themselves influence the allelochemical background of a system. It is well known that mycorrhizae alter the chemistry of plant roots. Preliminary work in our laboratory shows that they influence leaf chemistry as well, producing a shift away from common monoterpenes toward unidentified compounds that may be di- or sesquiterpenes (5). The effect that such shifts may have on herbivory, decomposition and allelochemical interactions is not known. Mycorrhizae have been shown to retard litter decomposition (43), an effect that could alter the production of allelochemicals, particularly those that result from microbial activity. It seems almost certain that mycorrhizal fungi are active participants in, rather than passive prisoners of, the allelochemical dynamics of ecosystems.

### Applications in Forestry and Agriculture

Because of their role as interfaces between plant roots and the soil environment, mycorrhizae have the potential to contribute significantly to the success or failure of agroecosystems. Selection of the proper fungal symbiont may reduce allelopathic effects of weeds or of one crop plant on another in mixed-species systems. Cultivation of mycorrhizal diversity and selection of specific host-fungal combinations could enhance exploitation of soil resources. Genetic selection for allelochemical-tolerant strains of mycorrhizal fungi is probably feasible. Before mycorrhizae can become a significant management tool, however, much research is needed on the basic biology and ecology of their interaction with allelochemicals, and how this influences community development.

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## Chapter 18

# Allelopathic Interference of Black Walnut Trees with Nitrogen-Fixing Plants in Mixed Plantings

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The concentration of juglone (5-hydroxy-1, 4-naphthoquinone), an allelopathic chemical produced by black walnut (Juglans nigra L.), was significantly lower in soil beneath plots of walnut trees mixed with autumn-olive (Elaeagnus umbellata Thunb.) than beneath plots of walnuts mixed with European alder (Alnus glutinosa (L.) Gaertn.) or walnuts alone. The number of nitrifying bacteria varied among treatments. Significantly more Nitrobacter inhabited the soil in plots of walnut mixed with European alder than in plots of walnut mixed with autumn-olive or walnut alone. Apparently juglone concentrations were not sufficient to inhibit populations of these nitrifying microorganisms.

A tree incorporates nutrients, water, light, and metabolites to control its health and growth. Additionally, the tree must adapt to the presence of other organisms and to its environment. The direct or indirect inhibitory or stimulatory effect of one plant (including microorganisms) on another through the production of chemical compounds that escape into the environment is termed allelopathy (1). One of the most prominent allelopathic responses in forestry is the effect of juglone from black walnut (Juglans nigra L.) on associated vegetation. The implications of juglone in black walnut management are of interest because black walnut has a high timber value and because some of the fastest-growing, plantation, black walnuts are planted in mixtures with nitrogen-fixing species such as autumn-olive (Elaeagnus umbellata Thunb.), European alder (Alnus glutinosa (L.) Gaertn.), or herbaceous legumes. Therefore, interference may affect the growth of walnut. The term "interference" includes the overall deleterious effects of both competition and allelopathy in interplant relationships (2).

Early evidence of black walnut allelopathy. As early as 77 A.D., the Juglans genus was cited as having a poisonous effect on other plants. An even earlier account was recorded by Pliny the Elder in

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his Naturalis Historia about 1 A.D., when he wrote: "the shade of the walnut even caused headaches in man and injury to anything planted in the vicinity" (3). Juglone, a pigment in black walnut, was first suggested to be the phytotoxic agent by Massey (4); this was later confirmed by Davis (5). Chemically, the active ingredient is a phenolic compound that has the chemical formula 5-hydroxy-1,4-naphthoquinone. Juglone and/or its immediate precursors are present in the green leaves, green hulls, bark, bud scales, flowers, phloem, and roots of trees in the walnut family.

The degree of juglone toxicity differs greatly depending upon the intercepting species. For example, under field conditions, juglone is highly toxic to tomato (Lycopersicon spp.) (6), but beans (Phaseolus spp.) may (7) or may not be affected (8). But when injected, juglone affects bean and tomatoes similarly, by reducing their respiration rates. The differential toxicity of walnut trees on other vegetation in the field may be due to differences in the ability of plants to release juglone from the glucoside form in which it normally occurs in walnut tissue (9). Some plants may avoid exposure to juglone because their root systems are too shallow to come into contact with walnut roots; others may be able to withstand the strong oxidizing effect of the phytotoxin (10).

Toxic effects due to juglone persist in nature even after the walnut trees are dead (11). Juglone is released either by leaching and exudation from foliage, the fruits, the bark, and the roots or by decomposition. In the soil, juglone may be decomposed by microorganisms or adsorbed onto clays or organic matter. Thus, the soil is an important factor in the allelopathic interactions caused by juglone (12).

#### Allelopathic effects of juglone on nitrogen-fixing microorganisms.

Growth of black walnut increases when planted with trees such as black locust (Robinia pseudoacacia L.) (13), European black alder (14), and the shrub autumn-olive (15), which form a symbiotic association with soil microorganisms to fix nitrogen. Although black walnut is known to inhibit the growth of associated plants, the effects of juglone on symbiotic nitrogen-fixing soil microorganisms have not been investigated extensively. The nitrogen-fixing microorganisms associated with the roots of autumn-olive and European alder belong to the genus Frankia. Dawson et al. (16) reported that the growth of Frankia cultures decreased as the juglone concentration increased. Vogel and Dawson (17) also studied the effects of juglone on Frankia isolates in culture and on the nodulation of European alder in soil. They reported that the total protein content of the isolates was reduced significantly by juglone at  $10^{-4}$  M concentration, but the degree of growth inhibition varied with Frankia isolates. Nodulation of black alder in prairie soil inoculated with a Frankia isolate from red alder (A. rubra Bong.) was significantly decreased at juglone concentrations of  $10^{-3}$  and  $10^{-4}$  M.

Allelopathic effects of juglone on nitrifying bacteria. I am unaware of any reports dealing specifically with juglone as an inhibitor of nitrification, but there have been several reports of inhibition of nitrification in grassland and a few reports indicating inhibition in forest soil. The causal agents in both situations have been

identified as phenolic acids or phenolic glycosides (18-20). These aromatic compounds completely inhibited the oxidation of ammonium-nitrogen to nitrite-nitrogen by Nitrosomonas at concentrations as low as  $10^{-6}$  to  $10^{-8}$  M (20). Oxidation of nitrite-nitrogen to nitrate-nitrogen by Nitrobacter required much higher concentrations of the inhibitors.

Changes in Nitrosomonas and Nitrobacter populations have been documented with changes in the successional development of old fields to climax vegetation (20,21). The number of nitrifiers was high in the first successional stage and decreased to a very low number in the climax. It appears that inhibition of nitrification starts during old-field succession and increases in intensity as succession proceeds towards climax. Rice (22,23) found that many plants important in old-field succession are very inhibitory to selected strains of nitrogen-fixing and nitrifying bacteria and concluded that most inhibitors identified were phenolics. Thus, juglone might affect the growth of higher plants through its influence on soil microorganisms in addition to its direct effect on the plants.

Allelopathic effects of juglone on young seedlings. Juglone may retard growth and inhibit seed germination by disrupting cell division. Rietveld (24) conducted experiments to determine juglone sensitivity of 16 plant species that were among those being considered for interplanting with black walnut as nurse species. He reported that seed germination and radicle elongation were affected by juglone in 6 and 11 species, respectively, mainly by the higher concentrations ( $10^{-3}$  and  $10^{-4}$  M). Of the species tested, European alder and autumn-olive were most sensitive.

European alder has been observed to decline at age 8 years in plantings when mixed with black walnut. It has been surmised that black walnut allelopathy was the most likely cause for the black alder decline (25).

To gain more understanding of the European alder decline and because of our concern about the future growth of black walnut planted with nitrogen-fixing species, a study was initiated to measure soil juglone concentration and to estimate the number of Nitrobacter and Nitrosomonas bacteria in a black walnut plantation containing plots of black walnut alone and in mixture with European alder and autumn-olive.

#### Methods and Materials

The absence of data at planting for parameters to be measured in the study limits comparisons to current conditions in the plantation. The study was conducted on soil of a 15-year-old bottomland forest planting in southern Illinois containing plots of walnut planted alone and mixed with either European alder or autumn-olive. European alder decline and mortality were already evident. Rows within the 29.6- x 38.4-m plots were 3.7 m apart. Trees within the rows of walnut-alone plots were 4.9 m apart. In mixed plots, the walnut trees and autumn-olive or European alder were spaced 2.4 m apart, thus achieving a 1:1 mixture of walnut to nitrogen-fixer. The height of walnut trees ranged from 1.5 m in walnut-alone plots to 5.5 m in mixed plots. The height of autumn-olive and European alder averaged 5 and 7 m, respectively.



Soil samples were collected in mid-November at a distance of 0.9 m from each walnut tree at depths of 0-8, 8-16, and 16-30 cm for juglone estimates. Special effort was taken to avoid locating sample plots near dead or declining European alder trees. Another set of samples was collected at 0.9 m from walnut trees in the same plots at depths of 0-4, 4-8, 8-16, and 16-24 cm for Nitrobacter and Nitrosomonas counts.

To determine juglone, soil was sieved through a 40-mesh sieve and dried for 24 h at 40° C. Soil was leached with chloroform and the leachate was banded on silica gel G TLC plates and chromatographed with a solvent mixture of cyclohexane, chloroform, and glacial acetic acid (70:20:10) (26). Juglone was observed as a visible yellow-orange band at  $R_f = 0.40$  (27). The band was scraped from the plate, the juglone eluted from the gel with chloroform, and the solution filtered. Juglone concentrations were measured spectrophotometrically with a Bausch and Lomb Spectronic 20 at 420 nm. The most probable number technique (MPN) was used to estimate the number of Nitrosomonas and Nitrobacter in samples (28). Counts were made after inoculated tubes were incubated for 3 weeks at 28° C.

Total and nitrate nitrogen were determined by Kjeldahl and colorimetric methods, respectively (29,30). Organic matter was determined by the wet oxidation method of Walkley (31). Soil pH was determined with a glass electrode in a 2:1 mixture of water and soil.

## Results

The mean juglone concentration in soil beneath plots of autumn-olive/black walnut was significantly lower than in soil beneath European alder/black walnut and black walnut-alone plots (Table I). Juglone concentrations also differed with sampled depth (Table I). Significantly higher concentrations were present at the 0-8 cm depth than at lower depths.

Although the number of Nitrosomonas bacteria was greater than the number of Nitrobacter in each treatment plots, only Nitrobacter differed significantly among treatments (Table II). Both Nitrosomonas and Nitrobacter counts decreased significantly with sampled depths.

Soil beneath European alder/black walnut plots had higher nitrate nitrogen levels than soils beneath autumn-olive/black walnut or walnut-alone plots (Table III). Mean total nitrogen did not differ significantly between treatments.

Mean organic matter was greatest in the European alder/black walnut treatment, followed by the walnut-alone and autumn-olive/black walnut treatments (Table III). The mean pH was lowest in the autumn-olive/black walnut treatment, followed by the European alder/black walnut and walnut-alone treatments.

## Discussion

The higher juglone concentrations in the surface soil and within a few centimeters of the soil surface beneath the walnut was due to the presence of leaves, fruit, and roots. Juglone concentrations in this bottomland plantation followed a pattern similar to treatment differences reported for a companion upland mixed planting (26). However, results from the present investigation showed juglone

Table I. Juglone Concentration in Soil According to Depth and Treatment in a Mixed Planting of Black Walnut and Nitrogen-fixing Species

Depth (cm)	Juglone concentration ( $\mu\text{g/g}$ soil)		
	European alder and walnut	Autumn-olive and walnut	Walnut alone
0- 8	3.72 a	2.25 a	3.65 a
8-16	0.85 b	0.50 b	0.95 b
16-30	0.65 b	0.25 b	0.55 b
Mean	1.74 a	1.00 b	1.72 a

Means followed by the same letter are not significantly different at the 0.05 level according to Scheffe's method of contrast.

Table II. Mean Number of *Nitrosomonas* and *Nitrobacter* in Soil in a Mixed Planting of Black Walnut and Nitrogen-fixing Species According to Depth and Treatment

Treatment	Soil Depth (cm)	<i>Nitrosomonas</i> (MPN/g soil)	<i>Nitrobacter</i> (MPN/g soil)
European alder and walnut	0- 4	2880	2325
	4- 8	1460	235
	8-16	793	85
	16-24	495	20
	Mean	1407 a	666 a
Autumn-olive and walnut	0- 4	2108	606
	4- 8	1735	42
	8-16	478	13
	16-24	205	3
	Mean	1131 a	166 b
Walnut alone	0- 4	2028	429
	4- 8	1223	29
	8-16	683	10
	16-24	322	4
	Mean	1064 a	118 b

Means followed by the same letter are not significantly different at the 0.05 level according to Scheffe's method of contrast.

Table III. Mean Total and Nitrate Nitrogen, Organic Matter, and pH in Soil Beneath Black Walnut Planted in Mixture with Nitrogen-fixing Species

Depth (cm)	European alder and walnut	Autumn-olive and walnut	Walnut alone
Total Nitrogen (%)			
0- 8	0.18	0.14	0.15
8-16	0.09	0.10	0.10
16-30	0.09	0.09	0.09
52-61	0.07	0.07	0.08
Mean	0.11 a	0.10 a	0.11 a
Nitrate Nitrogen (ppm)			
0- 8	3.1	2.5	2.1
8-16	2.9	2.0	1.5
16-30	2.4	1.6	1.5
52-61	1.8	1.3	1.3
Mean	2.6 a	1.8 b	1.6 b
Organic Matter (%)			
0- 4	3.50	2.07	2.51
4- 8	2.00	0.75	1.29
8-16	1.59	0.74	1.24
16-24	1.40	0.80	1.16
Mean	2.12 a	1.09 c	1.55 b
pH			
0- 4	4.60	4.27	4.53
4- 8	4.73	4.60	4.77
8-16	4.87	4.80	5.03
16-24	5.03	4.90	5.20
Mean	4.81 a	4.64 b	4.88 a

Means followed by the same letter are not significantly different at the 0.05 level according to Scheffe's method of contrast.

concentrations to be higher in the 0-8 cm soil layer for the autumn-olive/walnut treatment than in the upland plantation.

The lower mean juglone concentration in soil under autumn-olive (Table I), when compared with European alder, is probably associated with microenvironmental conditions that enhance the breakdown of plant organs and result in oxidation of juglone in the soil. The canopy in plots mixed with European alder was more open than in plots mixed with autumn-olive, and more herbaceous vegetation was present in these plots than in autumn-olive plots. European alder/black walnut plots contained clumps of fescue (*Festuca arundinacea* Schreb.), blackberry (*Rubus* spp.), scattered grasses, and broadleaf weeds. Ground cover in autumn-olive plots consisted of small herbaceous plants and a 2- to 3-cm-thick leaf litter layer. The shade and closed canopy provided by autumn-olive reduced water loss due to transpiration and evaporation immediately above the soil, keeping temperatures about 2° C lower in the summer and about 3° C warmer in the winter (15). All these differences in plot conditions associated with treatments are believed to account for differences in soil juglone concentrations that are reflected in the mortality of the European alder (25).

The higher nitrifying bacteria populations in the European alder/black walnut plots compared to autumn-olive/black walnut and walnut-alone plots suggest that more juglone should be metabolized. However, this does not appear to be the case. Apparently, the difference in bacterial populations is not related to any direct detrimental effect of juglone on the bacteria because relative *Nitrobacter* counts were highest in European alder/black walnut plots where the juglone concentration was also highest.

Treatment means for pH and organic matter varied significantly between treatments, except for the pH between European alder/black walnut and walnut-alone. According to Alexander (32), nitrification is almost negligible at pH 5.0. Nitrification in soil beneath red alder (*Alnus rubra* Bong.) was reported at a pH as low as 3.5 (33). Obviously nitrification has not been eliminated in soil beneath treatment plots in our study.

The higher nitrate-nitrogen concentration in European alder/black walnut plots (Table III) appears to be consistent with the higher nitrate-nitrogen and nitrifying capacity of red alder in mixed plantings or alone (34,35). These authors concluded that nitrification was better in soils beneath red alder because of better soil fertility, especially higher nitrogen content. However, we found that although nitrate-nitrogen was highest in European alder/black walnut plots, total soil nitrogen did not differ significantly between treatments. Therefore, soil fertility alone, as suggested by total nitrogen, does not appear to be responsible for the higher *Nitrobacter* counts in European alder/black walnut plots than in other treatments.

It seems that the high nitrate-nitrogen concentration in the European alder/black walnut plots is the result of a high nitrifying bacterial population that can be attributed to understory vegetation differences between treatments. Lodhi (34) reported that the number of *Nitrosomonas* and *Nitrobacter* showed a direct relationship with amounts of nitrate-nitrogen but an inverse relationship with ammonium-nitrogen, and the relative amounts of ammonium and nitrate nitrogen are influenced by the successional stage of the vegetation on the

site. Rice and Pancholy (36) reported that the numbers of Nitrobacter per gram of soil were generally highest in the first successional stage, intermediate in the second successional stage, and lowest in the climax. The dominant vegetation in walnut-alone plots was broomsedge (Andropogon virginicus L.). Several plants of this genus inhabit old fields and have been shown to be very inhibitory to Nitrobacter and Nitrosomonas (37).

The almost bare to sparsely covered understory under autumn-olive in autumn-olive/black walnut plots has been attributed to reduced light intensity caused by the dense shade. The understory conditions rapidly approach those of a hardwood "forest" compared to the open European alder/black walnut and walnut-alone plots (38). Thus, the autumn-olive shorten the time it would normally take old-field conditions to disappear (37). The overall height of autumn-olive and the understory plot conditions have not changed in the last 4 or 5 years, while growth of the black walnut has continued at an improved rate (39).

European alder/black walnut plots, compared to the other treatments, are undergoing vegetational disturbances due to the decline and death of European alder. The disturbance is probably responsible for the overall higher number of Nitrosomonas and significantly higher Nitrobacter counts. The number of nitrifiers and rate of nitrification increase with disturbance in the ecosystem (1). Nitrosomonas and Nitrobacter increased by 18 and 34 times, respectively, after a forest clearcut in Connecticut (40). It appears quite likely that, in our study, vegetational disturbance has eliminated or prevented the establishment of plant species that may be inhibitory to nitrifying bacteria. Also, the larger nitrifying population in European alder/black walnut plots is consistent with the higher nitrate-nitrogen concentration in the same plots compared to autumn-olive/black walnut or walnut alone.

In summary, there is little reason to be concerned about allelopathy in mixed plantations where walnut is grown for timber as the harvested crop. Allelopathy does appear to be a factor to consider before planting European alder as a nurse crop with black walnut. Black walnut has had no apparent effect on the autumn-olive but is probably responsible for the decline and mortality of the European alder. These results present interesting possibilities for future research on the plant/soil/microbial relationships related to the metabolism of aromatics.

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## Chapter 19

# Allelopathic Interference with Regeneration of the Allegheny Hardwood Forest

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Herbaceous weeds were a major cause of regeneration failure in some stands following turn-of-the-century logging. Forest openings became dominated by species of fern, grass, goldenrod, and aster, which are present today, 60 or more years after logging. Studies of the reasons for regeneration failure showed strong evidence of allelopathic interference between the weed species and black cherry. Today, in second-growth stands, other species of ferns and grass are a critical barrier to regeneration. A study to separate the independent effects of weeds and herbivory by deer showed that ferns and grass are the primary reason for regeneration failure. Deer had no direct effect on desirable species because the weeds prevented them from growing enough to emerge from the herbaceous cover where these species could be seen and browsed. Blackberries, raspberries, yellow and black birches, and pin cherry that did emerge from the herbaceous cover were browsed by deer.

Interference between weeds and forest tree seedlings is a significant cause of regeneration failure in the cherry-maple (*Prunus-Acer*) Allegheny hardwood forest found along the northern tier counties of Pennsylvania and the southern tier counties of New York. Difficulties in regenerating black cherry (*Prunus serotina*) were encountered on some areas at the turn of the century when the original forest was cut (1). Weeds are a critical barrier to regeneration of many stands today as the second-growth forest is cut (2, 3). This paper summarizes work conducted to determine the reasons for regeneration failure after cutting both the original forest and second-growth Allegheny hardwood stands.

### Interference with Regeneration after Cutting of the Original Forest

Most of the Allegheny Plateau was cut over between 1890 and 1930. On most areas, new fast-growing stands of black cherry, red maple

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(*Acer rubrum*), sugar maple (*Acer saccharum*), white ash (*Fraxinus americana*), and beech (*Fagus grandifolia*) developed and are present today; but on some areas, new forests did not develop. Typically, these areas that failed to regenerate are found on extremely acid, imperfectly drained soil in stream bottoms or on high flats that were cut after 1920 when deer browsing had become a serious obstacle to forest regeneration. All of these sites had been burned by slash fires that were common at the time (4, 5). Locally, these areas are called orchard stands if they contain a few scattered black cherry and red maple trees, or savannahs if they are treeless. Deer and fire apparently prevented initial regeneration of orchard stands and savannahs, and allowed development of a dense herbaceous ground cover dominated by bracken fern (*Pteridium aquilinum*), wild oat grass (*Danthonia compressa*), rough-stemmed goldenrod (*Solidago rugosa*), and flat-topped aster (*Aster umbellatus*). These species are present today, more than 60 years after harvest cutting, and there is little evidence of succession to longer lived species. Young black cherry seedlings are present beneath the crowns of the widely spaced overstory trees, but they live only a few years and grow an average of only 3 or 4 cm per year.

In a series of field and greenhouse experiments, a number of possible sources of interference with black cherry growth were eliminated or shown to have minimal effect. White-tailed deer (*Odocoileus virginianus*) undoubtedly played a role in the establishment of orchard stands, but they are not responsible for their maintenance. About 30% of the seedlings we marked were clipped, whether or not they were protected with wire cages. The clipping characteristics were those of small mammals, not deer.

Microclimate at the study site was moderate during the 2 years of measurement. Soil temperatures ranged from 18 to 20°C, whether or not weeds were present, and within this range would not cause slow seedling growth. Surface soil moisture was high, ranging from 30 to 38% by volume on weeded plots and 26 to 35% by volume on unweeded plots during both years. Soil samples showed a fragipan-like layer underlying the study site that caused soils to be imperfectly drained, despite the fact that the sandy loam texture would otherwise have allowed better drainage.

Herbaceous canopies reduced the quantity of sunlight by 70 to 80% at the seedling level. In a field experiment, 4 combinations of presence or absence of shade and weeds were evaluated: 1) shade and weeds present, 2) shade present, weeds absent, 3) shade absent, weeds present, and 4) shade and weeds absent. In treatment 2, weeds were removed and shade of similar intensity to that produced by the weeds was reimposed with Saran shade cloth. In treatment 3, shade was removed by restraining the herbaceous vegetation behind a barrier made of wood stakes and string that allowed seedlings to receive a 45° cone of light from above. Results of this experiment showed that during the 2 years that measurements were made, shading or competition for light did not cause a significant reduction in either shoot height increment or annual stem diameter increment. However, the physical presence of weeds resulted in significantly less increment in both of these measures in the second year of the experiment. Thus, weeds had an effect on the growth of black cherry seedlings that was independent of shading. Further work indicated that the action of the weeds was exerted through the soil.

It was not necessary for weeds to be continuously present to have this inhibiting effect. Seedlings removed from an orchard stand and grown in a greenhouse in the sandy loam soil from an orchard stand, without weed competition, grew the same amount as seedlings growing in the field. However, these seedlings were capable of a larger growth response. Another group of seedlings removed from the field and grown without competition in an agricultural sandy loam soil that did not have a history of weeds produced 10 times as much growth. If the weeds had depleted the soil of some essential nutrient, it should be possible to replace that nutrient by fertilization. Whether or not weeds were present, fertilization of planted black cherry seedlings with ammonium nitrate and triple superphosphate, an N, P, Ca treatment, in amounts capable of stimulating significant growth of black cherry seedlings on sites without a history of herbaceous plants, had no effect on the growth of black cherry seedlings (6). It was possible to obtain a response to added nutrients if they were applied repeatedly. Addition of a complete mineral nutrient solution to seedlings growing in a greenhouse in the sandy loam soil from an orchard stand resulted in a significant increase in seedling growth.

In a second experiment, we attempted to determine which nutrient elements gave the same quantitative response as the complete mineral nutrient solution. Results of this experiment demonstrated that nitrogen ( $\text{NO}_3$ ), phosphorus, and calcium, the elements applied in the field study, were the ones needed to produce a growth response quantitatively the same as the complete mineral nutrient solution. The need for repetitive application of these elements suggests that one or more may have a dual role, first as a nutrient and second as a soil conditioner that might exchange or complex with an inhibitory molecule deposited in the soil by the herbaceous weeds.

Additional studies gave strong evidence of allelopathic interference. The possibility of an allelopathic interaction was investigated at 3 physiological stages in the development of black cherry seedlings: during seed germination, growth on cotyledonary reserves, and growth once cotyledonary reserves were exhausted. Foliage extracts were produced by soaking 50 g fresh weight of whole leaves of fern, grass, goldenrod, or aster in 1 liter of distilled water at room temperature for 16 h. These extracts were diluted with distilled water or complete mineral nutrient solution to form a concentration series of solutions containing 0, 5, 25, 50, or 100% foliage extract, and used to moisten peat in which black cherry seeds were stratified, to water newly germinated seedlings growing on cotyledonary reserves, or to water 30-day-old seedlings that had exhausted cotyledonary reserves.

Results of these experiments showed that most concentrations of fern, goldenrod, and aster foliage extract caused significant reductions in the germination of black cherry seed. Grass extract inhibited germination at the lowest concentration, but not at higher concentrations.

During the cotyledonary reserve period, only aster foliage extract inhibited growth of black cherry seedlings. When 100% extract was applied in distilled water to seedlings growing in sand, there was 35% less shoot height growth and 58% fewer first-order

lateral roots on treated seedlings than on plants receiving only distilled water. Roots of aster-treated plants had brown, necrotic epidermal patches and dead terminal meristems. Similar reductions in shoot growth were obtained when 100% aster foliage extract (50 g of leaves soaked in 1 liter of distilled water) was applied to seedlings growing in the sandy loam soil from an orchard stand or an adjacent fully stocked forest stand that did not have a history of herbaceous plants. When the experiment was repeated in the same agricultural soil used earlier, inhibition was not evident. This soil had a higher organic matter content, higher pH, and evidence of activity of soil organisms. Thus, it is possible that the inhibitory constituents of the extract were bound by the soil or used as carbon sources by soil microorganisms.

Foliage extracts of all 4 species of plants inhibited the growth of plants that had exhausted cotyledonary reserves, whether the seedlings were grown in sand or in the sandy loam soil from an orchard stand. Foliage extracts were applied at 0, 5, 25, and 50% concentrations in the same complete mineral nutrient solution used earlier. Results for the effects of aster on height growth were typical. Both rate and duration of shoot growth were reduced as the concentration of foliage extract increased. Reductions in shoot growth were attributable to both production of fewer nodes and shorter internode length. Trends in shoot dry weight paralleled those of shoot growth. Root dry weight was reduced, but to a lesser extent than shoot dry weight.

Effects of root washings of the 4 herbaceous plants on black cherry seedling growth were investigated by the "stairstep" technique (7). Root washings of aster and goldenrod, but not fern and grass, caused significant interference with black cherry growth. Shoot growth was reduced 50% by either species. Root washings of a single aster plant reduced shoot and root dry weight by 75%.

Allelopathy appears to be the primary source of interference in orchard stands, though it is possible that other factors contribute to the problem.

It is probable that allelochemicals accumulate in the soil over a period of years so that even when the herbaceous plants were removed, growth responses of seedlings continued to be poor. In well-aerated soils, organic chemical residues are normally metabolized by indigenous soil microorganisms. This probably occurs only slowly in orchard stand soils and is abetted by the inherent soil characteristics, such as imperfect soil drainage, high soil acidity, and low organic matter content. Isolation and identification of allelochemicals from plants and soil and demonstration of their participation in growth inhibition of black cherry would confirm the presence of an allelopathic relationship.

#### Interference with Regeneration in Second-Growth Allegheny Hardwood Stands

Herbaceous plants are also playing a major role in the regeneration of many second-growth stands. Unlike other eastern hardwood forest types, natural regeneration frequently fails after clearcutting of the Allegheny hardwood forest; these areas are often dominated by ferns and grass. Studies of factors associated with successful regeneration after clearcutting have shown that the presence of

large numbers of hardwood seedlings before clearcutting was highly correlated with successful regeneration after clearcutting (8).

There is overwhelming evidence from a variety of studies that browsing by white-tailed deer is responsible for many of these failures (9). Where deer have been excluded by fencing, regeneration is often successful; but where they are not excluded, regeneration often fails.

Many uncut Allegheny hardwood stands also contain dense ground covers of ferns and grass, particularly hayscented fern (*Dennstaedtia punctilobula*), New York fern (*Thelypteris noveboracensis*), and short husk grass (*Brachyelytrum erectum*). When these plants are present in dense stands, they too have a substantial inhibiting effect on regeneration. In a survey of thinned stands, I found 50 to 80% fewer black cherry and other desirable hardwood seedlings where dense herbaceous plants were present than where they were absent, and 40 to 65% shorter seedling height (2).

Recently, shelterwood cutting has been proposed to regenerate Allegheny hardwood stands that do not have enough advance regeneration to qualify for clearcutting (10). In the shelterwood method, the first or seed cut removes a portion of the overstory. The moderate environment created is favorable for germination and early growth of black cherry, red and sugar maple, white ash, and other desirable hardwood species. The strategy is to build up the numbers of hardwood seedlings and then, when there is enough, to remove the overstory with a second or removal cut and allow these seedlings to grow rapidly out of reach of deer. Anything that prevents the early buildup in numbers of seedlings or their subsequent growth after the removal cut precludes the use of the shelterwood method.

Many Allegheny hardwood stands are exposed to heavy deer browsing and also contain dense fern or grass ground covers. Recently, we evaluated the relative importance of deer and weeds at the 2 critical stages in the regeneration process of shelterwood cut stands--after the seed cut and after the removal cut (3).

Identical experiments were performed in 2 stands which had both a dense herbaceous ground cover and a high deer population. One stand received a shelterwood seed cut; the other, which had received a seed cut 6 years earlier, received the shelterwood removal cut. The effects of weeds and deer were separated using 3 fencing and weeding treatments applied to clusters of three 1.83-m radius plots: 1) fencing to exclude deer, but not weeds (fenced), 2) fencing plus repeated hand weeding to exclude deer and weeds (fenced/weeded), and 3) no fencing or weeding, allowing access by both deer and weeds to the plots (control). The effects of deer were estimated as the difference between fenced and control plots. The effects of weeds were estimated as the difference between fenced/weeded and fenced plots. Both experiments ran for 5 years.

The stand receiving the shelterwood seed cut had a dense understory of hayscented fern covering nearly 80% of the ground. The overstory in this stand was primarily black cherry and red maple and these were the major species represented in the regeneration. A large black cherry seed crop during the study resulted in increased numbers of black cherry seedlings on all plots the following year. The amount of increase in numbers was 60% greater on fenced/weeded plots than on fenced or control plots. Seedlings on fenced/weeded

plots also survived better. There were only 2% fewer black cherry seedlings in the second year after seedfall. Both fenced and control plots had 48% fewer seedlings. Patterns in germination and survival were similar for red maple. A large red maple seed crop during the study resulted in a 244% increase in numbers of red maple seedlings on fenced/weeded plots versus a 34% increase on fenced or control plots. Survival of these seedlings was 90% on fenced/weeded plots compared with only 36% on fenced or control plots. The net effect was that 4 years after treatment there were 43,000 desirable seedlings per hectare on fenced/weeded plots, but only 8,000 per hectare on fenced or control plots. We concluded that the fern cover, but not deer, interfered with germination and survival of these small seedlings.

In addition to the reproduction of desirable species, dormant seed of pin cherry (Prunus pensylvanica) are typically stored in the litter of Northern hardwood stands and germinate after cutting or disturbance. Storage of pin cherry seed in the litter for periods in excess of 50 years is well known (11, 12). Many of these stored pin cherry seeds germinated on fenced/weeded plots. At the peak, 2 years after the study began, there were 64,000 seedlings/ha. These intolerant seedlings did not survive long; most died within a year or two.

This experiment does not tell us how fern removal affects germination of pin cherry seed. However, Auchmoody (13) reported that application of nitrate ion to the forest floor of Allegheny hardwood stands without physical disturbance resulted in the germination of many pin cherry seeds. Thus, it is possible that the ferns have a role in regulating soil nitrification, the biological process that converts ammonium-N to nitrate-N, and that their removal results in an increase in this process that may affect germination and growth of tree seedlings. Black cherry seedlings, for example, grow well when supplied nutrient solutions containing a nitrate-N source, but grow poorly when the N source is ammonium.

At the beginning of the experiment in the stand that received the shelterwood removal cut, one-half of the stand had a dense cover of grass, and the other half a dense cover of fern. After the removal cut, there were substantial changes in the herbaceous cover. Blackberry (Rubus allegheniensis) and raspberry (Rubus occidentalis), which made up less than 5% of the ground cover at the beginning of the experiment, increased to 70 to 75% coverage on fern-covered plots and 80 to 95% on grass-covered plots. As Rubus increased in ground coverage, fern cover decreased by 25 to 45% and grass cover decreased by 30 to 40%. Even though Rubus is a favorite deer food, deer had little lasting effect on Rubus cover in this stand, though in stands where Rubus is less plentiful, deer are able to remove much of it (14).

Hardwood seedling reproduction was affected by both the type of ground cover present before the study began and the treatment. About 90% of these seedlings were black cherry; the next most abundant species were black birch (Betula lenta) and yellow birch (Betula alleghaniensis). Before treatment, grass-covered plots had more than 3 times as many desirable seedlings as fern-covered plots, suggesting that fern interference was greater than grass interference. After the removal cut, the numbers of all species present at

the beginning of the experiment declined continuously over the next 4 years. Fencing had little effect on seedling numbers, but fencing and weeding resulted in significantly more seedlings 4 years after cutting. Thus, the presence of fern or grass, but not deer, affected numbers of seedlings.

Treatment effects on seedling height growth differed with species. Deer had no effect on height of black cherry seedlings. There was little difference between fenced and control plots with either fern or grass cover; the herbaceous cover prevented the seedlings from growing through it where they could be seen and browsed by deer. Fenced/weeded black cherry seedlings were significantly taller than seedlings on either fenced or control plots. Ferns were the more inhibitory of the 2 herbaceous species.

The birches were affected differently. Birch typically grew rapidly through fern or grass cover and was not affected by it. Height of birches was similar on fenced/weeded and fenced plots. Once the birches grew above the herbaceous cover where they could be seen by deer, they were invariably browsed. Deer had a direct effect on birch, but ferns and grass had no effect.

This study suggests that in Allegheny hardwood stands where both high deer populations and dense fern or grass cover are present, interference from ferns and grass is the primary factor that hinders the establishment of desirable species of regeneration, such as black cherry; deer browsing had little or no direct effect under these conditions because these seedlings were unable to grow above the herbaceous cover where they could be browsed. Where we have been able to compare the relative inhibition by ferns and grass, the ferns interfered more strongly than grass.

How do these ground cover plants, particularly the ferns, cause such strong interference with the establishment of black cherry seedlings? Previous research at our laboratory has shown that black cherry seedling growth can be modulated by supplies of nutrients, particularly nitrogen and phosphorus (15), water (16), light (16, 10), and allelochemicals (1, 2) within the range that these factors occur in the natural environment. Thus, ferns could interfere with the establishment of black cherry seedlings by interfering with the production, availability, or use of site or environmental resources.

We are conducting experiments to determine how hayscented fern interferes with the growth of black cherry seedlings in shelterwood seed cut Allegheny hardwood stands. These experiments measure the changes in site or environmental resources that occur in the presence and absence of hayscented fern cover, and the response of black cherry seedlings to these changes. We are investigating changes in light quantity and quality, plant water status, nitrogen production, and phosphorus and mycorrhizal status. Potential allelochemical effects of foliage leachings, root and rhizome washings, and foliage volatiles are being studied. The results of these studies should give us a clearer view of how hayscented fern interferes with the establishment of black cherry seedlings.

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## Chapter 20

# Conjugation of Allelochemicals by Plants

## Enzymatic Glucosylation of Salicylic Acid by *Avena sativa*

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Plants have the ability to conjugate endogenous compounds to most allelochemicals absorbed from the environment. Conjugation reactions are thought to be important for detoxication of secondary products such as allelochemicals because conjugation increases the water solubility and decreases the chemical reactivity of the parent compound. Glucosylation, conjugation with glucose, is one of the most common conjugation reactions in plants. Numerous glucosyltransferase enzymes have been extracted from plants. Uridine diphosphate glucose (UDPG) is the preferred glucose donor for these enzymes. The range of secondary products a particular glucosyltransferase can conjugate has not been determined nor has the ability of allelochemicals to induce different glucosyltransferases in plants. Roots of *Avena sativa* conjugated glucose to salicylic acid, a phenolic acid, when the allelochemical was present in solution bathing the tissue. The tissue's capacity to conjugate salicylic acid increased with time suggesting induction of glucosyltransferase activity in the tissue. A glucosyltransferase that transfers glucose from UDPG to the phenolic hydroxyl of salicylic acid was purified about 54-fold.

Organisms produce chemicals to protect themselves from other organisms and to give themselves advantage over other organisms (1). However, the fact that a chemical is produced and released by a donor does not mean that it will be effective on the receiver. In addition to loss to the environment during movement from the donor to the receiver, the chemical may become inactive after entering the receiver. This can occur by deposition of active ingredient in insensitive tissues and cellular compartments or by conversion to inactive compounds. Also, the receiver may excrete the compound as either

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active or inactive compounds. The basic principles of what happens to xenobiotic compounds when they enter an organism are the same for all organisms; only the details differ. For example, all organisms have mechanisms to remove xenobiotics from cellular cytoplasm. Higher animals do this by excretion in feces or urine. Plants have no circulation system or specific tissues involved in excretion; instead the compounds move into vacuoles or are integrated into insoluble residues such as lignin in the cell wall.

How quickly and to what extent an organism is able to reduce the concentration of an active chemical at the site of action is a major determinant of toxicity. Thus, differences in the capability of receivers to detoxify an active chemical is of major importance for selective action of allelochemicals. Selective toxicity will also be important if allelochemicals are to be put to practical use. For example, if allelochemicals are to be used as herbicides, the compounds will have to be toxic to some plants (weeds) but not toxic to others (the crop). Such selective toxicity could be accomplished if the crop, but not the weeds, had the ability to convert an administered allelochemical to nontoxic products.

The purposes of this paper are to review one of the common mechanisms of detoxication in plants, namely conjugation, and to present data showing that salicylic acid, an allelopathic phenolic acid, is enzymatically conjugated by oat roots.

#### Detoxication Reactions in Plants

The various types of reactions that lead to detoxication of xenobiotic compounds in plants have been categorized into three groups (2, 3). Phase I reactions include oxidation, reduction, and hydrolysis. Oxidation introduces an oxygen atom or a hydroxyl group into the molecule and is catalyzed by peroxidase, mono-oxygenase, or other oxygenase enzymes (2, 4, 5). Oxidation reactions are common in plants and can result in either the detoxication or the activation of xenobiotic compounds (3). Reduction is much less prevalent and occurs primarily with nitro groups (2). Hydrolysis of esters, amides, and nitriles is a common reaction in plants (2). Carboxylic acid esters are especially susceptible to hydrolysis to yield free acids. These free acid forms are thought to be more toxic than the esters (2). Phase I reactions can reduce significantly the toxicity of a xenobiotic compound and predispose it to Phase II reactions.

Phase II reactions are conjugation reactions. As applied to detoxication, conjugation can be defined as an *in vivo* reaction of a xenobiotic compound, or its primary metabolite resulting from a Phase I reaction, with an endogenous substrate to form a new compound of higher molecular weight (6). To aid in discussion of conjugates, Dorough (6) has defined "exocon" as that portion of the conjugate that is derived from the exogenous compound, and "endocon" as that portion derived from an endogenous compound. Conjugation reactions are catalyzed by many different enzymes depending upon the exocon and endocon being conjugated together.

Conjugation is a major determinant of the metabolic activity of a xenobiotic compound (7, 8). One reason is that conjugation significantly increases the water solubility of the exocon. An exocon that could previously diffuse across cellular membranes does not diffuse as readily once it is conjugated. Thus, the conjugate can be compart-

mentalized (9) more readily in the cell than can the exocon. Second, the conjugate is less reactive because at least one functional group that imparted chemical reactivity to the exocon has been blocked by the endocon.

Conjugates formed by Phase II reactions can have additional endocons added to them. The reactions responsible for such secondary conjugations have been classified as Phase III reactions (2, 3). In addition, primary and secondary conjugates can be incorporated into insoluble bound residues. The reactions responsible for production of these residues are also classified as Phase III reactions (2, 3). Phase III reactions occur primarily in plants presumably because excretion of Phase II conjugates is insignificant in plants. Phase III reactions are mechanisms whereby plants can reduce the effective concentration of xenobiotic compounds in the cytoplasm. Thus, conjugation reactions provide mechanisms for the elimination of xenobiotic compounds from sites of continuing metabolic activity in all organisms (6).

The above classification of detoxication reactions has been developed for the metabolism of synthetic pesticides in plants. However, the same reactions can occur with natural exocons, such as allelopathic compounds, that have the same functional groups as synthetic pesticides. Most allelopathic chemicals contain functional groups that can be conjugated by Phase II reactions. Thus, detoxication of allelopathic compounds can be expected to proceed by conjugation with the omission of Phase I reactions. The remainder of this review will be concerned with the conjugation of allelopathic compounds.

### Conjugation Of Allelochemicals

Most allelochemicals identified to date can be classified as secondary products because they are found only sporadically in nature and thus do not appear to be involved in the basic metabolism of organisms (10). That is not to say that these compounds do not serve a function in the organism producing them, and their involvement in both protective functions (11) and in metabolism (12) is now recognized. As many as 12,000 such compounds may be involved in ecological interactions between plants, microorganisms, and animals (13). The types of chemicals active in these interactions include straight-chain alcohols, aldehydes, ketones, carboxylic acids, quinones, terpenoids, steroids, coumarins, flavonoids, tannins, glucosinolates, and alkaloids (11, 14). The prominence of aliphatic hydroxyl, phenolic hydroxyl, and carboxyl groups in these chemicals is obvious. These functional groups, as well as amino and mercapto groups, can be involved in conjugation by plants. In fact, conjugates are the characteristic form in which phenols, aromatic acids, flavonoids, steroids, and many other secondary products exist in plants (7).

The endocons involved in these conjugations may be derived from monosaccharides (e.g., glucose, galactose, mannose, and apiose), disaccharides (e.g., gentiobioside and glucosylxylose), oligosaccharides, aromatic acids (e.g., benzoic acid, gallic acid, and caffeic acid), amines (e.g., putrescine and spermine), alkyl groups (e.g., methyl, acetyl, and dimethylallyl), amino acids and peptides (e.g., aspartic acid, glycine, and glutathione), aliphatic acids

(e.g., acetic, malic, and malonic acids) cyclic hydroxy compounds (e.g., shikimic acid), and inorganic acids (e.g., sulfuric and phosphoric acids) (7). This great diversity of endocons makes it difficult to predict what particular exocon-endocon conjugate will be present in a particular plant (15), and mixtures of conjugates can exist there.

Conjugates of secondary products with sugars are especially prevalent in plants (16, 17). Specific examples can be found in almost all classes of secondary products including glycosides of simple phenols (18) and phenolic acids (19), cyanogenic glycosides (20), flavonoid glycosides (22), cardiac glycosides (23), saponins (24), sesquiterpene lactone glycosides (25), quinone glycosides (26), and glucosinolates (27). In addition, glycosides are often formed when aglycones are fed to plant tissue (17). Such induced formation of glycosides is important with allelopathic compounds because the conjugate will most likely be less toxic to the plant than the aglycone (17).

#### Enzymology of Glucoside Formation

A large number of uridine diphosphate glucose glucosyl-transferases (EC 2.4.1.) (UDPG-GTases) that glucosylate secondary products to produce glucosides or glucose esters have been studied to varying levels of detail (8). I will summarize these studies, placing emphasis on those enzymes shown to glucosylate phenolic acids such as salicylic acid.

UDPG-GTases that use phenolic acids as acceptor molecules have been isolated from several plant tissues. These include leaves of sweet clover (28), geranium (29), *Cestrum euenthes* (30), oak (31, 32), unripe fruits of tomato (33, 34), entire seedlings of radish (35), and roots of oats (36).

Insofar as investigated, all the enzymes preferred UDPG as the sugar donor. In many of the studies, UDPG was the only donor used in the assay (28-30, 33, 34). An extensive study of sugar donors by Gross (32) showed that UDPG:gallic acid-GTase from oak leaves would not use ADP-, CDP-, GDP-, or TDP-glucose, nor UDP-galactose, -galacturonic acid, -glucuronic acid, -mannose, -xylose, or -N-acetylglucosamine. A UDPG:sinapic acid-GTase had the same donor specificity except that it used TDPG as well as UDPG (35).

Different GTases have different acceptor specificities (8). There are three potential classes of phenolic acids for UDPG:phenolic acid-GTases: true phenolic acids ( $C_6-C_1$ ), hydroxyphenylacetic acids ( $C_6-C_2$ ), and hydroxycinnamic acids ( $C_6-C_3$ ) (15). There are no reports of studies investigating the hydroxyphenylacetic acids as substrates for GTases. Hydroxybenzoic acids and especially hydroxycinnamic acids have been studied, often with inclusion of only one of the two classes. Kleinhofs et al. (37) extracted a UDPG-GTase from sweet clover that would glucosylate *o*-coumaric acid but not *o*-coumarinic acid; other phenolic acids were not tested as substrates. A UDPG-GTase from unripe tomato fruits was active with the cinnamic acids *p*-coumaric, ferulic, caffeic, and sinapic acids (33, 34). *p*-Coumaric acid was the best acceptor. Again no other phenolic acids were tested but the enzyme did glucosylate coumarins and flavonols. *p*-Coumaric acid was the only substrate tested as acceptor for the GTase extracted from *Cestrum euenthes* leaves (30).

For three UDPG-GTases, both hydroxybenzoic and hydroxycinnamic acids have been studied as substrates. UDPG-GTase activity from geranium leaves used both classes of phenolic acids (29), but no quantitation or relative activity with the two were stated. The GTase from radish seedling was active with both hydroxycinnamic acids and hydroxybenzoic acids (38). Compared to sinapic acid all other hydroxycinnamic acid derivatives and hydroxybenzoic acid derivatives showed only 30-40% as much activity. The only UDPG-GTase shown preferentially to use hydroxybenzoic acids was extracted from young leaves of oak trees (31, 32). Vanillic acid was the best acceptor of nine benzoic acid derivatives; all five cinnamic acid derivatives produced 35% of the activity that vanillic acid did. The enzyme showed no activity with salicylic acid.

With phenolic acids as substrates for a GTase, the question arises whether the glucoside (phenolic hydroxyl group) or the glucose ester (carboxylic hydroxyl group) is formed. It appears that both can be produced depending upon the substrate and the enzyme. Only glucose esters were formed from hydroxybenzoic and hydroxycinnamic acids by some GTases (29-32, 38). Both glucose esters and glucosides were formed from hydroxycinnamic acids by other enzymes (33, 34); glucosides were predominant over glucose esters (33), except for caffeic acid, from which only the ester was produced (34). *o*-Coumaric acid produced only the glucoside (28, 37), in agreement with feeding experiments (39). In the only *in vitro* study where salicylic acid was tested and found to be glucosylated, the glucoside, rather than the ester, was produced (29).

Two general points need to be made about all these UDPG-GTases. The first is that they may not be detoxication GTases. All these UDPG:phenolic acid-GTases are constitutive enzymes; it is probable that detoxication GTases are synthesized *de novo* only when the aglycone is present. Thus, induction of detoxication GTases in plants may be similar to detoxication UDPglucuronosyltransferases in animals in that the induced enzyme is different from constitutive glucuronosyltransferases (40).

The second point is the possibility that more than one GTase was present in the preparations used to investigate substrate specificity. In most of these studies, crude preparations were used and in no instance was gel electrophoresis used to assess purity. The greatest level of purification in these studies was 45-fold (32). Table I lists several additional GTases, the level of purification achieved, and the substrate specificity of the enzyme. In general, these more purified GTases displayed high substrate specificity; only substrates with very similar structures were glucosylated by the enzyme. The purity of the last two enzymes (Table I) was shown by gel electrophoresis. They both yielded one major protein band; the preparation from *Triglochin* also yielded one or two minor bands.

Because selective detoxication by a crop plant would be important if allelochemicals are to be useful as natural herbicides, the first objective of these studies was to determine if the cereal oats can metabolize exogenously applied salicylic acid, a known allelopathic agent. The second objective was to identify and partially purify any enzyme responsible for such metabolism of salicylic acid in oat roots.

Table I. Acceptor Specificity of Several Partially Purified Glucosyltransferases from Plants

Source <sup>a/</sup>	Purification	Acceptor Specificity			Ref.
		Class	Example	Relative Activity	
Qr leaves	45-X	C <sub>6</sub> -C <sub>1</sub>	vanillic acid	++	32
		C <sub>6</sub> -C <sub>1</sub>	salicylic acid	-	
		C <sub>6</sub> -C <sub>3</sub>	caffeic acid	-	
		C <sub>6</sub> -C <sub>3</sub>	ferulic acid	+	
Gj cells	110-X	C <sub>6</sub> -C <sub>1</sub>	salicyl alcohol	++	41
		C <sub>6</sub> -C <sub>1</sub>	p-OH-benzyl alcohol	-	
		C <sub>6</sub>	hydroquinone	-	
Ca roots	120-X	isoflavone	biochanin A	++	42
		C <sub>6</sub> -C <sub>1</sub>	benzoic acids	-	
		C <sub>6</sub> -C <sub>3</sub>	cinnamic acids	-	
Tm seedling	200-X	cyanohydrin C <sub>6</sub> -C <sub>1</sub>	4-OH-mandelonitrile	++	43
			p-OH-benzyl alcohol	-	
Pa sap	1700-X	C <sub>6</sub> -C <sub>3</sub>	coniferyl alcohol	++	44
		C <sub>6</sub> -C <sub>3</sub>	coniferaldehyde	+	
		C <sub>6</sub> -C <sub>1</sub>	p-OH-benzoic acid	-	
		C <sub>6</sub> -C <sub>3</sub>	cinnamic acid	-	
		flavonoid	naringenin	-	

<sup>a/</sup> Abbreviations of plant species: Qr, Quercus rubra; Gj, Gardenia jasminoides; Ca, Cicer arietinum; Tm, Triglochin maritima; Pa, Picea abies.

### Materials and Methods

Plant Materials. Oat seeds (Avena sativa L. cv. Goodfield) were germinated and grown aeroponically on moistened cheesecloth stretched across the top of a 4-L beaker containing 3 L 1 mM CaSO<sub>4</sub>. Plants were grown 5 days in the dark at room temperature (ca. 21 °C) with continuous aeration of the solution. The apical 5 cm of the roots were used to measure the absorption of salicylic acid. The entire, excised root was used for measurements of metabolism of salicylic acid and for protein extractions.

Salicylic Acid Absorption. The apical 5 cm of the primary and two seminal roots from each of three plants were cut into 1-cm segments to form an experimental unit (ca. 0.08 g). Incubation solution contained 0.5 mM KCl, 0.25 mM CaSO<sub>4</sub>, 0.5 mM salicylic acid, 10 nCi/mL [<sup>14</sup>C]-salicylic acid, with 25 mM Tris and 25 mM Mes buffers mixed to obtain pH 6.5. Because the salicylic acid was dissolved in absolute ethanol, the final concentration of ethanol in the incubation solution was 1% (v/v). Root segments were transferred to test tubes containing 10 mL continuously aerated incubation solution. After the predetermined absorption time, segments were collected from the incubation solution by rapid filtration on Whatman No. 2 filter paper,

washed for 2 min in unaerated, ice-cold, incubation solution lacking salicylic acid, collected again, and weighed. Radioactive content of the segments was determined by liquid scintillation spectroscopy using a dioxane/methylcellosolve scintillation fluid (45).

In Vivo Metabolism of Salicylic Acid. Entire oat roots were cut into segments 2-3 cm long and pooled to give 5 g for each experimental unit. Segments were incubated in 100 mL incubation solution containing 16 nCi/mL [ $^{14}$ C]-salicylic acid with aeration. At predetermined times, the segments were collected by filtration and washed as for the absorption experiments. Segments were frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was extracted with 10 mL 80% (v/v) methanol with gentle stirring for 30 min. The homogenate was centrifuged at  $20 \times 10^3$  xg for 20 min and the pellet was extracted twice more with 80% methanol. The methanol extracts were combined, concentrated with a flash evaporator, and evaporated to 1 to 1.5 mL under a stream of nitrogen gas. Aliquots of the concentrate containing at least 10 dpm were spotted on 250- $\mu$  cellulose-coated thin layer (250  $\mu$ ) chromatography plates and developed with either 6% (v/v) acetic acid or 6:1:2 1-butanol, acetic acid, water (BAW). Radioactive spots were located with a radiochromatogram scanner and  $R_f$  values were calculated. Radioactive content of the spots was quantified by liquid scintillation spectrometry after each spot was scraped off the plate.

Preparation of Protein Extracts. Excised intact roots (175 g) were incubated in 5.25 L incubation solution lacking radiolabelled salicylic acid for 20 hr with aeration. Incubation solution was decanted and the roots were rinsed in 5.25 L cold distilled water for 10 min. Water was decanted and the roots were frozen with liquid nitrogen and pulverized with a mortar and pestle. Extraction buffer (1.5 mL/g root) containing 10 mM 2-mercaptoethanol, and 25 mM Tris and 25 mM Mes mixed to give pH 7.0 was added and the homogenate was allowed to thaw at room temperature with gentle stirring. The homogenate was filtered immediately through four layers of cheesecloth and the supernatant was centrifuged at  $20 \times 10^3$  xg for 20 min. The resulting supernatant was brought to 50% (w/v) ammonium sulfate, stirred, and centrifuged again. Supernatant was brought to 65% (w/v) ammonium sulfate and centrifuged once more. The pellet was dissolved in 4 mL extraction buffer with the addition of NaCl to 0.8 mM and glycerol to 10% (v/v) (elution buffer). The protein solution was eluted through a G-100 Sephadex column (1.9 x 65 cm) with elution buffer at 8 mL/hr. Fractions containing GTase activity were pooled, concentrated to 10 mL by dialysis in polyethylene glycol 8000 MW, and eluted through G-75 Sephadex under the same conditions. Fractions containing GTase activity were pooled and applied to a DEAE-Sephacel column (1.22 x 14 cm), which was then washed with 80 ml elution buffer. Proteins were eluted from the column by linearly increasing the NaCl concentration in the elution buffer to 0.5 M over 10 h at 30 ml/h. Fractions containing GTase activity were pooled.

Protein content of various fractions was determined with Coomassie Blue by the method of Bradford (46) as refined by Spector (47).

In Vitro Salicylic Acid Metabolism. Protein fractions were assayed for their ability to produce the same metabolite of salicylic acid as the root tissue produced. Protein fractions (50-185  $\mu\text{L}$ ) were incubated in an assay mixture containing 0.4 mM salicylic acid, 25 pCi [ $^{14}\text{C}$ ]-salicylic acid, 1 mM UDPG, 25 mM Tris-Mes buffer to adjust to pH 7.0 (total volume 200  $\mu\text{L}$ ), for 1 h at 30  $^{\circ}\text{C}$ . The reaction was stopped by adding 200  $\mu\text{L}$  absolute methanol.

Salicylic acid and its metabolite were separated by two methods. The first was thin layer chromatography on cellulose with BAW solvent as for the in vivo metabolism studies. A quicker separation was achieved with a polyamide column. The entire 400  $\mu\text{L}$  from an individual assay was placed on top of a 0.8 x 2.0 cm column packed with Polyamide-6 (Accurate Chemical and Scientific Corp.). The salicylic acid metabolite was eluted with 6 mL water but salicylic acid was retained. 3a70B scintillation fluid (Research Product International Corp.) was used to determine the radioactive content of the entire 6 mL of eluant. Separation of salicylic acid and its metabolite by polyamide column chromatography was verified by thin layer chromatography.

The ability of an esterase or a  $\beta$ -glucosidase to hydrolyze the in vitro generated metabolite was tested. An assay mixture that had been incubated for 22 h (ca. 50% conversion of salicylic acid) was incubated with either 10 units of hog-liver esterase (E.C. 3.1.1.1, Sigma Chemical Co.) at pH 8.0 or 20 units of  $\beta$ -glucosidase (E.C. 3.2.1.21, Sigma) at pH 5.0 for 1 h at 37  $^{\circ}\text{C}$ . Salicylic acid and the metabolite were separated by thin layer chromatography with BAW and quantified by liquid scintillation chromatography.

## Results

Absorption of Salicylic Acid. Excised oat roots absorbed salicylic acid in two distinct phases (Figure 1). Upon exposure to salicylic acid the root segments rapidly absorbed the compound to attain a concentration of about 0.5  $\mu\text{mole/g}$  of tissue. On the assumption that 1 g of tissue equals 1 mL of tissue, this translates to 0.5 mM salicylic acid inside the tissue, the same concentration as the external solution. This concentration of salicylic acid was present in the tissue after 1 h and was maintained for over 3 h. By 4 h a second phase of absorption was evident (Figure 1). During the second phase, salicylic acid was absorbed at a greater rate that lasted for at least 24 h. At that time, enough salicylic acid had been absorbed that the concentration in the tissue was 8.0 mM. Thus, the tissue accumulated salicylic acid to concentrations greater than that in the external solution. An additional experiment (not shown) showed that the tissue would continue to absorb salicylic acid until the compound was depleted from the external solution.

In Vivo Metabolism of Salicylic Acid. All the salicylic acid absorbed by the tissue (Figure 1) did not remain as that acid. Initially (< 2 h), most of it remained as such (Table II). However, gradually more parent compound was converted to a metabolite, so that between 4 and 20 h most of the absorbed salicylic acid was thus converted. At 20 h, 6.79  $\mu\text{mole/g}$  of metabolite was present in the tissue. Thus, the accumulation of "salicylic acid" observed in the absorption

Table II. Distribution of  $^{14}\text{C}$  between Salicylic Acid and Metabolite in Oat Roots Following Exposure for Various Times <sup>a/</sup>

Exposure Time (h)	Salicylic acid	
	acid	Metabolite ( $\mu\text{mole/g}$ )
1	0.34	0.06
2	0.23	0.27
4	0.04	0.66
6	0.00	1.60
20	0.51	6.79

<sup>a/</sup> The radiolabelled compounds were separated by thin layer chromatography on cellulose using 6% acetic acid.  $R_f$  of salicylic acid was 0.63 and of the metabolite was 0.76.

experiment (Figure 1) resulted from metabolism of the parent compound as it continued to diffuse into the tissue. The extent of metabolism of absorbed salicylic acid increased from 0 to 6 h, and essentially all the absorbed salicylic acid was converted to the metabolite after 6 h (Table II).

In Vitro Metabolism of Salicylic Acid. The protein precipitating between 50 and 65% (w/v) ammonium sulfate possessed the capacity to produce the same metabolite as the oat root tissue (Table III). When the protein fraction was heated at 100 °C for 5 min it no longer converted salicylic acid to the metabolite. The enzyme that catalyzed

Table III. Salicylic Acid Metabolite Production by a 50-65% Ammonium Sulfate Fraction Extracted from Oat Roots Exposed to Salicylic Acid for 20 h <sup>a/</sup>

Assay Condition	Metabolite Production
+ [ $^{14}\text{C}$ ]-Salicylic acid, - UDPG	-
+ [ $^{14}\text{C}$ ]-Salicylic acid, + UDPG	+
+ Unlabelled salicylic acid, + UDP([ $^{14}\text{C}$ ]-G)	+
+ [ $^{14}\text{C}$ ]-Salicylic acid, + UDPG	
+ esterase <sup>b/</sup>	+
+ $\beta$ -glucosidase <sup>b/</sup>	-

<sup>a/</sup> Enzymatic activity was measured in the presence of various radio-labelled substrates. Salicylic acid and metabolite were separated on cellulose with BAW.  $R_f$ 's for salicylic acid, UDPG, and metabolite were 0.92, 0.00, and 0.68, respectively.

<sup>b/</sup> Esterase or  $\beta$ -glucosidase were added after the metabolite had formed.



the metabolite production required UDPG (Table III). The same metabolite was produced when the protein fraction was incubated with either [ $^{14}$ C]-salicylic acid and unlabelled UDPG or with UDP-([ $^{14}$ C]-glucose) and unlabelled salicylic acid. Addition of the  $\beta$ -glucosidase following production of the metabolite resulted in conversion of the metabolite back to salicylic acid. However, the esterase did not hydrolyze the metabolite. Thus, the metabolite appears to be  $\beta$ -glucosylsalicylic acid, which is produced by UDPG:salicylic acid glucosyltransferase (SA-GTase).

Partial Purification of SA-GTase. Proteins extracted from oat roots incubated for 20 h in salicylic acid were separated by salt precipitation and gel exclusion and anion exchange chromatography (Table IV). A 54-fold purification of the SA-GTase was achieved with this

Table IV. Partial Purification of the Glucosyltransferase <sup>a/</sup>

Fraction	Total Protein (mg)	Glucosyltransferase Activity			
		Total (mU)	Specific (mU/mg)	Yield (%)	Purification (fold)
Crude homogenate	142.4	259.0	1.82	100	1
50-65% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	56.1	159.2	2.83	62	1.6
Sephadex G-100	9.2	114.2	12.37	44	6.8
Sephadex G-75	2.3	64.1	27.45	25	15.1
DEAE-Sephacel	0.4	36.2	97.90	14	53.8

<sup>a/</sup> The metabolite and salicylic acid were separated on a polyamide column following incubation of the enzyme with radiolabelled salicylic acid and UDPG.

separation scheme. However, the SA-GTase was not homogeneous as shown by the presence of several protein bands on polyacrylamide gel electrophoresis. Total protein recovered from the anion exchange column was 0.4 mg and represented 14% of the SA-GTase activity present in the crude homogenate that contained 142 mg protein. Thus, less than 2.86 mg SA-GTase was present in the crude homogenate. Or, less than 2% of the protein extracted from the tissue was SA-GTase. Hence, the enzyme is a minor protein in the tissue.

### Discussion

These experiments demonstrate that oat roots can metabolize salicylic acid. Metabolism results in more salicylic acid being absorbed by the tissue and its accelerated metabolism with time of exposure to the parent compound (Figure 1, Table II). The appearance of only one metabolite (Table II) and the hydrolysis of the metabolite by  $\beta$ -glucosidase but not esterase (Table III) suggests that the glucoside and not the glucose ester of salicylic acid is produced by oat roots. Both metabolites of salicylic acid have been found in other plants (16). Some species produce only the glucose ester (48), others produce both the glucoside and the ester (49), and others

convert salicylic acid to gentisic acid and produce the glucoside of gentisic acid (19, 50). Positive identification of the metabolite from oats will require purification of sufficient amounts of the metabolite for structure elucidation.

The metabolite is enzymatically produced in the root tissue (Table III). In one other study (29), as already mentioned a protein extract from plants was shown to glucosylate salicylic acid; the glucoside rather than the glucose ester was produced. The protein extracts from oat roots also produced the glucoside and not the ester of salicylic acid (Table III). The enzyme uses UDPG as the glucose donor, but we do not yet know if UDPG is the preferred sugar donor. If so, the enzyme will be UDPG:salicylic acid glucosyltransferase. By analogy with similar enzymes (8), the enzyme probably catalyzes formation of a  $\beta$ -linkage between glucose and salicylic acid. Thus, the reaction can be visualized as shown in Figure 2. The enzyme has not yet been purified to homogeneity (Table IV). Because the enzyme represents < 2% of the extractable protein, further purification will require more starting tissue as well as additional techniques.

### Conclusion

Because of the presence of reactive hydroxyl groups on most allelochemicals identified to date, the likelihood is great that these compounds can be glycosylated by plants. Such glycosylation may result in detoxication of allelochemicals because of blockage of the reactive group and increased water solubility of the conjugate. Glycosyltransferases that catalyze glycosylation of secondary products in plants have been identified and the ability of such enzymes to glycosylate certain allelopathic compounds as they are absorbed by plants has been verified. The substrate range of glycosyltransferases has not been extensively studied. Thus, it is not known if a family of different glycosyltransferases are necessary to glycosylate the various allelochemicals plants might encounter.

Practical use of glycosyltransferases such as the glucosyltransferase that glucosylates salicylic acid in oat roots may be made to provide selective phytotoxicity for allelochemicals. Differences in the constitutive level of glycosyltransferases among crops and weeds might provide that selectivity. Selection of crop varieties with higher levels of glycosyltransferases could lead to crops more resistant to allelochemicals. More rapid induction of glycosyltransferases in desirable plants might be exploitable. And finally, transfer of genes encoding for glycosyltransferases with high activity toward a particular phytotoxic allelochemical might be possible in the future. Thus, modification of an allelochemical with a glycosyltransferase to detoxify the allelochemical may be a useful approach for making practical use of allelochemicals in agriculture. Further characterization of the types and activities of glycosyltransferases in plants must be done before such uses of these enzymes can be pursued.

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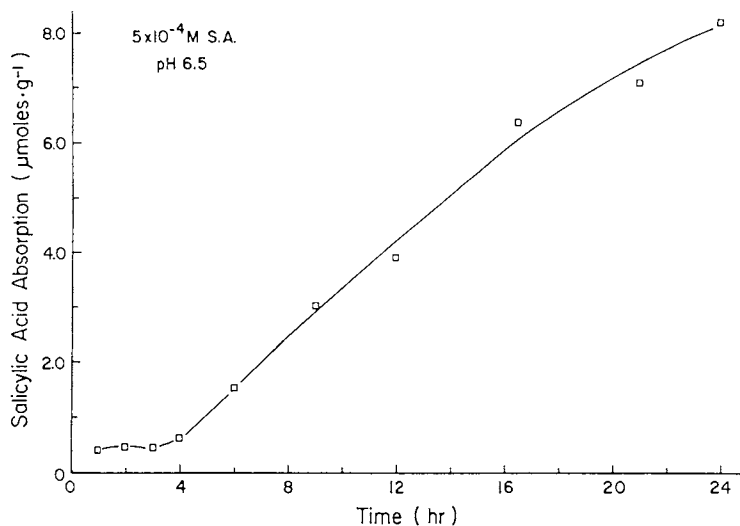


Figure 1. Absorption of salicylic acid for 1 to 24 hr by excised oat roots.

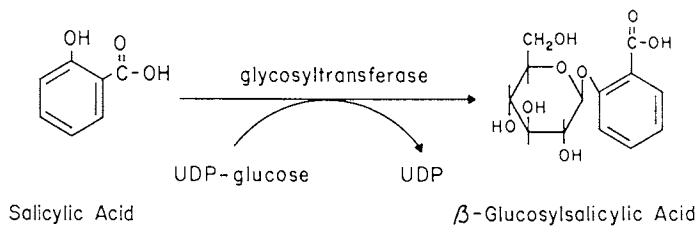


Figure 2. Reaction scheme for UDPG:salicylic acid glucosyltransferase extracted from oat roots incubated for 20 hr in solution containing salicylic acid.

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## Chapter 21

# The Role of Phytochemistry in Attacking the Leafy Spurge (*Euphorbia esula*) Problem

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Individual chemical constituents isolated and characterized from the noxious weed *Euphorbia esula* were assessed biologically and/or chemotaxonomically in relation to the allelopathy of *E. esula*, the allelopathy of *Antennaria microphylla* to *E. esula*, and the insect biological control of *E. esula*. Kaemferol 3-glucuronide and 1-hexacosanol obtained from aqueous extracts of *E. esula* are considered important allelochemicals in this weed. Biological assays, utilizing *E. esula* test systems, support hydroquinone and arbutin (obtained from extractives of *A. microphylla*) as major participants in the allelopathy of *A. microphylla* to *E. esula*. The distribution of triterpenes ( $\alpha$ ,  $\beta$ -amyrin and  $\delta$ -amyrenone) and unique jatrophone diterpenes characterized in *E. esula* leaf and root extracts have strong potential for the chemotaxonomic differentiation of *E. esula* varieties relative to insect biological control of this weed.

Leafy spurge (*Euphorbia esula*) is a dicotyledonous, herbaceous, deep-rooted, perennial noxious weed infesting more than four million acres of open rangeland in the upper great plains of the United States and the prairies of Canada. This lateciferous plant is toxic to livestock (1), allelopathic to desirable forage plants (2) and poses a serious threat to livestock production on open rangelands.

While leafy spurge can be controlled by herbicides (3) or vigorous cultivation, the cost of control is continuous since current chemical means do not eradicate this weed. More than 20 million dollars a year is spent for the control of this plant, and its agro-economic impact is greater than 12 million dollars per year in the state of North Dakota alone (4). Recent research efforts on the leafy spurge problem have concentrated on increased herbicide efficiency and the successful application of insect biological control methods.

This paper summarizes the results of phytochemical examinations of plant-plant and plant-insect interactions involving leafy spurge

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which could potentially provide new or improved methods for its control or eradication. These phytochemical investigations have resulted in the characterization of potential allelochemicals from leafy spurge and of constituents phytotoxic to spurge from Antennaria microphylla, a plant reported allelopathic to leafy spurge (2). Chemical examination of leafy spurge leaf and root material has also provided chemotaxonomic information of particular relevance in the selection of subspecies of the weed that are susceptible to insect biological control.

#### Plant-Plant Interactions (Leafy Spurge)

Several studies have examined the chemistry of Euphorbia species. A majority of these investigations have focused on the chemical character of the plant latex relative to taxonomy (5,6) or mammalian toxicity (7,8). Toxic ingenane diterpenes (phorbols) have also been obtained from the latex of E. esula (9-11). Other chemical investigations of the aerial portions of leafy spurge have led to the characterization of hydrocarbons (12), long-chain alcohols (13,14), long-chain aldehydes (14), triterpenes (12,15), flavonoids (16), and the description of an unidentified alkaloid (12). None of the chemical studies examined the chemical composition of leafy spurge in relation to allelopathy.

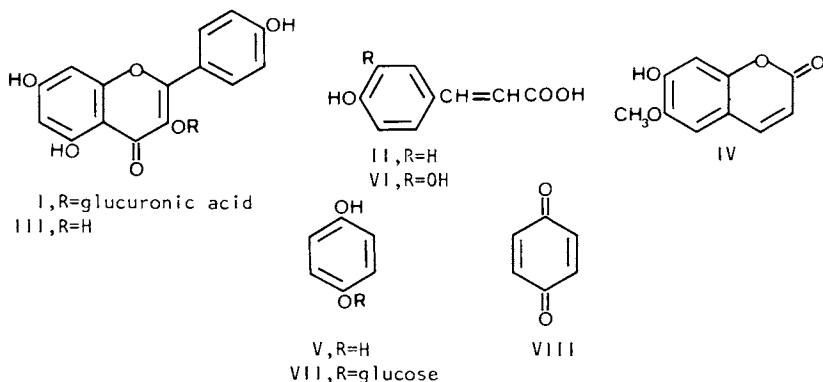
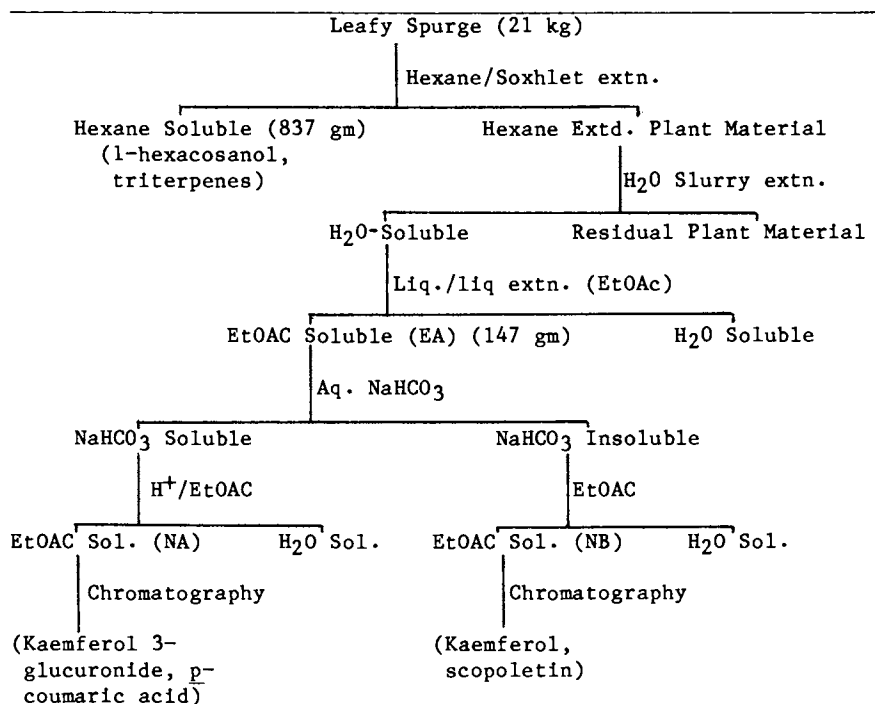
Allelopathy of Leafy Spurge. The lack of forbs and the occurrence of bare ground in stands of leafy spurge was considered evidence that the weed was allelopathic (1). The phytotoxicity of aqueous extracts of leafy spurge plant material and accompanying soil toward several test species verified the observed allelopathic behavior of the plant (2). The extracts were inhibitory to the germination and growth of forb test species. Aqueous leaf extracts were more toxic to the test species than the stem extracts while root extracts were the most toxic. Soil extracts were also toxic but at a lower level than the plant extracts. These results prompted our empirical phytochemical examination of leafy spurge plant material in an effort to define the chemical source of the observed allelopathy (17).

Air-dried aerial plant material was solvent extracted with hexane to remove nonpolar constituents and then extracted with water according to Scheme I. The water extract was liquid/liquid extracted with ethyl acetate. A lettuce seed bioassay of the crude ethyl acetate extract (EA) (dried in vacuo) showed a 22% reduction of root elongation at 200 ppm. The organic extract was extracted with aqueous sodium bicarbonate to produce acidic and basic fractions which were neutralized and extracted with ethyl acetate to achieve neutralized acidic (NA) and neutralized basic (NB) fractions. Fraction NA (dried in vacuo) maintained the previous level of phytotoxicity observed for fraction EA in a lettuce seed bioassay.

Preparative chromatographic fractionation of NA yielded kaemferol 3-glucuronide (K-3-G), previously described in E. esula (16), and p-coumaric acid (11) as major constituents. Lettuce seed root growth bioassay of K-3-G produced a 49% reduction of root elongation at 500 ppm. In contrast, kaemferol (III) (the aglycone of K-3-G) characterized in fraction NB showed a low level of stimulatory activity at the same concentration. The coumarin scopoletin (IV) was also isolated from fraction NB in low yield.

The observed biological activity of K-3-G, coupled with its relatively high yield ( $1.4 \times 10^{-4}$  moles/kg plant material) and water

Scheme I. The Chemical Separation of Leafy Spurge Plant Material



solubility, suggests the compound is an important chemical participant in the observed allelopathy of leafy spurge. The very high yield of nonpolar (hexane extract) constituents in the spurge plant material ( $\approx 5\%$  of total plant weight) also suggests their potential participation in the allelopathic complex of this weed. The hexane extract showed a remarkably high yield (15%,  $5 \times 10^{-3}$  moles/kg plant material) of a single long-chain alcohol (1-hexacosanol). Lettuce seed bioassay of this alcohol produced a 15% reduction in root length elongation at 500 ppm. The low solubility of this compound in the aqueous test system suggests the alcohol may function as a chronic allelochemical in leafy spurge stands.



Phytotoxicity to Leafy Spurge. The observed reduction of leafy spurge encroachment into increasingly dense stands of *Antennaria microphylla* (small everlasting) suggested that small everlasting was allelopathic to leafy spurge (1). Laboratory experiments with small everlasting plant and soil extracts conclusively showed inhibition of germination and seedling development of leafy spurge and the suppression of spurge growth in competition with small everlasting (2). The experimental observations coupled with the noncompetitive physical character of small everlasting (low growing, shallow-rooted) compared to leafy spurge (deep-rooted, vigorous root budding) suggested the observed dominance of small everlasting to be the result of an allelochemical effect rather than competition. However, characterization of small everlasting allelochemicals was not attempted in the study. Based upon the potential importance of biologically active chemical constituents of small everlasting as possible models for more efficient agents to control leafy spurge, a chemical investigation of small everlasting was undertaken (18).

Small everlasting plant material was collected, air dried, hammermilled and sequentially extracted with *n*-hexane, ethyl ether, acetone and methanol. Lettuce and leafy spurge root growth bioassays were used to determine which extract possessed significant biological activity (Table I). These results showed that the ether extract (dried *in vacuo*) of small everlasting reduced the growth of lettuce and leafy spurge seedlings by 74% and 76% respectively at 500 ppm. Much lower reductions of spurge root growth were observed for the hexane, acetone and methanol extracts (dried *in vacuo*). On the basis of the bioassay results, the ether extract was chosen as the most probable source of allelochemicals in small everlasting.

A portion of the dried ether extract was fractionated on a Sephadex LH-20 chromatographic column. Lettuce root growth bio-assay showed significant activity (68% and 77% reduction of root elongation) in two fractions. Each of these fractions was semi-preparatively chromatographed on a silica HPLC column. Three phenolic compounds, hydroquinone [V, 0.002%(w/w) of dry plant wt.], caffeic acid [VI, 0.012%(w/w)] and arbutin [VII, 0.034%(w/w)] were

Table I. The Effect of Small Everlasting Extracts on the Growth of Lettuce Roots and the Elongation of Leafy Spurge Roots

Extract	Lettuce Conc.(ppm)			Leafy Spurge Conc.(ppm)		Solubility Factor <sup>a</sup>
	100	300	500	300	500	
	growth, % of control			growth, % of control		
Hexane	91	100	97	78	82	0
Ether	77	41	26	36	24	2
Acetone	92	84	63	62	58	1
Methanol	81	69	66	65	60	3

<sup>a</sup>Empirical solubility of extract: 0 = insoluble, 1 = mostly insoluble, 2 = mostly soluble, 3 = totally soluble in aqueous test system. Source: Reproduced with permission from reference 18. Copyright 1986 Weed Science Society of America.

isolated, crystallized and characterized. Pure commercial samples of each of the three phenols and *p*-benzoquinone (VIII), the oxidation product of hydroquinone (included to evaluate the possible *in vivo* oxidation of hydroquinone), were tested in lettuce and leafy spurge root elongation tests and in leafy spurge cell cultures (Table II).

The high level of toxicity of both hydroquinone and *p*-benzoquinone toward leafy spurge was evident in both the root elongation and cell culture bioassays, with root length reductions of 93% and 90% and cell culture reductions of 80% and 64% respectively at 300 ppm. The observed phytotoxicity of arbutin to spurge seedlings (51% reduction in spurge root length at 300 ppm) was in contrast to the observed growth stimulation effect of arbutin on lettuce root growth. Caffeic acid showed a lower inhibition of spurge root elongation (64% at 300 ppm) with some mild stimulation appearing at low concentrations. The cell culture bioassay of caffeic acid supported the root growth bioassay results.

Table II. The Effect of Chemical Constituents Occurring in Small Everlasting on the Growth of Lettuce Roots, Root Elongation and Cell Culture Growth of Leafy Spurge

Compound	Lettuce roots				Spurge roots			Spurge cells		
	50	100	300	500	50	100	300	200	300	500
	growth, % of control				growth, % of control			growth, % of control		
Hydroquinone	19	12	9	0	36	7	0	79	20	24
Caffeic acid	51	48	18	13	110	81	36	106	105	103
Arbutin	115	114	113	119	81	70	49	87	86	74
<i>p</i> -Benzoquinone	11	9	6	0	34	10	0	87	36	19

Source: Reproduced with permission from reference 18. Copyright 1986 Weed Science Society of America.

Hydroquinone, arbutin and caffeic acid have been reported to occur in several plant families, but the appearance of hydroquinone and arbutin in small everlasting represents only the second reported occurrence of these compounds in Compositae. The two compounds have been previously reported in three species of *Serratula* (Compositae) (19).

Hydroquinone and arbutin have been considered as allelopathic agents in the aqueous leachates of species of manzanita (*Arctostaphylos*) (20,21) and chaparral (*Adenostoma*) (22). Because of the low toxicity of arbutin, it has been speculated (20,22) that hydroquinone and *p*-benzoquinone, originating from a large arbutin pool by hydrolysis and oxidation, were the chemical agents responsible for the observed allelopathy of manzanita and chaparral. Tests of hydroquinone on wild oats (*Avena fatua*) and brome grass (*Bromus rigidus*) (20) showed significant radical growth suppression at 50 ppm.

The high water solubility of arbutin provides efficient leachability of the compound from plant tissues and subsequent transport and absorption by other plants. The ultimate chemical form (hydroquinone vs. arbutin) available for absorption would be dependent upon the extent of decomposition (i.e. hydrolysis) affecting arbutin transport.

Glass and Bohm (23) showed arbutin and hydroquinone to be readily and continuously absorbed by the roots of barley plants. This study showed that hydroquinone was glycosated by the barley to form arbutin and was therefore effectively detoxified. If the equilibrium of the detoxification mechanism of a plant is sensitive to an oversupply of the toxic and detoxified compound, an oversupply of a detoxified compound could produce equilibrium amounts of the toxic compound. Cell culture bioassay (Table II) showed that hydroquinone is not significantly detoxified *in vivo* in leafy spurge, indicating the susceptibility of the plant to low levels of hydroquinone which could originate from an oversupply of arbutin. The observed toxicity of *p*-benzoquinone in the cell cultures and seed bioassays also indicates that oxidation processes affecting hydroquinone will not detoxify the compound *in vivo*.

This investigation has shown hydroquinone to be a potent phytotoxin toward developing leafy spurge roots. Hydroquinone, originating from a chronic oversupply of arbutin, is considered to play a significant role in the observed allelopathy of small everlasting toward leafy spurge.

#### Plant-Insect Interactions (Leafy Spurge)

The high cost of chemically controlling leafy spurge and the inability to eradicate the weed by chemical means has prompted the consideration of biological control by an insect predator as a viable alternative. *Euphorbia esula* has been successfully controlled by natural insect predators in Europe (24). However, these predators have not been successfully utilized in North America (25). The recognition of several morphologically different accessions of leafy spurge in North America (26,27) suggests either the occurrence of separate North American and European leafy spurge species or subspecies that can not be differentiated morphologically or intraspecies physiological and/or chemical differences. Intraspecies chemical and biochemical comparisons of leafy spurge accessions should provide important information for the chemical taxonomic differentiation of the accessions and the chemical relationship to insect predation. Our recent chemical examination of leafy spurge leaf (28) and root (29) material provides new information about this weed with primary application to its taxonomic differentiation as it relates to biological control.

Leafy Spurge Wax. Leaves of four North American accessions and one European accession of *Euphorbia esula* were dipped in chloroform (30 sec.) to obtain leaf wax samples for analytical gas chromatography/mass spectrometry (GC/MS) analysis (28). The wax samples were partitioned into acetone soluble and acetone-insoluble fractions. The GC/MS analysis showed the acetone-insoluble portion to contain hydrocarbons, long-chain aldehydes and alcohols, fatty acids and fatty acid esters while the acetone soluble portion contained terpenes and terpene esters. The yields of the general chemical classes as determined in the analysis of the five samples are summarized in Table III. A high yield of long-chain alcohols (primarily 1-hexacosanol) is found in all the accessions. While the yields are generally comparable in the North American samples, a significantly

lower occurrence of alcohols and a higher level of hydrocarbons appeared in the European sample.

Table III. Percent Composition and Yield of Epicuticular Waxes of Five Accessions of Euphorbia esula

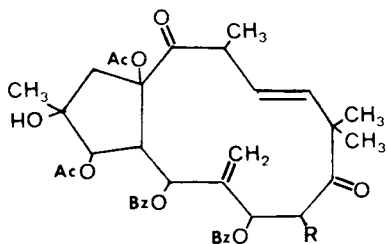
Component	Accession				Austrian no. 10
	North American				
	no. 5	no. 13	no. 14	no. 17	
Hydrocarbons	12	18	16	14	25
Free alcohols	54	52	53	57	29
Aldehydes	1	1	2	1	4
Free acids	3	2	3	2	4
Esters	17	13	7	10	18
Triterpenes	3	5	10	7	11
Triterpene esters	2	2	4	3	2
Unidentified	8	7	5	6	7
Yield (% dry wt.)	0.9	1.1	1.2	1.2	0.1
Acetone-sol.(%)	5.8	7.8	13.7	10.6	11.5
Acetone-insol.(%)	94.2	91.2	86.3	89.4	88.5

Table IV. Percent Composition of Free Triterpenes of Five accessions of Euphorbia esula

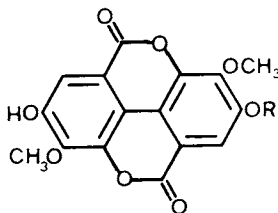
Terpene	Accession				Austrian no. 10
	North American				
	no. 5	no. 13	no. 14	no. 17	
$\beta$ -Amyrin	5	4	9	4	44
$\delta$ -Amyrenone	11	7	7	8	--
$\alpha$ -Amyrin	37	41	67	55	22
24-Me-Cycloartenol	28	31	5	19	23
Lupeyl acetate	4	5	1	4	2
Unidentified	15	12	11	7	9

Five triterpenes were identified and quantified in the acetone-soluble portion of the spurge leaf wax (Table IV). Except for a reduced amount of 24-methylene-cycloartenol in accession 14, general comparability of triterpene composition can be seen among the four North American spurge accessions. In contrast, the Austrian leafy spurge accession shows a greater amount of  $\beta$ -amyrin and a much lower amount of  $\alpha$ -amyrin and a total lack of  $\delta$ -amyrenone. These dramatic differences in triterpene occurrence support the suggested designation of North American leafy spurge as an intraspecies hybrid of E. esula and E. virgata (26) and substantiate the potential importance of chemical analysis for taxonomic differentiation within Euphorbiaceae.

Leafy Spurge Root Extractives. Prior chemical examinations of leafy spurge have considered only aerial portions of the plant. Our recent chemical examination of root material relative to mammalian toxicity and/or allelopathy (29) resulted in the isolation and characterization of two new jatrophone diterpenes (esulone A (IX) and esulone B (X)) from the ether extract of the roots. Biological assay of esulone A showed it to be moderately phytotoxic (29% root length reduction (lettuce seeds) at 250 ppm), moderately toxic (LD<sub>50</sub> 78 ± 23 mg/kg) and mildly inflammatory (10<sup>-5</sup> to 10<sup>-7</sup>M, dermal) to mammals with no hyperplasia.



IX, R=OH, H  
X, R=OAc, H



XI, R=xylose  
XII, R=glucose

Jatrophone diterpenes have not been previously described in *E. esula*, although twelve of the compounds have been described in other species of *Euphorbia* (31-37) and *Jatropha gossypifolia* (38, 39). The unique structural character of these diterpenes and their exclusive distribution in the family Euphorbiaceae may allow their use as specific chemotaxonomic markers within the family. Application of analytical HPLC methods developed in the isolation of the jatrophone diterpenes to extracts of other *E. esula* accessions has revealed distinctively different unidentified jatrophone diterpenes (30) among the accessions. The rarity and exclusivity of these compounds within Euphorbiaceae warrants continued chemical examination and differentiation of *E. esula* accessions in relationship to successful insect biological control.

A preliminary chromatographic examination of the acetone extractives of leafy spurge roots resulted in the isolation and characterization of 3,3'-di-O-methylellagic acid 4-β-D-xyloside (XI) (30). This ellagic acid glycoside has been previously characterized from the bark of the Indian timber tree *Anogeissus latifolia* (40). A second ellagic acid glycoside obtained from the leafy spurge root acetone extract was tentatively identified as 3,3'-di-O-methylellagic acid 4-D-glucoside (XII) of the basis of spectral data and a comparison of physical characteristics of this compound with those reported for its occurrence in the heartwood of the Indian tree *Terminalia paniculata* (41). The solubility characteristics of these compounds prevented their biological assessment as phytotoxins. Characterization of these ellagic acid glycosides is the first report of ellagic acid derivatives in *Euphorbia esula*.

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## Chapter 22

# A Search for the Allelopathic Agents in Diffuse Knapweed

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The allelopathic potential of knapweed (*Centaurea diffusa*) was investigated in field and pot trials. Field studies failed to show any phytotoxic effects from diffuse knapweed litter. In pot trials, knapweed seedlings did not inhibit seed germination or seedling development of three rangeland grass species. Under rangeland conditions, knapweed did not appear to influence soil microbial activity or affect levels of soil phenolics. Phenolic acids or sesquiterpene lactones were not detected in root exudates of knapweed. These results suggest that allelopathy is not a significant factor in the spread of knapweed.

Diffuse (*Centaurea diffusa*) and spotted knapweed (*C. maculosa*) are two introduced weeds that have expanded to occupy large areas of rangeland in the dry Interior of the Pacific Northwest. The apparent ability of knapweed to invade established, productive rangeland has been attributed to the action of allelochemicals produced by knapweed (1,2). The potential allelopathic effect of plants such as knapweed is an area of considerable interest, with researchers looking to exploit this phenomenon in agricultural situations, or simply to explain patterns of plant distribution (3).

In 1963, Fletcher and Renney (2) detected a plant growth inhibitor in knapweed and they suggested an allelopathic role for the substance(s) that could promote the spread of knapweed. Although these results are widely quoted, the inhibitor has never been identified nor has its existence been confirmed. Twenty years later, knapweed allelopathy was reexamined in our laboratory. A series of diffuse knapweed isolates were found to be inhibitory to ryegrass germination in petri dish assays

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(4). These studies indicated that the shoots of diffuse knapweed contained significant quantities of sesquiterpene lactones, with the germacranolide cnicin the most abundant. The sesquiterpene lactone fraction was inhibitory in both seed germination and seedling growth assays but the purified cnicin was largely inactive at the concentrations tested (400 ppm). A polar fraction was also found to inhibit ryegrass seed germination. The inhibitory effect of the polar fraction was not due to osmotic effects.

It soon became apparent that before a chemical agent could be implicated under field conditions, the subtle nature of the allelopathic effect would have to be carefully elucidated. It should be possible to demonstrate the release of the chemical agent into the soil environment and its accumulation to phytotoxic concentrations. An example of this phenomena is the accumulation of the thiophene,  $\alpha$ -terthienyl, in the root zone of common marigolds (*Tagetes erecta*) (5). If knapweed is allelopathic, is the effect present at all stages of plant development or is the effect most evident at the vulnerable seedling stage?

The main objectives in the present study were to: (a) determine the phytotoxicity of knapweed litter, (b) test the inhibitory effect of knapweed seedlings on the growth of grass seedlings, and (c) determine the effect of knapweed on soil microbial respiration and soil phenol concentrations.

### Methods

Knapweed litter, collected from a severely infested site, was air-dried and ground to pass a 20-mesh screen. To determine the phytotoxicity of the litter, the ground material was applied to  $m^2$  plots on a pasture site in 1981 and on two rangeland sites in 1982. Sand (650 g) was combined with each knapweed treatment to produce an even dispersal of litter. The litter was applied in November and grass yields, estimated on a dry matter basis, were determined in the following June.

A series of pot trials using rangeland soil was conducted to determine if knapweed seedlings could inhibit the growth of grass seedlings. Knapweed seeds and seeds of the following grasses were grown in 6 cm diameter pots: crested wheatgrass (*Agropyron cristatum*), Whitmar wheatgrass (*A. spicatum* var. *inerme*) and Sherman big bluegrass (*Poa ampla*). Whitmar wheatgrass and the bluegrass are commercial selections derived from native stock and crested wheatgrass is an introduced species, well adapted to Interior rangelands. Each grass was grown alone (10 seedlings per pot) or as a companion species with knapweed, in which case there were 5 grass seedlings and 5 knapweed seedlings per pot to keep plant densities uniform. Emergence rates were cumulated on a daily basis (6) and the seedlings were harvested and weighed after

six weeks. The two wheatgrasses were also germinated in soil collected from the root zone of knapweed. Soil from the root zone of native grasses was used as a control. This experiment was repeated using soils collected at a second site.

The methods for determining soil respiration and phenols were similar to those described previously (7). Air-dried soil samples (40 g) from the root zone were saturated with water, incubated in sealed containers at 25°C and respiration rates were determined by trapping evolved carbon dioxide in alkali. Soil phenols were determined by treating soil samples with 2 N NaOH for 1 hr at room temperature and measuring the extracted phenolics colorimetrically (8). Organic matter was determined by the Walkley-Black method (9).

### Results and Discussion

The rapid expansion of knapweed infestations, the development of knapweed monocultures, and the flush of grass after application of the herbicide picloram (4-amino-3,5,6-trichloropicolinic acid) are field observations often given as evidence for knapweed allelopathy. However, careful examination of a number of sites where diffuse knapweed has recently appeared failed to reveal any of the classic symptoms of allelopathy. There was no evidence of systematic plant distribution, zones of inhibition, or obvious signs of toxic effects on other plants. A detrimental impact of knapweed on forage production has been suggested (10,11) but these studies were conducted on rangeland already depleted with a history of severe overgrazing. Furthermore, the duration of these studies was too short to establish a definite correlation between knapweed invasion and a decline in forage yield.

When knapweed litter was applied to rangeland sites (Table I), there was no significant decrease in grass yield in the following spring. The data suggested the opposite, possibly due to a mulch effect. In a dense stand of knapweed, its above-ground biomass is approximately 200 g/m<sup>2</sup> in the fall. At rates three times this level no inhibition was observed, and therefore it is unlikely that accumulated litter transfers an inhibitory agent to the soil.

In pot trials there was no significant inhibition ( $P > 0.05$ ) of grass seedling emergence rates when the three grasses were germinated in the presence of knapweed seed (Table II). Knapweed had a significantly higher germination rate and its seedlings emerged faster than the grass species. This should give knapweed a significant advantage in the field. There was a significant decline ( $P < 0.05$ ) in the yield of Sherman big bluegrass but not for the wheatgrasses (Table III). The pot trials were observed for six weeks and there were no obvious signs of phytotoxicity. When the root zones

TABLE I. The Effect of Fall Application of Air-dried, Ground Knapweed on the Yield of Grasses in Spring

Knapweed application rate (g/m <sup>2</sup> )	Grass yield <sup>a</sup> (g/m <sup>2</sup> )		
	Crested wheatgrass (pasture)	Crested wheatgrass (rangeland)	Bluebunch wheatgrass (rangeland)
100	158	124	165
200	129	94	99
400	160	93	134
600	148	119	127
Mean	149	108	131
Control	142	71	110

<sup>a</sup> Each value is the mean of three determinations. Treated plots were not significantly different ( $P > 0.05$ ) from the controls using Duncans Multiple Range test.

TABLE II. Emergence Rates of Three Grasses Grown in Pots in the Presence and Absence of Knapweed

Companion species	Emergence Rate <sup>a</sup> (seedlings/day)			
	Knapweed	Crested <sup>b</sup> wheatgrass	Whitmar <sup>b</sup> wheatgrass	Sherman <sup>b</sup> big bluegrass
Knapweed	5	3	4	2
Crested wheatgrass	6	1		
Whitmar wheatgrass	5		3	
Sherman big bluegrass	6			3

<sup>a</sup> Based on five seeds of each species per pot.

<sup>b</sup> Each value is the mean of five determinations. T-tests were used to compare means. Significant differences ( $P > 0.05$ ) were not detected.

TABLE III. Yields of Three Grasses Grown in Pots in the Presence and Absence of Knapweed

Companion species	Yield ( mg/plant )			
	Knapweed	Crested <sup>a</sup> wheatgrass	Whitmar <sup>a</sup> wheatgrass	Sherman <sup>a</sup> big bluegrass
Knapweed	83	53	45	14
Crested wheatgrass	68	72		
Whitmar wheatgrass	88		37	
Sherman big bluegrass	107			22

<sup>a</sup> Each value is the mean of five determinations. T-tests were used to compare means. Significant differences were only detected for Sherman big bluegrass yields ( $P < 0.05$ ).

were examined the roots of knapweed and the grass species were well developed and intermingled. Similar results were also observed in a second trial (data not shown). When seed germination in knapweed and non-knapweed soils (from two sites) was compared, there was no significant difference between sites or soil zones. However, there was a significant difference in the levels of extractable phenols between zones ( $2.59 \mu\text{M/g}$  for knapweed versus  $3.08 \mu\text{M/g}$  for non-knapweed soil), and between sites ( $2.38 \mu\text{M/g}$  versus  $3.29 \mu\text{M/g}$  soil).

The field and pot trials with knapweed could not demonstrate a simple allelopathic effect. However, it is possible that knapweed exerts a chemical influence on its surrounding soil environment and presumably this would be a cumulative effect producing a competitive advantage. The mode of action in the soil might be a direct one through the excretion of phenolics or other toxic compounds. However, in petri dish assays inhibitory activity was not detected in acetonitrile or ether extracts of soil collected from the knapweed root zone. Alternatively, an indirect effect could be achieved by the excretion of chemical agents that modify soil microbial activity to enhance the growth of knapweed or suppress the growth of adjacent plants. Therefore soil respiration was measured to estimate the level of

microbial activity in the soil. If knapweed was modifying populations of soil microorganisms, this might produce a change in the rate of soil respiration (i.e. promote or suppress microbial activity). Extractable phenols and organic matter were also determined to detect corresponding changes in soil chemical properties that might be attributed to knapweed.

Chromatographic analysis of the soil extracts revealed *p*-coumaric acid as the major phenolic component. Results on extractable phenols, soil respiration rates, and organic matter in the root zone are shown in Table IV. In 1983 rates of soil respiration, extractable phenols, and organic matter were higher in the non-knapweed soil samples than in the knapweed soils. The soil respiration data suggested that knapweed had a negative impact on soil microbial activity. The higher levels of alkali-extractable soil phenols found in the non-knapweed soils were opposite to what would be expected if knapweed were excreting phytotoxic levels of *p*-coumaric acid. When respiration rates and phenolic acid levels were compared to the organic matter content of these soils it appeared that these variables reflected the different levels of organic matter. There was a significant correlation ( $P < 0.05$ ) between the level of extractable phenols and the soil organic matter. Results

TABLE IV. Respiration Rates, Phenolic Acid Levels and Organic Matter in Knapweed and Non-knapweed Soils.

	Knapweed Soils <sup>a</sup>		Non-knapweed Soils	
	1983	1984	1983	1984
Soil respiration (mg CO <sub>2</sub> /day)	15.1	19.1	23.5 <sup>b</sup>	22.7
Equivalentents of <i>p</i> -coumaric acid (μM/g soil)	2.80	3.16	3.98 <sup>c</sup>	3.23
Organic matter (%)	3.53	3.41	4.49 <sup>c</sup>	4.28 <sup>b</sup>

<sup>a</sup> Each value is the mean of 10 determinations in 1983 and six in 1984. T-tests were used to compare means between soils within years.

<sup>b</sup> Significant difference at  $P < 0.05$

<sup>c</sup> Significant difference at  $P < 0.01$

from a second site (data not shown) gave essentially the same pattern except that the soil organic matter and extractable soil phenols were approximately one third lower for both knapweed and non-knapweed soil samples. These experiments were repeated at both sites in 1984 with more analytical replication, but this time the results were inconclusive. Only the organic matter level at one site showed a significant difference between knapweed and non-knapweed soils (Table IV). These experiments were also repeated with freshly collected soils at field moisture levels, and the results were essentially the same. We concluded that knapweed seedling survival may be greater in soils with lower organic matter ( i.e. in the spaces between established plants), but mature knapweed plants do not have a significant impact on soil microbial activity or soil phenolics.

To determine the degree to which soluble soil phenols must be elevated to produce a biological effect, soils were irrigated with *p*-coumaric acid and then seeded to knapweed, crested wheatgrass, and Whitmar wheatgrass. A significant effect on seedling germination, emergence rate, or seedling biomass (Table V) was detected only at the highest level of *p*-coumaric acid (3  $\mu\text{M}$  / g). Since alkali-extractable phenols from soil are partially bound and are not completely available, an increase of at least

TABLE V. The Effect of Four Levels of *p*-Coumaric acid on the Emergence Rate and Yield of Knapweed and Two Grasses Grown in Pots

	<i>p</i> -Coumaric acid ( $\mu\text{M}$ /g soil)	Emergence Rate (seedlings/day)	Seedling Yield (mg/pot)
Knapweed	0	8 <sup>a</sup>	82 <sup>a</sup>
	0.5	6	75
	1	6	73
	3	<1	3
Crested wheatgrass	0	3	50
	0.5	2	37
	1	3	55
	3	<1	6
Whitmar wheatgrass	0	2	50
	0.5	2	46
	1	2	49
	3	<1	5

<sup>a</sup> Each value is the mean of five pot trails using 10 seeds per pot.

100 % in soluble soil phenols would be required for significant inhibition of grass growth. These results suggest that these grasses are not particularly sensitive to small changes in the levels of endogenous soil phenols.

To determine if knapweed roots were releasing substances into the soil environment, knapweed plants were grown in sand culture and the organic component of the root exudate was adsorbed on XAD-4 resin according to the method of Tang and Young (12). The components of the exudate were eluted from the resin and examined by GC/MS. Root exudates of knapweed contained no detectable quantity of phenols, terpenes, or polyketides. Cnicin, the major sesquiterpene lactone present in diffuse knapweed shoots, was not detected in the roots nor in the root exudates. The major component of the root exudate fraction was a series of long-chain saturated and unsaturated carboxylic acids.

In summary, a number of hypotheses on knapweed allelopathy were tested and the conclusions from the various experiments were as follows:

(A) Field observations suggest that knapweed suppresses the growth of grasses. However, the apparent inhibition by knapweed under field conditions could not be reproduced in pot trials.

(B) Chemical substances can be extracted from knapweed that inhibit seed germination or seedling development of other species *in vitro*. However, application of air-dried knapweed to field plots had no effect on grass yield. It appears that under field conditions, knapweed leachates are not phytotoxic.

(C) Inhibitory compounds can be identified in knapweed and these are probably released into the soil environment. However, sesquiterpene lactones were not detected in root exudates, where the major components were long-chain carboxylic acids.

(D) In many examples of allelopathy, plant phenols are implicated as a component of the phytotoxin and therefore it would be reasonable to expect phenols to be involved in this situation. However, our studies failed to show an accumulation of soluble phenols in the soil. A substantial elevation in soil phenol levels would be required to inhibit seedling development, and phenolics were not detected in root exudates.

(E) Knapweed may have an indirect effect on other plants because it may modify the soil microbial environment to its advantage. However, studies on soil respiration indicated that there is no obvious enhancement or inhibition of soil microorganisms in the root zone of knapweed.

### Conclusion

The spread of diffuse knapweed on grasslands of the Pacific Northwest has the appearance of being assisted by allelopathy. The observations and experiments reported here indicate that allelopathy is unlikely to be a significant factor in the success of knapweed. Other factors such as its prolific production of viable seed, its apparent tolerance to drought, its monocarpic perennial character, and the absence (until recently) of native predators combine to give knapweed a significant advantage over the native vegetation it is apparently displacing.

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## Chapter 23

# Effects of Phenolic Acids, Coumarins, and Flavonoids on Isolated Chloroplasts and Mitochondria

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The effects of allelopathic plant phenolics and polyphenolics (benzoic and cinnamic acids, coumarins, and flavonoids) on electron transport and phosphorylation in chloroplasts and mitochondria were investigated. All chemicals inhibited  $\text{CO}_2$ -dependent oxygen evolution in intact chloroplasts.  $I_{50}$  concentrations ranged between  $<1$  and  $10$  mM. In thylakoids, the primary effect of the compounds, at low concentrations, was on the ATP-generating pathway. However, at higher concentrations, they also inhibited electron transport. On the basis of  $I_{50}$  values, the compounds exhibited the following order of inhibitory effectiveness: flavonoids  $>$  coumarins  $>$  cinnamates = benzoates. The compounds did not act like uncouplers. In studies with mung bean mitochondria, the compounds primarily acted as electron transport inhibitors. Malate oxidation was more sensitive than either succinate or NADH oxidation. The flavonoids were most inhibitory, with  $I_{50}$  values that ranged between  $10$  and  $80$   $\mu\text{M}$ . For the coumarins, cinnamates, and benzoates,  $I_{50}$  values ranged between  $1$  and  $20$  mM. The compounds did not act as uncouplers or directly inhibit ATP synthesis. However, naringenin, some of the flavones, and the cinnamates acids inhibited the hydrolysis of ATP catalyzed by mitochondrial  $\text{Mg}^{2+}$ -ATPase. The inhibition of substrate oxidation appears to result from alterations and perturbations induced in the inner membrane as evidenced by interference with carrier-mediated transport processes.

The biochemical mechanisms through which allelochemicals exert deleterious or toxic effects on plants are, for the most part, unknown (1). Some phenolic acids, cinnamic acids, coumarins, and flavonoids have been reported to inhibit photosynthesis and respiration of intact plants and microorganisms. However, the mechanisms, at the molecular level, through which the compounds interfere, remain to be ascertained. Some phenolic acids, coumarins, and flavonoids were reported to inhibit  $\text{CO}_2$ -dependent  $\text{O}_2$

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evolution and photophosphorylation of isolated chloroplasts (2). Kaempferol and quercetin were shown to inhibit coupled electron transport and photophosphorylation, but had a limited effect on basal and uncoupled electron transport of isolated thylakoids (3). The authors (3) postulated that the flavones acted as energy transfer inhibitors, but that the action was different from the reference standards phlorizin and chlorotributyltin.

In studies with isolated plant mitochondria, flavones, flavanones, cinnamic acids, and benzoic acids were shown to inhibit the oxidation of succinate, malate, and NADH (2,4-9). Inhibition was observed under both ADP-stimulated and uncoupled conditions. There was no evidence that the various compounds acted as uncouplers. Kaempferol was postulated to inhibit the phosphorylation mechanism, but the action was different from that of oligomycin (9). In studies with both chloroplasts and mitochondria, glycosides, in general, were less inhibitory than the corresponding aglycones.

The objectives of the studies reported herein were to: (a) compare the effects of a series of phenolic acids, coumarins, and flavonoids on whole chain electron transport and phosphorylation in isolated plant chloroplasts and mitochondria; and (b) identify specific sites of inhibition with polarographic and enzymatic techniques. Exploratory studies were conducted with the 20 compounds listed in Table I. The three glycosides are shown indented below the corresponding aglycones. Detailed studies were conducted with the six compounds, one representative member from each chemical family, designated with an asterisk.

#### Materials and Methods

Chloroplasts. Intact chloroplasts were isolated from freshly harvested growth chamber-grown spinach (*Spinacia oleracea* L.) as described by Lilley and Walker (10). Thylakoids were prepared by the method of Armond *et al.* (11). Chlorophyll concentrations were determined by the method of MacKinney (12). Photochemical reactions were conducted at 25°C with a photon fluence rate of 750  $\mu\text{mol}/\text{m}^2 \cdot \text{s}$  (PAR).  $\text{CO}_2$ -dependent oxygen evolution of intact chloroplasts was measured as described by Walker (13). Effects on

TABLE I: Allelochemicals Evaluated for Effects on Reactions Mediated by Isolated Chloroplasts and Mitochondria

Benzoic acids:	gallic salicylic syringic *vanillic	Coumarins:	coumarin esculetin esculin scopoletin *umbelliferone
Benzaldehyde:	*vanillin	Flavones:	flavone kaempferol *quercetin rutin
Cinnamic acids:	caffeic trans-cinnamic p-coumaric *ferulic	Flavanones:	*naringenin naringin

\*Representative compounds selected for detailed study.

electron transport and photophosphorylation were measured in a medium (2.0 ml volume) that contained 0.1 M sorbitol, 20 mM tricine-NaOH (pH 8.0), 1 mM  $\text{KH}_2\text{PO}_4$ , 1 mM ADP, 5 mM  $\text{MgCl}_2$ , 10 mM NaCl, 0.1 mM methyl viologen,<sup>2</sup> and thylakoids (40  $\mu\text{g}$  Chl). Esterification of Pi was measured by the method of Taussky and Shorr (14). Electron flow was monitored polarographically with a Clark-type platinum electrode as oxygen consumed during the auto-oxidation of reduced methyl viologen. Uncoupled electron transport was determined in the same medium with the addition of 5 mM  $\text{NH}_4\text{Cl}$ . Effects on different segments of the electron transport chain, chlorophyll fluorescence, and binding studies with [ $^{14}\text{C}$ ]-atrazine were performed as described previously (15).

**Mitochondria.** Mitochondria were prepared from 3-day-old dark-grown mung bean (*Vigna radiata* Roxb.) hypocotyls. The isolation procedure, measurements of oxygen utilization, and effects of the test compounds on respiratory states were conducted as described previously (16). In accordance with the terminology of Chance and Williams (17), the ADP-stimulated rate of respiration will be referred to as state 3 and ADP-limited respiration as state 4. The mitochondria had respiratory control (state 3/state 4) ratios that averaged  $4.2 \pm 0.4$ ,  $3.4 \pm 0.2$ , and  $2.3 \pm 0.2$ , and calculated ADP/O ratios that averaged  $2.3 \pm 0.1$ ,  $1.3 \pm 0.1$ , and  $1.5 \pm 0.1$  for the oxidation of malate, NADH, and succinate, respectively.

Effects on  $\text{Mg}^{2+}$ -ATPase activity were determined with mitochondria that were ruptured by freezing ( $-20^\circ\text{C}$ ) and subsequent thawing (room temperature). The assay medium and procedure was essentially that of Blackmon and Moreland (18) except that DNP was omitted and the  $\text{MgCl}_2$  concentration was 5 mM.

**Osmotic swelling.** Changes in the osmotic stability of mitochondria were monitored spectrophotometrically at 520 nm. The 2.0 ml reaction mixture contained 10 mM Hepes-NaOH (pH 7.1) and 0.15 M KCl. Mitochondria (0.4 mg protein) were added to give an initial absorbance of 0.8 A. In studies that involved effects on valinomycin-induced swelling, the test compound was added 30 s prior to the introduction of the ionophore (0.1  $\mu\text{M}$ ). Effects on the phosphate/hydroxyl antiporter and the proline uniporter were measured by the ammonium swelling technique (19,20). The KCl in the reaction mixture identified above was replaced by ammonium phosphate (0.1125 M) and proline (0.2 M), respectively. Swelling was monitored beginning 6 s after injection of the mitochondria to the stirred reaction mixture containing the test compound.

**Test chemicals.** The allelochemicals were obtained from Sigma Chemical Co. Stock solutions of the desired concentrations were prepared as follows: most benzoic and cinnamic acids were prepared as the Na salts in  $\text{H}_2\text{O}$ ; salicylic acid, *trans*-cinnamic acid, flavone, kaempferol, and vanillin were dissolved in acetone; the other chemicals were dissolved in DMSO. The final concentration of acetone and DMSO was held constant at 1% (v/v) in all assays including the controls. Data presented were averaged from determinations made with three separate replications and isolations.

## Results and Discussion

Chloroplast and thylakoid responses. In exploratory studies, all of the allelochemicals inhibited  $\text{CO}_2$ -dependent  $\text{O}_2$  evolution of intact spinach chloroplasts.  $I_{50}$  values for the six representative compounds are shown in Table II. The flavones represented by quercetin were the most inhibitory with the flavanone naringenin being somewhat less active. The coumarins, cinnamic acids, benzoic acids, and the benzaldehyde were considerably less active. The glycosides were also inhibitory, but at higher concentrations than the corresponding aglycones.

The exploratory studies, as conducted, did not distinguish between effects imposed on the stromal-associated  $\text{CO}_2$  fixation (Calvin cycle) reactions or on the light reactions associated with the thylakoids. Consequently, studies were conducted on light-induced electron transport and ATP synthesis associated with isolated spinach thylakoid membranes.

Electron transport and photophosphorylation. Shown in Table II are  $I_{50}$  values for inhibition by the six representative compounds of coupled electron transport (column 3) and phosphorylation (column 4), and of uncoupled electron transport (last column) with spinach thylakoids. The flavonoids were the most inhibitory with naringenin being somewhat less active than quercetin. The coumarins, cinnamic acids, and vanillin were equally inhibitory at somewhat higher concentrations. The benzoic acids were the weakest inhibitors. For all compounds, the phosphorylation reaction was most sensitive, followed by the coupled electron transport. Uncoupled electron transport was least sensitive. Inhibition of coupled electron transport can result indirectly from an effect imposed on the phosphorylation pathway or directly by action on a component of the electron transport pathway. The two effects can be differentiated by using an uncoupler such as  $\text{NH}_4\text{Cl}$  or FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) to dissipate the energized state of the membrane (21). If the inhibition of electron transport coupled with ATP generation is caused by energy transfer inhibition, the inhibition should be circumvented by the uncoupler. However, if electron transport inhibition is the cause, the addition of an uncoupler will not circumvent the inhibition. As shown in Table III (column 3 versus column 5), inhibition of the uncoupled electron transport rate was only partially relieved. Thus, the compounds appear to have two effects: (a) the more sensitive is an effect on the ATP-generating pathway; and (b) a second, but weaker, effect involved the electron-transport pathway.

Energy transfer and uncoupling. Because of the suggestion that the allelochemicals might interfere with energy transfer, their action was compared to that of known energy transfer inhibitors of thylakoid-synthesized ATP (22). A series of  $\text{O}_2$ -consumption polarographic traces in which  $\text{H}_2\text{O}$  served as the electron donor and methyl viologen served as the electron acceptor are shown in Figure 1. Chlorotributyltin (Trace A), phlorizin (Trace B), and DCCD (*N,N'*-dicyclohexylcarbodiimide) (Trace C) strongly inhibited the rate of ADP-stimulated oxygen uptake. For all compounds,

TABLE II. Inhibition by Selected Allelochemicals of CO<sub>2</sub>-dependent O<sub>2</sub> Evolution by Intact Spinach Chloroplasts, of Coupled Electron Transport and Phosphorylation, and of Uncoupled Electron Transport by Spinach Thylakoids

Allelochemical	Assay			
	CO <sub>2</sub> dependent O <sub>2</sub> evolution <sup>a</sup>	O <sub>2</sub> uptake	Coupled <sup>b</sup> Phosphorylation	Uncoupled O <sub>2</sub> uptake
		I <sub>50</sub> (mM)		
Vanillic acid	4.63 ± 1.46	35 @ 25 <sup>c</sup>	19 @ 25 <sup>c</sup>	0 @ 25 <sup>c</sup>
Vanillin	4.77 ± 0.64	2.67 ± .06	2.20 ± .26	14 @ 4 <sup>c</sup>
Ferulic acid	4.47 ± 1.46	2.97 ± .12	2.13 ± .15	6.30 ± .52
Umbelliferone	4.23 ± 0.59	2.63 ± .06	1.87 ± .31	7.03 ± .12
Quercetin	0.05 ± 0.01	0.07 ± .01	0.04 ± .01	0.20 ± .02
Naringenin	0.56 ± 0.07	0.26 ± .03	0.21 ± .05	0.84 ± .05

<sup>a</sup>O<sub>2</sub> evolution was measured with a Clark-type electrode. Specific activity of the untreated controls averaged 40 ± 9 μmoles O<sub>2</sub> evolved/mg Chl · h. Data shown are arithmetic averages ± SD of determinations made with a minimum of three different isolations of chloroplasts.

<sup>b</sup>H<sub>2</sub>O served as the electron donor and methyl viologen as the electron acceptor. O<sub>2</sub> consumption was measured with a Clark-type electrode and phosphorylation was measured colorimetrically. Data are presented as averaged I<sub>50</sub> values ± SD obtained with three isolations of thylakoids. Average specific activities were: 89 ± 3 μmoles O<sub>2</sub> consumed and 171 ± 16 μmoles Pi esterified/mg Chl · h for the coupled reactions, and 223 ± 5 μmoles O<sub>2</sub> consumed/mg Chl · h for the uncoupled reaction.

<sup>c</sup>Compounds did not inhibit by 50%. The data presented are the percentages of inhibition achieved at the highest concentration (mM) that could be tested.

TABLE III. Inhibition by Selected Allelochemicals of Malate Oxidation by Intact Mung Bean Mitochondria and of  $Mg^{2+}$ -ATPase Activity of Freeze-thawed Mung Bean Mitochondrial Preparations

Allelochemical	Malate oxidation <sup>a</sup>	$Mg^{2+}$ -ATPase activity <sup>b</sup>
	I <sub>50</sub> (mM)	
Vanillic acid	17.67 ± 2.57	2 @ 25 <sup>c</sup>
Vanillin	4.03 ± 0.65	36 @ 10 <sup>c</sup>
Ferulic acid	4.53 ± 0.25	2.90 ± 0.44
Umbelliferone	1.88 ± 0.33	15 @ 4 <sup>c</sup>
Quercetin	0.02 ± 0.00	0.04 ± 0.01
Naringenin	0.11 ± 0.03	0.62 ± 0.06

<sup>a</sup>  $O_2$  uptake was measured polarographically with a Clark-type electrode with nonlimiting concentrations of ADP (state 3 respiration). Specific activity for the rate of  $O_2$  utilization by untreated mitochondria averaged  $202 \pm 10$  nmoles/mg protein min. Values shown are averages ± SD obtained from determinations made with three isolations of mitochondria.

<sup>b</sup> Phosphate liberated by hydrolysis of ATP was measured colorimetrically. Data are presented as average I<sub>50</sub> values ± SD obtained with three isolations of mitochondria. The average specific activity was  $8.53 \pm 0.20$   $\mu$ moles Pi liberated/mg protein · hr.

<sup>c</sup> Compounds did not inhibit by 50%. The data presented are the percentages of inhibition achieved at the highest concentration (mM) that could be tested.

inhibition was not only circumvented completely by the addition of the uncoupler FCCP, but  $O_2$  utilization was increased above the ADP-stimulated rate.

Comparative responses obtained with quercetin and naringenin are shown in Traces D and E, respectively. Quercetin and naringenin, at concentrations that completely inhibited photophosphorylation, inhibited the ADP-stimulated rate of  $O_2$  utilization 70 and 82%, respectively. FCCP restored the rate to 12 and 42% of the original rate for quercetin and naringenin, respectively. The failure to obtain complete recovery can be attributed to interference with a component of the electron-transport pathway, at these same concentrations, as evidenced by data presented in Table II. Similar results were obtained with the remainder of the representative compounds. The results reinforce the postulate that the primary effect imposed by the allelochemicals on thylakoids is energy transfer inhibition with a secondary effect imposed on the electron-transport pathway.

Partial reactions. Through the use of various electron donors and acceptors, it is possible to bracket specific sites on the electron-transport pathway associated with the action of inhibitors (23-25). Attempts to identify the site(s) of interaction of the allelochemicals on the electron-transport pathway were generally

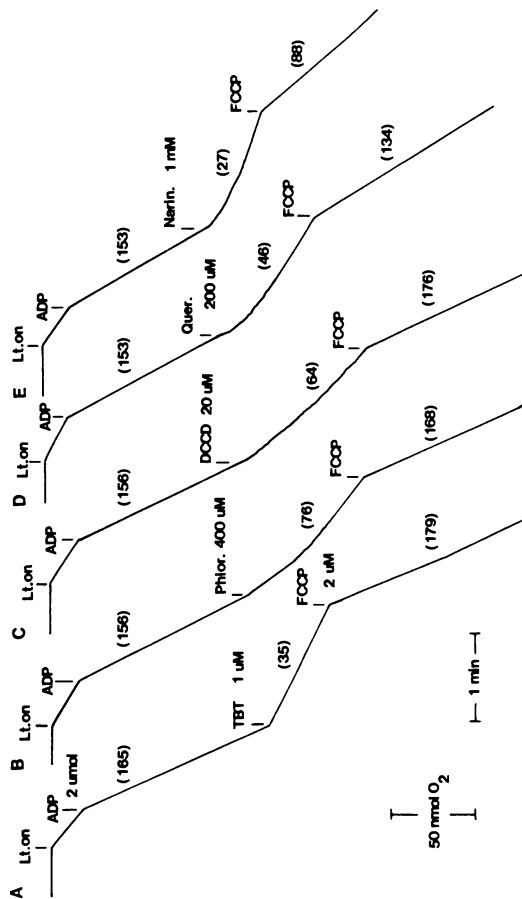


Figure 1. Representative polarographic traces that depict inhibition by energy transfer inhibitors and allelochemicals of ADP-stimulated electron transport in isolated spinach thylakoids and circumvention of the inhibition by an uncoupler (FCCP, 2  $\mu\text{M}$ ). Trace A: chlorotributyltin (TBT, 1  $\mu\text{M}$ ); trace B: phlorizin (400  $\mu\text{M}$ ); trace C: DCCD (20  $\mu\text{M}$ ); trace D: quercetin (200  $\mu\text{M}$ ); trace E: naringenin (1 mM). Water served as electron donor and methyl viologen as electron acceptor. Rates of oxygen utilization, that resulted from the autooxidation of methyl viologen, expressed as  $\mu\text{mol O}_2$  consumed/mg Chl  $\cdot$  h, are indicated parenthetically.

unsuccessful. Ferricyanide and DPIP were reduced chemically by some of the representative compounds (quercetin, naringenin, and ferulic acid), hence, could not be used as electron acceptors. Some, such as umbelliferone, fluoresced at the wavelength (420 nm) used to monitor ferricyanide reduction. DHQ (duroquinol) autooxidizes slowly and most of the allelochemicals greatly stimulated the rate of autooxidation. Consequently, DHQ could not be used as an electron donor.

Chlorophyll fluorescence analysis has been used to analyze effects imposed by various factors, including inhibitors, on the photochemical process associated with PS II and the plastoquinone pool (26-28). Unfortunately, most of the allelochemicals either absorbed at the excitation wavelengths or chemically quenched chlorophyll fluorescence.

Many inhibitors of photoinduced electron transport, including a large number of herbicides, have been shown to bind reversibly and competitively to the  $Q_B$  protein. The studies involve displacement of a radiolabeled inhibitor, such as [ $^{14}C$ ]-atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine], by an unlabeled inhibitor (29). Only marginal displacement of [ $^{14}C$ ]-atrazine was observed by high concentrations of umbelliferone and naringenin. The other allelochemicals produced no measurable effects.

Results obtained from the partial reactions, chlorophyll fluorescence, and binding studies did not provide any clues relative to interactive sites on the electron-transport pathway for the allelochemicals. No evidence was obtained to specifically implicate interference with the  $Q_B$  protein of PS II. Additionally, insofar as they could be analyzed, none of the allelochemicals affected PS I-associated electron transport between the site of donation by  $DPIP_2$  and acceptance by methyl viologen.

Mitochondrial responses. Studies similar to those involving isolated chloroplasts and thylakoids were conducted with isolated mung bean mitochondria in order to ascertain whether the effects imposed on thylakoid membranes were common to energy transducing membranes in general.

Electron transport. All the allelochemicals inhibited the oxidation of malate, succinate, and exogenous NADH under state 3 (ADP-stimulated) conditions.  $I_{50}$  values for inhibition of malate state 3 respiration by the six representative allelochemicals are compared in Table III. The order of inhibitory potency for the representative compounds, in ascending order of  $I_{50}$  values, was: quercetin > naringenin > umbelliferone > vanillin = ferulic acid > vanillic acid. For all compounds, malate oxidation was the most sensitive. In general, succinate oxidation was less sensitive and NADH oxidation was least sensitive. The order of inhibitory potency for the chemical families was similar to that obtained against the thylakoid system. The glycosides except for rutin were ineffective.

Uncoupling and energy transfer. Representative oxygen consumption polarographic traces that compare the effects of standard inhibitors with that of quercetin on the oxidation of malate are



shown in Figure 2. Uncouplers, such as FCCP, stimulate state 4 respiration (Trace A), but under the same conditions quercetin had no effect on state 4 respiration (Trace B). Energy transfer inhibitors, such as oligomycin, inhibit state 3 respiration much like electron-transport inhibitors. However, the inhibition imposed by energy transfer inhibitors, but not by electron-transport inhibitors, is circumvented by uncouplers. Inhibition of state 3 respiration by oligomycin and relief by FCCP is shown in Trace C. The failure of FCCP to circumvent inhibition of state 3 respiration by antimycin A, an electron-transport inhibitor, is shown in Trace D. FCCP also did not relieve the inhibition of state 3 respiration imposed by quercetin (Trace E). The evidence obtained from the above studies suggests that quercetin acted primarily as an electron-transport inhibitor in mitochondria. No indications were obtained for action either as an uncoupler or as an energy transfer inhibitor. The other allelochemicals acted similarly to quercetin.

In the experiments reported above, no strong indications were obtained that the allelochemicals interfered directly with the synthesis of ATP by acting on the energy transfer pathway ( $F_0-F_1$  complex). However, some of the compounds did inhibit ATP hydrolysis, as measured with preparations in which the mitochondria had been ruptured by freeze-thawing (Table III). The flavones (except flavone itself) and cinnamic acids strongly inhibited the  $Mg^{2+}$ -ATPase, whereas the benzoic acids and coumarins were weak inhibitors, i.e., less than 15% inhibition at 10 mM concentrations. Results obtained with quercetin (Table III) agree with published reports in which the compound was shown not to affect ATP synthesis, but to inhibit the hydrolysis of ATP by the mitochondrial  $Mg^{2+}$ -ATPase (30).

Inhibition of whole chain electron transport can result from: (a) interaction of the inhibitor with a redox component of the pathway; or (b) interaction with carrier systems that transport substrate molecules across the inner membrane. The latter interaction could be direct or indirect. Because electron transport associated with the oxidation of malate, succinate, and exogenous NADH were all inhibited, but to differing extents, a specific interaction with a single redox component of the inner mitochondrial membrane does not seem to be involved.

Transport systems. Partitioning of various types of molecules such as allelochemicals into the lipid bilayer of the mitochondrial inner membrane can perturb the membrane and alter the conformation, properties, and function of components of the membranes. Unfortunately, it is not always possible to demonstrate directly the existence of carrier systems, but indirect evidence can be obtained. Alterations induced to the membrane are sometimes reflected in the osmotic behavior of mitochondria. The inner membrane is relatively impermeable to many cations, including  $K^+$  and  $H^+$ , and many solutes (31). Hence, the organelles are osmotically stable under certain conditions. Indications were obtained that the allelochemicals inhibited the action of carrier-mediated transport processes associated with the mitochondrial inner membrane (as reflected in the osmotic behavior). Responses obtained with quercetin are shown in Figure 3. Mitochondria are osmotically

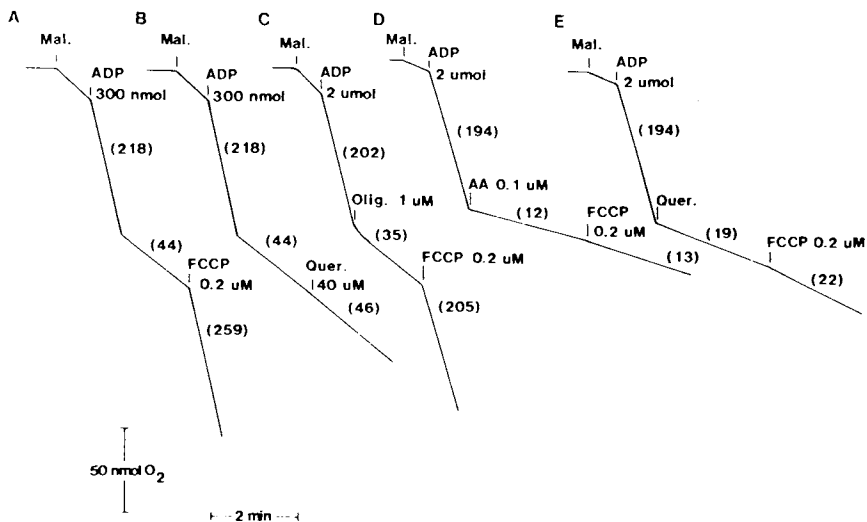


Figure 2. Representative polarographic traces that depict effects of quercetin (Quer.) on oxygen utilization by mung bean mitochondria with malate as substrate. Trace A, stimulation of state 4 respiration by FCCP; trace B, lack of state 4 stimulation by quercetin; trace C, circumvention of oligomycin (olig.)-inhibited state 3 respiration by FCCP; trace D, lack of circumvention of antimycin A-inhibited state 3 respiration by FCCP; and trace E, lack of circumvention of quercetin-inhibited state 3 respiration by FCCP. Rates of oxygen utilization, expressed as nmoles  $O_2$ /mg protein  $\cdot$  min, are indicated parenthetically.

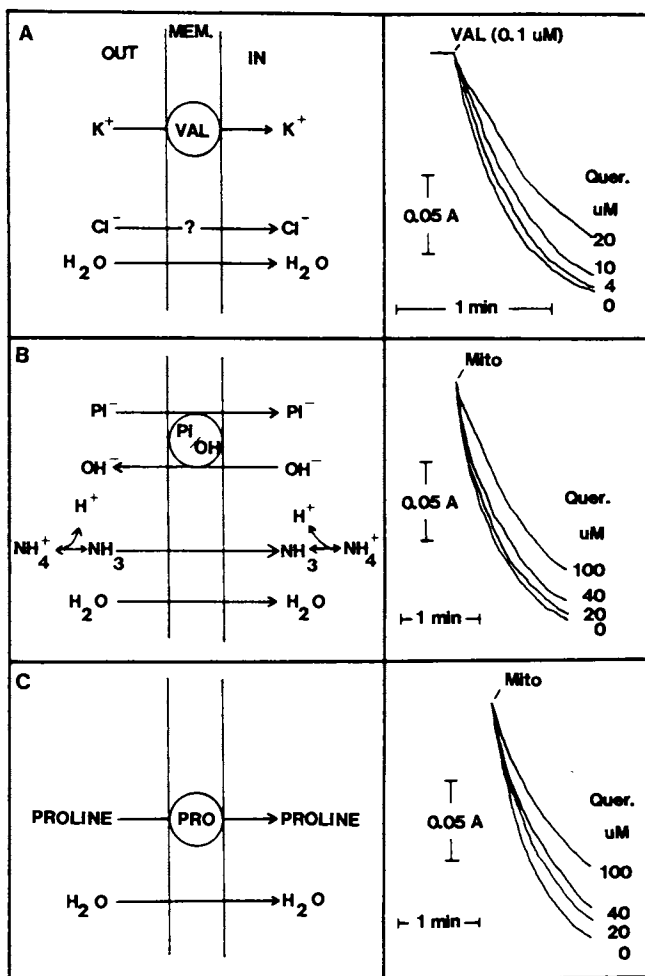


Figure 3. Diagrammatic models and representative traces of absorbance changes that show the kinetics and inhibition by quercetin (Quer.) of (A) valinomycin-induced swelling of mung bean mitochondria suspended in isosmotic KCl; (B) swelling of mung bean mitochondria suspended in isosmotic ammonium phosphate; and (C) swelling of mung bean mitochondria suspended in isosmotic proline.

stable when suspended in isotonic KCl. However,  $K^+$  permeability can be induced artificially by ionophores such as valinomycin (32). As depicted in Scheme A, valinomycin forms a lipid-soluble complex with  $K^+$  and transports it across the membrane. An increase in internal  $K^+$  will be accompanied by the movement (diffusion) of the  $Cl^-$  counter ion, in some as yet unknown manner, to maintain electroneutrality. The increase in the matrix concentration of KCl will result in the osmotic influx of water. Swelling is measured as a decrease in absorbance. The extent of swelling under control conditions is shown in the 0 trace. Quercetin inhibited, in a concentration-dependent manner, the extent of osmotic swelling.

By means of the ammonium swelling technique, the existence of a  $PI^-/OH^-$  exchange carrier can be demonstrated (19). As shown in Figure 3B, external  $NH_4^+$  dissociates into  $NH_3$  and  $H^+$ . The neutral ammonia ( $NH_3$ ) passes across the inner membrane down a concentration gradient. Inside the matrix,  $NH_3$  associates to equilibrium with protons ( $H^+$ ) to form the ammonium ion ( $NH_4^+$ ) and  $OH^-$ . The  $OH^-$  exchanges for phosphate on the  $PI^-/OH^-$  antiporter. The increased osmotic pressure induces spontaneous swelling that is reflected as a decrease in absorbance. This is shown in the 0 (control) trace. As before, quercetin, in a concentration-dependent manner, inhibited the magnitude of the spontaneous swelling.

In isosmotic solutions, movement of neutral amino acids such as proline across the inner membrane into the matrix results in swelling of mitochondria (20). As shown in Figure 3C, the movement of proline is thought to occur via a uniport. The increased concentration of proline in the matrix produces an osmotic-induced swelling. Kinetics of the swelling response is shown in the 0 (control) trace. Again, quercetin inhibited this response in a concentration-dependent manner.

The other representative allelochemicals inhibited the three transport processes such as quercetin did. The concentration ranges at which the allelochemicals produced interference were similar to those that inhibited whole-chain electron transport.

The inhibition of multisubstrate oxidation that involved complexes I and II, and the ubiquinone pool, observed with the allelochemicals, can best be explained by alterations and perturbations induced to the inner membrane. No clear-cut evidence was obtained for interactions with specific complexes of the membranes.

Inhibition of transport processes may result from: (a) alterations and perturbations induced to the inner membrane by the allelochemicals, or (b) prevention of the development of an electrochemical potential difference across the membrane (energization of the membrane). An energized state is required for the transport of ions, or the cotransport of ions with other organic molecules, including substrates, across the membrane. The allelochemicals could interfere with transport through both mechanisms. By inhibiting electron transport, the allelochemicals would prevent energization of the membrane. However, the membrane transport processes examined above do not require an energized membrane. The recorded interferences reflect alterations induced in the behavior of the carrier systems either through a direct interaction with the proteins themselves, or to the fluidity or integrity of the membrane within which the porters operate.

### Conclusions

In the studies with isolated chloroplasts and thylakoids, the primary effect of the phenolic allelochemicals was on the ATP-generating pathway, i.e., energy transfer inhibition. The compounds acted on the electron-transport pathway at higher concentrations, but the exact site(s) remain to be identified. These may be located on the oxidizing side of PS II or around the PQ pool. The sites are not associated with PS I. The compounds did not act as uncouplers.

In mitochondria, the allelochemicals acted primarily as electron transport inhibitors. Malate oxidation was more sensitive than either succinate or NADH oxidation. No evidence for interaction with a specific membrane complex was obtained. Instead, inhibition of substrate oxidation seems to result from alterations and perturbations produced in the inner membrane as reflected in interference with the behavior of transport processes. The compounds did not act as uncouplers or directly inhibit ATP synthesis. However, naringenin, some of the flavones, and the cinnamic acids did inhibit the hydrolysis of ATP catalyzed by mitochondrial  $Mg^{2+}$ -ATPase.

The concentrations of phenolic and polyphenolic allelochemicals that inhibited the various reactions of isolated chloroplasts and mitochondria have been reported to occur, either singularly or in combination, in organic litter (1). The studies conducted herein were short-term, on the order of several minutes, in which immediate responses were measured. Preincubation of the allelochemicals with the test systems could be expected to lower the concentrations required to produce inhibition.

Effects reported herein on interference with chloroplast and mitochondrial electron transport and phosphorylation by polyphenolic allelochemicals are in agreement with results obtained by other investigators (2-9). Inhibition of photosynthesis and respiration of intact plants and microorganisms (1) can be explained by the interferences measured with the isolated organelles. However, details of the biochemical mechanisms involved remain to be identified. Conceivably, the phenolic allelochemicals may perturb other cellular membranes (plasmalemma, tonoplast, nuclear, and endoplasmic reticulum) as they did the mitochondrial membrane. Alterations to the permeability of membranes by polyphenolic and other allelochemicals have been reported (1). Transport or cotransport of many ions and organic molecules across semipermeable membranes requires energy or an energized state of the membrane. The energy is provided directly or indirectly by ATP. Phenolic allelochemicals would limit the availability of mitochondrial and chloroplast-generated ATP by acting on either, or both, the electron transport or energy generating pathways. Quercetin has been shown to inhibit  $Na^+/K^+$ -ATPases as well as mitochondrial  $Mg^{2+}$ -ATPase (30). Naringenin, other flavones, and the cinnamic acids could behave like quercetin. Hence, at least some of the phenolic allelochemicals could prevent the utilization of ATP energy required for transport of materials across cellular membranes by inhibiting the hydrolysis of ATP. Conceivably, alterations induced to the permeability of organelle membranes coupled

with a reduction in the availability of chloroplast and mitochondrially generated ATP is involved in the biochemical mechanisms of action associated with phenolic allelochemicals.

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## Chapter 24

# Allelopathic Interference in a Wild Mustard (*Brassica campestris* L.) and Broccoli (*Brassica oleracea* L. var. *italica*) Intercrop Agroecosystem

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Non-crop plants associated with the crop species offer possibilities for allelopathic weed control. In this study *Brassica campestris* (wild mustard), which is an important weed in Santa Cruz County, and broccoli, a common crop, were intercropped. The allelopathic potential of both species and the changes in this potential throughout their life cycle were demonstrated with experiments in the laboratory. Effects of different planting densities and sowing time of *B. campestris* on the crop yield are analyzed. Preliminary steps to separate the physiologically active compound(s) are described. The possibilities for the use of *Brassica campestris* in agroecosystem design as a non-crop plant that promotes pest control are described.

From the point of view of the evolution of agroecosystems, weeds are a good example of the capacity of organisms to adapt to the continued disturbances that humans produce in their environment. A very important component of the aggressive nature of weeds is allelopathic interference, the full potential of which is just being realized in the management of agroecosystems (1). Allelopathy refers to biochemical effects, both detrimental and beneficial of one plant (including microorganisms) on the germination, growth, or development of another plant (2).

Studying the practices of peasants for cultivating land in Tabasco, Mexico, Chacon and Gliessman (3) suggested the possibility that certain beneficial weeds (non-crop plants) might be able to repress harmful weeds through allelopathic interactions. An example of this could be weedy *Brassica campestris*. Tarahumara Indians in northern Mexico (Chihuahua) indicate that a plot of fertilized soil which was planted to mustard during the previous year will have a less dense stand of weeds. Such a statement suggests that residues or excretions of the mustard inhibit the germination and growth of other plants (4), suggesting its use as a weed controller.

*B. campestris* is a common weed in Santa Cruz County, CA. This

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State produces 95% of the broccoli grown commercially in the United States (5) and it has been reported that residues of broccoli are phytotoxic and inhibit the establishment of other crops (6). Both species, *B. campestris* and *B. oleracea* var. *italica*, belong to the Brassicaceae, which contain as a characteristic chemical compounds the glucosinolates (7, 8). Species from the Brassicaceae family have been infrequently reported as plants containing phytotoxins that can affect germination, establishment, and growth of other species in natural systems (9), but frequently reported in agroecosystems (10, 16).

In order to design more diverse agroecosystems with pest control mechanisms built in, the incorporation of some non-crop plants such as *B. campestris* should be considered and the allelopathic potential of the species involved must be determined. The objectives of the study were: (a) to determine the possible allelopathic potential of *B. campestris* and *B. oleracea* var. *italica* throughout their life cycle on different crop species and noxious weeds growing in the area; (b) to assess the density in which *B. campestris* could be planted without lowering broccoli production; (c) to determine what was the best sowing time of the non-crop plant; and (d) to find out if research on allelopathic crop/weed interactions can contribute significantly on establishing alternative weed control methods that do not depend only on petrochemical-based herbicides.

#### Materials and Methods

The experiments were done at the Farm Facility of the Agroecology Program at the University of California at Santa Cruz, where there are facilities for laboratory, greenhouse, and field research. The climate is Mediterranean, and averages 40 inches of rainfall annually.

#### Laboratory Experiments

To determine the allelopathic potential of *B. campestris* and *B. oleracea* var. *italica*, leachates from each species were made. Extracts were prepared by soaking, for two hr, weighed amounts of fresh or dried material in sufficient doubly distilled water to prepare a 10% extract (of fresh material) or 1.5% extract (of dry material). Extracts were vacuum filtered through paper (Whatman #1). The osmotic concentration was measured with a freezing-point osmometer (0 to 500 mosm) and indicator seeds were soaked in 10 mL of the extract for an hour prior to planting.

Extracts (12 mL each) were added to Petri dishes 10 cm in diameter containing 50 g of 30-mesh washed sand covered with filter paper circle (7-cm diameter, Whatman #1). Controls were moistened with doubly distilled water. Ten indicator seeds were placed on the filter paper in each dish with the embryo down and the hypocotyl pointed to the center of the Petri dish. Each indicator/extract combination had 3 replicates. The Petri dishes were kept in a dark growth chamber for approximately 72 hr at 25°C. The radicle length of each germinated seedling was measured at 72 hr.

Table I shows the number of experiments done, age of plants, kind of extract, its concentration, and indicator species utilized in each experiment.

Bioassays with different plant parts of both species were done

Table I. Number of Experiments, Kind and Concentration of Extracts, Ages of Plants, and Indicator Species Utilized to Detect Allelopathic Potential of Brassica campestris L. and Brassica oleracea var. italica

No. of exp.	Mustard		Broccoli		Age (days)	Age (days)	Indicator species	Season
	fresh (10%)	dry (1.5%)	fresh (10%)	dry (1.5%)				
I	X	-	-	-	26,39,51, 27	-	Barley Vetch	Fall 1982
	X	-	-	-	18,31,45, 59	-	Barley Broccoli Collards Ryegrass	Winter 1983
III	X	-	X	-	10,20,30, 39,46,57	34,43,53, 63,72,79, 90	Barley Vetch Ryegrass Radish	Summer 1983
	X	X	X	-	18,20,24, 28,33,38, 43,46,51, 53,54,56, 58	43,45,51, 55,59,64, 69,72,73 77,79,80 82,84	Wild radish Lettuce	
IV	-	-	-	X	45,51,55, 59,73,79, 80,82,84		Barley Vetch Ryegrass Radish	Fall 1983

to detect which of them contained the inhibitor(s). Extraction of the allelochemicals from B. campestris leaves was done with solvents of different polarity (chloroform, acetone, methanol, and water). When all the fractions were ready, 0.09 g were dissolved in 21 mL of the solvent with which they were extracted and 3 mL used to moisten a filter paper circle (7-cm diameter, Whatman #1). Controls were moistened with 3 mL of the solvent alone. The solvents were allowed to evaporate and the filter papers then placed in the center of Petri dishes containing 50 g of 30-mesh washed sand. The, 12 mL of doubly distilled water were added to each dish in which 10 seeds were set.

Some assays were done using sinigrin (Sarsyntex brand) and with the chloroform-soluble fraction obtained from B. campestris, at the same concentration (0.013 g per Petri dish), using as indicator species: Hordeum vulgare, Lolium multiflorum, Raphanus sativus, and B. oleracea var. italica.

All the data from the experiments were analyzed through an ANOVA program for a randomized complete block design (17) and comparisons were made between treatments. The percentages of inhibition were calculated by considering the control as zero.

#### Experiments in the Field

Summer, 1983. In order to determine the best density at which broccoli and B. campestris could be planted without affecting production of broccoli, an experiment was conducted in a complete randomized block design with five treatments: 0, 2, 4, and 8 B. campestris plants/m<sup>2</sup> interplanted with broccoli at a density of 4.5 plant/m<sup>2</sup> and a control planting of B. campestris alone at the same density.

Five weeks after sowing, the broccoli was transplanted into the field while wild mustard was planted directly on the date of broccoli transplant. The area was irrigated every week with overhead sprinklers throughout the experiment and fertilized 10.1 L/ha fish emulsion ("Grow Force" brand) at 30 and 57 days after set-up of the experiment. The plots were hand weeded selectively every 15 days, samples of the volunteer weeds were taken through the plot method (18), and the number of different species, number of individuals of each species, and biomass (dry weight) were recorded for each plot. The dominance, frequency, density, and importance value were calculated for each species in each plot.

Some 90 days after the start of the experiment the broccoli was harvested and the mean diameter of each broccoli head and its mean weight were recorded as well as number of individuals with harvestable heads.

Fall, 1983. Taking into account the results obtained in the first experiment, another experiment with four treatments in a complete randomized block design was done to determine the effect of B. campestris sowing time on broccoli production. The treatments were: (a) broccoli alone, clean weeded throughout the experiment; (b) broccoli alone, unweeded; (c) B. campestris planted on October 7 and broccoli on October 15, clean weeded (d) B. campestris and broccoli planted on October 15, clean weeded.

The same procedures (fertilization, weedings, and harvest) were followed as in the summer experiment.

Production data from both experiments were analyzed with an ANOVA

for a complete randomized block design and Duncan's comparisons of means between treatments were done.

## Results

Broccoli production. Table II shows results for the different treatments tested in summer 1983. All the parameters (mean number of harvestable heads, mean diameter of the inflorescence, and total biomass produced) indicate that growth of broccoli was stimulated by B. campestris.

In the fall of 1983, broccoli production was very different between treatments (Table III). B. campestris planted on October 15 + broccoli produced a yield like that of weeded broccoli monoculture, but production diminished when B. campestris was planted on Oct. 7 and broccoli on Oct. 15.

Allelopathic activity of B. campestris and broccoli. Table IV shows the allelopathic activity of extracts of fresh B. campestris and broccoli on various indicator species. The osmotic concentration of these extracts ranged from 0 to 25 mosm/L; according to Bell (19) and Anaya and Rovalo (20) these concentrations are not likely to account for inhibition of germination or radicle growth. Nevertheless inhibition of such growth was sometimes observed, in general more in monocots (barley and rye grass) than in dicots. The extracts from B. campestris produced stronger inhibition than the broccoli extracts, and in several cases the species tested were stimulated by broccoli rather than inhibited. The greatest allelopathic potential of wild mustard was just before and during the early part of the flowering stage. The radicular growth of species from the same genus (broccoli and collards) was stimulated rather than inhibited by fresh extracts of wild mustard. These results support the idea of interplanting B. campestris with a species from the same genus.

Table V summarizes the effects of extracts of dry tissues of broccoli and wild mustard on the radicular growth of indicator species (H. vulgare, L. multiflorum, V. atropurpurea and R. sativus). The first general characteristic is that the inhibition produced by B. campestris was stronger than that produced by the broccoli. The changes in percentages of inhibition depended on the age of the plant as well as on the occurrence of rainfall. When the material was collected after a rainy day and put to dry, the inhibition was not as strong as before or at the beginning of the rain. The differences of inhibition before and after a rain are very clear in the extracts obtained from broccoli. As with the fresh extracts, monocots (L. multiflorum and H. vulgare) were more inhibited than dicots.

Figure 1 shows the percentage of inhibition produced by extracts of different parts of dry and fresh material of B. campestris on the radicular growth of H. vulgare. All the extracts of dry material (1.5%) significantly inhibited radicular growth, but only extracts (10%) of fresh leaves had this effect, as can be seen in Figure 1. V. atropurpurea was less affected than H. vulgare. Only the extracts of dry leaves and fresh roots inhibited its radicular growth.

Percentages of inhibition of radicular growth of both R. sativus and H. vulgare produced by extracts made with nonaqueous solvents are in Table VI. Treatment moistened with the solvents alone inhibited such growth of the indicator species, but not significantly as com-

Table II. Broccoli Production in Different Treatments, Summer 1983

Treatment	Mean number of individuals per plot	Mean diameter (cm)	Mean weight (g)	Total yield per plot (tons/acre)
1	73 A	13.55 A	353.61 A	4.408
2	89 AB	14.16 A	332.82 A	5.021
3	101 B	14.62 A	358.35 A	6.254
4	99 B	15.83 A	406.35 b	6.742

Means with the same letters are not significantly different,  $p = 0.05\%$ .

1 = Broccoli monoculture

2 = 2 *B. campestris* plants/m<sup>2</sup> + broccoli

3 = 4 *B. campestris* plants/m<sup>2</sup> + broccoli

4 = 8 *B. campestris* plants/m<sup>2</sup> + broccoli

Table III. Harvestable Broccoli Production in Different Treatments, Fall 1983

Treatment	Mean number of individuals per plot	Mean diameter (cm)	Mean weight (g)	Total yield per plot (tons/acre)
1	70.33 A	7.37 A	68.81 A	0.840
2	69.33 A	6.08 B	48.64 B	0.585
3	69.67 A	6.85 A	59.58 AB	0.721
4	71.33 A	6.18 B	52.57 B	0.652

Means with the same letters are not significantly different,

$p = 0.05\%$

1 = Broccoli alone, clean-weeded

2 = Broccoli alone, unweeded

3 = Broccoli + *B. campestris* planted on October 15

4 = Broccoli planted on October 15 + *B. campestris* planted on Oct. 7.

Table IV. Age at Which *B. campestris* or *B. oleracea* var. *italica* Showed Allelopathic Activity (fresh material extracts, 10g/100 mL) on Radicular Growth of *Hordeum vulgare*, *Vicia atropurpurea*, *Lolium multiflorum*, *Raphanus raphanistrum*, *R. sativus*, *B. oleracea* var. *italica*, *B. oleracea* var. *Georgia*, and *Lactuca sativa*.  $N = 30$ . Numbers show percent of inhibition compared to control.

Age of plants (days)	Season and year	Phenological stage of		Indicator Species													
		Brassica	Brassica	Barley	Vetch	Ryegrass	Wild radish	Radish	Broccoli	Collards	Lettuce						
51	Fall 1982	VG		19.00*	<i>Brassica campestris</i> 12.00*	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
57	Fall 1982	F		-3.07	17.00*	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
45	Winter 1983	VG		13.00*	DT	0	DT	DT	DT	DT	-1.38	DT	-10.00	DT	DT	DT	DT
59	Winter 1983	F		38.00*	DT	19.36	DT	DT	DT	DT	10.59	DT	-11.66	DT	DT	DT	DT
20	Summer 1983	VG		10.07	0	30.69*	8.47	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
30	Summer 1983	F		40.03*	-30.38*	-7.83	-8.00	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
39	Summer 1983	F		-6.15	0	-19.66	22.26*	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
46	Summer 1983	F		DT	27.07*	9	14.93	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
57	Summer 1983	F		0	16.06	11.85	7.65	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
18	Fall 1983	VG		11.50*	DT	-12.00	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
47	Fall 1983	F		-10.40	6.1	-13.00	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
34	Summer 1983	VG		16.48*	17.16*	26.3*	-9.37	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
43	Summer 1983	VG		-17.50*	0	-9.66	-15.43	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
63	Summer 1983	VG		0	17.21	6.30	35.48*	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
79	Summer 1983	F		17.02*	9.85	-19.60	0	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
43	Fall 1983	VG		-21.90*	0	-22.50*	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT
45	Fall 1983	VG		-20.50*	-30.00*	8.00	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT	DT

\*Differences among indicator species for this age are significant at  $p = 0.05\%$ . DT = did not test; VG = vegetative growth; F = flowering.

Table V. Age at Which *B. campestris* or *B. oleracea* var. *italica* Showed Allelopathic Activity (dry material 1.5 g/100 mL) on Radicular Growth of *Hordeum vulgare*, *Lolium multiflorum*, *Vicia atropurpurea* and *Lactuca sativa*.  $N = 30$ . Numbers show percent of inhibition as compared to controls Fall, 1983.

Age of Plants (days)	Pattern of rainfall	Indicator Species			
		Barley	Ryegrass	Vetch	Radish
<u><i>Brassica campestris</i></u>					
18	rained 1 day	28*	DT	DT	25
20		42*	DT	DT	24
24		56*	DT	DT	43*
28	rained 3 days	35*	57*	18*	23*
33		41*	17	30*	52*
38		45*	65*	24*	46*
43		45*	60*	17*	90*
46		38*	80*	30*	28*
47	rained 1 day	44*	48*	24*	29*
51	rained 4 days	35*	29*	26*	27*
53		50*	44*	10	31
54		48*	42*	24*	13
56		40*	48*	17*	28*
58		47*	DT	24*	34*
<u><i>B. oleracea</i> var. <i>italica</i></u>					
43	rained 1 day	6	DT	DT	8
45		36*	DT	DT	13
51		27	DT	DT	21*
55	rained 3 days	11	25	DT	7
59		41*	6	-5	32*
64		32*	59*	33*	30*
69		50*	33*	24*	28*
72		50*	67*	16*	8
73	rained 1 day	28*	48*	9*	15
77	rained 4 days	-5	10	14*	15
79		28*	1	7	12
80		32	10	4	5
82		14	6	13	-1
84		30*	DT	12	31*

\*Differences among indicator species for this age are significant at  $p = 0.05\%$ .

pared to a doubly distilled water control. Solvents with the extracts were significantly different from the control. The fraction that produced the stronger inhibition in both species was the one made with chloroform. H. vulgare was more sensitive than R. sativus.

Because of the fraction in which the inhibitor(s) were present, the allelochemicals in B. campestris are most likely isothiocyanate derivatives such as allyl isothiocyanate, a breakdown product of the thioglucoside, sinigrin (21).

Table VI. Comparative Effects of Solvents Alone and with Extracted Material from B. campestris Leaves on the Radicular Growth of R. sativus and H. vulgare

Treatment	Indicator Species	
	<u>H. vulgare</u>	<u>R. sativus</u>
Control	0.0 A	0.0 A
Control chloroform	11.0 A	3.1 A
Control acetone	4.0 A	13.0 A
Control methanol	7.0 A	19.3 B
Chloroform fraction (4.2 mg/mL)	53.4 C	35.9 C
Acetone fraction (4.2 mg/mL)	15.4 B	25.6 B
Methanol fraction (4.2 mg/mL)	8.0 A	19.3 B
Aqueous fraction (4.2 mg/mL)	17.3 B	22.4 B
Total extract (4.2 mg/mL)	14.4 B	10.7 A

Percentages of inhibition with different letters differ significantly at  $p = 0.05\%$  by Duncan's multiple-range test. Percentages were compared only between the same species.  $N = 30$ . 3 mL of the soluble fractions were put in each Petri dish and the solvents were allowed to evaporate.

Figure 2 shows the inhibition produced by sinigrin (0.013 g per Petri dish) compared with that produced by the chloroform fraction of B. campestris. It is clear that the fraction from B. campestris produced more inhibition than sinigrin, and only R. sativus was not inhibited by either sinigrin or the chloroform fraction.

Effects on weed species. In the field experiments of summer, 1983, the total weed biomass was not significantly different among treatments, and in the first weeding (15 days after the beginning of the experiment) all the treatments agreed on the main weeds present, which were from the Brassicaceae (volunteer B. campestris and R. raphanistrum). In subsequent weedings there were no particular patterns; the main weeds were: Spergula arvensis, Amaranthus retroflexus, Convolvulus arvensis, Malva parviflora, Medicago sp., Chenopodium album, Plantago lanceolata, Sonchus sp., Erodium sp., Anagallis arvensis, and Capsella bursa-pastoris. In the fall of 1983, weed samples were not taken because weeds (Spergula arvensis, Amaranthus retroflexus, and Chenopodium album) left in the field from previous experiments generated considerable heterogeneity in the weed seed bank. Weed species were very patchy in the different plots, so the samples did not truly represent the treatments.



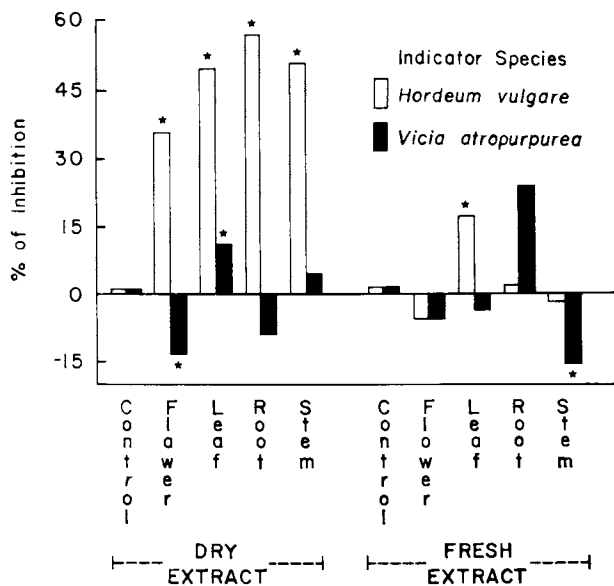


Figure 1. Percentage of Inhibition of Radicular Growth of *Hordeum vulgare* and *Vicia atropurpurea* Produced by Extracts from Dry and Fresh Material of Different Parts of *Brassica campestris*.  $N = 30$ . \* $p = 0.05\%$

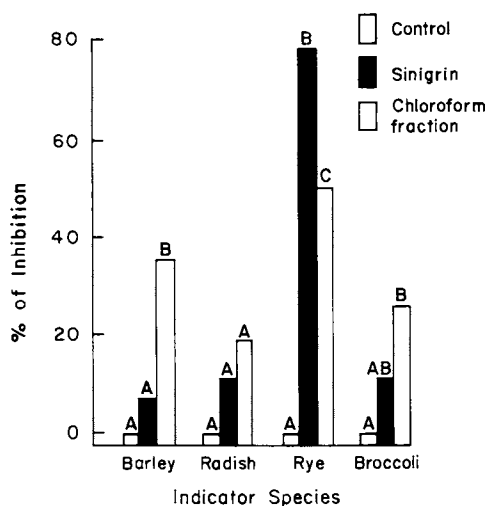


Figure 2. Percentage of Inhibition of Radicular Growth of Several Species Produced by Sinigrin and the Chloroform Fraction (0.013 g) Extracted from *B. campestris*.  $N = 30$ . Treatments with different letters differ significantly at  $p = 0.05\%$  by Duncan's multiple range test.

## Discussion

Other studies have provided evidence for the allelopathic potential of wild mustard (4) and broccoli (10, 14, 22). The results from the laboratory experiments in this study demonstrate that both species can produce allelochemicals throughout their life cycle.

It is very likely that the inhibitors are breakdown products of glucosinolates, which are considered nontoxic in themselves (23) but can yield physiologically active products upon hydrolysis by the enzyme myrosinase. Through hydrolysis glucosinolates can yield isothiocyanates (24-28). Ju et al. (29) demonstrated the capacity of thiocyanates as allelopathic agents, and isothiocyanates also have allelopathic activity (30).

The water-soluble extracts of wild mustard and broccoli plants were species specific, as shown by the results obtained by other authors (22, 31, 32). The greater insensitivity of crucifers is apparently related to the presence of specific myrosinases which are capable of transforming the breakdown products of the glucosinolates (33).

The strongest inhibition produced by extracts of fresh material of wild mustard occurred when it was bolting or just at the beginning of flowering, while inhibition by fresh extracts of broccoli did not show any such correlation. Nevertheless, this does not mean that allelopathic interference cannot take place earlier in the life cycle of the plant. Jiménez-Osornio and Gliessman (34) have demonstrated that seeds of crucifers can inhibit the radicular growth of some other species during simultaneous germination as early as 60 minutes after sowing.

Mustard extracts produced stronger inhibition than broccoli extracts (Tables IV and V). Although both species belong to the same genus, glucosinolate content undoubtedly differs in both quantity and quality, between species, as well as among varieties (35-40). Broccoli and wild mustard are of different species; in addition, people have been isolating chemical constituents of broccoli for a long time. On the other hand *B. campestris* has been considered a weed and its chemical constituents have not been studied as in the case of broccoli.

Inhibition of radicular growth was stronger with extracts of dry material than those of fresh material and the osmotic pressures of dry material extracts were higher than from fresh material, indicating better extraction of compounds.

Effects of extracts with dry material and with fresh material were not correlated. This may be because drying plant material causes hydrolysis of glucosinolates to isothiocyanates, while autolysis of fresh crucifers yields predominantly nitriles (28).

Field studies do not demonstrate conclusive allelopathic inhibition of weeds by wild mustard or broccoli, but there are some indications of allelopathic interference. First, the main weeds in the first weeding were crucifers in all treatments, but not in the following weedings. They were stimulated to germinate only at that time. Second, broccoli production was affected by *B. campestris*: yields were increased during the summer. Earlier planting of mustard in the fall inhibited broccoli yields, but had no effect when mustard was planted at the same time broccoli was transplanted. In addition, stimulatory effects of crucifers on other crucifers or other crops has been observed before (10, 16, 41).

In this study some assays showed stimulation by wild mustard extracts on broccoli radicular growth, and field experiments demonstrated that broccoli production can increase in the presence of the non-crop plant. Mean production of broccoli for Santa Cruz County varies from 4 to 6 tons/acre (42). The yield obtained when *B. campestris* was at a density of 8 plants/m<sup>2</sup> was 6.74 tons/acre even with broccoli planted in lower density than in conventional monocrops. During the fall, broccoli production was reduced significantly, yet it is important to mention that the variety (Premium Crop) is not the one most recommended for this season.

Increasing diversity in agroecosystems is important not only in space but also in time, since cultivars may produce long-lasting effects on soils and soil fungi. The results obtained during the summer experiment suggest the use of *B. campestris* intercropped with broccoli during the summer but not during the fall.

In order to manage a non-crop plant it is very important to understand its allelopathic potential and the chemical structure of the active chemical(s) as well as specific effects, spatial dynamics, and active life in the substrate. All this can give us a clear idea of the ecological significance of the allelochemicals and thus allow us to assess accurately the value of the chemical interference of some plants with others in an agroecosystem. With such information we can begin to make suggestions for future management and research in those systems when plant/plant interactions are integral such as in intercropping and rotational plantings.

It seems that progress towards a sustainable agriculture might benefit from a study of the naturally evolved chemical defenses of plants and their beneficial management through intercropping or planting crops with non-crop plants. It is possible that humans, by selecting crops over centuries, have created cultivars that have lost many of their allelopathic substances (41). Utilization of non-crop plants in agroecosystems can incorporate these chemicals back into the system. High intraspecific and intraspecific chemical diversity are likely to have several beneficial consequences, including allelopathic interference with harmful weeds. According to the data presented by Josefsson (43) from 12 thioglucosides analyzed, a broccoli monocrop will have 6 of them, whereas the intercrop of wild mustard and broccoli permits the presence of 11 of them. The presence of a non-crop plant in a agroecosystem incorporates structural complexity and variety of secondary chemicals.

Research of allelopathic interference is necessary in the design of agroecosystems in order to lessen dependence on petrochemical-based pesticides. The intercropping of non-crop species into crop systems offers alternatives for the design of more diverse and sustainable agroecosystems.

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## Chapter 25

# Specificity of Action of Allelochemicals: Diversification of Glycosides

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Diversification of structure of glycosides often accompanies coradiation of plant and herbivore species. In the evolutionary development of insecticidal chemistries, plants utilize differences in glycosylation patterns as well as aglycone structure to deter herbivory. Examples of glucosinolate, cyanogenic glycoside and triterpene glycoside ramification in the Passifloraceae, Brassicaceae and Cactaceae are correlated to insect host-plant specificity. Evidence for the presence of a specific glucosidase-mediated toxification mechanism is presented, and is considered to represent the proximal basis for the selection of altered glycosylation patterns.

Glycosides are elaborated by a large number of plants, and within families are often diversified in structure considerably. This diversification seems correlated with the coevolution of groups of plants with specialist herbivores. Examples are found in the *Passiflora-Heliconius* interaction (1,2) and in the association of *Pieris* and *Brassica* (3,4). The same phenomenon has recently been determined to occur in the *Drosophila*-cactus-yeast coevolved system (5,6) and may be present in the Danaiid-*Asclepias* and other systems (7).

Glycosides themselves are generally regarded as representing simple storage products, accumulable derivatives of the aglycone moiety (8). The fact of glycosidation is often disregarded as irrelevant in terms of potential biological activity, except in reducing same (9). It is certainly appreciated that glycosidase-mediated enzymatic hydrolysis of glycosides (8) releases the aglycone whereby these glycosides may become active plant toxic principles, but this process is also regarded as essentially a storage-release phenomenon.

$\beta$ -Glucosidases and other glycosidases have been determined to often show an exceedingly high degree of specificity toward a particular substrate (10,11). In recent work (12,13) this specificity has been shown to be attributable to the structure of the aglycone.

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Several recent reviews (14-16) have indicated that one can generally expect to find a specific glycosidase corresponding to a specific glycoside structure being present in the same plant. However, this necessary coelaboration has been frequently overlooked as a functional mechanism of evolutionary change in diversified systems of coevolved plants and insects.

I will argue in this chapter that the very process of hydrolysis is an event under the evolutionary control of the competing interests of plant and insect species, and that "toxic" plant glycosides should be regarded as such only in terms of an inseparable, targeted glycoside-glycosidase system.

I will extend this argument to encompass the diversification of such systems in lineages of plants in an effort to correlate enzyme-mediated glycoside toxicity with the evolution of host plant specificity and the coevolution of plants and insects.

### The *Passiflora-Heliconius* Interaction

The specificity of  $\beta$ -glucosidases toward their substrates has been confirmed in the production of cyanide from cyanogenic glycosides of *Passiflora* and its relatives (Table I). While ecologically significant quantitative variation in specificity exists (1,2), the more obvious degree of specificity shown by these enzymes toward cyanogenic glycosides will suffice for the present discussion. The specificity is such that even a crudely purified enzyme preparation (2,15) can be used to identify biosynthetic and structural types of cyanogens.

The peculiar observation that particular combinations of  $\beta$ -glucosidases from various related species, or combinations of  $\beta$ -glucosidases and substrate substitutes, frequently prevented expected hydrolysis (Table II), gave the first indication that the interaction of plant enzymes could be of importance in the *Passiflora-Heliconius* system. The same inhibitory interaction has since been observed in insect-plant glucosidase combinations (17).

The cyclopentenoid cyanogenic glycosides undergo hydrolysis according to the reaction illustrated in Figure 1. This two-step process is entirely consistent with that determined for other cyanogenic glycosides (18). The second step is thermodynamically favored, and occurs rapidly at normal cell pH even in the absence of  $\alpha$ -hydroxynitrile lyase.

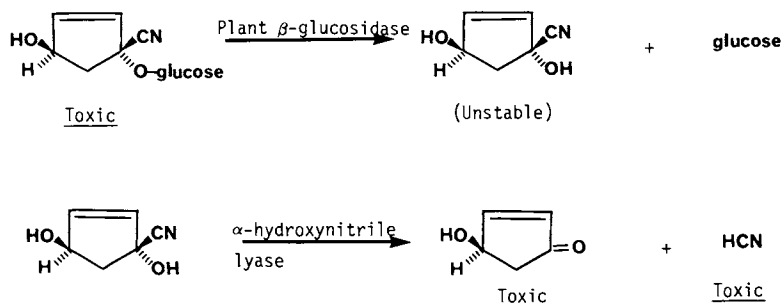
The production of a 2-cyclopentenyl ketone is unique to cyclopentenoid hydrolysis. This fact is of biological importance, as the ketone is an  $\alpha,\beta$ -unsaturated compound, and has been determined to be a powerful alkylating agent (Figure 2) (6,19). While HCN is a general toxin which reduces fitness in many organisms (6,17,20 and references therein), it is the ketone moiety which confers specific toxicity upon the *Passiflora* cyanogenic glycosides. Preliminary data indicate that the alkylation reaction is so rapid and non-specific as to theoretically preclude the successful development of a specific post-hydrolysis resistance in an herbivore. The insect species is therefore highly induced to produce a defensive capability that prevents hydrolysis altogether. This selective pressure is not at all necessarily the same as would induce the development of a defense against HCN.

Table I. Specificity of  $\beta$ -Glucosidases of *Passiflora* and Related Species

Enzyme Preparation	Compound										
	linamarin	prunasin	tetraphyllin B sulfate	deidacilin	tetraphyllin B	gynocardin	passibiflorin	passitrifasciatin	passisuberosin	passicoriacin	amygdalin
<i>Turnera ulmifolia</i>	-	-	-	+++	+	-	+	-	-	-	-
<i>Passiflora foetida</i>	-	-	+++	+++	+++	-	+++	-	-	-	-
<i>P. X alatocaerulea</i>	-	-	+++	-	+	-	+	-	-	-	-
<i>P. caerulea</i>	-	-	+++	-	+	-	+	-	-	-	-
<i>P. trifasciata</i>	-	-	-	-	-	-	-	+++	-	-	-
<i>P. suberosa</i>	-	-	-	-	-	-	-	-	+++	+	-
<i>P. coriacin</i>	-	-	-	-	-	-	-	-	+	+++	-
Emulsin (Sigma)	-	+++	-	-	-	-	-	-	-	-	+++
Linamarase	+++	-	-	-	-	-	-	-	-	-	-
<i>Gynocardia odorata</i>	-	-	-	-	-	+++	-	-	-	-	-

Table II. Inhibition of Natural  $\beta$ -Glucosidase Activity by Addition of Similar Glucosidases of Competing Substrates

Combinations of Enzyme Preparations and Cyanogenic Compounds	Compound									
	deicalin	tetraphyllin B sulfate	prunasin	linamarin	gynocardin	passibiflorin	passitrifasciatin	passisuberosin	passicoriacin	amygdalin
<i>T. ulmifolia</i> + <i>P. X alatocaerulea</i>	-	-	-	-	-	-	-	-	-	-
<i>T. ulmifolia</i> + emulsin	+	-	-	-	-	-	-	-	-	-
<i>T. ulmifolia</i> + linamarase	(+)	-	-	(+)	-	-	-	-	-	-
<i>T. ulmifolia</i> + <i>P. foetida</i>	-	-	-	-	-	-	-	-	-	-
<i>P. X alatocaerulea</i> + <i>P. foetida</i>	-	(+)	-	-	-	-	-	-	-	-
<i>P. foetida</i> + emulsin	-	(+)	-	-	-	-	-	-	-	-
<i>P. biflora</i> + <i>P. trifasciata</i>	-	-	-	-	-	-	-	-	-	-
<i>P. suberosa</i> + <i>P. biflora</i>	-	-	-	-	-	-	-	-	-	-
<i>P. coriacea</i> + <i>P. trifasciata</i>	-	-	-	-	-	-	-	-	-	-
<i>T. ulmifolia</i> + amygdalin	-	-	-	-	-	-	-	-	-	-
Emulsin + tetraphyllin B	-	-	-	-	-	-	-	-	-	-
<i>G. odorata</i> + linamarin	-	-	-	-	-	-	-	-	-	-
<i>P. X alatocaerulea</i> + prunasin	-	-	-	-	-	-	-	-	-	-

Figure 1.  $\beta$ -Glucosidase-mediated hydrolysis of cyclopentenoid cyanogenic glycosides.



*In vitro* studies in this laboratory have established that specific resistances to toxification by cyclopentenoid cyanogenic glycosides exist in *Heliconius* and its relatives, and that the process involves the specific inhibition of the hydrolysis of the compounds present in the host plant (1,2). While the actual mechanism has not been satisfactorily quantitatively demonstrated, our data are consistent with the models illustrated in Figure 3. In the first reaction, the *Heliconius*  $\beta$ -glucosidase binds to the plant substrate-enzyme complex, either during or after complex formation, in a competitive manner. The one-substrate, two-enzyme complex precipitates out of solution. In the second reaction, the *Heliconius*  $\beta$ -glucosidase and/or other glycosidases actually attack and hydrolyze the plant  $\beta$ -glucosidase. Both reactions have been measured *in vitro* and appear to occur *in vivo* (6,17). Both reactions are highly substrate specific, and both result in the inactivation of the plant  $\beta$ -glucosidase and prevent hydrolysis. The specificity of the reaction is such that the production of *Heliconius* inhibitory enzymes must be regarded as a coevolved response to the specific *Passiflora* toxification system (cyanogenic glycoside plus  $\beta$ -glucosidase) which is targeted against it.

The interaction of the *Passiflora*  $\beta$ -glucosidase and cyanogenic glycoside with a specialized *Heliconius* herbivore is summarized in Figure 4. Glucose is assumed as the model sugar moiety. Here, in response to the development of an insect  $\beta$ -glucosidase capable of inactivating the plant toxification syndrome, the plant species may evolve any one or more of the following changes: 1) modification of aglycone structure. This occurs in *Passiflora* through the attachment of different substituents or replacement of the double bond with a single bond or epoxide, or through changes in symmetry. 2) Modification of the sugar moiety, through a change in number, type or linkage of sugar substituents. 3) Change in cyanogenic glycoside skeletal type through an alteration in the biosynthetic pathway to another precursor to yield an alternate type cyanogenic glycoside (i.e. cyclopentenoid becomes aromatic as 2-cyclopentenylglycine is replaced with phenylalanine as precursor). 4) Production of ionically destabilized cyanogenic glycosides; in *Passiflora* by attachment of a sulfate at C-4. 5) Production of cyanohydrins through the omission of the final glycosylation step in biosynthesis. This results in an exceedingly unstable form of cyanogenic compound ( $\alpha$ -hydroxynitrile) which, having no sugar moiety, no longer requires a  $\beta$ -glucosidase for hydrolysis. 6) Change in structure of the plant  $\beta$ -glucosidase complement: a) to facilitate hydrolysis of an altered structure, b) to resist binding and inactivation by a given insect  $\beta$ -glucosidase, c) to hydrolyze insect  $\beta$ -glucosidases (not yet observed).

#### The Pierid-Cruciferae Interaction

Given the rapid radiation of many specific plant glucosidase-substrate systems and the coradiation of specific insect adaptations in the *Passiflora*-*Heliconius* coevolutionary interaction, it seems quite reasonable to expect that such a process would occur in other systems.

As the discovery of the diversification of cyanogenic glycosides in *Passiflora* was the prerequisite for interpreting the co-

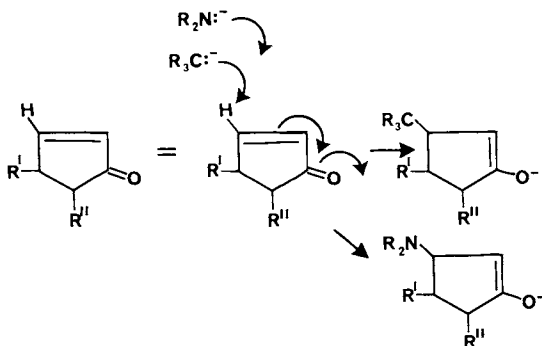


Figure 2.  $\beta$ -Alkylating property of 2-cyclopentenones derived from hydrolysis of cyclopentenoid cyanogenic glycosides.

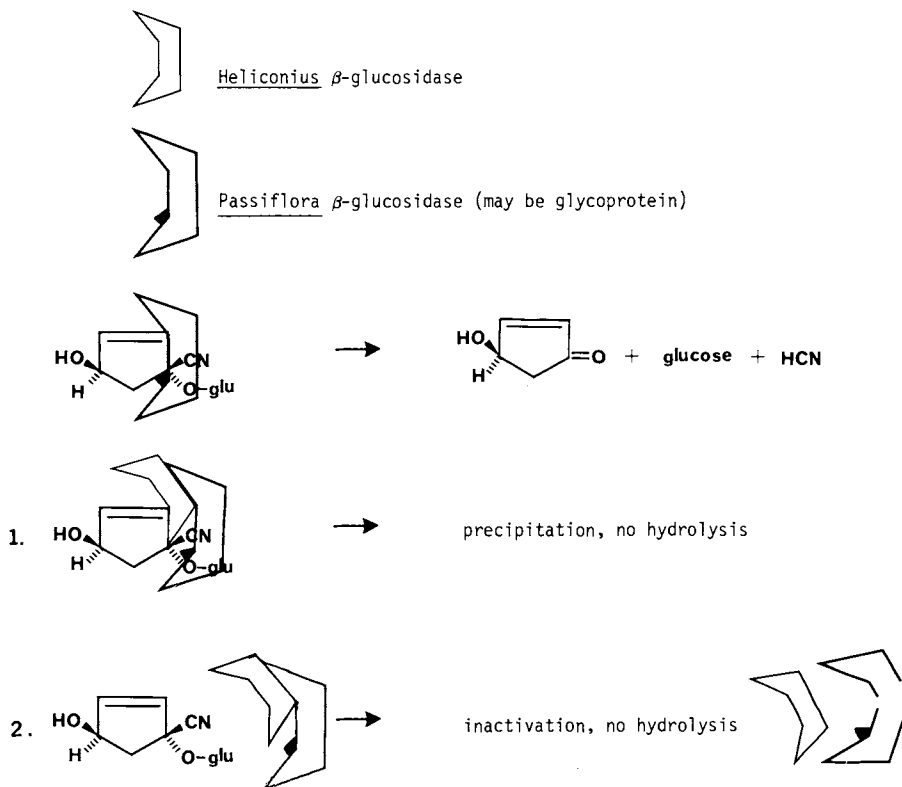


Figure 3. Two hypothetical mechanisms by which *Heliconius* may protect itself from toxification by *Passiflora* cyanogenic glycosides.

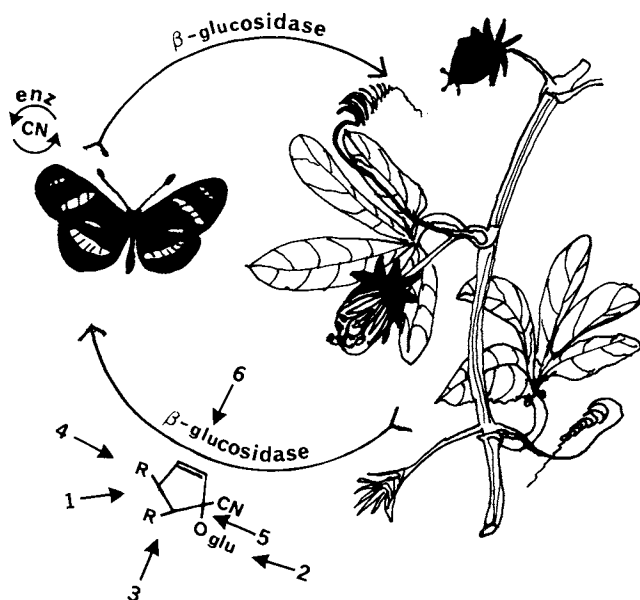


Figure 4. Chemical responses of *Passiflora* to specialization by *Heliconius*. See text for explanation of numbered responses.

evolution of *Passiflora* and *Heliconius*, a similar coevolved system with a diversified glycoside chemistry should provide a logical comparison of toxification processes.

The crucifer-Pierid interaction is particularly well studied (3,4,21) and is thought to represent an intricately coevolved system. Species in the Cruciferae characteristically elaborate glucosinolates (thioglycosides). A large number (>75) have been described from the family (22,23), representing a considerable diversification in structure when compared to glucosinolate production in other families (4).

These compounds are biosynthesized in a pathway entirely analogous to that of cyanogenic glycosides (24), and are hydrolyzed to toxic (25) isothiocyanates by  $\beta$ -thioglycosidase in a process analogous to the hydrolysis of cyanogenic glycosides (26). The substrate and enzyme may also be compartmentalized within tissues in the same way as cyanogenic glycosides and glucosidase (28-31).  $\beta$ -Thioglycosidase (myrosinase) has long been considered, at least *de facto*, to consist of only a single enzyme activity (4). However, myrosinase has been determined to be a family of isoenzymes (also glycoproteins) (27).

While the host specificity of *Pieris* and its relatives to crucifer species has been interpreted as a stepwise reciprocal selective response to evolutionary changes in glucosinolate chemistry (32), further synthesis has proved difficult. Because of the interpretation of the toxic activity as residing in the glucosinolates alone, as released by a nonspecific activating enzyme, analyses become compounded by the apparently widespread distribution of many compounds in various combinations. The defensive value of the glucosinolate arrays, and their importance as selective agents in the coevolution of the Cruciferae and the Pieridae, have been well explored (33-35). However, a plausible mechanism for recognition and tolerance of these compounds remains elusive.

Isolations were made of thioglycosidase fractions from eight plants in the Cruciferae and several others known to produce glucosinolates. Each showed a number of specific thioglycosidase activities to be present upon separation by gel electrophoresis and assay for  $\text{SCN}^-$  release after treatment with a variety of substrates.

Combinations of enzymes inhibited expected hydrolysis of a substrate in an experiment constructed according to Table II. Enzyme fractions from different plant species release  $\text{SCN}^-$  at different rates when sinigrin, sinalbin, benzyl glucosinolate, and glucosinolate-containing fractions of each plant were used as substrate. The specificities showed were significant but were not as restricted as was observed for glucosidases in Table I. This is to be expected as a far greater number of glucosides and enzymes appear to be present in the latter samples than in the previous experiment.

Enzyme preparations of three *Pieris* species each contained  $\beta$ -thioglycosidase activity, a fact previously reported (36), and each inhibited hydrolysis in one or more combinations with a plant enzyme-substrate system. These data are being quantified and extended to include insect/preferred host-plant pairs.

The *Pieris*-crucifer interaction therefore seems to involve the same biochemical parameters as the *Passiflora*-*Heliconius* interaction, and it is proposed that the evolution of host plant specificity has proceeded in an analogous fashion in both systems.

### The *Drosophila*-Cactus-Yeast Interaction

The interaction between *Drosophila*, yeasts and columnar cacti of the Sonoran Desert has been the subject of much recent interest (37). As a coevolved system, perhaps more is known about this interaction than any other. The chemistry of the cacti (70 spp.) has been postulated to play a significant role in the establishment of this system (38), but this was based upon reports of a relatively small number of relatively simple alkaloids, and a small number of terpenoid compounds. Only recently, the diversification of plant compounds has been discovered to be much greater (5). In their section on alkaloids in the above work, Bajaj and McLaughlin report the presence of some thirty-five structures. In addition, I have been able to isolate some sixty triterpenoid glycosides (structures were not determined) and more were detected but not isolated.

Previously (39), some sixteen distinct triterpene skeletons in two classes were isolated and reported from this group of cacti. Standard isolation procedure for these compounds requires hydrolysis of sugars, so the glycosidic structures have not been described. As the data presented previously imply, individual glycosides are the compounds of ecological interest.

A laboratory study was undertaken to determine the likelihood that cactus triterpenoid glycosides are important factors in the host-plant choice of desert *Drosophila* (6).

A feeding experiment was conducted using field concentrations of the alkaloidal fraction and the total triterpenoid glycoside fraction of thirty related species of columnar cacti (most in the Pachycereeae). Survivorship was measured as + or - and indicates successful development, pupation and emergence after eggs were laid by several *D. melanogaster* or *D. mojavensis* females. The latter species is a desert fly known to specialize on several species of Pachycereeae; the former is a nonspecialized, nondesert species. *Heliothis zea* larvae were also used in a separate bioassay of toxicity where compounds were added to commercial diet.

Triterpenoid glycosides are hydrolyzed to yield aglycone ketones and diols by  $\beta$ -glucosidases. Assays of plant materials showed these enzymes to be present. It is known that cactophilic yeasts are able to hydrolyze terpenoid glycosides (38). It was determined that plants and yeasts hydrolyze different glycosides at different rates. In the present experiment, commercial baker's yeast was utilized for *Drosophila* feeding trials. It was possible to detect hydrolysis of glycosides and arrays of glycosides in triterpenoid fractions through the production of free sugars and the degradation of individual compounds as revealed by specific color reagents and TLC. Many triterpenoids underwent hydrolysis, but alkaloids did not.

Specific differences in toxicity toward each test species were discovered between the various plant chemical arrays. Table III lists survivorship for these species for alkaloid-producing cacti. Additionally, data was obtained for 20 species in which alkaloids were not detected. The three insect species showed consistent differences in tolerance toward given plant species, with no clear phylogenetic pattern accounting for this. Insects also responded differently to alkaloidal fractions versus triterpenoid glycoside fractions. Alkaloids were found to be generally not toxic to *D.*

Table III. Survivorship of *Drosophila* and *Heliothis* Upon Alkaloidal and Triterpenoid Glycoside Extracts of Alkaloid-Producing Columnar Cacti

		ALK			TRI		
		<i>D mel</i>	<i>D moj</i>	<i>H zea</i>	<i>D mel</i>	<i>D moj</i>	<i>H zea</i>
<i>Stenocereus</i>	<i>stellatus</i>	-	-	-	-	+	-
	<i>treleasei</i>	-	+	-	-	+	+
	<i>beneckeii</i>	+	+	+	+	-	+
	<i>quevedonis</i>	-	+	+	-	+	+
	<i>dumortieri</i>	-	+	-	+	-	-
<i>Polaskia</i>	<i>chende</i>	-	+	+	-	+	+
<i>Escontria</i>	<i>chiotilla</i>	-	+	-	-	-	-
<i>Lemaireocereus</i>	<i>hollianus</i>	-	+	+	-	-	-
	<i>humilis</i>	+	-	-	+	-	+
<i>Pterocereus</i>	<i>gauneri</i>	+	+	-	+	-	-

*mojavensis* (tolerated 90% of plant species), yet triterpenoid glycosides from 60% of the alkaloid-producing and from 80% of non-alkaloid-producing plants were lethal. *D. melanogaster* grew successfully upon 40% of all triterpenoid fractions, and somewhat less (30%) of alkaloid fractions. *H. zea* performed consistently better on triterpene fractions (60%+) than on alkaloid fractions (40%+).

In terms of comparative performance upon extracts from individual plant species, *D. melanogaster* and *D. mojavensis* showed different tolerance on 80% of plant species whether tested against triterpenoids or alkaloids.

From these preliminary data, it can be concluded that: 1) triterpenoid glycosides are hydrolyzed by yeasts to yield aglycones that are selectively toxic to *D. mojavensis* at field levels. 2) Cactus alkaloid arrays are not generally toxic to *D. mojavensis* at field levels. 3) Non-adapted and non-specialized insects do not exhibit selective tolerance toward any given compound arrays, and are susceptible to toxification by either alkaloids or triterpenoid glycosides or both. 4) Triterpenoid glycosides may be specifically targeted against *Drosophila* more than other insects, and alkaloids may be less specialized toxins. This would therefore be analogous to the separation of specific (aglycone) and general (HCN) toxic principles in the *Passiflora-Heliconius* interaction.

Further work is being conducted using specialized yeasts and other species of *Drosophila* and quantifying differences in hydrolysis and toxicity of glycosides under these conditions.

We may hypothesize for the present that columnar cacti produce alkaloids as a general deterrent to herbivory, and triterpenoid glycosides and associated hydrolytic glycosidases as a specific toxification mechanism against specialist *Drosophila* species. Radiation and diversification of triterpenoids may have occurred in response to continued interaction between the cacti and *Drosophila*. This process is dependent upon coevolution with specialized yeasts which may interfere with hydrolysis of triterpenoids or hydrolyze individual compounds selectively.

### Other Systems

Glycoside diversification also has occurred in the coevolution of monarch butterflies and milkweeds (7). It may be desirable to relate the toxicity of cardenolides to the hydrolytic capabilities of susceptible and nonsusceptible insects. Cardenolides from *Asclepias* species can be hydrolyzed by  $\beta$ -glucosidases present in the plant (6), yet specialized *Danaus* species are able to sequester these compounds, a process which requires control of hydrolysis.

Plant  $\beta$ -glucosidases also cause hydrolysis of iridoid glycosides, and are highly specific in activity (6). Several insect species that are able to tolerate iridoids have been found to contain inhibitory  $\beta$ -glucosidases. These and other systems are under continuing investigation in this laboratory.

### Ecoregulatory Processes

Recent work (40) has shown that plant tannins are capable of inactivating plant  $\beta$ -glucosidases *in vitro*. It has been determined (6) that insect  $\beta$ -glucosidases are also inactivated by plant tannins at

field levels (Figure 5). *Heliconius*  $\beta$ -glucosidase fractions have been assayed for their ability to inhibit *in vitro* *Passiflora*  $\beta$ -glucosidase hydrolysis of cyclopentenoid cyanogenic glycosides (6, 17). After treatment with tannins, the insect enzyme fractions were dialyzed to recover soluble glucosidase activity. The remaining inhibitory ability was determined through quantitative measurement of HCN release in the test reaction.

It is possible that these ecoregulatory enzymes, used by the insect to inhibit targeted plant toxification systems, may themselves be the principal target of the production of tannins by plants. The discovery of other examples of secondary chemical interactions with enzymes dedicated to the regulation of toxification and detoxification mechanisms may be expected as our knowledge of chemically mediated plant-insect interaction expands.

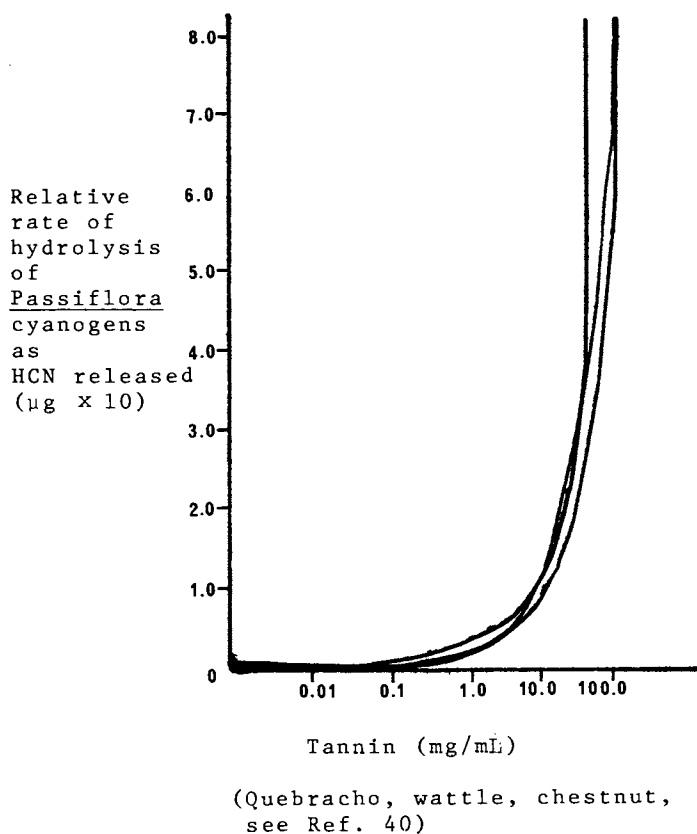


Figure 5. Tannin inhibition of *Heliconius*  $\beta$ -glucosidases that interfere with cyanogenesis of *Passiflora*.



### Summary

Plant lineages exhibiting diversification of glycosides should more properly be regarded as having produced a diverse set of glycoside/glycosidase systems. These are generally toxic, owing their toxicity to the structures of one or more of the hydrolysis products and to the fact of successful hydrolysis. The effectiveness of the glycoside-derived toxin also depends upon inhibitory glycosidases present in the target insect digestive system which can inhibit hydrolysis through competitive interaction with the substrate-enzyme complex, or through direct hydrolytic action against the plant enzyme. Plant tannins may interact directly with the enzymes responsible for hydrolysis, and may therefore be targeted against insect ecoregulatory enzymes. This underscores the importance of regarding plant allelochemicals as one part of complex targeted toxification systems.

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## Chapter 26

# Purine Alkaloids in Tea Seeds During Germination

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During imbibition of whole tea seeds (6 days) two purine alkaloids, caffeine and theobromine, did not decrease in the seed coats and there was no increase in the seeds. In parallel with and after the breaking seed coats there was a gradual release of caffeine from coats of germinating seeds. By contrast, when the seed was freed from the outer seed coat and soaked, imbibition of the seed required only two days and simultaneously caffeine was released from the inner seed coat. In such seeds, but not in whole seeds, growth of embryonic tissues (roots and shoots) was inhibited after the breaking of the inner seed coats. Nevertheless, caffeine increased more in such roots of the seedlings of decoated seeds than in roots of normal seedlings.

Studies of caffeine (1,3,7-trimethylxanthine; Figure 1) in the coffee plant have shown that it undergoes a variety of metabolic changes (1-3), and has an ecological role rather than one as a nitrogen reserve (4-9). We (10) reported that two purine alkaloids, caffeine and theobromine (3,7-dimethylxanthine; Figure 1), in leaves and shoots of tea (*Camellia sinensis*) decreased significantly in August, October, and November in Japan. This suggests that the alkaloids have no role in the storage of nitrogen in tea leaves during winter months. The biosynthesis of caffeine in coffee and tea plants proceeds through the steps: purine nucleotides in the nucleotide pool (AMP and/or GMP) → XMP → xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine (3,11,12).

In contrast with the seed caffeine of *Coffea* species, relatively little attention has been paid to that of tea. This is in part because the fruit of tea, including the seeds, is of minor economic importance compared with that of coffee; moreover earlier studies revealed little caffeine in the tea seed (13,14). Recently we (15) found that the pericarp contains the greatest concentrations of alkaloids in the dry fruit of tea, and that appreciable amounts occur in the seeds, especially in the coats. Thus, from physiological and ecological viewpoints, our concerns are the roles of purine alkaloids and seed coats of tea during fruit development (seed formation) and seed germination. Caffeine in *Coffea arabica* seed is synthesized in the pericarp, transported to the seed, and accumulated there during fruit

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development (16), whereas the caffeine in both the pericarp and seed coats of tea is synthesized and accumulated in the same tissues (15). Allelopathic reactions of coffee seeds and fruits containing these alkaloids have been described (7,17,18). The behavior of two purine alkaloids in tea during germination, and their physiological and ecological functions are described.

### Materials and Methods

Seeds of tea (*Camellia sinensis* L.) were surface-sterilized in a saturated solution of calcium hypochlorite for 30 min, and soaked for 30 min in running water. The whole seeds, or else seed that had been decorticated, i.e. removed from the hard testae, were sown in moist sea sand and allowed to germinate and grow in dark at 28 °C.

The mixture of alkaloids present was extracted and analyzed as described previously (2,3).

### Results

After the flowering in October, fruit growth of tea is almost unchanged until spring, and then proceeds progressively until the fruit is full-ripened and dried (15). The seeds are then shed in November, and lose viability after several months. The contents of purine alkaloids in the seeds of coffee and tea plants are given in Table I.

When the whole tea seed was used, imbibition required about 6 days, and then the seed broke out of the outer seed coat 6-10 days after soaking (Figures 2 & 3). During imbibition alkaloid concentrations were unchanged in the seed coat although caffeine is readily water-soluble (Figure 4). During and after the breaking of seed coats caffeine was gradually released from the coats of germinating seeds (Figure 3). By contrast, when the seed was removed from the outer seed coat and soaked, imbibition required only 2 days, and concurrently caffeine was released from the inner seed coat (Figures 2 & 3). In such seeds, but not in whole ones, growth inhibition of embryonic tissues (roots and shoots) occurred after the breaking of the inner seed coats. Nevertheless, caffeine increased more in roots of the decoated seed than in normal seedling roots. Table II shows results on growth inhibition and increments of caffeine in the roots of 5-week-old tea seedlings, when the seeds were freed from the outer seed coats, allowed to imbibe, and incubated in water extracts from seed coats.

### Discussion

From these studies and the work of others (4-9,17,18), we conclude that caffeine found in the seed coats of tea seeds (Table I) has no nutritive function and that it is phytotoxic and autotoxic i.e., inhibits growth of germinating tea seedlings (Table II). The seed coats are the barriers between the embryo and its immediate environment. As dead tissues, the seed coats of the mature seed protect the enclosed embryo. Equally important are the nutritive and regulatory functions of the living seed coats during embryo development (19-23). The developing tea seed has a more highly developed seed coat than the coffee seed (15). The coat functions as a good reservoir of the toxic alkaloids (caffeine and theobromine) (Table I), but prevents autotoxic hazards from occurring during fruit development in the tea plant.

The seed coat of tea regulates imbibition of tea seed (Figure 2) and releases caffeine during germination (Figure 3). These processes may be dependent upon

	Trivial name	R <sup>1</sup>	R <sup>3</sup>	R <sup>7</sup>
Xanthine		H	H	H
1,3-Dimethylxanthine	Theophylline	CH <sub>3</sub>	CH <sub>3</sub>	H
3,7-Dimethylxanthine	Theobromine	H	CH <sub>3</sub>	CH <sub>3</sub>
1,7-Dimethylxanthine	Paraxanthine	CH <sub>3</sub>	H	CH <sub>3</sub>
1,3,7-Trimethylxanthine	Caffeine	CH <sub>3</sub>	CH <sub>3</sub>	CH <sub>3</sub>

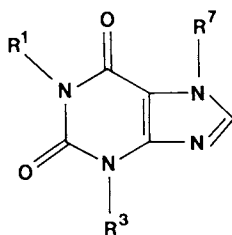


Figure 1. Some Naturally Occurring Methylated Xanthines.

Table I. Caffeine (Cf) and Theobromine (Tb)  
Contents of Coffee and Tea Seeds

<i>C. arabica</i> seed <sup>a</sup>		<i>C. sinensis</i> seed <sup>b</sup>			
		Seed coat		Cotyledon	
Cf	Tb	Cf	Tb	Cf	Tb
(μg/g fresh wt.)					
3893	54	2343	143	100	<10

<sup>a</sup>Suzuki and Waller (15).<sup>b</sup>From the ripened dry fruits almost ready to drop.

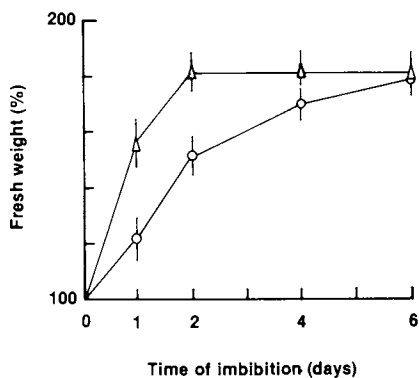


Figure 2. Imbibition of Tea Seeds at 28 °C Expressed as Percentage of the Original Weight of the Seeds (mean  $\pm$  S. D.;  $N = 6$ ). Whole seeds (O), and seeds removed from the outer seed coats prior to germination ( $\Delta$ ).

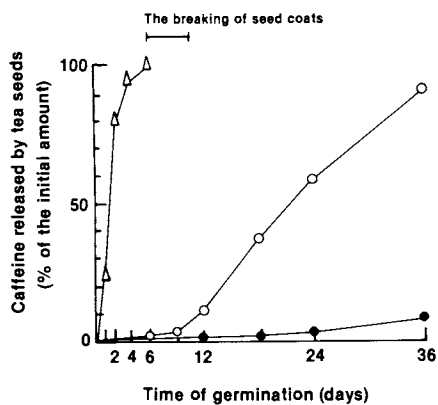


Figure 3. Caffeine Leakage from Germinating Normal Tea Seeds (●), Seed Coats (O), Seeds removed from the Outer Seed Coats prior to Germination ( $\Delta$ ) into Surrounding Sand.

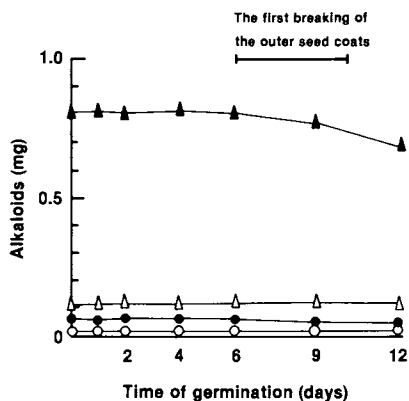


Figure 4. Levels of Caffeine ( $\Delta$ , $\blacktriangle$ ) and Theobromine (O, $\bullet$ ) in Seed Coats ( $\blacktriangle$ , $\bullet$ ) and Cotyledons ( $\Delta$ ,O) of Tea during Imbibition and Early Germination.

Table II. Growth and Caffeine Content of Roots and Shoots of 5-week-old Tea Seedlings, when Seeds were Separated from the Seed Coats and Incubated in Water Extracts from Tea Seed Coats<sup>a</sup>

Growth		Caffeine content	
Roots	Shoots	Roots	Shoots
(% of control)			
65 $\pm$ 10	60 $\pm$ 8	165 $\pm$ 25	65 $\pm$ 10

<sup>a</sup> Data represent percentages of growth (length) and caffeine content of the control (in distilled water); average of 10 seedlings ( $\pm$  s. e.). Seeds freed from the outer seed coats were allowed to imbibe for 2 days, and then were freed from the inner seed coats and incubated in water extracts for 33 days.

the embryo, since removal of the outer seed coats prior to inhibition resulted in release of caffeine from imbibing seeds and growth inhibition of embryonic tissues. Inhibited roots of seedlings from the decoated seed had higher concentrations of caffeine than roots of normal seedlings. Water extracts from seed coats, containing caffeine, showed autotoxicity (Table II). These suggest an important protective role of the tea seed coats during germination, i.e., to release caffeine gradually and thereby prevent its autotoxic (allelopathic) action.

In conclusion: (a) caffeine has ecological significance in both tea and coffee seeds, i.e., acting against predators and against competitors, (b) in contrast to coffee seed, tea seed localizes caffeine primarily in the seed coats whence the alkaloid is liberated gradually into the soil, and (c) such release may also be important for avoidance of caffeine autotoxicity to the germinating tea plant, because soil microflora use caffeine as a substrate, and thus prevent it from reentering the plants.

#### Acknowledgments

We are grateful to the Takeda Herbal Garden, Kyoto, Japan for the tea samples. We also thank Dr. Otis C. Dermer of the Oklahoma State University, Stillwater, Oklahoma for critical reading of this manuscript. This is Journal Article No. 4934 of the Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma 74078.

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## Chapter 27

# Increased Concentration of Myristicin and 6-Methoxymellein in Carrot Root upon Irradiation with UV Light

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Myristicin has not been reported to possess antifungal activity, and therefore is not a phytoalexin according to the standard interpretation of this term (1). It does, however, potentiate the activity of the insecticide, paraoxon, in flies by inhibiting its degradation (2), and may in similar manner potentiate the action of phytoalexins of carrot root (falcarinol, falcarindiol, 6-methoxymellein) (3,4). The presence of myristicin in carrots is of interest to nutritionists because of its biological properties (5), and its chemical similarity to safrole, a mild carcinogen (6).

Results of Wulf *et al* (7) show that carrot roots obtained from a supermarket contain myristicin; Imperator variety carrots contain an average of 15 parts per million (ppm). Recently harvested, unprocessed carrots only rarely contain myristicin (8). The presence of myristicin in supermarket carrots and its absence in recently harvested ones indicate that its increased concentration may have been induced by some elicitor following harvest. Solar radiation after harvest, or fluorescent lighting during display, may function as such an elicitor. Light is known to produce ethylene and is an activator of phenylalanine ammonia-lyase, one of the regulatory enzymes responsible for phenylpropanoid biosynthesis in plants (9).

In the following experiments carrot roots were exposed to various sources of ultraviolet light in the laboratory and set aside to allow time for enzyme synthesis. Following this period, changes in myristicin and phytoalexin levels were measured. All of these components of carrot root are measured in one assay. Myristicin and 6-methoxymellein concentrations increased in some samples after irradiation with ultraviolet light; falcarinol and falcarindiol concentration changes did not appear to be related to the ultraviolet light used in this study.

### Experimental

Plant source. Carrot roots were bought in a supermarket or grown in the Northern Regional Research Center field plot. Carrots from the supermarket were used without further cleaning; carrots harvested from the field plot were brushed lightly to remove soil and debris, and used without further cleaning.

Identical samples for UV radiation studies were prepared by halving longitudinally each of two carrots and pairing the different halves to give two samples, or by quartering longitudinally each of

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four carrots and combining a quarter of each carrot with others to give four samples.

Protocol. Following a 1-h irradiation period with ultraviolet light, carrots were placed in a polyethylene bag and kept in the dark at ambient temperature--along with an unirradiated control sample--for an induction period. Changes in carrot constituents were measured at the end of the induction period by analysis of purified dichloromethane extracts using gas-liquid chromatography (GLC) (8). Response factors for myristicin, falcarinol, and falcarindiol were determined by comparing the detector response of pure standards to that of methyl palmitate. The response factor of 6-methoxymellein was assumed to be one. Data from both low-polarity (SE-52) and intermediate-polarity (OV-17) columns were used for peak identification; data from the low-polarity column only were used for quantitation. 6-Methoxymellein was also identified by gas chromatography/mass spectrometry. Irradiated samples were compared to an identical, unirradiated control kept under the same induction conditions.

Light source. The most suitable light system examined was a Chromato-Vue Model C-3 from Ultra Violet Products, Inc., San Gabriel, CA. The light source was a GE G15T8, 15-W, Germicidal, 2537 bulb. The mercury lamp emitted radiation maxima at 254, 265, 280, 302, 313, 365, 405, and 436 nm. Radiation from this source passed through a filter which transmitted radiation only between 230 and 410 nm, with peak transmission at 360 nm. Total ultraviolet energy received by the carrots was approximately  $800 \text{ ergs cm}^{-2} \text{ s}^{-1}$  (Kettering Radiometer, Model 68, Charles F. Kettering Research Laboratory, Yellow Springs, Ohio).

#### Results and Discussion

A four-year study of field-grown commercial carrot roots revealed that recently harvested, unprocessed carrot roots contained 24 ppm falcarinol and 65 ppm falcarindiol (8). 6-Methoxymellein (6-MM) had not been identified by Yates *et al* (8) at that time, and was not measured in that study. Reexamination of data revealed that 6-MM was absent from most samples, but present in a few at concentrations of 2 to 8 ppm. Myristicin, 1 ppm, was detected in only one sample. Wulf *et al* 1978, reported that myristicin was present in supermarket carrots. Other studies have shown that certain brands of supermarket carrots contain myristicin while others do not (Yates, unpub.). The presence of myristicin in some samples from the supermarket and its absence in unprocessed carrots analyzed as soon after harvest as possible suggests that myristicin formation is induced during some stage of processing. Since light is known to be an elicitor of a plant system that results in the synthesis of phenylpropanoid compounds, a study of the effect of light on harvested carrot roots was undertaken.

Preliminary experiments, using a container with reflective surfaces, employed light from eight fluorescent bulbs that produced a preponderance of radiation at 310 nm. Myristicin increased in samples that were irradiated 1 h and then kept for a 24-h induction period. However, heat produced by this system and the resulting dehydration of carrot roots may have affected results. Later experiments, employing a Chromato-Vue box used for ultraviolet examination of TLC plates, avoided these problems. One-hour light

Table I. Changes in Carrot Root Irradiated One Hour with Ultraviolet Light:  
Myristicin and 6-Methoxymellein

Brand or Variety	Myristicin Concentration <sup>3</sup>				6-Methoxymellein Concentration <sup>3</sup>							
	Beginning (mg)	Dark			Beginning (mg)	Dark						
		Control (mg)	24 h (mg)	48 h (mg)		72 h (mg)	120 h (mg)	Control (mg)	24 h (mg)	48 h (mg)	72 h (mg)	120 h (mg)
Buni Luv <sup>1</sup>	NA	.058	.090	.102	.090	NA	NA	.302	.611	.965	.944	NA
Shirley Fresh <sup>1</sup>	NA	ND	ND	ND	NA	NA	NA	.126	.165	.120	.062	NA
Buni Luv <sup>1</sup>	ND	ND	ND	NA	NA	NA	.012	ND	.079	.211	NA	NA
Imperator 58 <sup>2</sup>	ND	ND	.068	NA	.124	NA	.112	.021	.046	NA	.565	NA
Imperator 58 <sup>2</sup>	ND	.028	ND	NA	.062	NA	ND	.017	ND	NA	.202	NA
Imperator 58 <sup>2</sup>	ND	ND	.070	NA	.033	NA	ND	.008	.173	NA	2.186	NA
Imperator 58 <sup>2</sup>	ND	ND	ND	NA	.058	NA	ND	.008	.088	NA	.722	NA
Imperator 58 <sup>2</sup>	ND	ND	NA	NA	ND	ND	.018	.014	NA	NA	.066	.049
Imperator 58 <sup>2</sup>	ND	.035	NA	NA	.031	.193	.016	.014	NA	NA	.155	.065

ND = not detected, NA = not analyzed. <sup>1</sup>Supermarket carrots. <sup>2</sup>Field plot carrots. <sup>3</sup>Per 25 g fresh carrot root.

Table II. Changes in Carrot Root Irradiated One Hour with Ultraviolet Light:  
Falcarinol and Falcarindiol

Brand or Variety	Falcarinol Concentration <sup>3</sup>					Falcarindiol Concentration <sup>3</sup>									
	Beginning (mg)	Dark Induction Period			Beginning (mg)	Dark Control (mg)	Dark Induction Period			Beginning (mg)	Dark Control (mg)	24 h (mg)	48 h (mg)	72 h (mg)	120 h (mg)
		24 h (mg)	48 h (mg)	72 h (mg)			24 h (mg)	48 h (mg)	72 h (mg)						
Buni Luv <sup>1</sup>	NA	.648	.464	.456	.516	NA	NA	NA	1.272	1.246	1.482	1.590	NA	NA	
Shirley Fresh <sup>1</sup>	NA	.236	.219	.219	.199	NA	NA	NA	1.013	.959	.972	1.034	NA	NA	
Buni Luv <sup>1</sup>	1.040	.982	.944	.834	NA	NA	NA	1.078	1.069	1.024	1.278	NA	NA	NA	
Imperator 58 <sup>2</sup>	.241	.202	.606	NA	.229	NA	NA	1.334	1.477	2.381	NA	1.799	NA	NA	
Imperator 58 <sup>2</sup>	.682	.625	.202	NA	.699	NA	NA	1.906	2.563	1.315	NA	3.287	NA	NA	
Imperator 58 <sup>2</sup>	.991	.450	1.280	NA	.564	NA	NA	2.274	1.965	3.719	NA	1.923	NA	NA	
Imperator 58 <sup>2</sup>	.725	.453	.608	NA	.428	NA	NA	2.217	1.526	1.773	NA	1.496	NA	NA	
Imperator 58 <sup>2</sup>	.320	.266	NA	NA	.300	.296	NA	1.244	1.557	NA	NA	1.459	1.372	NA	
Imperator 58 <sup>2</sup>	.688	.565	NA	NA	.610	.823	NA	1.614	2.021	NA	NA	1.663	3.445	NA	

ND = not detected, NA = not analyzed. <sup>1</sup>Supermarket carrots. <sup>2</sup>Field plot carrots. <sup>3</sup>Per 25 g fresh carrot root.

treatments using the Chromato-Vue box were followed by induction periods of 24 to 120 h. The last light-treated sample and nonirradiated control sample were analyzed at the same time.

Myristicin content of some carrot samples was increased two- to five-fold over nonirradiated controls (Table I). The increase in concentration of myristicin is presumed to be via the phenylpropanoid pathway; phenylalanine ammonia-lyase, an enzyme of that system, is activated by light (9). Failure of some samples exposed to UV light to synthesize myristicin may be due to the absence or inhibition of a key enzyme needed for myristicin synthesis.

The dramatic increase in 6-MM content--90 to 270 fold in two samples--was unexpected. 6-MM is a potent antifungal agent (4) and one of the most important carrot phytoalexins. Usually 6-MM, one of the components that contributes to the bitterness of stored carrots (10), is not detected in fresh carrots, but develops during storage. Biosynthetic studies indicate that 6-MM is synthesized via the acetate pathway and its production is stimulated by ethylene (11). Thus, UV light may trigger ethylene production in carrots which in turn leads to 6-MM accumulation.

Falcarinol and falcarindiol concentration changes are small compared to those in myristicin and 6-MM content, and do not appear related to radiation (Table II). These polyacetylenes are present in fresh carrots. Immediately upon wounding, they are translocated to the surface through oil ducts (3), and, therefore, their function as phytoalexins apparently does not depend upon *de novo* synthesis.

Although myristicin is not considered to be an antifungal agent, an increase in myristicin concentration in some carrot roots upon exposure to UV radiation indicates that it may have some protective function.

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## Chapter 28

# Interference Between Crops and Weeds

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The exact nature of weed interference between crops and weeds is still inadequately understood. It has been assumed that direct crop yield reductions from weed presence were the result of competition, of allelopathy, or of these two acting together. Further, many attempts have been made to explain the extent of crop yield reduction in terms of weed thresholds (numbers). Information is presented that suggests that neither numbers of weeds nor their influence through competition and allelopathy adequately explain the effects of weeds on crop yield. A third influence of weeds, what may be termed "direct feedback response to light," is introduced as a possible factor in yield reduction.

Why weeds reduce crop yields cannot be adequately answered. Considerable data have accumulated which relate duration of weed presence and weed density to crop yield. However, such data provide little explanation for why crop yields are reduced. The objectives of this paper are to 1) provide an overview of the time relationship of competition for growth factors and of allelopathy as factors in crop yield reduction and 2) suggest a direct feedback effect on reproduction in response to light as a possible third direct factor in explaining effects of weeds on crop yield.

It is commonly assumed that reductions in crop yield from weeds are the direct result of competition, of allelopathy, or of the two acting together. Competition between crops and weeds is generally for the growth factors available in the space occupied by these plants rather than for space itself, except in special situations such as in root crops. As applied to weed-crop relationships, competition implies the removal of an essential growth factor by neighboring plants. Theoretically, competition could occur for any of the growth factors--light, water, nutrients, oxygen, and carbon dioxide (1). Practically, environmental conditions commonly preclude competition for  $O_2$  and  $CO_2$ . This leaves light, water, and

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nutrients as the most likely sources of competition to explain reduction in crop yield due to weeds.

Based on current knowledge, the explanation for lower crop yield with weed presence is a matter of identifying which of these competed-for growth factors and/or allelopathy accounts for the observed reduction in crop yield at any given time during the growing season. Figure 1 is a generalized response curve which shows the effect of duration of weed presence on crop yield. This generalized curve may be skewed to the left or to the right depending upon the weed, the crop, growing conditions, and possible other factors, but the general relationship remains. Of particular interest is the fact that there is a period at the beginning of the crop's life cycle when yield is not reduced by weed presence (2). What causes the initial down-turn in the yield curve? Is it due to competition for light, water, or nutrients? Is it due to allelopathy? Is the cause(s) of the initial drop the same as the cause(s) for the continued drop in the midportion of the yield curve? Is the cause(s) of yield reduction toward the lower portion (later in the growing season) of the yield curve the same as that earlier in the growing season?

Available data do not allow precise, unequivocal answers. However, a comparison of usage patterns for specified growth factors with the weed presence--crop response curve provides a basis for broad generalizations explaining such crop yield reduction. The generalizations which follow are not intended to imply definitive answers to this extremely complex question so much as they are to provide a general perspective of "most likely explanations" and to identify areas in which additional research will likely be most helpful in our search for answers. Further, the question of why weeds reduce crop yields is approached from a broad, holistic perspective. This suggests that conclusions from specific studies, especially of single factors, may well differ from these generalizations.

#### Competition for Growth Factors

Phosphorus. Let us first consider the nutrients. Figure 2 shows the usage of phosphorus by corn (3) and soybeans (4) superimposed on the crop response--weed presence curve. The usage of phosphorus at any point is the percentage of the total taken up by the crop plants for the entire growing season. With both crops the first eight weeks are a time of relatively minor usage--about 25% of the total. For competition to occur there must be an overlapping of the root depletion zones of neighboring weed and crop plants. Since phosphorus moves almost solely by diffusion in soil, the potential depletion zone will be essentially no larger than the size of the root system (1). In effect this means there likely would need to be a co-mingling of weed and crop roots in sufficient density to deplete the available phosphorus for competition to occur. Although this could occur in the young plants early in the growing season, it is more apt to take place later when the respective root systems are fully elaborated. Furthermore, some of the phosphorus in weeds destroyed early, either mechanically or by herbicides and left on the soil surface, can be expected to be available to the crop that

same year as a result of microbial decomposition of young weeds. Phosphorus likely would not be released as readily from older less succulent weeds. The narrowness of root depletion zones, minor relative early need by the crop, and release of some phosphorus in tissues of young weeds suggest that competition, if it occurs, does so toward the end of the yield curve as shown by the vertical lines in Figure 2. The likelihood of competition occurring is indicated by the closeness of the vertical lines. Phosphorus competition, if it occurs, likely contributes only a small increment of the total crop yield reduction attributable to weeds.

Potassium. Figure 3 shows that only a small part, 7 to 8%, of the soybean crop's usage of potassium occurs in the first four weeks (4). At 8 to 10 weeks, usage still represents only 1/4 to 1/3 of total usage. Usage by corn occurs earlier in the life cycle than in soybeans. More than 50% of the corn plants need for K will have occurred by eight weeks after corn emergence (3). It can thus be theorized that competition for K could occur earlier in the growing season with corn than with soybeans. Even with a crop such as corn, it seems unlikely that competition for K could account for the yield reduction from weeds present only during the early part of the crop's life cycle. There are several reasons for suggesting that competition for K likely does not occur early in the crop's life cycle. As discussed earlier, for competition of a soil-supplied growth factor to occur, there must be an overlapping of depletion zones of neighboring plants. As is true of P, K moves in soil mainly by diffusion, which is a slow process. Thus, the depletion zones of neighboring plants will be narrow. Further, since K remains in solution in the plant and is not locked into organic compounds in the weed tissues, K in weed plants destroyed mechanically or by herbicides and left on the soil surface should be quickly available to crop plants. As was suggested for P, competition for K, if it occurs, is most apt to occur later as depicted by the vertical lines in Figure 3 and accounts for only a small part of the reduction due to weeds.

Water. As shown in Figure 4, usage of water by soybeans increases steadily to a maximum 12 to 14 weeks after emergence in Missouri. Water uptake is shown as a percent of the maximum quantity needed and is adapted from data on soil water depletion (5). Water differs from other soil-supplied growth factors in that it is continually being lost by evaporation. Any water lost by evaporation or plant use at any point in the growing season is permanently lost. Evaporation may result in water movement over considerable distances to plant roots in soil. The depletion zone for water may therefore extend well beyond the plant roots.

The combination of water usage, continual water loss, and potential for a soil depletion zone extending beyond the roots suggests that competition for water might occur earlier in the crop life cycle than for any other soil-supplied growth factor. Competition for water may account for a major part of the crop yield reduction from weeds. Realistically, it seems unlikely that competition for water explains the earliest observed reduction in crop yield. At least in humid areas, the likelihood of a relatively full soil



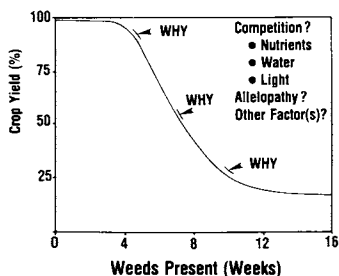


Figure 1. Generalized Crop Yield Response to Duration of Weed Presence.

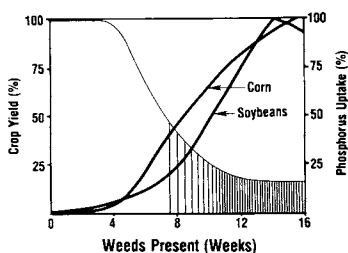


Figure 2. Crop Response to Weed Presence, left, and Relative Phosphorus Uptake, right. Closeness of the vertical lines indicates the relative possibility of competition for P being a factor in crop yield reduction.

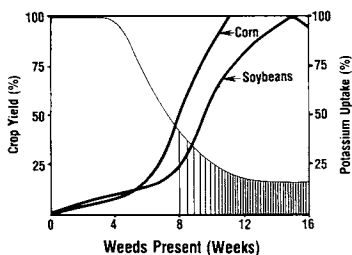


Figure 3. Crop Response to Weed Presence, left, and Relative Potassium Uptake, right. Closeness of the vertical lines indicates the relative possibility of competition for K being a factor in crop yield reduction.

water charge at crop planting and the extension of roots with moisture drawdown in the soil suggest that soil water should be adequate for both weeds and crops until some time later, possibly until about week 6 on the generalized crop yield--weed presence response curve.

Nitrogen. Total N used by a corn crop is relatively small during the first six to seven weeks (3) but increases rapidly thereafter as the corn plant approaches tasseling (Figure 5). Applied N, and that present in soil, may be in many different forms. Some forms are readily soluble and others are not. Sooner or later the organic and insoluble forms undergo nitrification to soluble inorganic forms in soil. Water-soluble N, unlike P and K, is freely mobile in soil. Thus, the depletion zone of N can extend well beyond the plant's roots in soil.

Therefore, as suggested by the vertical lines beginning at week 6, competition for N may occur earlier than for P and K but somewhat later than for water. Thus, competition for N may account for a significant part of the total crop yield reduction from weeds but likely accounts for little of the earliest observed crop yield reduction in nonleguminous crops. For the purpose of this discussion, it is assumed competition for nitrogen does not occur with leguminous crops.

Light. Light is the other growth factor for which weeds and crops may compete. With respect to competition, light is very different from the other growth factors because it is nontransferable in the plant whereas the other growth factors are relatively mobile. Therefore, anytime a single leaf or an entire plant is shaded, it in effect suffers competition for light. Such competition during the first few weeks of the crop's life cycle may not necessarily result in reduced crop yield because most crop plants will compensate for some early loss in total photosynthesizing area. Although as suggested in Figure 6, competition for light could occur throughout the time weed presence reduces crop yield, it seems unlikely that competition for light could by itself account for the earliest observed reduction in crop yield. However, competition for light may account for much of the remaining reduction in crop yield due to weeds.

#### Allelopathy

Allelopathy is the remaining direct factor currently used to explain crop yield reductions. For the purpose of placing allelopathy in context with competition in understanding the cause(s) of crop yield reduction from weed presence, only allelopathy associated with weeds present in the crop will be considered. For such weeds, at least in humid temperate regions, allelochemicals contained in weeds can be assumed to enter the crop's environment by exudation, leaching, decomposition or some combination of entry modes.

Evidence to date is conflicting relative to when allelopathy may be a factor in crop response to weed presence. In closed-system controlled studies, some results show seedling weeds to be allelopathic towards seedling crop plants (6) and some results show them not to be (7).

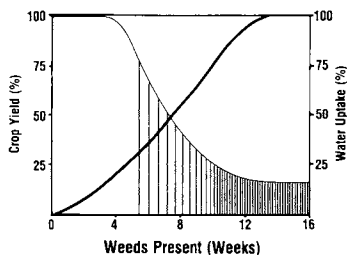


Figure 4. Crop Response to Weed Presence, left, and Relative Water Uptake by Soybeans, right. Closeness of the vertical lines indicates the relative possibility of competition for water being a factor in crop yield reduction.

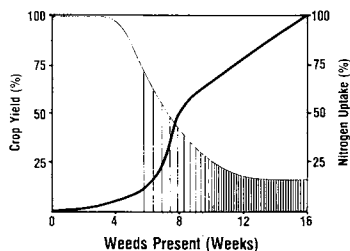


Figure 5. Crop Response to Weed Presence, left, and Relative Nitrogen Uptake by Corn, right. Closeness of the vertical lines indicates the relative possibility of competition for N being a factor in crop yield reduction.

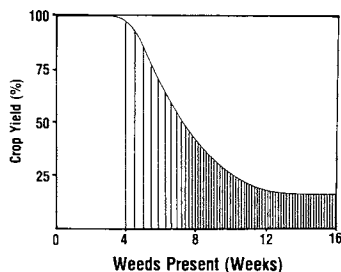


Figure 6. Crop Response to Weed Presence. Closeness of the vertical lines indicates the relative possibility of competition for light being a factor in crop yield reduction.

It has also been suggested by some researchers (8) that allelochemicals produced by velvetleaf which emerged with soybeans reduced soybean branching. Since branching is initiated relatively early (stage  $V_4 - V_5$  or 3 to 4 weeks after emergence), this provides circumstantial evidence for release of the allelochemicals by young velvetleaf plants. However, since the branches were counted at mid-season (10 to 12 weeks after emergence) and velvetleaf was present to soybean maturity, the possibility of reduced branching in response to interference for light cannot be ruled out.

On the basis of results to date, it is concluded, as shown in Figure 7, that allelopathy could be a source of crop yield reduction throughout the time crop yields are reduced by weed presence, but it is most apt to be a factor from the midpoint of the curve on. Thus, the contribution of allelopathy to crop yield reduction likely is less than that of competition for light, water, and nitrogen. The reduction in crop yield could be the result of inhibition in growth, inhibition in reproduction, or some combination of the two.

#### Direct Feedback Response to Light

The remainder of this discussion examines the possibility of a direct feedback mechanism in response to light as an explanation for crop yield reduction from early weed presence. Three types of data will be examined: 1) results of our research on velvetleaf interference with light in soybeans; 2) a comparison of observed and estimated soybean yield reductions for weed presence versus leaf removal; and 3) the poor correlation between weed control and crop yields.

Velvetleaf interference in soybeans. In 1984, a field study was conducted on the Agronomy farm near Columbia, Missouri to examine the effect of velvetleaf height and duration on soybean yields. Other weeds were kept out for the entire test period. Williams 82 soybeans were drilled in 10-inch rows May 24 and overseeded with velvetleaf the same day. The area was fertilized to excess prior to planting and irrigated regularly throughout the growing season to eliminate these as factors in soybean yield. Velvetleaf clipping to 75, 100, or 125% of the soybean height was begun the week of soybean emergence and continued for the prescribed 3 and 6 weeks. Since velvetleaf in the 125% height treatment did not attain this height by 3 weeks, only the 6-weeks data will be presented.

As can be seen in Figure 8, keeping velvetleaf 25% below the top of the soybean canopy for the first six weeks resulted in yields only slightly (7%) below yields of soybeans kept free of velvetleaf for the entire season. Maintaining velvetleaf at the same height as soybeans for six weeks resulted in a 16% reduction and overtopping velvetleaf a 64% reduction in soybean yields compared to weed-free status. Since it is assumed nutrient and water needs were fully met, this leaves allelopathy and light as possible factors to explain yield results. A comparison of soybean yields and velvetleaf weights at 6 weeks for 100% and 125% heights supports the conclusion that a direct effect of shading the top portion of the soybean plant, and not allelopathy, is responsible for the observed yield reduction. The dry weight of velvetleaf at 6 weeks was nearly

identical for the 100% and 125% heights (99.0 and 100.00 g/m<sup>2</sup>, respectively). Yet, as we saw in Figure 8, soybean yields where velvetleaf overtopped (125% height) were less than half those where weeds were at the same height as soybeans (100% height). Weight of velvetleaf at 6 weeks under the 100% height was not less than weight under the 125% height apparently because of increased lateral branching and growth in response to removal of some terminals on the main stem. There is evidence that an allelopathic effect is directly related to the biomass of the allelopathic species (9, 10, 11, 12) and a light effect to the relative heights of the involved species (1). Since the velvetleaf biomass was the same for the 100% and 125% heights, this suggests that a difference in light interference between the two velvetleaf heights was responsible for the lower yield under the 125% height. In addition to lower total yield, branches per soybean plant and number of pods per plant were less under the 125% height. As previously mentioned, other research (8) has shown allelopathy to be involved in reductions in soybean yields by velvetleaf. Since our research suggests that reductions in soybean yields from early velvetleaf presence are likely due to a direct effect of light interference, it is theorized that the observed allelopathic effect reported by Dekker and Meggitt (8) occurs later and is likely the result of overall inhibition of growth, not a direct effect on reproduction. Overall inhibition of growth could be expected to increase coincidentally with an increase in velvetleaf biomass associated with continued presence of velvetleaf (refer to the closeness of vertical lines in Figure 7 for a diagrammatic representation).

Weed presence versus leaf removal. A comparison of observed and expected reductions in soybean yields for periods of weed presence and leaf removal provides additional circumstantial evidence for a direct effect of light interference. Table I shows several such comparisons. The observed yield is that reported for a given weed present in soybeans for the indicated period following soybean emergence. The estimated yield is from the National Crop Insurance Association charts showing reduction for given degrees of plant damage at different stages of soybean plant development. The soybean stage of development is based on the average number of days required for development (13); an adjustment was made for determinant growth-type varieties. In every case, the observed yield reduction exceeded that estimated for 50% leaf removal. For venice mallow (*Hibiscus trionum*) and velvetleaf, observed reductions exceeded that estimated for 100% leaf removal for the same period.

Fifty percent leaf shading is suggested as an arbitrary degree of leaf shading one might expect with a dense stand of the weeds in Table I by 4 to 6 weeks after emergence. If anything, this likely is on the high side. For example, Oliver (14) found that a velvetleaf stand of 32,000 plants per hectare had less leaf area than soybeans 8 weeks after emergence for either early- or late-planted soybeans. Theoretically, the crop can suffer competition for light any time weeds shade its leaves. However, if the effect is that of simple competition for light, e.g., a reduction in photosynthesis, one might expect the observed yield to be somewhat comparable to that for 50% leaf removal. The observed reduction is consistently

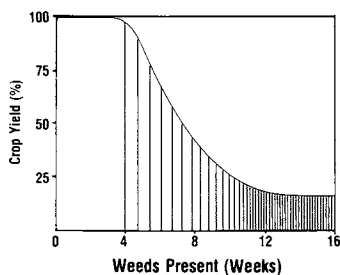


Figure 7. Crop Response to Weed Presence. Closeness of the vertical lines indicates the relative possibility of allelopathy being a factor in crop yield reduction.

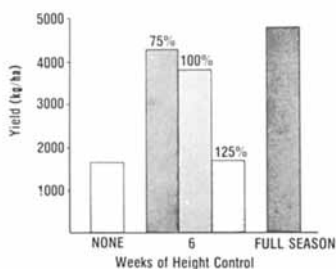


Figure 8. Effects on Soybean Yields of Three Velvetleaf Heights Maintained for the First Six Weeks after Soybean Emergence Compared to No Height Control (left bar) and Weed Removal for the Entire Season (right bar). Velvetleaf height (75%, 100% and 125%) is relative to soybean height.

Table I. Comparison of Observed Soybean Yield Loss from Weed Presence with Estimated Loss for Leaf Removal for the Same Period

Weed	Weeks Present	Soybean Stage	Soybean Yield Reduction		
			Observed	Estimated <sup>1/</sup>	
				50% leaf removal	100% leaf removal
			(%)		
Venice mallow	5	V <sub>7</sub> -V <sub>8</sub>	22 <sup>2/</sup>	6	17
Venice mallow	6	V <sub>8</sub> -V <sub>9</sub>	39 <sup>2/</sup>	6-7	17-20
Sicklepod	4	V <sub>6</sub>	14 <sup>3/</sup>	5	15
Tall morningglory	4	V <sub>6</sub>	6 <sup>4/</sup>	5	15
Tall morningglory	6	V <sub>8</sub> -V <sub>9</sub>	15 <sup>4/</sup>	6-7	17-20
Velvetleaf	4	V <sub>4</sub> -V <sub>5</sub>	34 <sup>5/</sup>	4-5	21-22

<sup>1/</sup>From National Crop Insurance Association "Soybean Loss Instructions," NCIA Publication 6302, Revised in 1979.  
<sup>2/</sup>From Eaton, et al. (15)  
<sup>3/</sup>From Thurlow and Buchanan (16)  
<sup>4/</sup>From Oliver, et al. (17)  
<sup>5/</sup>From Aldrich unreported data.

greater; by as much as 7 times in the case of velvetleaf. If the shaded 50% of the leaves serve as a sink for photosynthate produced by the remaining 50%, it could be theorized that the shaded leaves are acquiring photosynthate needed for branch and pod production and in this way are reducing yield. However, it has been shown that a mature soybean trifoliolate is an inconsequential sink for photosynthate produced in other trifoliolates (18). Thus, it is suggested that the reduction in soybean yields from early weed presence is not due to either allelopathy or simple competition for light. Rather, it may be due to a direct effect manifested through fewer branches and pods per soybean plant. Studies are underway to determine the effect on branching of shading specific trifoliolate leaves and combinations of trifoliolate leaves on the soybean plant.

Weed control and crop yield. One other type of circumstantial evidence emphasizes the importance of light in weed interference and suggests that the effect of shading may not be simple competition for light. If the effect of weeds was due to simple competition, one would expect a rather close relationship between extent of weed control and crop yield. An analysis of crop yields for post-emergence herbicide studies reported in the 1983 and 1984 North Central Weed Control Conference--Research Report shows that there are no differences in crop yield within the range of 70 to 90% or better control (Table II). Yield data from all reported experiments, which met certain constraints, were included regardless of crop. To eliminate as much confounding as possible, the following constraints were used in deciding what data to include: (a) only post-emergence treatments were included; (b) only data involving increasing rates of application of a single herbicide were used;

(c) only treatments made at the same stage of crop growth were used; and (d) treatments involving any additional weed control were excluded. Crop yields for reported weed control of 90% or better were compared with yields for reported control of 80% to 89%, 70% to 79%, and 50 to 69%. This provided 69, 51, and 25 yield comparisons for 90% or better versus 80 to 89% control, 70 to 79% control, and 50 to 69% control, respectively. The average yield for 90% or better control for the two years compared to the average yield for 80 to 89% control was 100.5; in 1983 it was 99.6 and in 1984 it was 101.2. Although no statistical treatment was attempted, the 0.5% difference certainly is inconsequential. The comparison of 90% or better control with 70 to 79% control was 100.3; it was 100.8 in 1983 and 99.1 in 1984. Here, too, the difference of 0.3% in yield in favor of 90% or better control is inconsequential. The comparison of 90% or better control with 50 to 69% control was 118.0 indicating an advantage for 90% or better control.

Table II. Relationship Between Percent Weed Control and Crop Yield, North Central Region, 1983 and 1984

Weed Control Range	Crop Yields for 90% or Better Weed Control Compared to 3 Lower Control Ranges <sup>a</sup>		
	1983	1984	Average (%)
80 to 89%	99.6	101.2	100.5 <sup>1/</sup>
70 to 79%	100.8	99.1	100.3 <sup>2/</sup>
50 to 69%	115.8	121.1	118.0 <sup>3/</sup>

<sup>a</sup>The average obtained after dividing the yield for 90% or better weed control by the yield in the given lower weed control range.

<sup>1/</sup>69 yield comparisons.

<sup>2/</sup>51 yield comparisons.

<sup>3/</sup>25 yield comparisons.

Why are crop yields not more closely related to weed control within the 70% or better range? It is suggested that treatments effective enough to provide 70% or better control sufficiently suppress height of the weeds not controlled to prevent their interfering with crop yield. There are some reports in the literature which support this suggestion. For example, it has been shown that broad-leaf weed escapes from herbicides in sugar beets are smaller than those in untreated plots and less damaging to sugar beet yields than untreated weeds (19). It has also been reported that giant foxtail suppressed early in the growing season by sublethal rates of herbicides applied post-emergence did not reduce soybean yields even though the giant foxtail dry weight exceeded that on the weedy check late in the growing season (20). For most of the yield comparisons in Table II, the herbicides were applied to relatively small weeds early in the crop's life cycle. The implication is that preventing



early shading of crop plants by weeds avoids any direct effect on reproduction.

### Summary

There is much circumstantial evidence that reduction in crop yield from weeds may not be adequately explained by either competition, as currently defined, or allelopathy, especially the reduction from weeds present only during the early part of the crop growing season. Specifically, the removal of light by weeds, in keeping with the current definition of competition, may not fully describe the effects of light interference. A direct effect of shading on reproduction is suggested as a possible third explanation in soybeans. Additional research is needed to prove or disprove this suggested explanation. Additional research is also needed to ascertain the specific reason for yield reductions due to allelopathy, e.g., is it due to direct inhibition of reproduction or indirectly due to overall inhibition of growth of the crop plant.

An alternative way of viewing the light effect on soybean yield is to consider competition as being of two types, what may be termed passive and active competition. Passive competition would apply to reduced crop growth, and thus yield, as a result of light capture (removal) by associated weeds. The effects on crop growth, and thereby yield, of such competition would increase steadily with continued shading by weeds. Active competition would apply to a direct and immediate inhibition of reproduction in response to altered light quality and/or quantity. The effects of active competition on crop yield would be as a consequence of immediately losing the potential yield from the new branch or pods prevented from forming.

The suggested overall chronology explaining crop yield reduction from weed presence in soybeans thus is summarized as follows:

Earliest yield reduction from weed presence	= direct effect of shading on axillary bud differentiation and development.
Yield reduction in the mid-portion of the yield drop curve	= direct shading effect, + competition (for light and water in that order) and + allelopathy.
Yield reduction in the last part of the yield-drop curve	= direct shading effect, + competition (for light, water, phosphorus, and potassium in that order) and + allelopathy.

This chronology of explanations helps understand why the curve relating crop yield to weed presence drops as steeply as it does. As indicated in the explanation, there is a compounding of effects. Earliest reduction in crop yield potential may be due to only one factor, e.g. direct feedback. The effect of this factor is visualized as persisting for the remainder of the crop growth cycle

although there may conceivably be some compensation depending upon the plant process(es) affected and when the weeds are removed. The second factor in yield adds to the effect of the first factor as does the third factor, and so on for all factors involved. The reverse would be true for the direct effect of shading; if this effect of light interference is avoided during this early period, it will not be a factor in crop yield and the potential yield will be proportionately higher. It remains for future research to corroborate, modify, or discard this proposed chronology of explanations.

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## Chapter 29

# Micro Liquid Chromatography-Mass Spectrometry Combination

## Application to Allelochemical Compounds

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Many nonvolatile and thermally labile allelochemicals can be well separated by liquid chromatography (LC). Identification of the separated components on-line by mass spectrometry (MS) is of great value. Fused-silica LC columns of 0.22 mm ID packed with small-particle material are used in the described LC/MS system. The shape of the column end allows direct connection to a electron impact ion source of a magnetic sector mass spectrometer. Separations by LC are reported and LC/MS mass spectra are shown for monoterpenes, diterpene acids, phenolic acids and cardiac glycosides. The LC/MS system provides identification capability and high-efficiency chromatography with a universal detector.

Allelochemicals found in extracts of such botanical materials as plant leaves can often be well separated by liquid chromatography (LC). Identification of the separated components on-line by mass spectrometry (MS) is of great value because LC has the ability to deliver samples into the ion source of the spectrometer with low or no thermal decomposition.

A mass spectrometer adapted for chemical ionization can tolerate a solvent flow rate into the vacuum system of 5-10  $\mu$ L/min. This suggested the use of a LC column operating at this low flow rate. A micropacked fused-silica column (1-3) is well suited for this application.

In this paper we describe how this type of column can be very simply interfaced to a mass spectrometer with an electron impact ionization source. We also report experimental results obtained with compounds of allelochemical interest.

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### LC/MS System

Fused-silica tubing (20-50 cm x 0.22 mm ID) is filled with 3-or 5-  $\mu\text{m}$  high-performance liquid chromatography (HPLC) packing material by using a high pressure slurry packing technique (3). In order to effect a simple and efficient coupling of the column to the ion source of the mass spectrometer, the column (for reasons described below) must end with a fine tip. Figure 1 and 2 shows two alternative designs of the columns.

Figure 1: The column tube end is drawn to a fine tip. To prevent fouling the ion source with packing material a small amount of coarser HPLC material is placed inside the tip. By coating with high-temperature epoxy glue (EPO-TEK 353ND) the tip is mechanically protected. This design gives the best chromatographic results. A 30-cm packed column with 3  $\mu\text{m}$  Spherisorb ODS particles gives almost  $5 \times 10^4$  theoretical plates (3). The separation impedance (unretained solute time per plate multiplied by the pressure drop per plate and divided by the viscosity of the eluent) has been calculated as 750.

Figure 2: In the end of the column a 50-  $\mu\text{m}$  ID fused-silica tube is cemented with high-temperature epoxy glue. A glass fibre filter frit in front of the tube hold the chromatographic bed. After the packing, the end tube is drawn out to a fine tip and protected with epoxy glue. Higher mechanical strength of the tip and not so high packing pressure is needed with this design.

A standard HPLC pump (Spectra Physics 8700) is used in constant-pressure mode and pulse-free flow rates from 1 to 5  $\mu\text{L}/\text{min}$  are obtained without any modifications of the pump.

A 0.5- $\mu\text{L}$  syringe-loaded micro injector (Rheodyne 7520) is used for sample injection. To minimize the dead volume ( $< 20 \text{ nL}$ ) the fused silica column is connected directly to the injector block.

The column is led into the ion source through a ball valve and a 0.5-mm ID stainless-steel tube (Figure 3). The tube terminates about 15 mm from one of the four sample ports in the ion source block. The high vacuum seal consists of the vespel (trademark of DuPont) ferule mounted on the ball valve. During operation the tip of the column can be easily adjusted for optimal signal-to-noise ratio. Exchange of LC columns or change-over to the gas chromatographic system can be done in a few minutes.

Ions are produced in a standard electron impact ion source (VG 70-70). The only modification necessary to obtain true electron impact spectra was to widen the LC inlet hole and uncover the other three inlet ports.

Vaporization is governed by four factors: the electrostatic field between the column tip and the ion source block, the configuration of the column tip, the pressure in the ion source housing, and the ambient temperature. The electrostatic field between the tip and

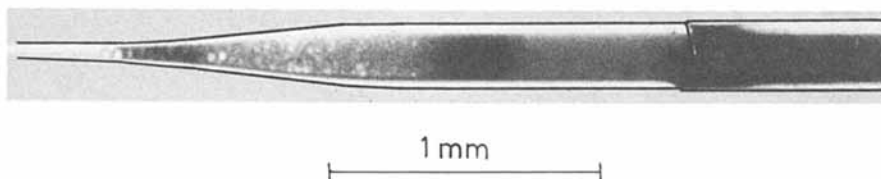


Figure 1. Column tip, first version. Column I.D. 0.22 mm; outlet I.D. 40  $\mu$ m; 1 mm of the column constriction is filled with coarse packing material (30-50  $\mu$ m).

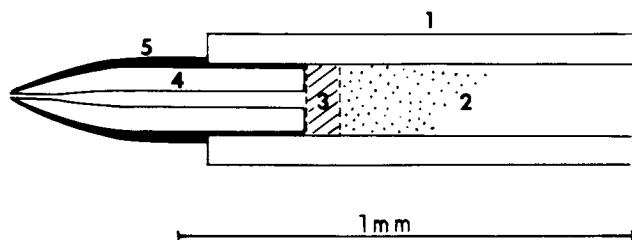


Figure 2. Schematic diagram of column tip, second version. 1, Fused-silica column I.D. 0.22 mm; 2, chromatographic bed; 3, glass fiber filter frit; 4, drawn-out 50  $\mu$ m fused-silica tube; 5, high temperature epoxy coating

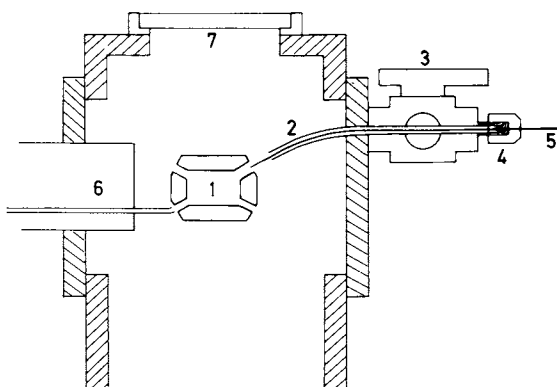


Figure 3. Schematic diagram of the LC/MS connection. 1, Ion source; 2, stainless-steel tube; 3, ball valve; 4, high vacuum seal; 5, LC column; 6, GC inlet; 7, window. Modified from ref. 3.

the ion source is the major factor for vaporization. Owing to this the eluate from the column leaves the tip in the form of very small invisible droplets. To prevent electrical spark the end part (at least 10 mm) of the column must be electrically isolated from ground. A fine column tip can be used with lower electric field than a coarse tip. Normal tip position is shown in Figure 3 and the electrostatic field obtained with the acceleration voltage (3-5 kV) is sufficient.

The mass spectrometer is a large-radius magnetic-sector instrument built in our department (3) for both GC/MS and LC/MS work. The capacity of the high vacuum system is similar to that of ordinary mass spectrometers adapted to chemical ionization.

## Results

Monoterpenes are often analyzed by capillary gas chromatography. LC/MS offers a new method for the separation and identification of members of this class of compounds (Figure 4). The separation occurs in a cold liquid phase, which eliminates the risk of thermal decomposition. Spectra obtained from the LC/MS system (Figure 5) can be interpreted by comparison with GC/MS reference spectra.

Phenolic acids are often found in plant tissue, and have been implicated in many cases of allelopathy (4). Figure 6 shows a separation of three free phenolic acids and Figure 7 shows mass spectra obtained from these compounds. These spectra give both molecular weight and structural information. Phenolic acids can easily be thermally decarboxylated. The height of the molecular ion peak varies owing to ion source temperature. The variation depends also to some extent on the composition of the LC eluent, and this will be further examined.

Chlorogenic acid, the most widespread depside in the plant kingdom, has often been associated with resistance of plants to fungal attack (5). Most phenolics occur in plant in water-soluble form as glycosides but hydroxycinnamic acids differ from most other phenols in occurring most frequently as quinic acid esters (6). The mass spectrum of chlorogenic acid is shown in Figure 8. The peak at  $m/z$  354 represents the molecular ion and only small peaks (e.g. M-18 at  $m/z$  336) are present in the high-mass region. The prominent peaks at  $m/z$  180 and 163 are related to the aromatic (caffeic acid) part of the structure and only small fragment peaks from the quinic acid part are present.

Resin acids. To obtain good mass spectra of resin acids it has previously been necessary to esterify the acids. Figure 9 shows a chromatogram of a crude resin sample from Pinus montana. The small peaks at the beginning of the chromatogram represents monoterpenes and the main

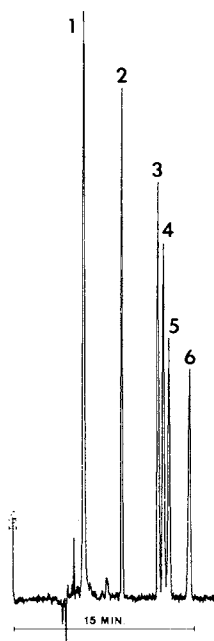


Figure 4. LC separation of a terpene mixture. 1, Thymol; 2,  $\rho$ -cymene; 3,  $\gamma$ -terpinene; 4,  $\alpha$ -terpinene; 5,  $\beta$ -pinene; 6,  $\alpha$ -pinene. Column: 30 cm x 0.22 mm I.D. 3- $\mu$ m Spherisorb ODS. Mobile phase: methanol-water (80:20) Detection: TIC (ions of  $m/z$  <40 suppressed).

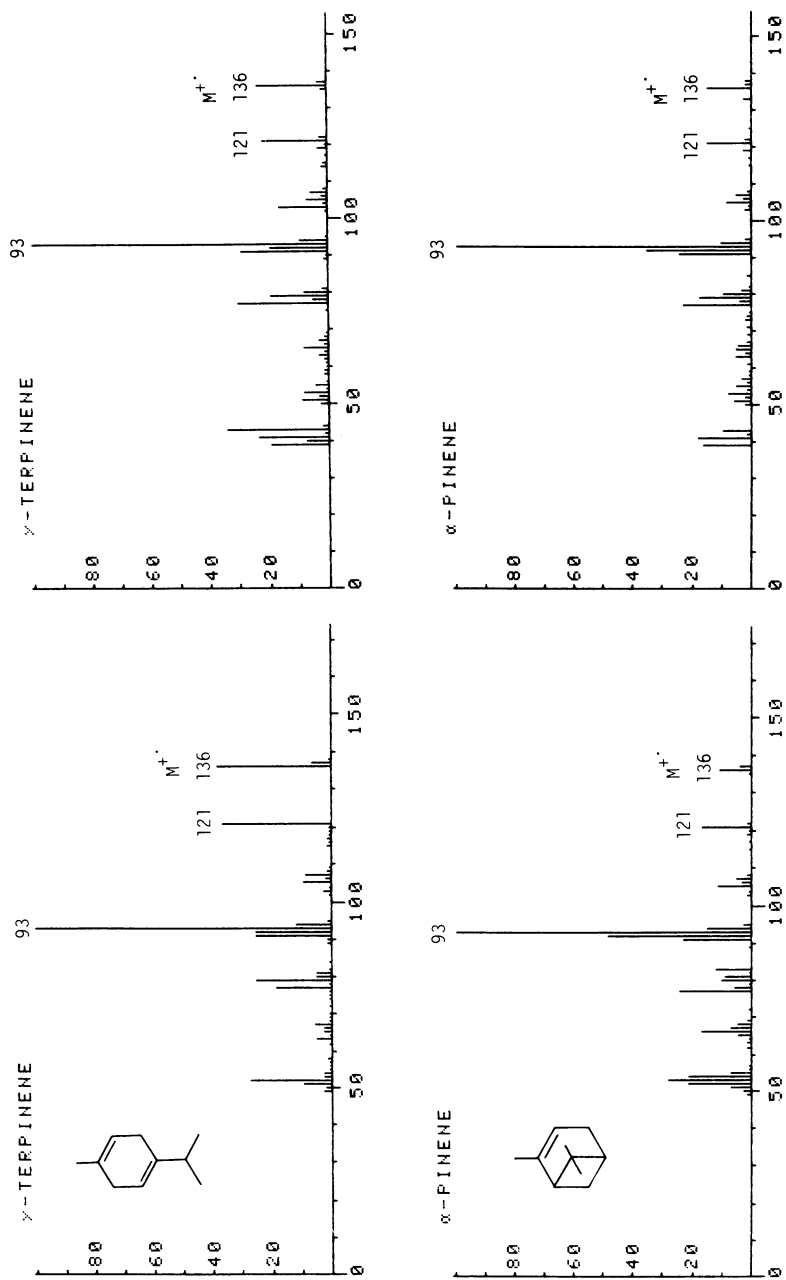


Figure 5. Comparison of mass spectra obtained by LC/MS and GC/MS.



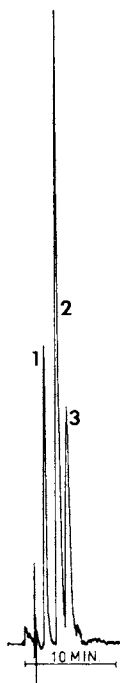


Figure 6. Separation of free phenolic acids. 1, Caffeic acid; 2, *p*-coumaric acid; 3, sinapic acid. Column 18 cm x 0.22 mm I.D. 3- $\mu$ m Spherisorb ODS. Mobile phase: methanol-water-acetic acid (20:75:5). Detection: TIC (ions of  $m/z$  <60 suppressed). Ion source temperature: 210 C.

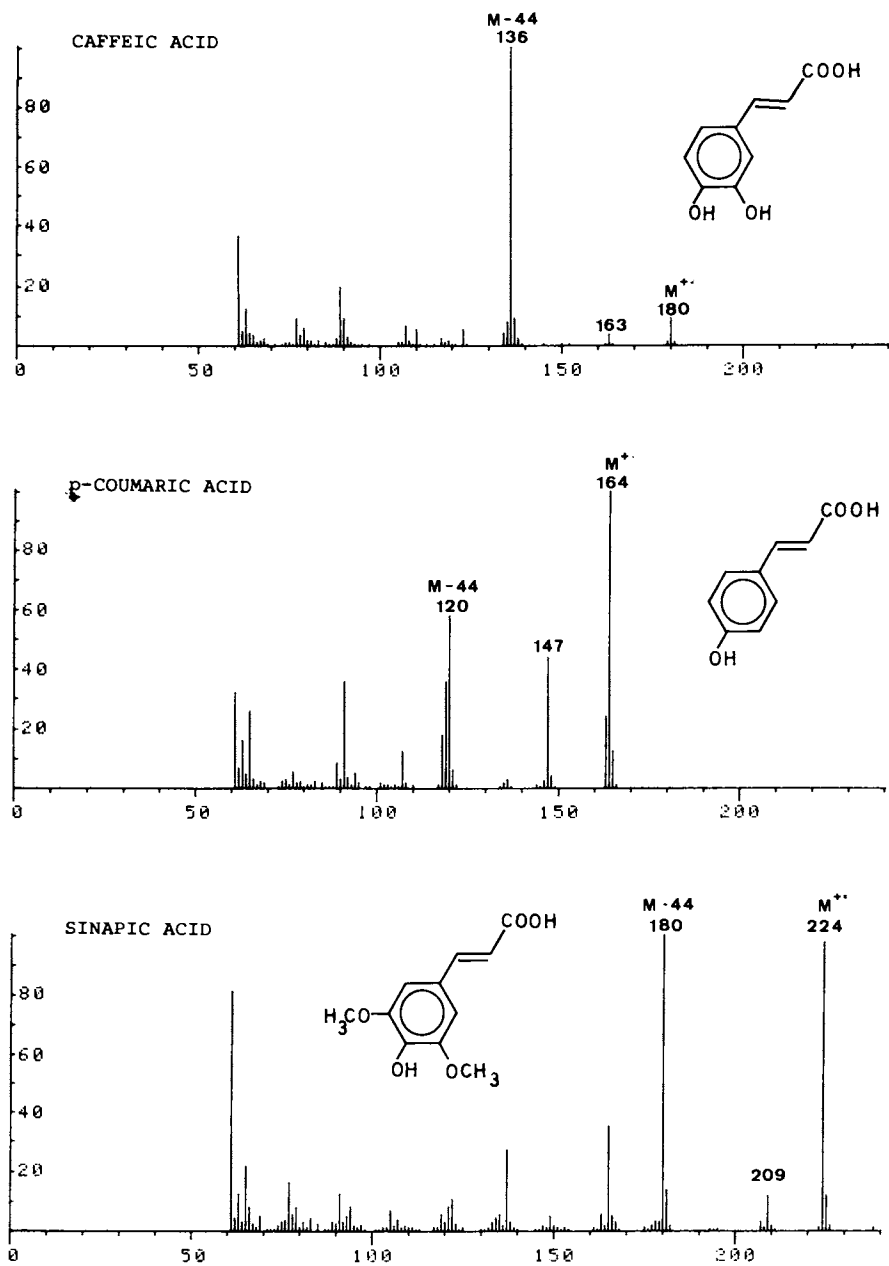


Figure 7. LC/mass spectra of phenolic acids. Conditions as in Figure 6.

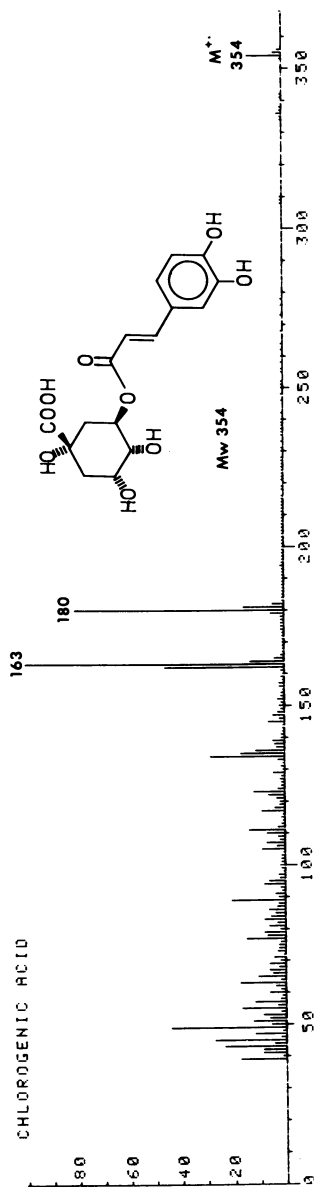


Figure 8. LC mass spectrum of chlorogenic acid. Ion source temperature: 200°C. LC mobile phase: methanol-water (80:20).

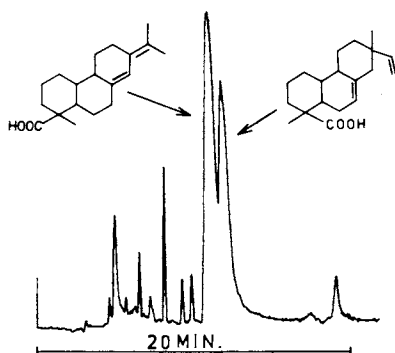


Figure 9. Liquid chromatogram of crude pine (Pinus montana) resin with tentative structures of the main components. Column: 25 cm x 0.22 mm I.D. 3- $\mu$ m Spherisorb ODS. Mobile phase: methanol-water (90:10) adjusted to pH 4 with formic acid. Detection: TIC (ions of  $m/z$  <40 suppressed). Ion source temperature: 230°C.

peaks, resin acids. The mass spectrum of one of these acids is shown (Figure 10) in comparison with the reference spectrum of the corresponding methyl ester (7). The identification is tentative but the great similarity in the fragmentation pattern is a strong indication of its correctness.

Cardiac glycosides have high biological activity not only medically but also as defense substances in both plants and insects (8,9). A separation of two cardiac glycosides is shown in Figure 11. To reduce the peak tailing the ion source temperature and the solvent flow rate were increased.

Mass spectra of cymarín and ouabain are shown in Figure 12. Peaks at  $m/z$  549 and 585 represent the protonated molecular ions and the other peaks in the high-mass region are due to successive elimination of water. The peaks in the middle region are related to the genin part, and some of those in the low region to the sugar part of the substances.

### Discussion

The mass spectrometer when used as a detector for liquid chromatography is a universal detector and in most instances has no influence on the chromatographic separation. LC/MS spectra obtained from both volatile and low-volatile compounds are similar to ordinary EI spectra and may be interpreted by comparison with normal reference spectra collections. Interpretable mass spectra can also be obtained from nonvolatile compounds.

This LC/MS system can be connected with only few modifications to most magnetic-sector mass spectrometers.

The system can tolerate a high amount of water in the eluate and also organic acids or ammonia for pH adjustment. Buffered solvent systems will increase the noise level and can be used only in low amounts. Gradient elution is important for complex samples and will be tested in the near future.

The sensitivity for volatiles is in the low nanogram range. To obtain good mass spectra of the most complex and labile compounds tested so far it has been necessary to inject up to a few micrograms. We believe that the sensitivity can be improved.

The LC column can operate for several weeks without any loss of separation efficiency. The flow rate of solvent into the mass spectrometer is 2-3  $\mu\text{L}/\text{min}$  under normal conditions. The pressure in the ion source housing is  $10^{-3}$  Pa and  $10^{-5}$  Pa in the analyzer. No deterioration of the vacuum system during two years of operation has been observed.

We suggest that this LC/MS system can be very useful in allelochemical research.

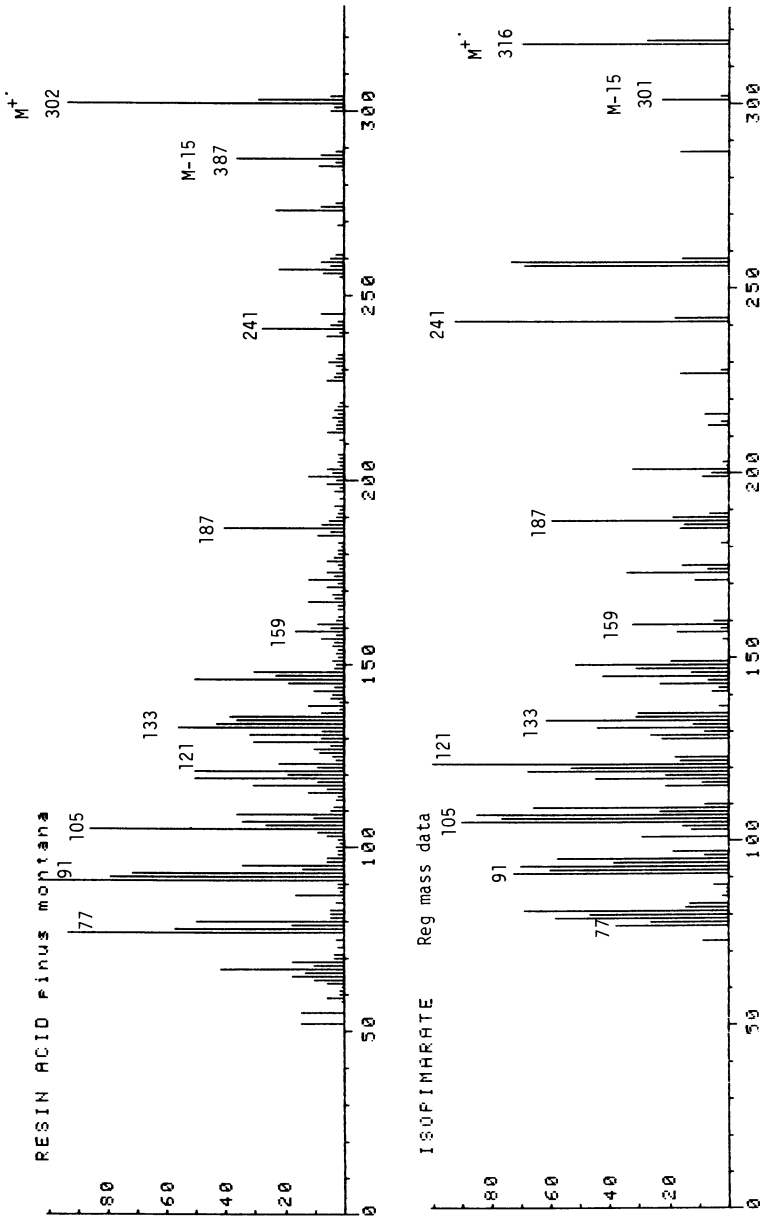


Figure 10. Mass spectrum of one of the main components of *Pinus montana* resin in comparison with reference spectrum of isopimarate. Conditions as in Figure 9. (Reproduced with permission from ref. 3. Copyright 1985, Elsevier.)

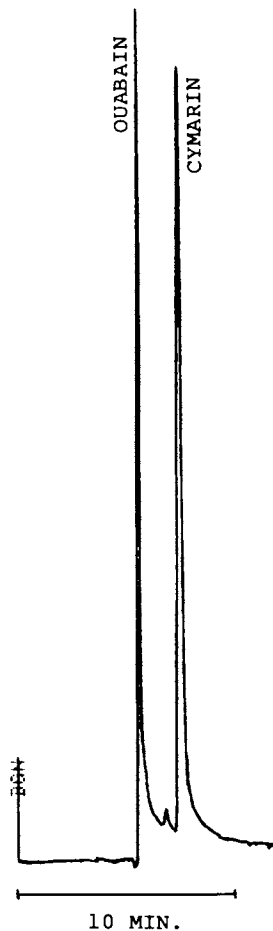


Figure 11. Separation of cardiac glycosides. Column: 30 cm x 0.22 mm I.D. 3- $\mu$ m Spherisorb ODS. Mobile phase: methanol-water (80:20) Detection: TIC (ions of  $m/z$  <40 suppressed). Ion source temperature: 280 C.

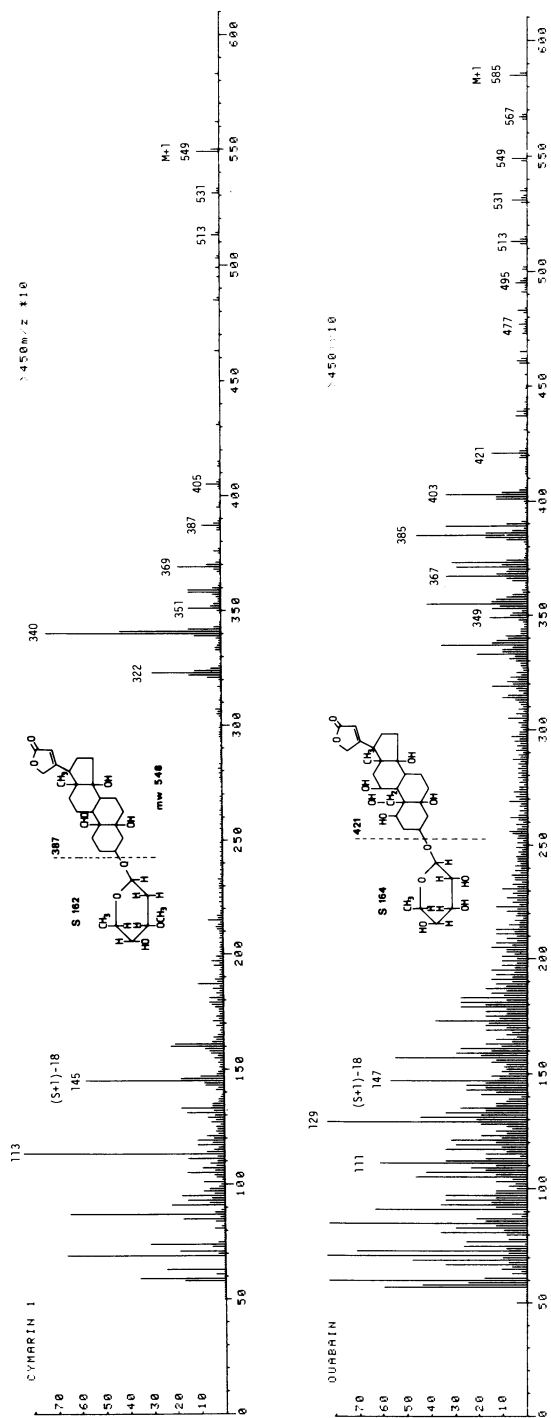


Figure 12. LC mass spectra of cymarin and ouabain. Conditions as in Figure 11. (Reproduced with permission from ref. 3. Copyright 1985, Elsevier.)



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## Chapter 30

# Isolation and Identification of Plant Growth Inhibitors from Leaves of the American Cranberry (*Vaccinium macrocarpon*)

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An extract of leaves of the American cranberry plant, *Vaccinium macrocarpon*, was examined for growth inhibitors. No free parasorbic acid, a known growth inhibitor from cranberry leaves, could be isolated in the absence of a preliminary acid or base treatment. The parasorbic acid was isolated from the extract in the form of its glucoside. It was shown that this glucoside could only account for a portion of the growth inhibition of the leaf extract.

Cranberry plants produce growth inhibitors requiring cranberry growers to remove fallen leaves and berries from their fields. If this dead material is allowed to remain it will cause reduced yields and growth. If it is allowed to accumulate over a period of years, it will result in dead areas in the bog.

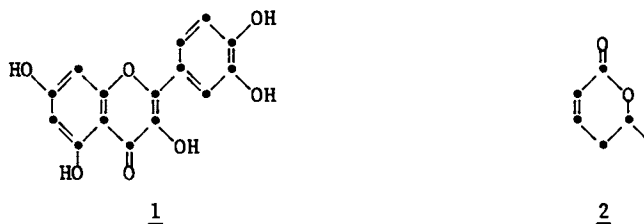
Devlin (1) reported that an aqueous extract of cranberry leaves inhibited the germination and growth of wheat. We became interested in identifying this growth inhibitor and thus examined the cranberry leaf extract. Vapor phase chromatographic analysis of the extract, prepared according to the procedure of Devlin, did not indicate the presence of abscisic acid, a growth inhibitor known to have this type of activity. Quercetin, 1, which has been reported to be a plant growth inhibitor (2) and has been found in cranberries (3), precipitated from one of our extracts. It was identified by comparison of the NMR, IR, and chemical ionization mass spectroscopic data with those of an authentic sample. The point of attachment and identity of the sugar residue was not determined. Although quercetin has been reported to be a plant growth inhibitor, we were unable to find any effect of this water insoluble glycoside or its aglycone on wheat seed germination and growth.

### Parasorbic Acid

While seeking to isolate the growth inhibitor from the cranberry leaf extract, Cardellina and Meinwald (4) reported the isolation of

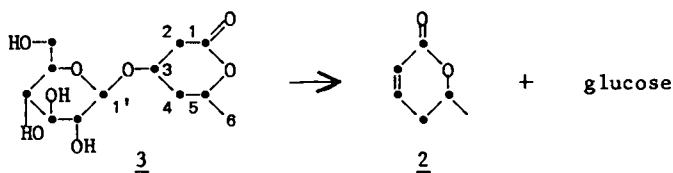
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parasorbic acid (actually a lactone), 2, from the cranberry plant. Since parasorbic acid is known to inhibit seed germination (5, 6), we sought to determine what role this acid (lactone) might play in the inhibition.



We prepared a sample of parasorbic acid by the method of Stafford (7). This allowed us to develop an analytical glc technique to determine the acid or its precursor in the leaf extract.

Since parasorbic acid was previously isolated by steam distillation of the juice of mountain ash berries (8), we steam distilled a sample of the cranberry leaf extract, but obtained little 2. The literature reports that before the ash berry juice was distilled, it was treated with calcium hydroxide to precipitate malic acid. Tschesche later showed that such treatment followed by acidification converted the glucoside of parasorbic acid, 3, into the free acid (lactone), 2, (9). This base treatment effects a  $\beta$ -elimination of the glucose fragment. In the absence of this base treatment, no free parasorbic acid was liberated from the berries.



Hence we sought to determine whether we simply were observing the effect of parasorbic acid, a known plant growth inhibitor, or its glucoside in our tests.

When the cranberry leaf extract was treated with calcium hydroxide followed by acidification and extraction with ether, parasorbic acid was indeed isolated. It was identified from the IR and NMR spectra of the crude extract and by identical retention time on two glc columns with an authentic sample of parasorbic acid. We then analyzed samples of the cranberry leaf extract by glc before and after treatment with calcium hydroxide (followed by acidification), samples of parasorbic acid alone, and extract plus parasorbic acid (in the presence of calcium hydroxide followed by acidification). Our results (Table I) show that parasorbic acid from the extract was obtained only upon calcium hydroxide treatment. This result agrees with Tschesche's observations on mountain ash berries.

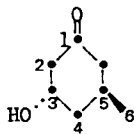
Table I. Parasorbic Acid Found After Calcium Hydroxide Treatment

Sample	Added Parasorbic Acid	Ca(OH) <sub>2</sub> Treatment	Parasorbic Acid Found
H <sub>2</sub> O	42 mg	+	29.5 mg
4.6 g extract	41 mg	+	35.6 mg
4.6 g extract	-- --	+	28.6 mg
4.6 g extract	-- --	-	0 mg

#### Isolation of Glucoside of Parasorbic Acid

These results raised the possibility that the glucoside of parasorbic acid was responsible for the biological activity of the extracts. Therefore, we set out to isolate the glucoside, determine its biological activity, and calculate whether it could account for the growth inhibition of the extract. Isolation of the glucoside was aided by the fact that Tschesche had accomplished this before and hydrolyzing the glucoside to parasorbic acid provided a very sensitive and rapid glc method for guiding the isolation. The successful isolation paralleled the isolation of an analogous glycoside, ranunculin (10, 11). The procedure was as follows. The leaves were extracted with 85% aqueous acetone and the extract filtered. The filtrate was freeze dried and the residue washed with ether. The solid residue was digested in acetone and the mixture filtered. The acetone filtrate was evaporated and the residue dissolved in water. The water was treated with charcoal, which absorbed the glucoside. The crude glucoside was eluted from the charcoal with 50% methanol, chromatographed on a silica gel column, and eluted with ethyl acetate and acetone. From the total of 200-400 g of leaves, we were able to isolate about 800 mg of glucoside, mp 131.5-132°C; an IR band at 1695<sup>-1</sup> cm.

Comparison of our glucoside with the material reported by Tschesche showed differences in the melting point and infrared spectrum. Tschesche reported a mp of 68-69°C for the hydrate and 143-144°C after heating at 80°C overnight. Since our glucoside was isolated directly as the anhydrous solid rather than the hydrate, differences in the melting point of the anhydrous solid might be expected. There were also some differences in chemical shifts in the NMR spectrum, but these were due to solvent effects. The multiplicity of the spectrum, however, was in agreement with the reported values and with the aglycone 4 (Table II). Since we knew



4

that parasorbic acid could be liberated upon calcium hydroxide treatment, the lactone moiety must be present.

Table II. Proton NMR Shifts (ppm) of Parasorbic Acid Glucoside, 3, and its Aglycone, 4

	H2	H3	H4	H5	H6	H1'
Glucoside <u>3</u> ( <u>8</u> )	2.85 (m,2)	----	----	----	----	4.80 (d)
Aglycone <u>4</u> ( <u>8</u> )	2.56 (m,2)	4.30 (m,1)	1.78 (m,2)	4.80 (m,1)	1.33 (d,3)	----
Found for	2.63	4.28	1.72	4.77	1.32	4.34
Glucoside <u>3</u>	(d,2)	(m,1)	(m,1)	(m,1)	(d,3)	(d,1)
			2.12 (m,1)			

While differences from the reported values were evident for the glucoside, they were removed upon its conversion to the tetra-acetate. This was effected, in 74% yield, by treating the glucoside with acetic anhydride and pyridine. The melting point and all other properties of the tetra-acetate were in agreement with those reported by Tschesche.

Having the glucoside, we could now determine its concentration in the leaves. Extract samples of varying sizes were subjected to calcium hydroxide treatment, acidification, extraction, and glc quantitation. The amounts of parasorbic acid obtained from the samples are shown in Figure 1 to be proportional to sample size.

In order to estimate the amount of parasorbic acid glucoside present, we needed to know the efficiency of recovery of parasorbic acid from the extracts being analyzed. To do so, a second set of samples was taken in which a constant weight of glucoside was added to each sample. These samples were similarly analyzed. The upper line indicates the amount of glucoside that was added to each sample with the displacement from the lower line being equal to the weight of added glucoside. Since the weight of glucoside was constant, the upper line is parallel to the first line. The center line gives the amounts found for this second set of samples. The difference between the center and the upper line gives the recovery, 63%. Applying this fraction to the cranberry leaf extract gives 31 mg  $\pm$  6.9 mg of glucoside per gram of extract or 6.8 mg  $\pm$  1.5 mg of glucoside per gram of leaves.

#### Wheat Seed Bioassay

The bioassay was performed by placing twenty wheat seeds (*Triticum aestivum* var. "Olaf" spring wheat) between two pieces of filter paper in plastic Petri dishes. The extract solutions were poured onto the paper, the dish covered, and the dishes placed in the dark for about 64 hours for the seeds to germinate. Checks were provided by using de-ionized water. The lengths of the primary roots were measured. Seeds with the five longest and five shortest roots were removed and the lengths of the primary roots of the ten remaining seeds recorded.

A range of concentrations were chosen so that the range of root inhibition would be between 50 and 90%. By having several points, a best-fit line could be found for the test sample and the ED<sub>50</sub>

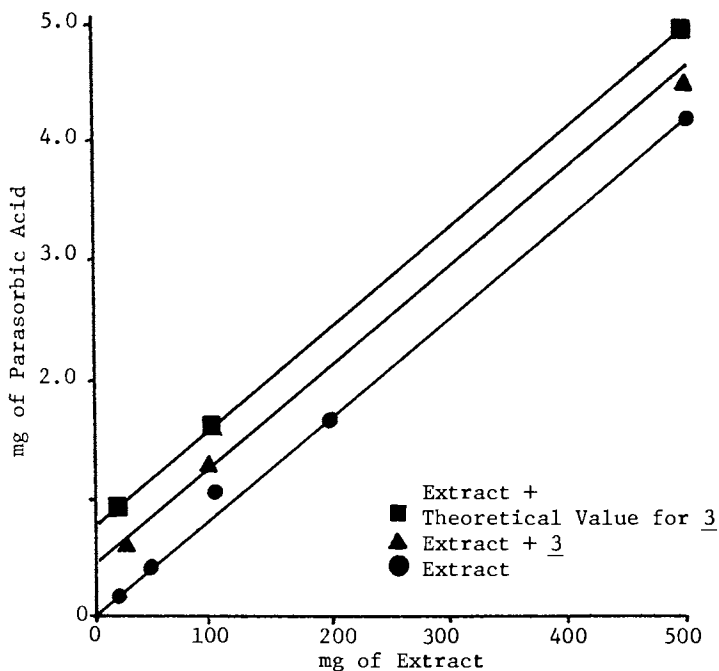


Figure 1. Recovery of Parasorbic Acid from Cranberry Leaf Extract

determined. The best fit line (10 g l/concentration versus root length) was found via a least squares analysis for a plot of the root length equal to one-half the water control (check) length. However, in some bioassays the check lengths were shorter than the test lengths obtained at high dilution. In those instances, the calculated length for zero concentration was used in place of the actual check length in determining the  $ED_{50}$ . This was most often the case when the check lengths were abnormally short and the root lengths at high dilutions about the same as the check lengths noted in other tests.

### Results and Conclusions

Bioassay of the glucoside gave results as follows: (concentration in mg/mL, root length in cm) 6.25, 1.18; 3.13, 2.07; 1.56, 2.46; 0.78, 3.88; 0.39, 2.77; 0.00 ( $H_2O$ ), 2.56. Thus the root length was 50% inhibited at about 5.75 mg/mL (19.6 millimolar concentration). As the glucoside was approximately 3.1% of the extract and 0.68% of the leaf, if it were solely responsible for the growth inhibition, then 193 mg extract/mL or 875 mg leaves/mL would be required to give the same biological response. Similar bioassay of residue produced by evaporation of the crude acetone extract of the leaves gave results as follows (concentration in mg/mL, root length in cm):

110, 0.14; 66, 0.40; 39.5, 1.15; 24, 1.22; 14, 1.43; 8.5, 1.63; 5, 1.48; 3, 1.66; 1.8, 1.31; 1.1, 1.78; 0.0 (H<sub>2</sub>O), 2.85. Thus the crude residue has an approximate ED<sub>50</sub> of 14.8 mg extract/mL which corresponds to 68 mg leaves/mL.<sup>50</sup> Therefore, the glucoside can account for only a fraction of the total activity.

Although the bioassay of racemic parasorbic acid showed good activity (wheat seed root growth was 50% inhibited with 0.25 mg/mL), the conclusion that little of the growth-retardant activity in cranberry leaves can be accounted for by parasorbic acid was confirmed by examination of Devlin's extract. In this case neither parasorbic acid nor its glucoside was present. Subsequent work by Hussain (12) has resulted in the isolation of two plant growth inhibitors identified as cinnamyl alcohol and 3-phenyl-1-propanol (hydrocinnamyl alcohol).

#### Acknowledgments

I must acknowledge the contributions of others: Robert Devlin, for alerting us to the problem and providing cranberry leaves and extract; Robert Templeton, for working out the bioassay; Laurie Lutz for doing the bioassay and especially for measuring the root lengths; James Clapp, for being skeptical about the bioassay; Marinus Los, who helped to make it happen; and Ronie Bilotto and Claire Perniciaro, for typing the manuscript. Finally, I thank American Cyanamid Company, whose support made this possible.

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## Chapter 31

# A Rapid Seedling Bioassay for the Study of Allelopathy

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A bioassay was developed to evaluate the biological activity of pure allelopathic compounds qualitatively and quantitatively and to monitor activity of interest during the purification of chemical components in extracts. The effects of allelopathic compounds and analogs on various growth parameters (GP) were measured using the test species Echinochloa crusgalli (L.) Beauvois and Sesbania exaltata (Raf.) Cory. Root length and root fresh weight were the most sensitive GP, but these measurements were extremely time consuming. Therefore, a model was developed to ascertain predicted shoot-plus-root fresh weight (PSRFW) from total plant fresh weight, which is less sensitive due to seed weight, but rapidly measured. It was concluded that a seedling bioassay using PSRFW is an efficient method to evaluate the phytotoxicity of biological samples.

The development of reliable bioassays is crucial to successful research in the rapidly growing field of allelopathy. The need for bioassays in the study of allelopathy is twofold. First, a bioassay is needed to determine if a specific plant-plant interaction has a chemical basis. Often in these studies, both the potential donor species [i.e., the plant releasing compound(s)] and the bioassay species (i.e., receptor) are studied in situ (1). In other cases, donor species are extracted with various solvents and the extracts are bioassayed (2). Although time-consuming, these studies are essential if an allelopathic interaction is to be demonstrated. Second, a bioassay is needed to help isolate and characterize the compound(s) causing the interaction. There are two considerations with these types of bioassays: the bioassay species and growth parameter(s) used to indicate biological activity. Nicollier et al. (3) used Lycopersicon esculentum (tomato) and Raphanus sativus (radish) as bioassay species to help in characterization of dhurrin isolated from Sorghum halepense rhizomes. However, these two species were not involved in the actual allelopathic association.

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To identify ferulic acid, Liebl and Worsham (4) used weed species in their bioassays that were actually growing in association with wheat (*Triticum aestivum* L.) in the environment. Thus, there is diversity in terms of which plant species are used in bioassays to indicate biological activity.

In most studies (2-10), a whole plant system is used to monitor the phytotoxic activity by measuring some aspect of growth (e.g., root length). Determining inhibition using a growth parameter such as root length can be tedious and time consuming. Time considerations become even more critical during the purification of chemical components in extracts due to the large number of samples generated. Bioassays that monitor specific biochemical (11, 12) or physiological (13-15) processes have also been developed. Although these types of bioassays are generally more sensitive than growth bioassays, they are specific in the types of biological activity detected. When extracts and/or compounds are unknown in terms of structure and/or biological activity, a bioassay that detects specific activity may not be useful because interesting activity may be missed.

The objective of this study was to develop an efficient whole plant bioassay that would identify diverse types of compounds that cause phytotoxic activity.

#### Materials and Methods

Light conditions and growth parameter evaluation. These experiments were conducted to determine which growth parameter(s) indicates the highest level of inhibition in response to a selected chemical(s) under light and dark conditions. Two compounds ( $\alpha$ -phenyllactic acid and *p*-ethoxybenzoic acid) were individually dissolved in acetone and the 2 mM solutions placed in 60 ml glass jars with Whatman #1 filter paper. After the solvent was evaporated, either 0.22 g (60 seed) of *Echinochloa crusgalli* L. Beauvois (barnyardgrass) or 0.40 g (30 seed) of *Sesbania exaltata* (Raf). Cory (hemp sesbania) were placed in jars. Three ml (for barnyardgrass) and 3.5 ml (for hemp sesbania) of 15 mM MES buffer [2-morpholinoethanesulfonic acid] adjusted to pH 6.0 was added to each jar. Seeds were germinated at 25°C either in continuous darkness or using a 12-h photoperiod. Controls consisted of acetone-treated filter paper alone. After 72 h of incubation, the following factors were measured and used to determine percent growth inhibition: percent germination, root length, shoot length, root fresh weight, shoot fresh weight, seed fresh weight and total plant fresh weight. After drying plant material for 48 h at 80°C, weights of shoots, roots, seeds, and total weight were again determined. Growth parameters were compared on the basis of their sensitivity to the test compounds under light and dark conditions. The term shoot is used for simplicity in this paper. Actually, in the case of barnyardgrass it refers to coleoptile and leaf tissue, and with hemp sesbania, hypocotyl tissue.

Development of model. Experiments were conducted to develop a model for predicting shoot-plus-root fresh weight (PSRFW) from total fresh weight (TFW). This seemed reasonable because of the apparent relationship between shoot fresh weight versus shoot length, and

root fresh weight versus root length. Both barnyardgrass and hemp sesbania were grown as previously described, but no chemicals were used. Plants were incubated at 25°C using a 12-h photoperiod and harvested at the following times: 2, 4, 6, 8, 10, 15, 40, 51, 69, and 92 h. At these times, fresh and dry weights of shoots, roots, and seeds were determined. Regression analysis was used to determine the best model. (See Statistics section).

Effects of allelopathic compounds. To compare the sensitivity of shoot and root length to PSRFW, 25 additional compounds were tested at 2 mM as described in the previous section. Hemp sesbania was harvested at 60 h and barnyardgrass at 72 h. These times were chosen in an attempt to maintain sufficient solution volume during the experiment. Root and shoot lengths, fresh and dry total weights, and percent germination were then determined. TFW was used to determine PSRFW.

Statistics. Data were subjected to analysis of variance and regression analysis by using the general linear model procedure of the Statistical Analysis System (16). Correlation coefficients between growth parameters were determined with the same system. Equations were best fitted to the data based on significance level of the terms of the equation and  $R^2$  values.

### Results and Discussion

Both barnyardgrass and hemp sesbania gave consistently good germination (barnyardgrass, 93%; hemp sesbania, 89%) and produced an easily measured dominant root under both light and dark conditions. In addition, within each population (i.e., within each jar) of both species, growth was relatively uniform. This was important, since it was not feasible to measure every plant. Furthermore, both are weed species and, therefore, observed inhibition of growth could represent activity of economic significance. Using a whole plant versus a specific biochemical bioassay also allows for the identification of useful biological activity (i.e., activity that translates to the whole plant level). The use of both a monocot and dicot would also indicate any specificity of activity.

Analogues of known natural products [ $\alpha$ -phenyllactic acid (17) and *p*-ethoxybenzoic acid (18)] were used to determine differential response to light and to compare the sensitivity of various growth parameters (Table I). There would be an advantage to evaluating chemicals and/or extracts of unknown structure and/or activity in the light with a nonspecific bioassay (i.e., whole plant). This type of bioassay would indicate not only growth inhibition, but also qualitative changes in plant pigmentation (e.g., bleaching and chlorosis). However, even with these stated advantages, there are differences in sensitivity. All measures of shoot growth (length and fresh dry weights) of barnyardgrass were inhibited more in the dark than under light conditions. This would be expected, as shoots are etiolated in the dark and grow more rapidly. Perhaps the potential for the appearance of a differential response was greater. The same trend was true for hemp sesbania, although the differences in sensitivity in the light and dark were not as dramatic. Root

growth was similar or more sensitive under light conditions for both species. With these two compounds there would be an advantage to evaluating inhibition in the dark; however, the ability to identify a diversity of biological activity would be sacrificed.

Overall, root growth was the most sensitive growth parameter measured. In the light, root fresh weight of barnyardgrass and root length of hemp sesbania were the most sensitive indicators of activity. Although these growth parameters were sensitive, their measurement was extremely time consuming. Total fresh weight (TFW) represents a growth parameter that is rapidly determined but less sensitive. The decrease in sensitivity was due partly to the fact that seed weight represented a tissue that was either nonresponsive or one that responded inversely to chemical inhibition (i.e., as the level of inhibition increased, the seed weight loss would potentially decrease). Note that for barnyardgrass grown in the light, average shoot-plus-root fresh weight (SRFW) was almost twice as sensitive as TFW (48 versus 26% inhibition, respectively).

To determine if TFW could be used to obtain predicted shoot-plus-root fresh weight (PSRFW), a time course study was conducted to ascertain the relationship between TFW, SRFW, and seed fresh weight (SFW). Figures 1 and 2 illustrate relationships for barnyardgrass and hemp sesbania, respectively. As indicated, the percentage of TFW that was due to SFW changes with time. It was assumed that changes in the relationship with time would simulate changes due to chemical inhibition. It was also apparent that the sensitivity of TFW (i.e., the need for PSRFW) decreases as chemical inhibition increases. This was caused by the fact that as the amount of seedling growth decreased, the amount of TFW represented by SFW increased. Thus, the need for a model to determine PSRFW was apparent if the sensitivity of fresh weight was to be acceptable.

With the aforementioned data, a linear regression was developed to determine the relationship between TFW and SRFW (Figures 3 and 4). With these equations, TFW could be used in future experiments to determine PSRFW without actually measuring it. The equations were tested for accuracy by solving the equations using TFW and correlating the results with actual shoot-plus-root fresh weight (ASRFW). Models were also determined for total dry weight and predicted shoot-plus-root dry weight, but the equations were not significant. The PSRFW correlated well with ASRFW (Table II) for both barnyardgrass and hemp sesbania (0.97 and 0.91, respectively) for these two compounds. PSRFW correlated well with average shoot-plus-root length (barnyardgrass; 0.86; hemp sesbania; 0.88) and root length alone (barnyardgrass; 0.84, hemp sesbania; 0.87).

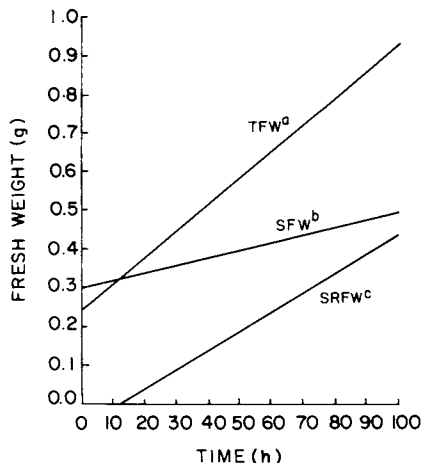
Once the model was tested empirically, 25 additional compounds (19) were evaluated to compare the sensitivity of PSRFW to root length (Table III). These data indicated that PSRFW was more sensitive than TFW, but it was not as sensitive as root length. Correlation coefficients also indicated the same trend (Table IV). There are two possible reasons for the lower sensitivity of PSRFW as compared to root length. First, PSRFW measured the growth of both shoot and root, and in most cases, root growth was more sensitive to the tested compounds than shoot growth. A second weakness of using fresh weights to quantitate phytotoxicity was caused by compounds that induced radial expansion of roots (root swelling). Coumarin

Table I. The effect of  $\alpha$ -phenyllactic acid and *p*-ethoxybenzoic acid on various growth parameters of barnyardgrass and hemp sesbania.<sup>a</sup>

Growth Parameters	Barnyardgrass		Hemp sesbania	
	Light	Dark	Light	Dark
	----- % inhibition <sup>b</sup> -----			
Germination	6*	10*	11*	11*
Shoot fresh weight	12*	48*	28*	33*
Root fresh weight	83	35*	52	34
Average shoot-plus-root fresh weight	48*	42*	40*	34*
Shoot dry weight	9*	31*	2*	14*
Root dry weight	53*	54*	44*	12*
Shoot length	15*	55*	18*	30*
Root length	70	73	61	67
Average shoot-plus-root length	42*	64*	40*	48*
Total fresh weight	26*	26*	25*	17*
Total dry weight	5	5	7	10

<sup>a</sup> Averaged across both chemicals at 2 mM.

<sup>b</sup> Values followed by asterisk are significantly different from the control at the 0.05 level according to the general linear model procedure.

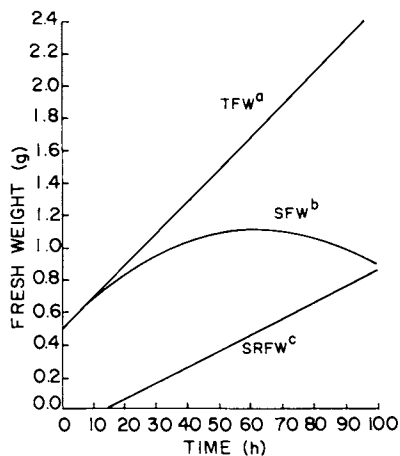


<sup>a</sup>  $TFW = 0.24 + 0.007 (\text{Time})$ ;  $F = 306.4$ ,  $P > F 0.0001$ ,  $R^2 = 0.92$ .

<sup>b</sup>  $SFW = 0.30 + 0.002 (\text{Time})$ ;  $F = 492.1$ ,  $P > F 0.0001$ ,  $R^2 = 0.95$ .

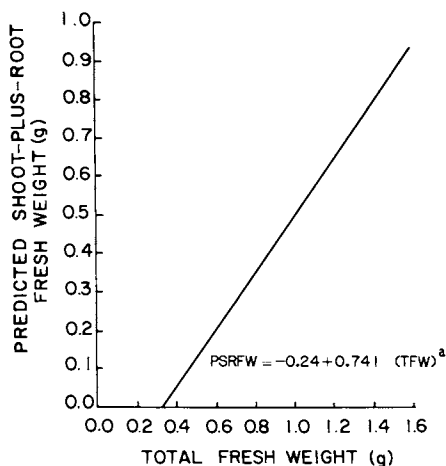
<sup>c</sup>  $SRFW = -0.06 + 0.005 (\text{Time})$ ;  $F = 183.9$ ,  $P > F 0.0001$ ,  $R^2 = 0.87$ .

Figure 1. The relationship between barnyardgrass total fresh weight (TFW), seed fresh weight (SFW), and shoot-plus-root fresh weight (SRFW) over time.



- <sup>a</sup>  $TFW = 0.49 + 0.02 (\text{Time})$ ;  $F = 1,461.7$ ,  $P > F 0.0001$ ,  $R^2 = 0.98$ .  
<sup>b</sup>  $SFW = 0.5_2 + 0.02 (\text{Time}) - 0.000161 (\text{Time})^2$ ;  $F = 92.1$ ,  $P > 0.0001$ ,  $R^2 = 0.87$ .  
<sup>c</sup>  $SRFW = -0.14 + 0.01 (\text{Time})$ ;  $F = 469.8$ ,  $P > F 0.0001$ ,  $R^2 = 0.94$ .

Figure 2. The relationship between hemp sesbania total fresh weight (TFW), seed fresh weight (SFW) and shoot-plus-root fresh weight (SRFW) over time.

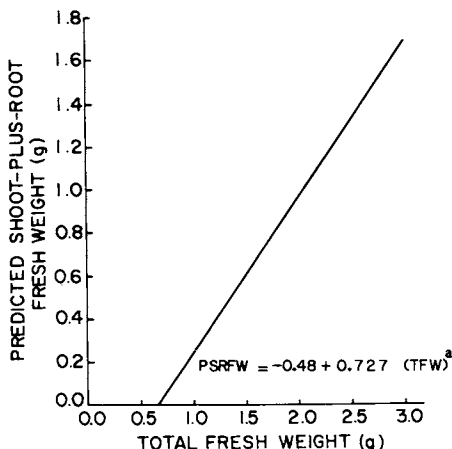


- <sup>a</sup>  $F = 2,532.8$ ,  $P > F 0.0001$ ,  $R^2 = 0.99$ .

Figure 3. Model for the determination of predicted shoot-plus-root fresh weight (PSRFW) from total fresh weight (TFW) for barnyardgrass.

caused this type of response in hemp sesbania. Root length was inhibited by 82% but PSRFW only 12%. In plants that were exposed to these types of compounds, the longitudinal growth (root length) was affected more than the overall fresh weight of the root. Therefore, when a qualitative assessment indicates this type of activity, a more appropriate growth measurement, such as root length, should be used to quantitate phytotoxicity.

It was evident from this study that a whole plant bioassay is more appropriate than a specific biochemical bioassay for the evaluation of diverse chemical types and/or extracts causing unknown



<sup>a</sup>  $F = 368.7$ ,  $P > F 0.0001$ ,  $R^2 = 0.93$ .

Figure 4. Model for the determination of predicted shoot-plus-root fresh weight (PSRFW) from total fresh weight (TFW) for hemp sesbania.

Table II. Correlation coefficients between growth parameters affected by  $\alpha$ -phenyllactic acid and  $p$ -ethoxybenzoic acid in the light <sup>a, b</sup>

Growth parameter	Barnyardgrass <sup>d</sup>		Hemp sesbania	
	TFW <sup>c</sup>	PSRFW <sup>d</sup>	TFW	PSRFW
Shoot fresh weight	0.82	0.69	0.79	0.79
Root fresh weight	0.92	0.95	0.77	0.77
Actual shoot-plus-root fresh weight	0.93	0.97	0.91	0.91
Shoot length	0.62	0.62	0.74	0.74
Root length	0.81	0.84	0.87	0.87
Average shoot-plus-root length	0.84	0.86	0.88	0.88
Total fresh weight	--	0.90	--	0.66

<sup>a</sup> Average for both chemicals at 0.5, 1.0 and 2.0 mM.

<sup>b</sup> All coefficients significant at the 0.05 level.

<sup>c</sup> Total fresh weight.

<sup>d</sup> Predicted shoot-plus-root fresh weight.

biological activity. Numerous qualitative observations were made which indicated varying modes of action for the compounds tested. Juglone caused blackening of the tips of barnyardgrass shoots and roots. Flavone caused severe bleaching in barnyardgrass shoots and coumarin caused root swelling. Some of these observations would not have been made if a specific biochemical bioassay were used. Although there are limitations, as indicated previously, the described bioassay appears to be an efficient method to evaluate the phytotoxicity of various samples, both qualitatively and quantitatively.

Table III. The effect of 27 compounds on four growth parameters of barnyardgrass and hemp sesbania

Chemical 2 mM	Barnyardgrass				Hemp sesbania			
	SL <sup>a</sup>	RL <sup>b</sup>	TFW <sup>c</sup>	PSRFW <sup>d</sup>	SL	RL	TFW	PSRFW
Benzoic acid	13*	17*	8*	10*	25*	69*	14*	20*
p-Ethoxybenzoic acid	18*	84*	29*	43*	22*	66*	27*	41*
p-Hydroxybenzoic acid	1	15*	0*	0*	12	0	0	0
p-Aminobenzoic acid	0	37*	14*	18*	0	25*	0*	0*
o-Ethoxybenzoic acid	16	27	12*	18*	30	81*	20*	47*
Vanillin	12	0	2	3	0	0	0	0
Vanillic acid	4	0	0	0	0	0	0	0
Gallic acid	7*	7	0	0	3	0	0	0
Shikimic acid	18*	0	12	16	15	10	3	4
Protocatechuic acid	20*	18	8	10	12	0	1	1
2,3-Dihydroxy- benzaldehyde	44*	45*	40*	51*	25	17	2	3
3-Ethoxy-4-hydroxy- benzaldehyde	15*	17*	15*	19*	0*	0*	0*	0*
t-Cinnamic acid	37*	97*	42*	56*	27*	91*	23*	34*
β-Phenyllactic acid	12*	9	20*	26*	0	0	3	4
Caffeic acid	14*	0	13*	16*	0	0*	0	0
Ferulic acid	27*	12	23*	30*	8	39*	0	0
o-Hydroxycinnamic acid	27*	80*	32*	41*	29*	49*	7*	10*
Coumarin	100*	100*	57*	96*	74*	82*	9	12
Scopoletin	20*	89*	37*	49*	1*	0*	0	0
Umbelliferone	40*	94*	51*	67*	52*	50*	9	23
4-Hydroxycoumarin	17*	40*	28*	36*	6	24	8	11
Flavanone	20*	4*	30*	39*	4	4	0	0
Flavone	55*	67*	51*	66*	0	0	5	7
2-Carboxy-5,7- dihydroxy-4-methoxy- isoflavone	23*	0	26*	34*	24*	13	7*	9*
Quercetin	11*	0*	9*	12*	13*	0*	7*	9*
Juglone	68*	91*	52*	76*	46*	53*	22*	35*
α-Phenyllactic acid	12	57*	22*	33*	13	56	22*	34*

<sup>a</sup>Shoot length. <sup>b</sup>Root length. <sup>c</sup>Total fresh weight. <sup>d</sup>Predicted shoot-plus-fresh weight. <sup>e</sup>Values followed by an asterisk are significantly different from the control at the 0.05 level according to general linear model procedure.

Table IV. Correlation coefficients between growth parameters of barnyardgrass and hemp sesbania averaged for all 27 compounds<sup>a</sup>

Growth parameter	Barnyardgrass		Hemp sesbania	
	TFW <sup>b</sup>	PSRFW <sup>c</sup>	TFW	PSRFW
Total fresh weight	--	0.93 <sup>d</sup>	--	0.97
Root length	0.71	0.84	0.80	0.81
Shoot length	0.90	0.87	0.47	0.43
Average root-plus-shoot length	0.86	0.94	0.73	0.72

<sup>a</sup>Compared at 2 mM only. <sup>b</sup>Total fresh weight. <sup>c</sup>Predicted shoot-plus-fresh weight. <sup>d</sup>All coefficients significant at the 0.05 level.

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## Chapter 32

# Interactions Among Allelochemicals and Other Stress Factors of the Plant Environment

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Current evidence indicates allelopathic inhibition most often results from the combined action of several different chemicals. A specific allelochemical may be present at a concentration below its growth inhibition threshold and still affect growth. Several combinations of allelochemicals have been shown to have either additive or synergistic action. Other work demonstrates that the action of phenolic acids is interrelated with nutrient conditions and temperature, moisture, and herbicide stress. Grain sorghum and soybean seedlings grown under relatively hot conditions exhibited a ferulic acid inhibition threshold at only one-half the concentration required under moderate temperatures, indicating stress interactions. Additive inhibition occurred when phenolic acids were tested in conjunction with moisture stress. Recent work showed that the growth of seedlings subjected to ferulic acid and atrazine together was suppressed more than with either alone. Allelochemicals may also promote damage from disease organisms. Hence, associated physical and chemical stress conditions may either enhance the inhibitory action of allelochemicals or result in an additive incremental detriment to plant growth.

It has been difficult to make an absolute connection between a suspected allelochemical inhibitor and the reduction in plant germination, growth, or function that characterizes a particular allelopathic situation. One reason this cause-effect relationship has been hard to establish is that the quantity of a biologically active compound recovered from the environment typically has been below the level required for inhibition in bioassays. Thus, a constant concern and argument against allelopathy has been that the level of an allelochemical in a natural setting is inadequate to be effective in growth regulation. However, the literature on allelopathy is replete with situations where several different

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chemicals have been identified and inferred as substances which could cause interference. In fact, in most of the cases where the putative chemicals have been sought a number of compounds with biological activity have been found. This suggests the possibility that allelopathic interference may be the result of the simultaneous action of several compounds.

Receiving plants often contact allelochemicals through the soil medium, yet the information on availability from the soil is minimal (1). Much of what is known concerning allelochemicals in the soil references phenolic acids and closely related phenolic structures. Because these compounds have received more scrutiny than others, they will be a central focus in the subsequent discussion. Reported concentrations of *p*-coumaric, ferulic, *p*-hydroxybenzoic, vanillic, and other phenolic acids in the soil have varied according to what allelopathic species colonized the area, abundance and duration of plant residue, soil type, environmental factors, time of year, and method of extraction. Although individual phenolic acids in the soil may exceed 1,000  $\mu\text{g/g}$  of soil (2-4), it is probable that only a fraction of this contributes to allelopathy, because a large percentage may not be biologically active. For example, Whitehead et al. (5) found that water extracts of *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids from soil under quackgrass [*Agropyron repens* (L.) Beauv.] were equivalent to one micromolar or less for each compound in the soil solution. An extraction that might simulate limed conditions, 5%  $\text{Ca(OH)}_2$ , gave values in the 10 to 100  $\mu\text{M}$  range. These values are typical, yet they are below concentrations that have been used in tests for biological activity.

A characteristic feature of allelopathy is that the inhibitory effects of allelopathic compounds are concentration dependent. Dose-response curves with known compounds show an inhibition threshold. Below this level either no measurable effect occurs, or stimulation may result. Although the concentration of a compound required to exceed the inhibition threshold varies extensively according to different sensitivities among species and also among phases of the growth cycle for higher plants, the concept of an inhibition threshold seems consistent. Thus, it is reasonable to evaluate how, and if, a subthreshold concentration of an allelochemical may contribute to allelopathic interference. Also in need of evaluation is how environmental conditions may influence the deleterious action of an allelochemical and the concentration required for an effect. Such interactions are especially pertinent for those environmental situations that place some degree of stress on plant functions.

This paper will review the literature on the cooperative action of known allelochemicals. It will also focus on the increasing evidence that the biological importance of these substances, especially in low concentrations, depends on associated environmental conditions.

#### Inhibition by Combinations of Allelochemicals

The allelopathic potential of plants has often been evaluated from tests of the biological activity of volatiles, leachates, and root exudates, or from aqueous extracts of the tissue. Alternatively, assessment of inhibitors in the soil associated with a suspected

allelopathic plant has been routine. Such evaluations almost always deal with a complex matrix of biochemicals. When the bioassays have demonstrated effects on germination or growth, subsequent work on identification of the responsible allelochemicals has often followed. Although these searches have seldom been exhaustive, they have consistently revealed more than one compound with biological activity. It has not been uncommon to isolate ten or more compounds which may include several different chemical classes (6-9). However, it has been difficult to determine the quantity of each that might be functional in the environment, and generally the relative contribution of each allelochemical to growth inhibition has not been evaluated.

Some investigators have tested equimolar mixtures of chemicals they have isolated as the agents in allelopathic situations (10-12), and a few have attempted to simulate combinations from field situations. Glass (13) grew plants hydroponically in a solution which reproduced the phenolic acid conditions found in the soil associated with *Pteridium aquilinum* (L.) Kuhn. This mixture of 39  $\mu\text{M}$  *p*-hydroxybenzoic acid, 49  $\mu\text{M}$  vanillic acid, 42  $\mu\text{M}$  *p*-hydroxycinnamic acid, and 4  $\mu\text{M}$  ferulic acid altered the root growth of barley (*Hordeum vulgare* L.) and several other species. In a study of hackberry (*Celtis laevigata* L.) allelopathy, Lodhi (3) found that the combined effect of *p*-coumaric, ferulic, and caffeic acids at the concentrations found in soil underneath these trees was much more inhibitory to seed germination than the effect of each chemical (at its soil concentration) tested separately. Weaver and Klarich (14) reported an increase in respiration rate in wheat plants that were exposed in the field to volatile substances, presumably monoterpenes, from *Artemisia tridentata* Nutt., but the relative amount of different terpenes was not ascertained. A recent study of *Lupinus albus* L. showed the allelopathic effects of a mixture of quinolizidine alkaloids which approximated that excreted from germinating seeds and seedlings (15).

More definitive efforts have been made to ascertain the concerted action of allelochemicals by quantitatively comparing the action of a mixture of substances with the activity of each component part (Table I). Although most of these studies have been with derivatives of benzoic acid, cinnamic acid, and coumarin, some evaluations of other compounds have occurred. Asplund (16) reported that the phytotoxic monoterpenes, camphor, pulegone, and borneol, exhibited marked synergistic action on root growth. Up to 100-fold enhancement was found using two compounds simultaneously, demonstrating that biological activity could occur with concentrations two orders of magnitude below the threshold for a single compound. Wallace and Whitehead (17) showed the synergistic action of volatile fatty acids, and their work demonstrated the value of recognizing that similar compounds may have different activities. It took ten times as much acetic acid to inhibit wheat (*Triticum aestivum* L.) as butyric or propionic acid.

We have investigated the concurrent action of some of the more commonly reported phenolic allelochemicals by testing these compounds at, or below, their inhibition threshold (18-21). Our first experiments showed that a combination of 5 mM each of *p*-coumaric and ferulic acids reduced grain sorghum [*Sorghum bicolor* (L.) Moench.] germination appreciably more than separate treatments

Table I. Quantitative Assessment of Effects of Combinations of Allelochemicals

Chemical Class	Conc. of Single Cpd. in Mixture	Bioassay <sup>a</sup>	Effect <sup>b</sup>	Ref.
Monoterpenes	0.017-0.68 $\mu$ M/L	G	Syn	<u>16</u>
Fatty Acids	0.27 - 3.2 mM	RE	Syn	<u>17</u>
<u>Phenolic Acids, etc.<sup>c</sup></u>				
FA, <i>p</i> CA	2.5 - 5.0 mM	G	Syn	<u>18</u>
	0.125-0.25 mM	SG	Syn	
VA, <i>p</i> HB	2.5 - 5.0 mM	G	Syn	<u>19</u>
	0.5 mM	SG	Syn	
FA,VA, <i>p</i> CA	3.3 mM	G,SG	Syn,Ant	<u>20</u>
<i>t</i> CnA, <i>p</i> CA,FA,CA	1.0 - 2.5 mM	G	Syn	<u>21</u>
	0.25 - 1.0 mM	RE	Syn	
	0.04 - 0.1 mM	SE	Syn	
Coumarin,CGA,FA, HCnA, <i>p</i> CA, <i>p</i> HBAL,PYR	1.0 mM	G	Several Add	<u>22</u>
CA,FA, <i>p</i> CA	1.0 - 3.0 mM	G	Add,Ant	<u>23</u>
CA,FA,VA	0.5 mM	RE	Ant	<u>24</u>
CA,FA, <i>p</i> CA, <i>p</i> HB, PRO,SIN,SYR,VA	0.125- 0.5 mM	SG	Syn,Add,Ant	<u>25</u>

<sup>a</sup>G = germination; RE = root elongation; SG = seedling growth

<sup>b</sup>Syn = synergistic; Add = additive; Ant = antagonistic

<sup>c</sup>CA = caffeic; CGA = chlorogenic; FA = ferulic;  
HCnA = hydrocinnamic; *p*CA = *p*-coumaric; *p*HB = *p*-hydroxybenzoic;  
*p*HBAL = *p*-hydroxybenzaldehyde; PRO = protocatechuic;  
PYR = pyrocatechol; SIN = sinapic; SYR = syringic;  
VA = vanillic; *t*CnA = *t*-cinnamic

of these chemicals (18). In tests with sorghum seedlings grown in nutrient solution amended with *p*-coumaric and ferulic acids, the threshold for growth reduction was less than 1/20th the level required to reduce germination. Cooperative inhibitory action of these phenolics was obvious since seedlings grown with 0.125 mM *p*-coumaric or ferulic acids were significantly stimulated, whereas growth of plants in a combination of the two was inhibited. Similar studies showed the three-way interaction of *p*-coumaric, ferulic, and vanillic acids on seed germination was synergistic, while vanillic acid seemed to antagonize some of the inhibition of the other two on shoot elongation (20). Colby's (26) analysis was used as an index for judging potential interactions among four cinnamic acids (21). Based on this criterion, concentrations of 0.04 mM *t*-cinnamic acid and 0.1 mM ferulic, *p*-coumaric, and caffeic acids had synergistic effects on sorghum growth when applied in combinations of two, three, and all four. Similar cooperative effects may also occur with mixtures of allelochemicals of different chemical categories. Work now in progress (unpublished data) has shown synergistic inhibition of sorghum germination and growth by a three-way combination of a flavonoid (rutin), a coumarin (umbelliferone), and a benzoic acid (salicylic acid). Antagonism among these three occurred in *Lemna minor* L. bioassays, demonstrating that species vary in their response.

Investigations using above-threshold concentrations also indicate that several phenolic compounds in a mixture can have at least a cumulative effect. Duke et al. (23) concluded from probit analysis of data on lettuce seed germination that *p*-coumaric and ferulic acids produced additive inhibition. Blum et al. (25) tested eight phenolics in various combinations of two or three on cucumber (*Cucumis sativa* L.) leaf expansion, and applied regression analysis. The effects of mixtures tested ranged from synergistic to antagonistic, and the authors concluded that the nature of the response depended on the magnitude of inhibition associated with each compound, the compounds in the mixture, and the factor measured.

Methods for assessing the joint action of inhibitors have many difficulties, especially when used for studying the relatively low concentrations that are most probable in a field situation. As pointed out by Morse (27) and Nash (28), nearly all methods have their shortcomings and even an unambiguous definition of terms such as synergism has remained elusive. However, these shortcomings and problems in communication should not be allowed to detract from the biological importance of the joint action of chemicals functioning in allelopathy. The preponderance of evidence indicates: (a) an allelochemical seldom acts alone, (b) concentrations considerably below inhibition thresholds in bioassays may be biologically active, and (c) the joint action of several compounds can influence plant growth and functions. Viewed in this manner, an allelopathic substance that is released into the environment can justly be considered one of several stresses that may influence plant distribution and vigor of plant growth.

#### Interaction Between Allelopathy and Mineral Nutrition

An early observation that allelopathic effects might be subject to other environmental conditions was the influence noted

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from adding nutrients (29). Several investigations in the last decade have demonstrated that phenolic allelopathy may be more severe under low fertility, and raising the nutrient level can suppress some of the allelochemical effect (13,30). Stowe and Osborn (30) reported that toxicity of vanillic (25 and 50 ppm) and *p*-coumaric (5 and 10 ppm) acids to barley plants depended intimately on nutrient concentrations. Two-way analysis of variance showed a definite interaction between phenolic treatments and nitrogen and phosphorus levels. Vanillic acid inhibited barley growth in a manner dependent upon phosphorus supply, and *p*-coumaric acid effects were dependent upon nitrogen levels. At low nutrient levels both phenolics were significantly inhibitory, suggesting soil fertility might be very important in phenolic allelopathy. Hall et al. (31) found pigweed (*Amaranthus retroflexus* L.) grown in soil amended with chlorogenic acid was stunted and the plants had a reduced phosphorus content, but these effects were overcome by adding a nitrogen-phosphorus-potassium supplement. Indeed, case studies indicate inputs of nitrogen and phosphorus have alleviated allelopathic inhibition from goldenrod (*Solidago canadensis* L.), tall fescue (*Festuca arundinacea* Schreb.), and sunflower (*Helianthus annuus* L.) (31-33).

Several phenolic acids and many nonspecific allelopathic conditions have been shown to alter the mineral content of plants, and certainly phenolic allelochemicals may perturb cellular functions in a number of ways that are of importance to plant nutrition (34,35). However, raising fertility does not always suppress allelopathic inhibition, and the interrelationships between these two factors are still not clear. Bhowmik and Doll (36) showed that allelopathic inhibition of corn and soybeans by residues of five annual weeds was not alleviated by supplemental nitrogen or phosphorus. Similarly, an increase in fertilizer did not overcome inhibition of corn by quackgrass or circumvent the autotoxicity of berseem clover (*Trifolium alexandrinum* L.) (37,38). Even when raising nutrient levels releases inhibition, it does not mean that allelopathy was inoperative under the original conditions. These instances simply illustrate the importance of the interaction of the two stress conditions.

#### Enhancement of Allelochemical Effects by Temperature Stress

I often observed that under greenhouse conditions there was considerable variation in the effect a particular concentration of a phenolic inhibitor had on the growth of seedlings. Since procedures used in these bioassays were quite uniform, it was logical that environmental factors were influencing the results. This was verified when we tested the hypothesis that temperature of the growth environment modified allelochemical action (39). Grain sorghum and soybean [*Glycine max* (L.) Merr.] seedlings were treated with several levels of ferulic acid, then each treatment group was subdivided and the two subsets held at different temperatures with light intensity the same for the two environments. The two temperature regimes in each experiment were within the normal range the seedlings might experience under field conditions. However, the higher temperatures would generally be considered more stressful.

Seedling response over a 10-day treatment period showed a significant interaction effect (two-way analysis of variance) between temperature and ferulic acid. Although several morphological features were different, the dry weights of control plants in the two temperature regimes were equivalent at the end of each experiment. Effects of equimolar concentrations of ferulic acid were more severe at the higher temperatures. The threshold concentration for inhibition of sorghum growth was 0.2 mM ferulic acid at 37° C average day temperature, while 0.4 mM ferulic acid was required for inhibition with an average day temperature of 29° C. Both shoot and root weight reductions evidenced this difference in an inhibition threshold. Soybeans were more sensitive than sorghum to both temperature and ferulic acid, but a similar interaction between temperature and ferulic acid occurred in these experiments. Soybeans grown with a day temperature of 34° C and 0.1 mM ferulic acid were significantly inhibited, weighing 63% as much as control plants, while at 23° C even 0.25 mM ferulic acid-treated plants were not stunted as severely. These experiments clearly established that relatively hot environmental conditions enhanced allelochemical inhibition.

Several other evidences that temperature variation can alter the extent of allelopathic inhibition have been reported. Glass (13) subjected barley seedlings to a mixture of phenolic acids with subgroups at 5, 10, 15, 20, 25, and 30° C. The phenolic acid mixture suppressed root growth (fresh weight) in each environment over a 14-day growth period, but the extent of inhibition was more severe at the extremes of low and high temperature. Steinsiek et al. (40) reported leachates from wheat straw caused a more marked inhibition of germination and growth of sensitive weeds, such as ivyleaf morningglory [*Ipomoea hederacea* (L.) Jacq.], when incubated at 35° C than at 30 or 25° C. Both temperature and photosynthetic photon flux density altered the allelopathic effects of residues of redroot pigweed (*Amaranthus retroflexus* L.) and yellow foxtail [*Setaria glauca* (L.) Beauv.] on corn (41). The inhibitory effects of these weed residues were less when corn was grown with moderate light and at 30/20° C light/dark conditions, as compared to a lower light level and temperature. However, allelopathic effects of the weed residues on soybean were not overcome at the more moderate conditions.

#### Concurrent Action of Allelochemicals and Moisture Stress

Phenolic acids interfere with many major physiological processes of higher plants (35). These disruptions of function include an alteration of plant water balance. We found depression of leaf water potential to be an early indicator of allelochemical stress from ferulic and *p*-coumaric acids (42). Likewise one mechanism of allelopathic action by cultivated sunflower, velvetleaf (*Abutilon theophrasti* Medic.), *Kochia* [*Kochia scoparia* (L.) Schrad.], and several other weeds was water stress (43-45). Since some allelochemicals interfere with plant-water relationships, it seemed logical that their action might be most critical at times when plants are under water stress from other causes.

We initiated several experiments to determine the impact of allelochemicals acting simultaneously with moisture stress (46).

Seeds were germinated in vermiculite for five days, then transplanted to 80-ml opaque vials containing a complete nutrient medium. The following day, seedlings were treated by transferring them to nutrient solutions amended with ferulic acid (0.1 or 0.25 mM), an osmoticum (e.g., -0.2 MPa), or both. After ten days growth in the greenhouse, plant dry weights were compared by analysis of variance and Colby's (26) analysis was applied for evaluating potential interactions. All osmotic agents may create some ancillary effects, but at the levels used in these experiments equivalent results were obtained with either polyethylene glycol 4000, KCl, or NaCl.

The results of these experiments clearly established that the simultaneous actions of water stress and an allelochemical are more deleterious than either alone. This combination effect was especially evident with relatively minor stresses from each source, as shown with data from one experiment (Table II). Treatment of 0.1 mM ferulic acid had no effect on growth, while nutrient media with an osmotic adjustment of -0.2 MPa caused some suppression of sorghum seedlings. These two together significantly reduced sorghum below the effect caused by the NaCl alone. Apparently moisture-stressed plants were more sensitive to ferulic acid. Dry weights of plants grown in the combination of 0.25 mM ferulic acid and -0.2 MPa were significantly below those observed in the separate treatments, and Colby's analysis suggested the combined action at these levels was more than additive. Replicate experiments gave similar results. We found comparable cooperative effects in studies of seed germination, except that both a lower water potential and higher phenolic acid concentration were required to achieve a germination inhibition threshold.

Experiments using a matrix of four levels of ferulic acid and four levels of moisture stress demonstrated that the combined action was additive under more stressful levels of the individual factors than in the previous tests. Duke et al. (23) tested the germination of lettuce seeds treated with phenolic acids (1 mM) at water potentials (D-mannitol) of 0, -0.2, -0.4, and -0.6 MPa. The combined action of low water potential and exposure to phenolic acids resulted in an additive detriment to germination, and the authors concluded from probit analysis that the mechanism of action from these sources was similar. Whatever their mechanisms, moisture stress and phenolic acids appear to work together in limiting growth of plants.

Although indirect and probably quite rare, another route has been reported for allelochemical interference with plant-water relationships. Lovett and Duffield (47) identified benzylamine as an allelochemical in the leaf washings from the cruciferous weed *Camelina sativa* (L.) Crantz. Subsequent work showed benzylamine induced hydrophobic conditions in the soil, and these conditions could reduce water availability for plant growth (48). Thus, indirect action through changes in soil structure could be partially responsible for adverse effects on linseed (*Linseed usitatissimum* L.) and could enhance more direct allelopathic effects.

#### Joint Action of Herbicides and Allelochemicals

Opportunity exists in agroecosystems for two sources of chemical interference, natural and synthetic. The origin of allelochemicals



Table II. Effects of Moisture Stress and Ferulic Acid Separately and Together on Sorghum Seedlings

Treatment -MPa <sup>a</sup> mM FA	Dry Weight <sup>b</sup> (mg + SE)		Plant	% of Control Plant Wt.	% E <sup>c</sup> in Comb.	Dif. <sup>d</sup>
	Root	Shoot				
-	208.5 <sub>±</sub> 4.2a	264.0 <sub>±</sub> 6.5ab	472.5 <sub>±</sub> 7.4a	100.0		
0.2	153.4 <sub>±</sub> 5.6cd	197.2 <sub>±</sub> 6.0c	350.6 <sub>±</sub> 11.0b	74.2		
-	192.6 <sub>±</sub> 7.0ab	271.5 <sub>±</sub> 7.0a	464.1 <sub>±</sub> 12.4a	98.2		
-	176.2 <sub>±</sub> 17.0bc	240.1 <sub>±</sub> 15.7b	416.3 <sub>±</sub> 30.7a	88.1		
0.2	129.5 <sub>±</sub> 13.5de	171.1 <sub>±</sub> 12.9cd	300.6 <sub>±</sub> 25.9bc	63.6	72.9	+9.3
0.2	112.3 <sub>±</sub> 8.9e	155.6 <sub>±</sub> 11.9d	267.9 <sub>±</sub> 20.3c	56.7	65.4	+8.7

<sup>a</sup>Water potential in negative megapascal units (-MPa).

<sup>b</sup>Means (N = 15) in a column not followed by the same letter are significantly different, P < 0.05, ANOVA with Duncan's multiple range test.

<sup>c</sup>Expected using Colby's (26) analysis; product of % of control for single treatments divided by 100.

<sup>d</sup>Positive difference from predicted = synergism.

may be weeds, crop plants, residue from these plants, or microbial activity in the decomposition process (49). The use of market products for weed control always carries some risk of herbicide stress to crop growth. This impact may occur when the herbicide has some deleterious side effects on the immediate crop, or may be carried over to harm the subsequent crop. Certainly the extent of any such problem varies greatly, ranging from no apparent injury to severe carryover problems for certain crop sequences. The latter is especially important in dry years. Herbicide difficulties may also be encountered when these compounds are readily water soluble and enter irrigation water. It is a reasonable conjecture that in some situations allelochemicals and herbicide stress may operate simultaneously.

Atrazine, a triazine compound, is extensively used as a selective herbicide on corn and sorghum fields for the control of broadleaf and grassy weeds. Depending on soil properties and climatic effects, its persistence from recommended application rates in north central states may extend well beyond one year and crop injury may result when sensitive species are in the rotation (50). We designed experiments to determine if residual concentrations of atrazine and near-threshold levels of phenolic acids have a cooperative action (51). Procedures in these studies were similar to the ones used in investigations described earlier for determining moisture-allelochemical interference, except oat (*Avena sativa* L.) seedlings were utilized and these were treated 9 days after germination. Ferulic acid was chosen as a representative allelochemical. Treatments of atrazine and ferulic acid were made as amendments to the nutrient solution in which the oat plants were grown.

Treatments of 1 and 10 ppb atrazine did not affect oat seedling weight over the 10-day growth period (Table III). A ferulic acid level of 0.25 mM caused some inhibition of growth, although the plants did not show other injury symptoms. The combinations of ferulic acid and either concentration of atrazine caused more inhibition than either treatment alone. Thus, these treatment levels clearly demonstrated the concerted inhibitory action of the two chemicals. Reduction of shoots was greater than of roots, although both were affected. In a replicate experiment, plants in each atrazine treatment were inhibited and those grown with 10 ppb atrazine, as well as those with 10 ppb atrazine plus ferulic acid, were dead by the end of the experiment. Oat seedlings subjected to the combination of 1 ppb atrazine with ferulic acid were more inhibited than with either single treatment, confirming the evidence found in the first trial. Higher light and temperatures occurred in the greenhouse during the second experiment, and it is probable that these environmental differences contributed to the increased sensitivity of oats to atrazine.

The levels of atrazine tested in this study were within a range of residual quantities that might occur under some cropping situations. Atrazine in irrigation wells in a corn producing area of Nebraska ranged from 0.01 to 8.29 ppb, with the deeper wells typically below 0.5 ppb (52). The significance of the data on combination effects is that a 1-ppb, or lower, level of atrazine may not be detrimental alone, but its potential biological effects may be enhanced by the presence of a second source of stress, allelochemicals.

Table III. Effects of Atrazine (AT) and Ferulic Acid (FA) Separately and Together on Growth of Oats

Treatment ppb AT	mM FA	Dry Weight <sup>a</sup> (mg + SE)			Plant Wt.	% of Control Plant	% E <sup>b</sup> in Comb.	Dif. <sup>c</sup>
		Root	Shoot					
-	-	38.3+ 3.a	88.7+ 8.1a	127.0+11.2a	100.0			
1	-	40.4+ 4.7a	93.1+11.1a	133.5+15.6a	105.0			
10	-	35.7+ 2.3ab	80.3+ 7.9a	116.0+ 9.9a	91.3			
-	0.25	33.2+ 2.0abc	54.7+ 3.9b	88.0+ 5.8b	69.3			
1	0.25	28.8+ 1.8bc	47.6+ 4.9b	76.3+ 6.9b	60.0	72.8	+12.8	
10	0.25	25.8+ 2.0c	42.1+ 3.1b	67.9+ 5.1b	53.5	63.3	+ 9.8	

<sup>a</sup>Means (N = 12) in a column not followed by the same letter are significantly different, P<0.05, ANOVA with Duncan's multiple range test.

<sup>b</sup>Expected using Colby's (26) analysis; product of % of control for single treatments divided by 100.

<sup>c</sup>Positive difference from predicted = synergism.

Implications of these experiments with atrazine and ferulic acid go beyond the specific compounds tested. They provide the first evidence that allelochemical action can either synergize or supplement the activity of a herbicide. Work now in progress in our laboratory suggests that other herbicides may interact with allelochemicals similarly. This information is especially timely and pertinent to agricultural management practices. Conservation tillage was used on over 30% of the cropland in the U.S. in 1982, and such practices are predicted to increase (53). Reduced tillage operations will often result in an elevation of levels of both natural and synthetic chemicals on and in the soil. Increased quantities of residue on the soil surface raise the potential for allelopathy as substances are released from the residue or from microbial metabolism. Reduced tillage operations have often relied heavily on herbicides. Thus, these conditions may result in the combined action of herbicides and allelochemicals causing stress on crop plants. Alternatively, it is possible that such a joint action might reduce the quantities of herbicides necessary for adequate weed control. Certainly current agricultural practices present a need for understanding herbicide-allelochemical interactions.

#### Interactions of Allelochemicals and Disease

Allelopathic substances have often been implicated as agents that increase the susceptibility of higher plants to disease. Toussoun and Patrick (54) found products from plant residues decomposing under field conditions greatly enhanced the pathogenesis of *Fusarium solani* f. *phaseoli* on beans, with an increase in permeability of host tissue noted as one action of the toxins. The phenomenon of root rot enhancement by decomposing residue has been observed in a variety of other situations (55). Even concentrations of toxins that did not seem to directly injure plants previous to inoculation were effective in promoting fungal invasion and colonization of tissue when root pathogens were introduced.

Recent work on asparagus (*Asparagus officinalis* L.) allelopathy and autotoxicity gave evidence of an interaction between *Fusarium* spp. infection and the direct inhibitory activity of asparagus tissue (56). Allelopathic substances from dried asparagus tissue dramatically enhanced severity of root rot and significantly decreased dry weight in test asparagus seedlings. In another case history, Lynch (57-61) determined that a similar combination of factors was responsible for interference with grass and cereal crop establishment caused by straw residues in certain locations in the United Kingdom. Planting wheat after wheat without tilling has been a particularly severe problem in wet soils. Acetic acid can be formed in phytotoxic concentrations by microorganisms in the soil, and it has a synergistic effect with *Fusarium* spp. Although relatively few studies have attempted to quantify allelochemical-disease interactions, it is apparent that this type of joint action is the mode of interference in certain situations. It is certainly one way that the biological action of relatively noninhibitory concentrations of allelochemicals may be magnified.

### Conclusions

Several lines of evidence support the conclusion that allelopathic inhibition of germination and plant growth typically occurs from the joint action of several allelochemicals. Additive or synergistic effects have been shown in bioassays with combinations of monoterpenes, organic acids, and several classes of phenolic compounds. These experiments demonstrate that a specific compound may be present below its threshold for inhibition and still be active in allelopathic interference by its effect in concert with other allelochemicals.

Stresses from a variety of associated conditions may also augment the inhibitory effects of allelopathic chemicals. The data show that higher plants growing with suboptimal nutrients, moisture, or temperature conditions are more sensitive to allelochemicals than those grown in nonstress environments. This may even occur when these stresses are insufficient to have any measurable effect on growth. Likewise, studies of combinations of ferulic acid and residual herbicide levels indicate these two chemical sources may supplement each other. The interaction of other stresses with allelochemicals may be synergistic or additive, depending on the magnitude of these stresses, and probably their mode of action. Other work indicates that some cases of higher plant allelopathy are mediated by a combination of direct allelochemical effects operating in conjunction with enhancement of the severity of infection from root rot organisms. These observations make it clear that the biological activity of allelochemicals is closely tied with other facets of the environment.

Thus, the potential impact of an allelochemical on plant growth should be evaluated with regard to both the presence of associated allelopathic compounds and the influence of other chemical and physical conditions in the environment. Certainly allelochemical action is not an isolated event, and from the standpoint of plant functions, the controversy between competitive and allelochemical interference loses some of its significance. Allelochemical action needs to be regarded with a holistic view where one stress may reinforce, or magnify, another. From this perspective, inhibition of plant growth is not so much a matter of which factor is most detrimental; instead it is determined by the interaction of multiple stresses.

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## Chapter 33

# Sorption and Mineralization of Plant Phenolic Acids in Soil

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A discussion of the chemical and biological processes affecting phenolic compounds in soil is made, suggesting the possible importance of these processes in regulating the fate of other allelochemicals. Reference is also made to the composition of the complex soil environment with an emphasis on relevant properties governing allelochemicals' behavior. A recommendation of agronomic research needs is outlined stressing the importance of improved understanding of the carbon cycle as it relates to the productivity of our land resource under conservation production systems.

The impact of allelopathy on crop production has received increasing attention recently (1-3). A number of potentially growth-inhibitory organic compounds may be released from plants into their immediate environment by leaching and volatilization from plant foliage, via exudation from and slough-off of plant roots and from the decomposition of dead plant residues. Many of these natural products enter the soil medium (4). Expression of phytotoxicity depends largely upon the environmental chemistry and persistence of these chemicals in soil. Some of the most thoroughly studied allelochemical substances are those derived from the shikimate biosynthetic pathway, including phenolic acids and their derivatives: terpenoids, coumarins, flavonoids, alkaloids and cyanohydrins and tannins (2). Phenolic acids have been associated with the toxic effects of a plant or plant parts on another plant or to itself in several crop rotation systems and monocultures (5-12). This allelopathic phenomenon has been implicated in conservation tillage systems commonly practiced in the Great Plains of the U.S. (13, 14). The primary objective of this review is to highlight the processes governing the behavior of phenolic compounds in soil (Figure 1) and to illustrate the possible importance of these processes in regulating the behavior and fate of allelochemical substances in the soil environment.

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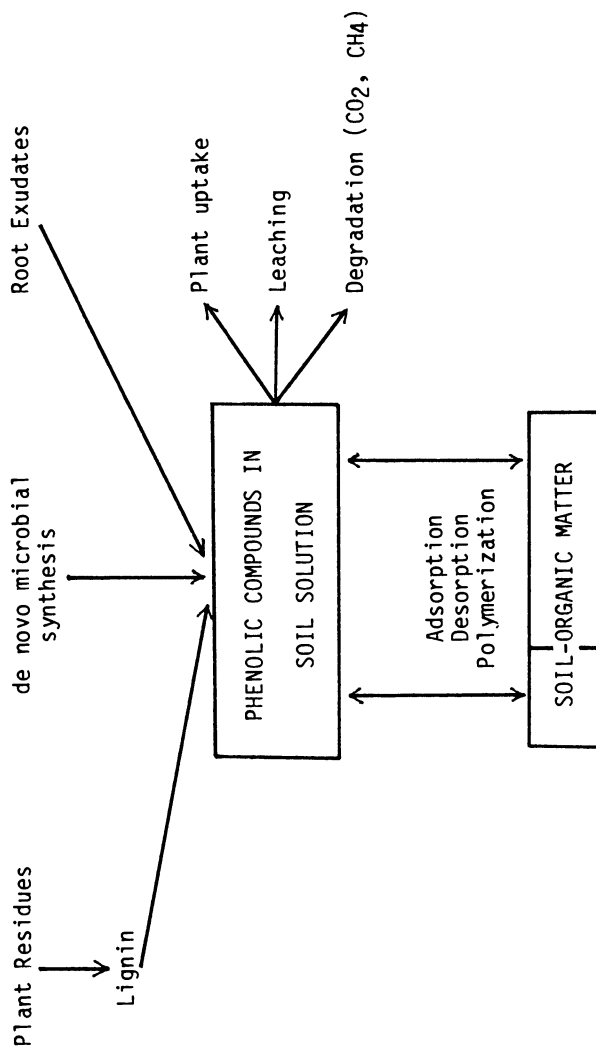


Figure 1. Selected sources and fate of phenolic compounds in soil.

### Agricultural Conservation Production Systems

Conservation agroecosystems developed in the Great Plains of the U.S. to control soil erosion are characterized by the presence of varying quantities of plant residues on the soil surface. This residue mulch protects the soil from the erosive forces of wind and water, resulting in improved stream water quality and soil conservation. Conservation tillage systems also help maintain soil productivity and reduce energy requirements of crop production (15). However, crop yield reduction has been observed with conservation wheat production in some areas of the U.S. (16-18) and with rice culture in the Far East (19, 20).

Unger and McCalla have pointed out factors (21) which could contribute to the observed yield reductions including lack of proper expertise and equipment to manage surface residues, lack of pest and disease control, inadequate nutrient availability, particularly nitrogen, major alterations in biological properties of soil, and production of phytotoxic chemicals.

While much research on the nature and impact of phytotoxins has been conducted, many questions remain unanswered. Little is known about the rate of phytotoxin production and accumulation, localized concentration, threshold soil concentration for expression of bioactivity, duration of bioactivity, stability in soil, and environmental redistribution. Although the fate of specific chemicals under defined experimental conditions is discussed, it is hoped that the principles involved can be extended to provide a basic understanding of the fate of the wide range of allelochemical substances in the soil.

### The Complex Soil Environment

Organic chemicals in the soil participate in many interactions and transformations between the gaseous, liquid, solid, and biological phases. The chemical finds its environment consisting of solid particles ranging in size from one to several millimeters down to sub-microscopic colloidal materials. The solid matrices may be coated with aqueous films or be part of gas-solid interfaces. Microorganisms are present in the voids and the fluid medium, forming the living phase of the complex environment (22-24).

The mineral phase. Mineral colloids are composed of layered silicates and amorphous metal hydroxides. The two basic building layers of the silicates are (i) a tetrahedral silicon dioxide layer modified by occasional substitution by  $Al^{3+}$  and (ii) an octahedral Al oxyhydroxide layer with occasional substitution by  $Mg^{2+}$ ,  $Fe^{2+}$ , or  $Fe^{3+}$ . The two types of layers can be found in a 1:1 arrangement as in kaolinite and halloysite clay minerals, or in a 2:1 arrangement as in montmorillonite, vermiculite, illite, and chlorite. Dissociation of edge hydroxyl groups and the substitution of  $Si^{4+}$  and/or  $Al^{3+}$  by lower-valency cations within the tetrahedral/octahedral layers result in a net negative charge. Moreover the layered silicates are often hydrated by a thin film of water. The water molecules in this film are highly structured and in conjunction with counter-ions in the bulk solution give rise to the very low surface pH. This high acidity of clay surfaces plays an important role in

the adsorption and catalytic reactions of phenolic compounds as will be shown later.

The organic phase. Soil organic matter that is in intimate contact with the mineral phase may be subdivided into two major fractions: (i) fresh or partly decayed plant or animal residues and (ii) humified or completely altered or resynthesized substances (24). The first group is referred to as nonhumic substances, and contains organic chemicals belonging to such classes as amino acids, carbohydrates, lipids, pigments and other low-molecular weight compounds. The second group, known as humic substances, are high-molecular weight, dark-colored substances formed by secondary synthesis reactions. Humic substances commonly are classified into three subcategories on the basis of solubility characteristics: (i) humic acid, dark-colored substance soluble in alkali but insoluble in acid, (ii) fulvic acid, the colored material which remains in solution or colloidal dispersion, after precipitation of humic acid by acidification, and (iii) humin, the acid and alkali-insoluble fraction. A variety of functional groups, including carboxyl, phenolic hydroxyl, alcoholic hydroxyl, enolic hydroxyl, quinone, lactone, and ether have been shown to be present in the humic substances. Carboxyl and phenolic hydroxyl groups are the source of the cation exchange capacity of the organic matter. Differences in degree of humification of organic matter are reflected in differences in the degree of reactivity and adsorptive behavior of the various fractions of the soil.

#### Behavior and fate of phenolic compounds in soil

The behavior of phenolic compounds derived from decaying plant residues, or released from degrading humic substances, is dictated by the physico-chemical processes of adsorption and desorption. Equilibria between these processes determine the concentration of phenolic compounds in the soil solution and consequently the bioactivity, movement, and persistence of these substances in the soil. Surface interactions between phenolic compounds and colloidal matrices may promote their polymerization (25, 26) or protect them from microbial degradation and mineralization.

The nature of soil-phenolic acid interaction: adsorption-desorption. Adsorption of a solute from solution onto a solid matrix results in a higher solute concentration at the fluid-solid interface than in the solution. Huang and coworkers (27) observed a high sorption capacity of the mineral fraction of four latosols for phenolic acids. On the basis of their results, distribution coefficients,  $K_d$ , or the ratios of solution-phase solute concentration and adsorbed-phase concentration were calculated to estimate the relative affinity of the soils for phenolic acids. The  $K_d$  values for p-hydroxybenzoic acid, p-coumaric, vanillic, ferulic, and syringic acids were 67, 75, 69, 92 and 376, respectively for a 48-hr equilibration of  $0.1 \mu\text{mol mL}^{-1}$  phenolic acid solution with a sample of an alfisol preextracted in boiling water. The sorption capacity was greatly reduced by pretreatment of soil samples with sodium acetate-hydrogen peroxide to remove organic matter and metal sesquioxides.  $K_d$  values were 49, 32, 61, 37 and 92 respectively for the phenolic

acids named, further treatment to remove more sesquioxide reduced the  $K_d$  values to 16, 20, 39, 30, and 54 respectively. Huang and coworkers postulated that complexation of the phenolic acids by noncrystalline sesquioxides was attributable to the interaction of phenolic hydroxyl and carboxyl groups with positively charged  $Al-OH_2^{0.5+}$  and  $Fe-OH_2^{0.5+}$  sites.

Approximately 40 to 50% of the total amount of phenolics sorbed was retained by the organic matter fraction (27). In surface soil layers, organic matter is frequently intimately associated with the mineral components present, providing a large surface area and reactive sites for surface interaction. Soil acidity has a major influence on phenolic adsorption by the organic carbon fraction, since the degree of dissociation of the phenolic acids is pH-dependent. Whitehead and coworkers (28) observed that the extractability of several phenolic acids was highly dependent upon the extractant pH between pH 6 and 14. The amount extractable continually increased with extractant pH; thus the extracted acids could not be readily classified into distinct fractions.

The adsorption of cinnamic acid on Renfrow silt loam was measured in our laboratory using the batch equilibration method and a liquid chromatographic technique. Freundlich adsorption coefficients were 2.0, 1.7, and 0.98 ( $N = 0.92, 0.94, \text{ and } 0.74$ ) at soil suspension pH of 4.5, 5.0, and 5.5. Our data agreed with the trend of extractability observed by Whitehead and co-workers (28). The adsorption of cinnamic acid decreased with increasing pH of the soil suspension. The magnitude of the partition coefficients indicated a low to intermediate adsorption potential for cinnamic acid. However, the observed high capacity of soils for retention of phenolic compounds (stabilization) may have in fact been due to their sorption on soil colloids. The term stabilization as used in the humus literature is to be interpreted as the overall disappearance of phenolic acids during contact with the soil medium. Thus the phenomenon includes the effects of adsorption, desorption, surface-induced polymerization, enzymatic polymerization, and immobilization in microbial tissues. Adsorption on colloidal surfaces may promote the formation of humic polymers by inducing favorable conformational arrangement for catalysis by metal sesquioxides (29) or by participation of free radical groups of soil organic matter (30, 31). Because of the reactivity of phenolic substances in soils, the processes of oxidative polymerization and degradation must be uncoupled from adsorption to properly assess the latter process. Several attempts have been made to obtain detailed kinetics of adsorption in microbially active systems. Ogram and co-workers (32) have shown that the herbicide 2,4-D can be microbially degraded only in the solution phase and by non-adsorbed degraders. Dao and Lavy (33) have observed that adsorption of reactive solutes such as phenol, aniline, and benzoic acid on soil was very rapid; equilibrium was attained in a few minutes. Degradation or surface-induced transformation would lead to erroneous estimates of adsorption. Experimental and mathematical approaches to uncoupling these two processes have been described (32, 33).

Although not specifically applicable to phenolic acids, hydrophobic adsorption of many xenobiotic compounds has been reported, such adsorption being dependent on the organic carbon content of the sorbing medium (34, 35). A liquid-liquid partition model has been

extensively used to describe this partitioning of organic solutes between organic matter and an aqueous solution. The 1-octanol-water model was found to provide satisfactory preliminary indices of adsorption potential for a wide variety of organic chemicals in soil (36, 37). Recently, computation and predictive correlations using linear free-energy relationships have become increasingly accurate alternatives to experimental measurements of partition coefficients (37, 38). Liquid chromatographic techniques have also been used to estimate the partitioning behavior of neutral as well as weakly ionizable chemicals (39, 40). Therefore, varied mechanisms of adsorption, ranging from physical dipole-to-dipole interaction and hydrogen bonding to oxidation-reduction, can effectively dictate the behavior of allelochemicals in soil.

Irrespective of the sources of phenolic compounds in soil, adsorption and desorption from soil colloids will determine their solution-phase concentration. Both processes are described by the same mathematical models, but they are not necessarily completely reversible. Complete reversibility refers to singular adsorption-desorption, an equilibrium in which the adsorbate is fully desorbed, with release as easy as retention. In non-singular adsorption-desorption equilibria, the release of the adsorbate may involve a different mechanism requiring a higher activation energy, resulting in different reaction kinetics and desorption coefficients. This phenomenon is commonly observed with pesticides (41, 42). An acute need exists for experimental data on the adsorption, desorption, and equilibria for phenolic compounds to properly assess their environmental chemistry in soil.

Adsorption has a significant impact on the movement of allelochemical substances in soil. Such movement in soil by water is important from the standpoint of mechanism of phytotoxin activity in the receiving species at a site remote from the donor plant. Adsorption reduces the solute concentration in the soil solution and consequently minimizes redistribution in the environment. Solute transport has been described by Fick's second law of diffusion and the kinetic models for adsorption and degradation of reactive solutes (43, 44). The contribution of adsorption is measured and expressed as the retardation factor,  $R$ .

$$R = 1 + \rho/\theta \cdot K \cdot \frac{C}{C_0} (N-1)$$

where  $C$  = solution-phase solute concentration,  $K$  and  $N$  = Freundlich adsorption coefficients,  $\rho$  = soil bulk density, and  $\theta$  = volumetric water content.

For singular adsorption-desorption processes where  $N = 1$ ,  $R$  becomes

$$R = 1 + \rho/\theta \cdot K$$

Thus unbound phenolic acids should be easily transported by convection. Shindo and Kuwatsuka (45) have observed this in leaching experiments.

Phenolic acid metabolism. During the last 30 years, much has been learned about the production of phytotoxins during plant residue decomposition in the laboratory and in the field (6-8, 18). However, little is known about the fate of these molecules in the soil.

Some clues may be available from studies of the decomposition of lignin. Lignin constitutes the second most abundant carbon polymer on earth after cellulose (46). The understanding of biodegradative pathways of lignin and lignin-cellulosic polymers may elucidate the problems of reduced plant productivity associated with surface residues in conservation production systems.

a. Lignin biodegradation as a source of phenolic acids in soils. Plants synthesize phenolic acids, which are then combined to form polymers such as lignin, lignin-celluloses, flavonoids, and tannins. The flavonoids and tannins occur in plants as pigments. Lignin and lignin-celluloses are important for the structural integrity of the plant and impart rigidity to cell walls, decrease water permeation across cell walls, and resist microbial invasion of plant tissues (46). When the plant senesces and decays, the phenolic polymers are attacked by fungi of various genera, including Aspergillus, Aureobasidium, Basidiomycetes, Cephalosporium, Fusarium, Hemicola, Neurospora, and Schizophyllum (47). These polymers and their degradation products may also be degraded by bacteria, most notably the gram-negative strains (47).

Soil microorganisms degrade the polymers by cleaving off sub-units consisting of one, two, or at most three phenolic acid moieties. Fungal degradation of lignins appears to be essentially oxidative and decayed lignins exhibited three main changes from the parent lignin: (i) oxidation of the substituted side chains, (ii) oxidation of the alpha-carbon in the propanoid side chains, and (iii) cleavage of aromatic rings still attached to the polymer (47). Colberg and Young (48) showed that the extent of degradation of lignins in a given time increased as molecular size decreased. Relatively large fractions having an average molecular size of 1,000 to 1,400 lost 21% of the total available carbon as gaseous products, while molecules having an average molecular size of 400 to 1,000 lost 32% of the available carbon. Molecules of average molecular size less than 400 had 40% of the carbon oxidized to CO<sub>2</sub>. These results agreed with those of Crawford and co-workers (49) who found that larger phenolic molecules, particularly polymeric ones, are more stable than small, free monomeric phenolic acids. Because of the stability of large polymers, the rate-determining steps in the biological transformation of lignins to gaseous products occur at the stage where lignins are broken down into the monomeric phenolic acids (50).

Often this decomposition and its rate-determining steps are expressed in terms of first-order kinetics (51). Of course the decomposition rate coefficient is affected by various factors such as temperature, pH, soil moisture, and substrate concentration and composition. Many studies have shown that plant residues are first decomposed rapidly, then more slowly. The soluble fraction is rapidly metabolized, followed by the cellulose fraction of the lignin-celluloses, then the relatively slow-degrading lignin component. Crawford (52) showed that the cellulose component can decompose four to ten times as fast as the corresponding lignin. Since decomposition is an enzymatic process in microorganisms, factors that adversely affect the microorganisms or the enzymes decrease the decomposition rate. For instance, as the temperature decreases, soil dries, or pH changes from the optimum pH level for the enzyme system, decomposition becomes slower (51).

The monomeric phenolic compounds released during lignin degradation have been shown to consist mainly of benzoic and cinnamic acids and their derivatives, including the aldehyde forms (47, 53). Colberg and Young (54) isolated ten monoaromatic compounds produced in lignin microbial degradation, including catechol, phenylacetic, benzoic, 3-phenylpropionic, cinnamic, syringic, vanillic, ferulic and caffeic acids, and vanillin. Cinnamic, benzoic, caffeic, vanillic, and ferulic acids were found in the largest amounts.

Regardless of the source, phenolic acids are ultimately broken down to gaseous products such as CO<sub>2</sub> and methane. This breakdown occurs by three general methods: (i) aerobic respiration, using molecular oxygen as an electron acceptor, the end product being CO<sub>2</sub>, (ii) anaerobic respiration with electron acceptors such as nitrate and (iii) anaerobic fermentation with phosphorylation reactions involving no external electron acceptor (50).

b. *Aerobic catabolism of phenolic compounds.* The aerobic breakdown of phenolics compounds generally following the pathways outlined by Gibson (55). Degradation of the side chain occurs via a beta oxidation reaction with the enzymatic removal of two-carbon-atom units. Thus phenolic acids with an odd number of carbon atoms in the side chain ultimately yield hydroxybenzoic acids, while those with an even number of atoms in the carbon side chain are converted to hydroxyphenylacetic acids. The phenolic acid is then further hydroxylated and possibly decarboxylated to form a ring cleavage precursor, such as catechol or protocatechuic acid in the case of hydroxybenzoic acid, and 2,5-dihydroxyphenylacetic acid or 3,4-dihydroxyphenylacetic acid in the case of hydroxyphenylacetic acid. The hydroxylated benzene ring then undergoes fission by ring opening between the two hydroxyl groups with a dioxygenase to yield aliphatic acids which are then oxidized to CO<sub>2</sub> (56).

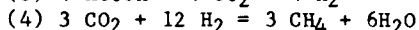
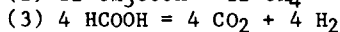
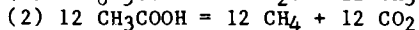
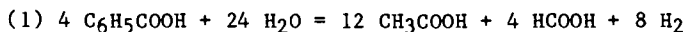
Once lignin has been degraded into monomeric products, the degradation of the individual aromatic monomers proceeds quite rapidly. Haider and Martin (53) have shown that <sup>14</sup>C-labeled benzoic and cinnamic acids and their derivatives can be aerobically mineralized in the first two weeks, and some of the compounds had over 90% of their carbon converted to CO<sub>2</sub> in one week.

Since these reactions are relatively rapid, i.e., phenolic acids are rapidly degraded aerobically, their presence in the soil under these conditions appears transitory. It has been difficult to detect unbound phenolic acids in the soil solution and the compounds do not appear to accumulate in appreciable amounts under aerobic conditions. However, the soil is a heterogeneous medium consisting of loci or microenvironments that are at times completely opposite in character, i.e., anerobic microsities in a well-aerated soil (57). The phytotoxicity problem should be viewed in the context of a spacially variable environment.

c. *Anaerobic catabolism.* In soil, free phenolic acids may encounter anaerobic conditions, particularly when the soil has poor drainage, has been temporarily flooded, or is in the center of soil aggregates. Sommers and co-workers (51) note that as soil water potential decreases different groups of phenolic-degrading microorganisms become active. They noted at soil water potentials in the

range of plant growth (-0.03 to -1.5 MPa), microbial metabolism takes place under aerobic conditions with oxidation of carbon sources to CO<sub>2</sub>. As the water content of the soil increases, the microbial population shifts to facultative anaerobic organisms that degrade the phenolic compounds through fermentation reactions with production of organic acids, alcohols, and other partially oxidized carbon compounds. At even lower water potential, as oxygen becomes limiting, the microbial population shifts to obligate anaerobic organisms. As the soil becomes more reduced, the electron acceptors used by the organisms change, usually following the sequence of (i) O<sub>2</sub>, (ii) NO<sub>3</sub><sup>-</sup>, (iii) Mn<sup>3+</sup>, (iv) Fe<sup>3+</sup>, (v) SO<sub>4</sub><sup>2-</sup>, (vi) H<sub>2</sub>, and (vii) CO<sub>2</sub>. The latter electron acceptors result in the formation of gaseous products characteristic of anaerobic systems such as CH<sub>4</sub>, N<sub>2</sub>, N<sub>2</sub>O, H<sub>2</sub>, and H<sub>2</sub>S.

Evans (50) has shown that anaerobic fermentation is often a two-stage reaction. A consortium of microorganisms mineralizes the aromatic compounds to methane and CO<sub>2</sub>. First the benzene ring is reduced to a cyclohexane ring and then cleaved to aliphatic acids such as adipate, heptanoate and heptanedioate, (pimelate) by gram-negative organisms. These acids are then broken down to form acetate and formate, which in turn are mineralized by methagenic bacteria to CO<sub>2</sub> and methane. Ferry and Wolfe (cited in 50) showed that the conversion of phenolic acids to methane followed the reductive pathway for benzoate:



for a total reaction of  $4 \text{ C}_6\text{H}_5\text{COOH} + 18 \text{ H}_2\text{O} = 15 \text{ CH}_4 + 13 \text{ CO}_2$ .

Healy and Young (58) observed that the conversion of vanillic and ferulic acids under anaerobic conditions to methane and CO<sub>2</sub> was nearly stoichiometric. More than half of the organic carbon could potentially be converted to methane. This could have great importance in studies where the degradation of phenolic compounds are studied by trapping the evolved CO<sub>2</sub>. Under anaerobic conditions, part of the normal CO<sub>2</sub> evolution may be shifted to methane production with a subsequent low reporting of CO<sub>2</sub> evolved, and an underestimation of microbial activity in the soil (51).

Colberg and Young (48) have also shown that there could be an effect on the degradation of lignin itself, because under anaerobic conditions the methagenic consortium can break the beta-aryl bond, the most common linkage of aromatic monomers in lignins, to release phenolic compounds for further degradation. These processes occur at a lower rate than under aerobic conditions, so some of these phenolic acids and their breakdown products may accumulate in the soil.

d. Synthesis of humic acids by oxidative polymerization of phenolic acids. Phenolic acids and their polymers in the soil are in a continual state of flux, constantly being polymerized and partially degraded, broken down and resynthesized and adsorbed and released as they are eventually immobilized and mineralized by soil microorganisms.



Phenolic compounds can be condensed forming aryl-aryl and aryl-oxygen-aryl (ether linkages) bonds to yield diaryl and diaryl ether polymers (59). These are in many ways similar to natural humic acids, confirming earlier research by others (60-62) that humic acids are formed from the copolymerization of phenolic compounds with amino acids, peptides, and amino sugars.

The rate at which phenolic acid units are incorporated into humic fractions depends on many factors. Berry and Boyd (63) used a peroxidase enzyme from horseradish to study the oxidative coupling of phenols and anilines and found that the reaction rates were on the order of 10 to 185  $\mu$ moles/s for a number of methyl- and methoxy-phenols and anilines at 20°C. They suggested that the degree to which such compounds polymerize through enzymatic oxidative coupling reactions would be affected by substituent groups on the aromatic ring. Electron-withdrawing functional groups would inhibit the polymerization whereas electron-donating groups (such as OCH<sub>3</sub>) that commonly occur on lignin-derived phenols would enhance it. The reactivity of substituted phenols and anilines were predictable from the position and nature of the substituent group, as reflected by the Hammett constant (64).

The yield of humic acid was found to be higher when the concentration of phenolic acids was low; when the concentration of unbound acids was high, they were used as a substrate for microbial mineralization, with a subsequent reduction in humic acid synthesis (65). Kassim and co-workers (31) further showed that a significant portion of intact <sup>14</sup>C-labeled ferulic acid was stabilized into soil humic substances. Two percent of the added carbon-14 remained in the soil biomass after one year, representing 5 to 7% of the added ferulic acid. The degree of stabilization is related to the ease of free radical formation via the activity of phenolase or peroxidase enzymes. Solid humic acid was found to exhibit paramagnetic resonance due to the presence of unpaired electrons (30). Stable organic free radicals occur in humic acid on the order of 10<sup>8</sup> radical/g and appear to be an integral part of the humic acid structure. Their presence points to a humic acid biosynthetic pathway based on oxidative coupling of phenolic compounds via semiquinone or quinhydrone-type free radicals (30, 59).

Phenolic compounds have also been oxidatively polymerized to humic substances by clay minerals (29) and by the mineral fraction of a latasol (66). After a 10-day equilibration period, montmorillonite and illite clay minerals yielded 44 to 47% of the total added phenolic acids as humic substances whereas quartz gave only 9%. Samples of a latasol yielded over 63% of the total amount, from mixtures in varied proportion, of mono-, di- and trihydroxy phenolic compounds as humic substances (66). Extractions of the reaction products yielded humic, fulvic, and humin fractions that resembled soil natural fractions in color, in acid-base solubility, and in infrared absorption spectra. Wang and co-workers (67) further showed that the catalytic polymerization of catechol to humic substances was enhanced by the presence of Al oxide and increased with pH in the 5.0 to 7.0 range. Thus the normally very reactive products of lignin degradation can be linked into very stable humic acid polymers which will maintain a pool of potentially reactive phytotoxins in the soil.

### Summary and Conclusions

In summary, much is known about the sources, the behavior, and fate of phenolic substances in soil. However, much of this information has been gained under relatively harsh and unnatural conditions. Improved extraction and isolation methodology must be developed for allelochemical studies in soil. Knowledge of the relationship between extracted and actual bioactive chemical entities is critical in assessing allelopathic interactions. Process models will also provide much insight for describing, predicting the presence and effective concentration of allelochemical substances, and relating them to the expression of a toxic response in higher plants. New understanding of the microbiology of the lignin-cellulosic and humic polymers in natural soil environment and the degradation of the lignin component in agricultural systems is needed. The productivity of millions of acres of rangelands and cultivated lands under conservation production practices hinges on the improved understanding of the turnover of these polymeric components of the carbon cycle. Research efforts may provide some answers to questions of allelopathy involving plant residues in conservation production systems, crop-residue and weed-residue interactions, and the biocontrol potential in many of these agroecosystems.

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## Chapter 34

# Allelopathic Influences on No Tillage Versus Conventional Tillage in Wheat Production

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Incorporating allelopathy into agricultural management may reduce the use of herbicides, cause less pollution, and diminish autotoxic hazards. Authentic inhibitors isolated from plant material have been subjects for examination *in vitro*, but attempts to compare their effects in soils are limited. Soils contain a heterogeneous collection of organic matter of various origins. Organic solvents and water extracts prepared from monoculture wheat soils under conventional tillage (CT) and no tillage (NT) indicated that both soils contain some inhibitory compounds. The CGC/MS/DA of some of the organics is presented. Selected organics from CT and NT as well as allelopathic and autotoxic effects are described and discussed. The relationship between the wheat yields in CT and NT and the possible biological stress is indicated.

Conservation tillage practices in wheat production have increased steadily from about 15% in 1971 to 30% in 1985. These practices are attractive to growers for soil and moisture conservation and fuel savings, but there may be drawbacks. Variations in yields of forage and grain compared with those of conventional tillage differ according to rainfall conditions and geographic location. These erratic results are probably also partly attributable to biological factors such as diseases, insect damage, and allelopathy, which can vary substantially from season to season. Researchers elsewhere have generally shown that allelopathy from wheat residue reduces the subsequent wheat yield (1-3). There is reason to believe that the same phenomenon is occurring in the Great Plains Area as reported here, though under some circumstances it may be masked by the favorable effects of no-till growing such as greater retention of soil moisture. Allelopathic chemicals from soils, crop residues, and weeds are known to reduce the growth of several crops, and there are numerous examples of allelopathy among wild plants. We are studying the allelopathic effects of wheat residue and soil on the germination and growth of wheat under Oklahoma conventional-tillage and no-tillage conditions. We see this as basic research aimed at determining the presence and magnitude of any allelopathic effects of wheat on itself, as well as the identity of the chemicals causing them. This would, of course, be a necessary first step in remedying allelopathic effects and increasing wheat yield.

Whittaker (4), Waller and Nowacki (5), and Rabotnov (6) discussed the evolution of stable plant communities and species susceptible to allelopathic chemicals that were released by other plants. Such plants would have been eliminated by natural selection, and allelopathically neutral or allelopathically

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tolerant plant communities would result. Allelopathy is often more evident in disturbed plant communities, such as agricultural ones.

McCalla and coworkers (1-3, 7) did pioneer research on the wheat crop in the eastern Nebraska area. They showed that water-soluble substances in crop residues reduced the germination and growth of seedlings of wheat, corn, sorghum, and other crops. The water extracts of the seeds had the least effect and the stem extracts had the greatest inhibitory effect on wheat seedlings. Wheat as well as other crops were shown to contain a number of phenolic acids and the five most dominant ones were: ferulic, *p*-coumaric, syringic, vanillic, and *p*-hydroxybenzoic acids. These were quantitatively estimated in the crop residues; e.g., the total amount of phenolic acids from wheat left on the field was 1.5 tons/acre under no-tillage conditions. McCalla and Norstadt (3) worked extensively on the antibiotic patulin (C<sub>7</sub>H<sub>8</sub>O<sub>4</sub>; M.W. 156) produced by *Penicillium urticae* Bainer, which was found in wheat soil, and found that the severity of visible symptoms of phytotoxicity to winter wheat (no-tillage) corresponded to the concentration of patulin. Elliott et al. provided a review of phytotoxicity in 1978 (8) and Elliott et al. (9) showed that bacterial colonization of plant roots can cause a 25% increase in the release of allelochemical compounds produced. Putman and DeFrank (10) made use of phytotoxic plant residues for selective weed control. Lehle and Putman (11) used sorghum plants to show that the self-inhibitory activity (autotoxicity) varied widely depending upon the stage of development. Schilling et al. (12) showed that compounds, some identified ( $\beta$ -phenyllactic acid,  $\beta$ -hydroxybutyric acid) and some not identified, were effective in the suppression of certain weeds by rye and wheat mulches in no-till crops.

The data otherwise accumulated by the Oklahoma Agricultural Experiment Station so far indicate that for 6 years the wheat yield obtained in studies comparing conventional-tillage and no-tillage averages is 42 bu/acre for each. This is independent of location. However, there is an indication that the soil systems have not stabilized with the change in tillage practices; thus, one would not expect to see any real differences in the soil as it relates to the wheat crop yields between the tillage treatments. Factors essential to maintain wheat crop yields are the availability of water and nutrients, control of insects, and disease. We have found that the forage yield (hay) from the wheat crop taken at the early jointing stage for the conventional-tillage system is twice that for no-tillage. If this difference continues to be found in future years then it provides some evidence for an allelopathic effect occurring early in the growing season.

Our objective was to study in detail these systems by identifying the allelochemicals involved, their primary modes of action as mediated by soils, and as appropriate the microflora, microclimate, soil moisture and other factors that may influence allelopathy.

### Experimental

Sampling of the Soil. Representative soil samples were taken from conventional and no-tillage plots before planting and afterward at intervals of 1 month since April, 1985. The soil samples were placed in quart jars, frozen immediately by using dry ice, and stored at -18 °C.

Extraction of Organic Compounds from the Soil. Soil biochemicals that are free or absorbed loosely, but not bound to the humus, were extracted by the following procedures:

- A) A 100-200-g sample of soil was thawed, placed in an extraction thimble, and extracted with redistilled isopropyl alcohol in a Soxhlet extractor for 48 h. Some of these alcohol extracts were analyzed for

their biological activity and others were evaporated and the residue was extracted with water at 60 °C, 3 times, and the mixture filtered through 7-mm Whatman 41 filter paper; the insoluble part was extracted with methyl alcohol at room temperature, and those compounds that remained were redissolved in isopropyl alcohol. Each extract was weighed and bioassayed.

- B) A 200-g sample of soil was loosely packed in a 24 x 40 cm chromatography column and sequentially extracted with redistilled organic solvents at room temperature and a flow rate of 0.5 mL per minute in the following order; 200 mL hexane; 100 mL hexane + 100 mL methylene chloride; 200 mL methylene chloride; 100 mL methylene chloride + 100 mL ethyl acetate; 200 mL ethyl acetate; 100 mL ethyl acetate + 100 mL methyl alcohol; 200 mL methyl alcohol; 100 mL methyl alcohol + 100 mL triply distilled water; 800 mL triply distilled water. Each extract was taken to dryness over nitrogen gas except the aqueous one, which was evaporated with a rotary evaporator at 45 °C. Each residue was weighed and bioassayed.
- C) Steam distillation, extraction, and evaporation (Waller et al. [13, 14]) were completed and the resulting mixtures bioassayed.

Analysis of the Mixture of Organic Compounds from the Soil. The crude fractions were analyzed using a LKB-2091 capillary gas chromatograph/mass spectrometer/data analysis system (CGC/MS/DA). The capillary column used was a J & W DB-1, 60 m x 0.32 mm, connected directly to the ion source of the mass spectrometer. Up to 1.0 µL of a solution of the sample in an appropriate solvent was injected directly onto the column at 40 °C, whereupon the column temperature was immediately raised to 100 °C for 4 min, and programmed to 310 °C at a rate of 10°/min. and held there for 30 min.

Bioassay of Organic Compounds from the Soil. Bioassay experiments measured the germination and early growth (generally the most sensitive time of any plant's life) of wheat. The methods are similar to those of McPherson and Muller (15) and others in the field, and are summarized below.

Containers, media and seeds. Glass Petri dishes, 100 x 15 cm, were used with two sheets of 75-mm Whatman 41 filter paper as the absorptive medium. Ten seeds of TAM105 wheat were placed in a radial pattern with the micropyle end toward the center between the two sheets of filter paper. Seeds were hand-selected for normal size and absence of damage. TAM105 was selected because it is the variety used in the ongoing field research on conservation tillage practices. The bottom section of each Petri dish cover was covered with a square of kitchen-type plastic wrap to retard moisture loss before the lid was pressed on.

Allelopathic test materials and controls. Some 2.5 mL of aqueous or organic extracts were required for thorough saturation. Water-soluble or partially water-soluble extracts were applied directly to the filter paper. Distilled water controls were used. With organic solvent-soluble extracts, the solution was applied to the filter paper and allowed to dry, then distilled water was added to support germination. Controls having pure solvent applied were similarly allowed to dry before the distilled water was added. Quantification of the amount of allelopathic material applied to each sample

was made by weighing the amount of extract so that a consistent ratio could be maintained. Records of the amounts were kept so that a consistent calculation of concentrations could be made.

Incubation conditions. Preliminary trials indicated that incubation at 20 °C for 72 h in darkness is optimal. This relatively low temperature allows adequate wheat growth while retarding mold development.

Replication. Six Petri dishes each containing ten seeds were used for each control and for each treatment. Controls accompanied all experiments.

Results, parameters, and measurements. Counts of germinated vs. ungerminated seed, length and width of coleoptile, and length of central root and stem were recorded. Means per dish and per treatment (four to six dishes) were calculated and standard statistical tests were used in the analysis.

## Results

This paper reports the initial results of a new study. We collected soil samples from the Agronomy Farm at Stillwater at monthly intervals (April-July, 1985) and extracted them with isopropyl alcohol in a Soxhlet apparatus. The bioassay of the dried extracts of soil from no-tillage vs conventional-tillage plots is shown in Tables I, IIa and III. Surprisingly both types of soils were inhibitory to the growth of wheat. The Soxhlet extract of June soil was dried in a rotary evaporator and then subjected to successive methyl alcohol and water extractions. The bioassay results are shown in Table IIb and show that the conventional-tillage soil contained no more allelopathic material than did the no-tillage soil. In view of a laboratory error that influenced these results, we believe that the extract of no-tillage soil may have equalled or exceeded the conventional-tillage soil in allelopathic potency.

Soil collected at other locations (El Reno and Altus) were extracted and steam distilled as described in Experimental. No-tillage samples of soil were used; however, they varied from Altus plots devoted to monoculture for 10 years previously, to soil that had been used to grow wheat only one previous year (El Reno) which had been part of a virgin prairie until cultivation. The results, shown in Table IV, again indicate that both these no-tillage soils are allelopathic toward wheat. Products of steam distillation, although this is a severe treatment, showed slight growth inhibition by the initial (pH 6.0) fraction, whereas those from the highly basic soil suspensions were markedly inhibitory. Table V shows the number of compounds obtained from Tillman soil obtained by steam distillation as identified by the CGC/MS/DA system; the soil has quite an array of organic compounds -- some quite complex (13, 14). In the milder treatment by solvent extraction for El Reno soil, shown in Table IVb, the aqueous fraction was more allelopathic than the ethyl acetate/methylene chloride fraction. The duplicate plots (Table IVb, no-till I & II) showed some difference in bioassay results which cannot be explained just now.

Also shown in Tables I-IV are the amounts of crude organic extract and the amount of soil extracted; each represents the quantity that was bioassayed per wheat seed. These amounts represent less than the soil mass in the normal seedling environment. The quantity of organic matter that is present in the soil around the germinating seed and seedling is striking. It strongly suggests that this soil organic matter is a subject about which scientists should be concerned. In fact, solvent extraction (Table IVb) shows the quantity of organics in the soil and is representative of what would be found in nature.

Presented in Figures 1a and 2a are reconstructed partial total ion current chromatograms obtained by the CGC/MS/DA run on the May, 1985 samples. Figures 1b and 2b show mass spectra taken at a certain specified time and peak number. Figure 1b shows the mass spectrum of phthalate plasticizer in the soil



Table I. Wheat Bioassay Of Soxhlet Extracts of Wheat Soil  
Collected April 9, 1985

Experimental Soil	Root Length (mm)	Shoot Length (mm)	Inhibition %		Amount of Crude Organic Extract (mg/seed)	Amount of Soil Extracted (g/seed)
			Root	Shoot		
Control, Dist. H <sub>2</sub> O	23.1 ± 0.9	6.6 ± 0.2				
No-Till, Aq. Ext.	15.5 ± 2.9 <sup>b</sup>	5.3 ± 0.7 <sup>b</sup>	32	19	0.76	3.5
Conv-Till, Aq. Ext.	20.7 ± 4.2 <sup>c</sup>	6.5 ± 0.8 <sup>c</sup>	10	2	0.75	3.6

<sup>b</sup> Significantly different from control at 95% level of confidence or better (t-test).

<sup>c</sup> Not significantly different from control (t-test).

Table II. Wheat Bioassay Of Soxhlet Extracts of Wheat Soil  
Collected June 10, 1985

A	Experimental Soil	Root Length (mm)	Shoot Length (mm)	$\frac{\text{Inhibition \%}}{\text{Root}}$	$\frac{\text{Shoot}}{\text{Shoot}}$	Amount of Crude Organic Extract (mg/seed)	Amount of Soil Extracted (g/seed)
	Control, Dist. H <sub>2</sub> O	18.0 ± 1.7	5.7 ± 0.1				
	No-Till, Aq. Ext.	4.9 ± 1.3 <sup>a</sup>	3.5 ± 0.5 <sup>a</sup>	83	39	0.7*	2.8
	Conv-Till, Aq. Ext.	3.3 ± 0.5 <sup>a</sup>	2.9 ± 0.3 <sup>a</sup>	92	49	1.4	2.5

B	Experimental Soil	Root Length (mm)	Shoot Length (mm)	$\frac{\text{Inhibition \%}}{\text{Root Shoot}}$	Amount of Crude Organic Extract (mg/seed)	Amount of Soil Extracted (g/seed)
	Control, Dist. H <sub>2</sub> O and MeOH Ext.	23.8 ± 4.6	6.5 ± 0.7			
	No-Till, MeOH Ext.	22.8 ± 1.7 <sup>c</sup>	6.2 ± 0.5 <sup>c</sup>	4	0.7*	2.8
	Conv-Till, MeOH Ext.	18.2 ± 3.3 <sup>b</sup>	5.0 ± 0.9 <sup>b</sup>	24	1.4	2.5

\*Lost about one-half of extract by accident.

<sup>a</sup> Significantly different from control at 99.9% level of confidence or better (t-test).

<sup>b</sup> Significantly different from control at 95% level of confidence or better (t-test).

<sup>c</sup> Not significantly different from the control (t-test).

Table III. Wheat Bioassay Of Soxhlet Extract of Wheat Soil  
Collected July 9, 1985

Experimental Soil	Root Length (mm)	Shoot Length (mm)	Inhibition % Root      Shoot	Amount of Crude Organic Extract (mg/seed)	Amount of Soil Extracted (g/seed)
Control, Dist. H <sub>2</sub> O	23.8 ± 4.6	6.5 ± 0.7			
No-Till, Aq. Ext.	16.6 <sup>b</sup> ± 1.9 <sup>b</sup>	5.2 ± 0.3 <sup>b</sup>	30      20	0.45	1.8
Conv-Till, Aq. Ext.	18.7 ± 2.5 <sup>b</sup>	5.2 ± 0.6 <sup>b</sup>	22      20	0.58	2.0

<sup>b</sup> Significantly different from control at 95% level of confidence or better (t-test).

Table IV. Wheat Bioassay Of (A) Fractions Of Steam Distillates of Altus, OK and (B) Solvent Extracts of El Reno, OK Wheat Soil

Method of Obtaining Organics from Experimental Soil	Root Length (mm)	Shoot Length (mm)	$\frac{\text{Inhibition}}{\text{Root Shoot}} \%$	Amount of Crude Organic Extract (mg/seed)	Amount of Soil Extracted (g/seed)
<b>A- Steam Distillation, Extraction, Evaporation (2-kg sample)</b>					
Control, Dist.H <sub>2</sub> O	24.4 ± 0.7	7.2 ± 0.6			
No-Till Made at pH 5.9 (natural)	23.7 ± 1.7 <sup>c</sup>	6.5 ± 0.3 <sup>c</sup>	10	2.9	67
No-Till Made at pH 11	3.9 ± 1.4 <sup>a</sup>	2.7 ± 0.6 <sup>a</sup>	85	3.5	67

Continued on next page

Table IV. Continued

Method of Obtaining Organics from Experimental Soil	Root Length (mm)	Shoot Length	Inhibition % Root Shoot	Amount of Crude Organic Extract (mg/seed)	Amount of Soil Extracted (g/seed)
<b>B-Solvent Extraction and Evaporation (200-g sample)</b>					
Control, Dist. H <sub>2</sub> O	24.4 ± 0.7	7.2 ± 0.6			
No-Till-Plot I Aq. Ext.	16.2 ± 2.5 <sup>b</sup>	6.3 ± 0.7 <sup>b</sup>	39	2.5	6.7
No-Till-Plot II Aq. Ext.	21.8 ± 5.2 <sup>c</sup>	7.3 ± 0.7 <sup>c</sup>	17	2.5	6.7
No-Till-Plot I EtOAc + CH <sub>2</sub> Cl <sub>2</sub>	23.4 ± 0.2 <sup>c</sup>	7.2 ± 0.6 <sup>c</sup>	11		6.7

<sup>a</sup> Significantly different from control at 99.9% level of confidence or better (t-test).

<sup>b</sup> Significantly different from control at 95% level of confidence or better (t-test).

<sup>c</sup> Not significantly different from control (t-test).

Table V. Compound Groups Obtained From Soil By Steam Distillation As Identified By CGC/MS/DA System

	<u>Initial</u>	<u>Acidic</u>	<u>Basic</u>
Fatty acids	8	20	0
Fatty acid esters	0	3*	0
Alcohols	1	2	3
Aldehydes	3	6	8
Ketones	1	4	2
C-N and other N-contg. compounds	4	5	17
S-contg. compounds	1	1	2
Cl-contg. compounds	2	0	0
Aromatics not otherwise included	7	1	23
Aliphatics not otherwise included	11	33	16
	39	75	75
Totals	39	75	75
*All ethyl esters			

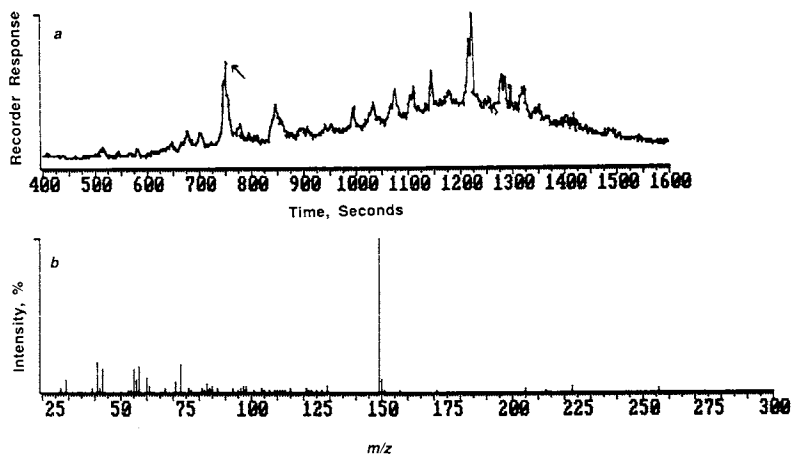


Figure 1. a) Reconstructed Part of the Total Ion Current Chromatogram of a No-Tillage Soil Extract (LKB-2091 CGC/MS/DA): Peaks Represent Compounds. Soil Sample: May 9, 1985

b) Mass Spectrum of a Phthalate Plasticizer Present in the Soxhlet Soil Extract That Corresponds to Peak (750 s) Marked with the Cursor.

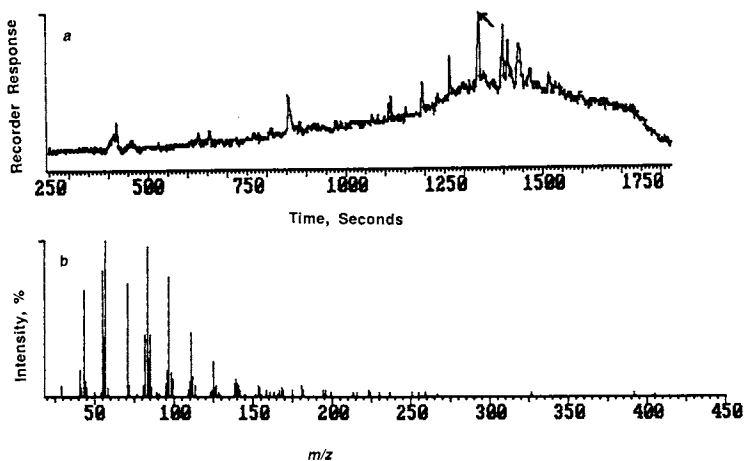


Figure 2. a) Reconstructed Part of the Total Ion Current Chromatogram of a Conventional-Tillage Soil Extract (LKB-2091 CGC/MS/DA): Peaks Represent Compounds. Soil Sample: May 9, 1985

b) Mass Spectrum of a Hydrocarbon Present in the Soxhlet Soil Extract That Corresponds to Peak (1338 s) Marked with the Cursor.



extract giving the peak at 749 s. We believe it to be an actual soil component and not an artifact of our work since our handling of the sample used all-glass equipment with Teflon stopcocks and closures without stopcock grease. We have not determined whether or not it is phytotoxic. Figure 2b is a mass spectrum of a hydrocarbon from the soil extract. It is probably not an allelopathic compound.

Allelopathic activity toward germinating wheat was clearly demonstrated with extracts of Oklahoma soils. However, a convincing difference in activities in no-till and conventional-till soil has not yet appeared. The total ion current chromatograms (Figures 1a and 2a) of the CGC/MS/DA illustrate the complexity of the soil extracts. Some of these compounds, if isolated, may serve as new effective biodegradable insecticides, herbicides, or fungicides.

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## Chapter 35

# Studies on the Fulvic and Humic Acids of Minnesota Peat

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Fulvic and humic acids have been investigated with carbon-13 and proton nuclear magnetic resonance spectrometry, GC/MS, and IR spectroscopy. The fulvic and humic acids were found to be predominantly carboxylic and aromatic with a high proportion of O- and N-substituted carbon atoms, although aliphatic ones were also observed.

Humic substances constitute a very important class of natural products, especially fulvic and humic acids. These are present in soil, water, and coal throughout the world (1) and participate in many significant agricultural, geochemical, and environmental processes (1-4). Examination of recent publications shows that there is an increasing interest in these materials by chemists, soil scientists, hydrologists, organic geochemists, and others in environmental sciences. Chemical investigations on humic substances have occupied the attention of scientists for more than 200 years (1,2); but relatively little progress has been made in the elucidation of their chemical nature as compared to that of other natural products such as proteins, polynucleotides, and polysaccharides. However, this is not to imply that no progress has been made at all in the study of these substances. A vast amount of information has been accumulated on the chemical, physical, biological, geochemical and agricultural aspects of humic substances, but it has not been possible to integrate this knowledge within a satisfactory conceptual framework of the nature of these substances.

Although soil organic matter has been extensively studied by spectroscopic methods, very few investigators have studied peat as a source of humic substances (5-8). This paper presents carbon-13, proton nuclear magnetic resonance (<sup>13</sup>C-NMR and <sup>1</sup>H-NMR) spectra,

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proton nuclear magnetic resonance ( $^{13}\text{C}$ -NMR and  $^1\text{H}$ -NMR) spectra, GC/MS, and IR absorption spectra of fulvic and humic acids of Minnesota peat, and conclusions about the nature of both types of acids.

### Materials and Methods

Materials. The peat, taken from St. Louis County, Minnesota, is derived from mosses of the genus *Sphagnum*. It also contains remains of some ericaceous shrubs and few forbs and sedges. It was air-dried to approximately 15% moisture, coarse-screened to remove woody particles, and pulverized in a laboratory Wiley mill fitted with a 20-mesh screen. All solvents were distilled before use.

Preparation of Fulvic and Humic Acids. Waxes, resins, and other substances soluble in organic solvents were removed by successive extractions with petroleum ether (35-60°), chloroform, and ethyl acetate. These extractions removed 4% of the original material. The residual peat was air-dried to remove solvents. Wax and resin-free peat (50 g) was stirred with 1 liter of 0.5N NaOH for 24 h. The insoluble material was removed by centrifuging at 5000 rpm for 15 min in a Beckman Model No J-6B centrifuge. This extraction with alkali was repeated twice more and the solutions of sodium salts of acids were collected. Humic acids were precipitated from the combined NaOH solutions by adjusting the pH to 1 with 2N HCl slowly with stirring and the mixture was left overnight. The precipitated humic acids were collected by filtration through Whatman 1MM paper and washed with 0.1N HCl. The filtrates were extracted three times with ethyl acetate and the extracts dried over sodium sulfate and evaporated, the residue constituting the fulvic acids. Both fulvic and humic acids (precipitates) were air-dried, and then dried in a vacuum desiccator over phosphorus pentoxide at room temperature. The yields were 3.5 g and 18.9 g respectively.

Samples of both fulvic and humic acids were suspended in methanol and methylated with diazomethane. Both  $^1\text{H}$  and  $^{13}\text{C}$  spectra of the free acids were obtained, at 299.94 MHz and 75.42 MHz respectively, on a Varian XL-300 spectrometer having a Nicolet TT-100 PET accessory. Spectra were obtained in  $\text{D}_2\text{O}$ , in a 12-mm tube, with deuterated TSP (sodium 3-(trimethylsilyl)propionate- $2,2,3,3\text{-d}_4$ ) added as internal reference. GC/MS of methylated acids was conducted on a Hewlett-Packard Model No 5995 GC/MS/DA system equipped with a fused silica capillary column (12 m x .020 mm ID, Hewlett Packard) internally coated with crosslinked methylene silicone. Infrared spectra were obtained with solid samples dispersed in KBr pellets, by using a Beckman IR-33 spectrophotometer. The various absorption peaks in IR and NMR were interpreted conventionally (9-10).

## Results

The  $^{13}\text{C}$ -NMR spectra of the fulvic and humic acids are presented in Figures 1 and 2 respectively. An important region for absorption was the 171-184 ppm range; a particularly sharp signal at 176 ppm in fulvic acid is characteristic of carboxylic groups. The region between 171 and 184 ppm is representative of carbonyl carbons. These can be contained in free acids, esters, salts, amides, aldehydes, and ketones. The region between 113 and 145 ppm represents aromatic, heteroaromatic, and olefinic constituents. Although olefinic carbons are generally not major constituents to these acids, aromatic ones are expected to be major contributors to peaks in this region. The largest peaks are present in the aromatic carbon region, which spans the region from 119 to 132 ppm in both acids; thus they appear to represent predominantly aromatic structures. The peaks at  $\approx 58$  ppm are most likely due to methoxy groups, while those around 59-64 ppm would also include contributions from N-substituted carbons of amino acids (11). Both spectra show resonances in the 19-40 ppm region characteristic of aliphatic moieties. Significant differences exist between the two types of acids, fulvic acid spectra showing more prominent resonances. Both acids, however, are rich in carbon resonating at  $\approx 30$  ppm. These may include  $-\text{CH}_2-$  groups in long-chain fatty acids. The peak at 19 ppm in fulvic acid (Figure 1) is assigned to the terminal methyl in aliphatic chains. There is less aliphatic carbon than aromatic in both acids. The spectra, particularly that of fulvic acid (Figure 1), also show the presence of acetal groups at 101 ppm.

The  $^1\text{H}$ -NMR spectrum of fulvic acid is shown in Figure 3. The signal at 0.94 ppm is the methyl  $^1\text{H}$  resonance, while that at 1.24 ppm is due to methylene protons. The strong signal at  $\approx 1.97$  ppm can arise from the resonance of methylene groups  $\beta$  to Ph,  $\text{CH}=\text{CH}_2$ , OH,  $-\text{O}-$ ,  $\text{OCOR}$ ,  $\text{COPh}$ ,  $\text{CHO}$ ,  $\text{COOH}$ ,  $\text{COCH}_3$ ,  $\text{COOR}$ , or  $\text{CONH}_2$  groups. Although aliphatic carbons  $\alpha$  to carboxyl, carbonyl, or carboxamide groups yield signals that are not shifted downfield of the aliphatic carbon region, they produce a  $^1\text{H}$ -NMR signal at  $\approx 2.24$  ppm that is clearly resolved from that of other aliphatic protons, such as from amino acids. A wide region between 2.99 ppm and 4.0 ppm shows the presence of methylene and methyl groups linked to electronegative atoms, very likely oxygen, and/or the proton of a  $\text{Ph}-\text{CH}_2$  fragment  $\alpha$  to carbonyl and/or carboxylic groups (12-13). The broadness is attributable to the large variety of oxygen and nitrogen compounds in fulvic acids, the most likely ones containing ether, amino acid, and peptide groups. Aromatic or conjugated olefinic proton signals were present between 6.64 to 8.54 ppm. The sharp signal at 8.54 ppm can be attributed to aromatic protons. The prominent peak at approximately 5 ppm obviously shows the presence of OH protons in  $\text{D}_2\text{O}$ .

The  $^1\text{H}$ -NMR spectrum of humic acids is shown in Figure 4. It is similar to that of fulvic acids except there is no sharp peak at 8.54 ppm corresponding to aromatic protons. The peaks for aliphatic protons were at 0.90, 1.28, 1.34, 1.93, 1.94, 2.19, 2.22, 3.37 to

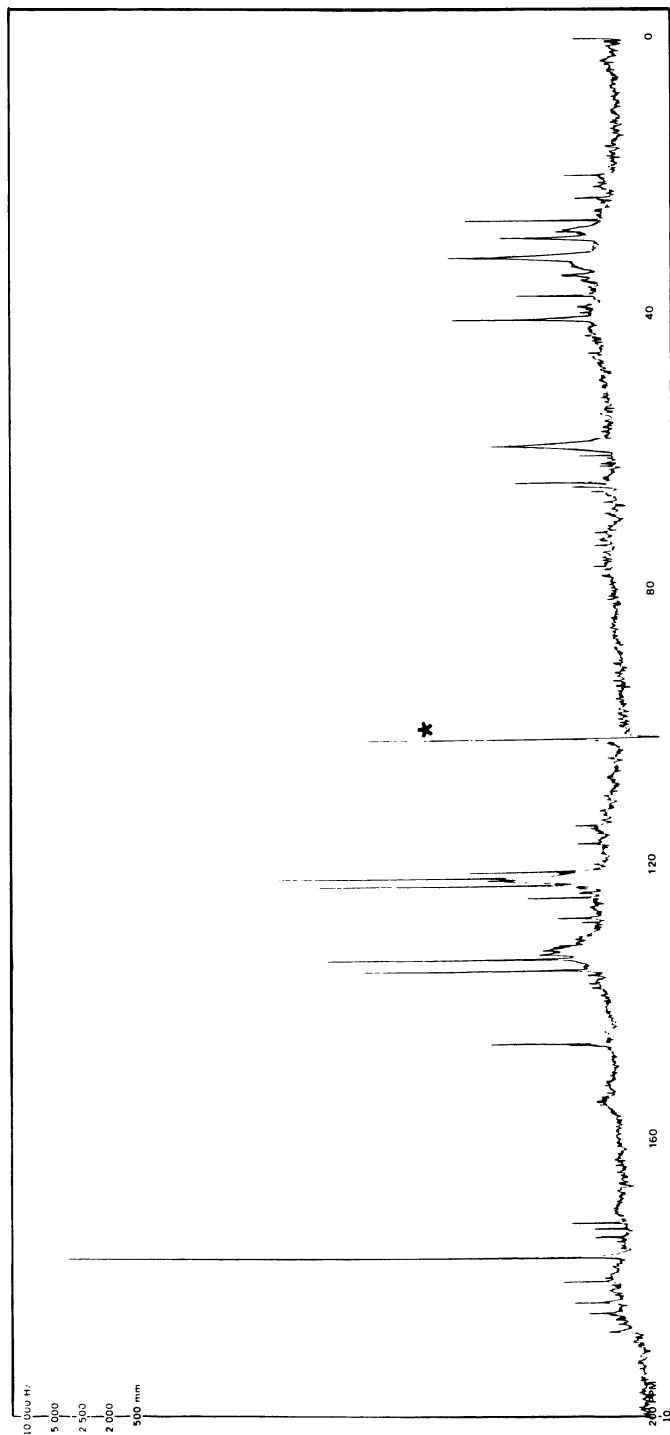


Figure 1.  $^{13}\text{C}$ -NMR of Fulvic Acid of Minnesota Peat (\* indicates instrumental artifact)

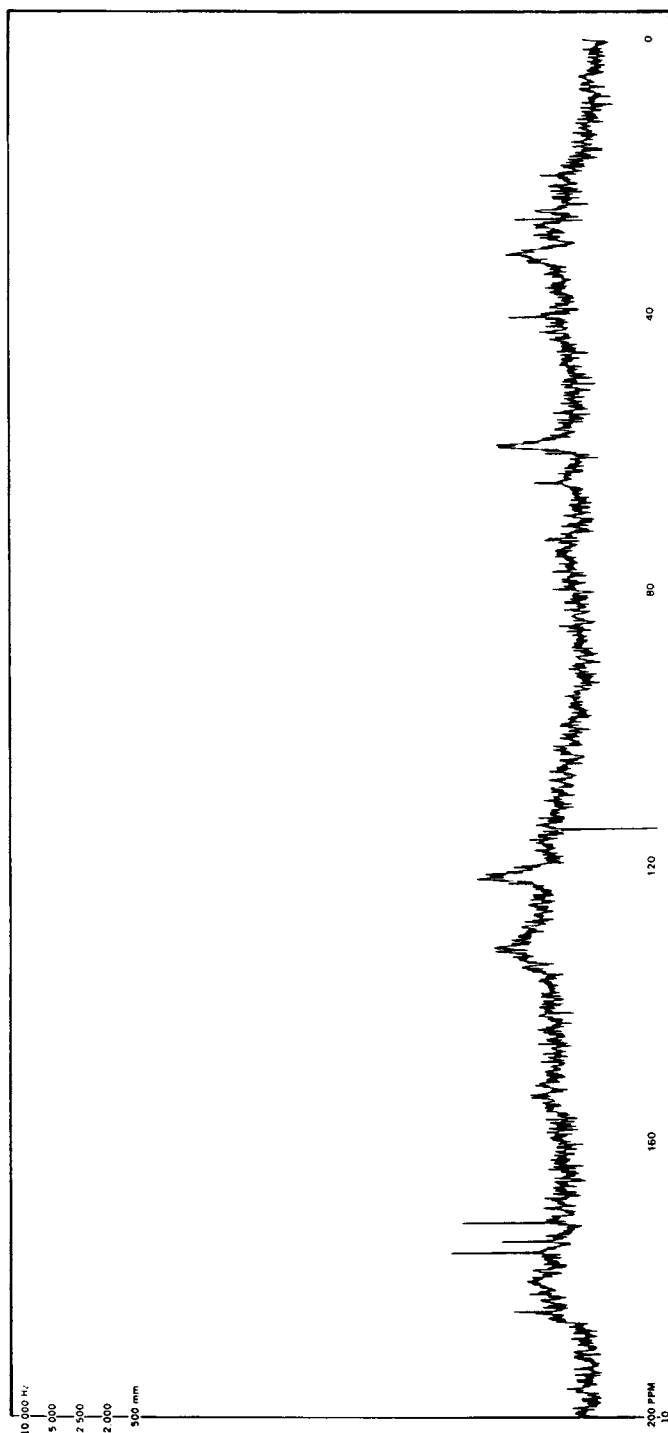
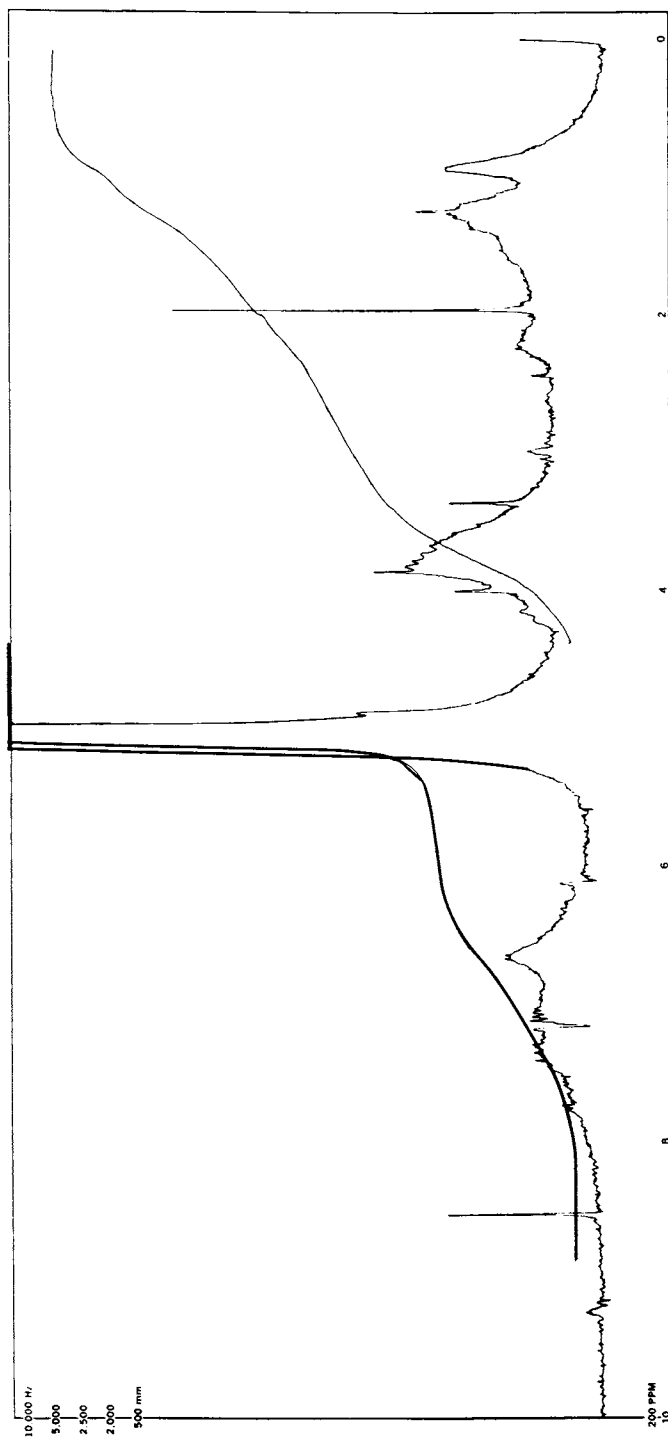


Figure 2.  $^{13}\text{C}$ -NMR of Humic Acid of Minnesota Peat

Figure 3.  $^1\text{H}$  NMR of Fulvic Acid of Minnesota Peat

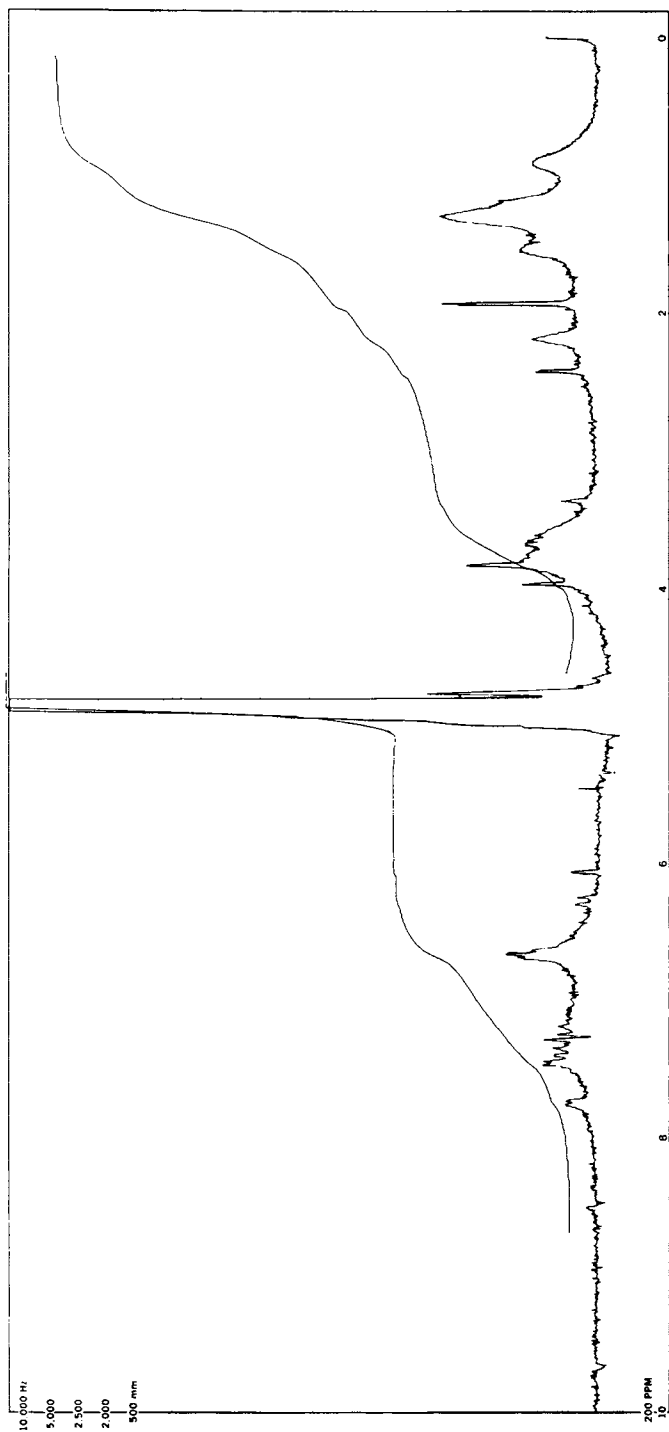


Figure 4.  $^1\text{H}$  NMR of Humic Acid of Minnesota Peat



3.83 (broad), and 3.97 ppm. Olefinic and aromatic protons were indicated by peaks at 6.05, 6.24, 6.30, 6.65, 7.20-7.52 and 7.72-7.77 ppm.

The GC/MS study of methylated fulvic acid showed the presence of *p*-hydroxybenzoic acid ( $M^+$  166), vanillic acid ( $M^+$  196), a methylaldihydroxybenzoic acid ( $M^+$  210), coumaric acid ( $M^+$  192), syringic acid ( $M^+$  226), caffeic acid ( $M^+$  222), ferulic acid ( $M^+$  222), and stearic acid ( $M^+$  298). The same compounds were present in humic acid, along with palmitic acid ( $M^+$  270). The presence of these compounds was confirmed by TLC, using standard compounds.

A typical IR spectrum of fulvic acid (Figure 5a) shows the following features: a wide and intense band from stretching of the H-bonded OH groups at  $3280\text{ cm}^{-1}$ , and a weak band near  $2960\text{ cm}^{-1}$  due to aliphatic C-H stretching. A  $1740\text{ cm}^{-1}$  absorption is due to C=O stretching in carboxylic and/or carbonyl groups. The  $1615\text{ cm}^{-1}$  band is attributed to vibrations of aromatic C=C and/or carbonyl C=O groups that form hydrogen bonds, and/or of double bonds conjugated with carbonyl groups. The absorption at  $1520\text{ cm}^{-1}$  is probably due to aromatic C=C stretching, while the bands at  $1445\text{ cm}^{-1}$  and  $1380\text{ cm}^{-1}$  are likely due to  $\text{CH}_2$  scissor deformation and  $\text{CH}_3$  symmetric deformation. Bands were also exhibited near  $1215\text{ cm}^{-1}$  and  $1025\text{ cm}^{-1}$ , likely due to C-O stretching of esters and ethers and OH deformation in carboxylic groups.

The IR spectrum of humic acid is shown in Figure 5b. As for fulvic acid, it shows broad bands in the  $3380\text{ cm}^{-1}$  region (hydrogen-bonded OH groups), a weak band near  $2920\text{ cm}^{-1}$  (aliphatic C-H stretch), a well defined peak near  $1710\text{ cm}^{-1}$  (C=O stretching frequency of COOH; C=O stretch of ketonic carbonyl), medium-sized bands near  $1650\text{ cm}^{-1}$  (probably due to aromatic C=C bonds conjugated with C=O and/or COO<sup>-</sup>),  $1500\text{ cm}^{-1}$  (aromatic C=C stretching), and  $1445\text{ cm}^{-1}$  ( $\text{CH}_2$  deformation). The bands at  $1220\text{ cm}^{-1}$  and  $1020\text{ cm}^{-1}$  are due to C-O stretching of esters and ethers and to OH deformation of carboxylic group; those at  $825\text{ cm}^{-1}$  and  $685\text{ cm}^{-1}$  might be aliphatic  $\text{CH}_2$  chain or aromatic bands.

## Discussion

A number of investigators (14-20) have recorded  $^{13}\text{C}$ -NMR spectra of humic acid and observed signals from aromatic carbons, but others (21-22) found no such signals in the  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectrum.

Signals in the aromatic or conjugated olefinic region between 6 and 8.5 ppm in  $^1\text{H}$ -NMR and 101-145 ppm in  $^{13}\text{C}$ -NMR are present. The presence of aromatics was confirmed by GC/MS of methylated samples showing the presence of a number of phenolic acids, and the presence of 1650, 1500,  $1520\text{ cm}^{-1}$  bands in the IR spectrum. Aromatic resonances have been observed for both acids and are predominant in both  $^{13}\text{C}$  spectra. The confirmed presence of phenolic acids is in agreement with the discovery of lignin in humic substances (23).  $^1\text{H}$  resonances in the 0-2.4 ppm region characterize aliphatic protons,

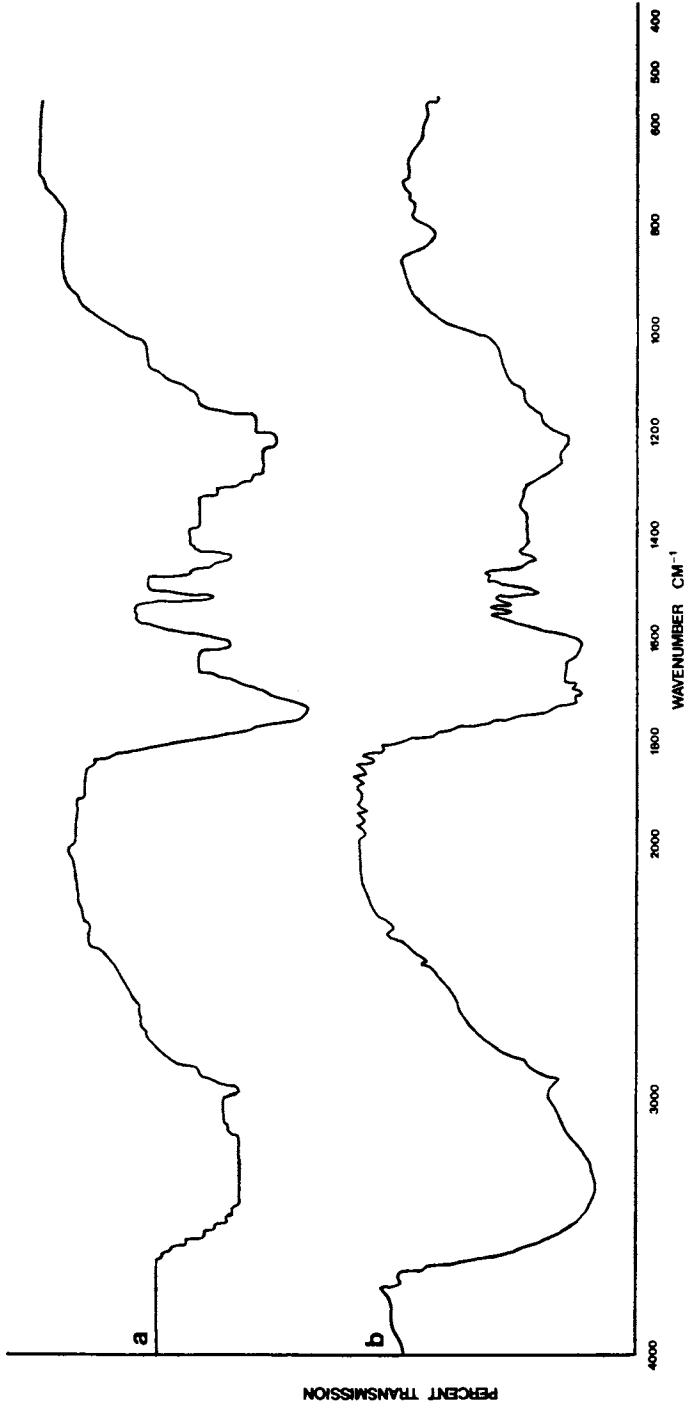


Figure 5. Infrared Spectra of a) Fulvic Acid b) Humic Acid of Minnesota Peat

while  $^{13}\text{C}$  resonances at 0-50 ppm show the corresponding aliphatic carbons. The presence of fatty acids, palmitic and stearic, was shown by GC/MS and IR spectra. It is clear from the work of Schnitzer and Vendette (24) that fatty acids were present in arctic soil samples. Other spectra of soil humic acids obtained in NaOD solution (25) were quite informative, as both aromatic and aliphatic protons were shown present. No thorough study of fulvic and humic acids was conducted by Hatcher and associates (26-28) using  $^1\text{H}$  and  $^{13}\text{C}$ -NMR. The experiments reported here show that there are more aromatic and less aliphatic components in fulvic and humic acids of Minnesota peat.

### Conclusion

Both  $^1\text{H}$  and  $^{13}\text{C}$  NMR showed the presence of aromatic and aliphatic components. In  $^{13}\text{C}$ -NMR, resonances at  $\approx 58$  ppm indicate the presence of many  $\text{OCH}_3$  groups, such as those occurring in syringic, vanillic, and ferulic acids. Spectra clearly show the presence of palmitic and stearic acids by GC/MS, IR, and NMR data. The fulvic and humic acids are predominantly made up of phenolic and fatty acid units. These are highly aromatic because lignin residues have been incorporated in the humification process.

### Acknowledgments

This work was supported by a grant from Minnesota Department of Natural Resources, St. Paul, Minnesota.

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## Chapter 36

# Natural Plant Compounds Useful in Insect Control

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Extracts of plants have been used as insecticides by humans since before the time of the Romans. Some of these extracts have yielded compounds useful as sources (e.g., pyrethrins, rotenoids, alkaloids), others as models (e.g., pyrethrins, physostigmine) of commercial insecticides. Recent technological advances which facilitate the isolation and identification of the bioactive constituents of plants should ensure the continued usefulness of plant compounds in commercial insect control, both as sources and models of new insect control agents and also as components in host plant resistance mechanisms. The focus in this paper will be on several classes of compounds, including limonoids, chromenes, ellagitannins, and methyl ketones, which were found to be components of the natural defenses of both wild and cultivated plants and which may be useful in commercial insect control.

Insect resistance and environmental pollution due to the repeated application of persistent synthetic chemical insecticides have led to an increased interest in the discovery of new chemicals with which to control insect pests. Synthetic insecticides, including chlorinated hydrocarbons, organophosphorus esters, carbamates, and synthetic pyrethroids, will continue to contribute greatly to the increases in the world food production realized over the past few decades. The dollar benefit of these chemicals has been estimated at about \$4 per \$1 cost (1). Nevertheless, the repeated and continuous annual use in the United States of almost 400 million pounds of these chemicals, predominantly in the mass agricultural insecticide market (2), has become problematic. Many key species of insect pests have become resistant to these chemicals, while a number of secondary species now thrive due to the decimation of their natural enemies by these nonspecific neurotoxic insecticides. Additionally, these compounds sometimes persist in the environment as toxic residues, well beyond the time of their intended use. New chemicals are therefore needed which are not only effective pest

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control agents, but which are safe, selective, biodegradable, environmentally acceptable, economically viable, and suitable for use in programs of integrated pest management (3).

One approach to the discovery of new insecticides which fulfill the criteria of efficacy, safety, selectivity, etc., is through the study of the natural chemical defenses of plants. Extracts of plants have been used as insecticides by humans since before the time of the ancient Romans, a practice that continues today with many of the 2000 species of plants known to have insecticidal properties (4-5). The use of "insecticidal" plants is especially prevalent among subsistence farmers since plants grown locally are cheaper, and sometimes more accessible, than synthetic chemical pesticides. Commercially, however, only a few of these plants, including those containing pyrethrins, rotenoids, and alkaloids, have been used to any extent in the United States as sources of insecticides (6-8).

The most economically important group of natural plant insecticides are the pyrethrins, a group of six closely related esters extracted from pyrethrum (Chrysanthemum cinerariaefolium) flower heads (Figure 1). Pyrethrum has been used as an insecticide since at least the early 1800's in Persia and Yugoslavia. By 1828 pyrethrum was being processed for commercial insect control, and by 1939 imports of pyrethrum into the United States reached a peak of 13.5 million pounds. Use of the natural product declined in the early 1950's because of the advent of synthetic pyrethroid analogs (for example, allethrin), which were both more stable and more effective in the field. The present worldwide demand for pyrethrum flowers remains in excess of 25,000 tons annually and is satisfied by the estimated 150 million flowers still hand-harvested daily, predominantly in natural stands and cultivated fields in Kenya, Tanzania, and Ecuador (9).

Rotenone and the rotenoids (Figure 2) have long been used as insecticides and piscicides (fish poisons). By the early 1950's more than 7 million pounds of Leguminosae roots (Derris, Lonchocarpus, and Tephrosia spp.) containing these insecticides were imported annually into the United States. In 1972, about 1.5 million pounds of the roots were used in the United States for pest control in the home and garden markets and to control ectoparasites on animals (10).

Among the most important of the natural alkaloids used in insect control have been nicotine and the related compound nornicotine (Figure 3). The use of these insecticidal alkaloids dates back to the 1600's and grew to 5 million pounds by the mid-1900's. Since then, the annual worldwide production of nicotine has dropped to about 1,250,000 pounds of nicotine sulfate and 150,000 pounds of nicotine alkaloid because of the high cost of production, disagreeable odor, extreme toxicity to mammals, and limited insecticidal activity (10-11). The structurally related compounds anabasine (necotinine) (Figure 3) is currently in commercial use in the Soviet Union (6). Other less important insecticidal alkaloids include veratrine [a mixture of alkaloids (cevadine, veratridine, and sabacilline)] and ryanodine. Physostigmine (Figure 4), another alkaloid that is isolated from the calabar bean (Physostigma venenosum), served as a model compound for the development of the carbamate insecticides (12-13).

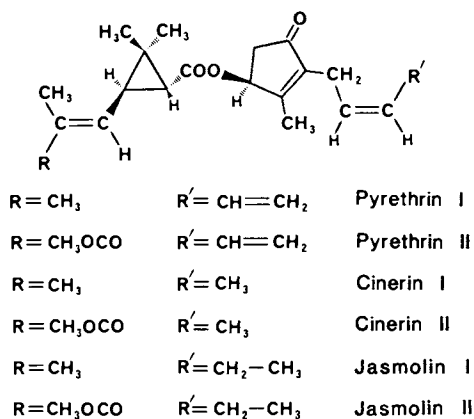


Figure 1. Structures of Six Insecticidal Esters Extracted from Pyrethrum (Chrysanthemum cinerariaefolium)

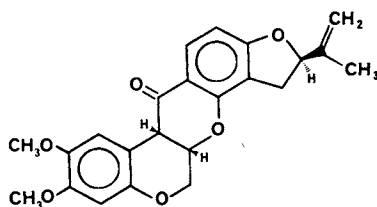
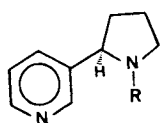
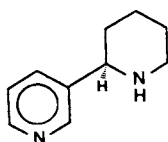


Figure 2. Structure of Rotenone, an Insecticide and Piscicide Isolated from Leguminosae Roots



$R = \text{CH}_3$  NICOTINE

$R = \text{H}$  NORNICOTINE



ANABASINE

Figure 3. Structures of Some Plant Alkaloids Used in Insect Control

Insect growth regulators, including analogs and antagonists of endogenous hormones, have also been identified in plants. Prominent among these are the analogs of two insect hormones (juvenile hormone and molting hormone) and the antagonist for juvenile hormone. Analogs of juvenile hormones found in plants include the juvocimenes in Ocimum basilicum, juvabione in Abies balsamea, and farnesol in many plant oils (14). These natural plant products have never been used commercially as a source of insecticides, but they have served as model compounds for the development of synthetic juvenile hormone analogs such as kinoprene and methoprene (Figure 5).

Chemicals structurally similar or identical to the insect molting hormone (ecdysterone) have been found in many plants, especially in ferns and yews (15). One example is ponasterone A, which differs structurally from the insect molting hormone only in the absence of a hydroxyl group at C-25 (Figure 6). Ponasterone A caused severe disruption of the final stages of molting (termed ecdysis) when fed at 2 ppm in artificial diet to pink bollworm (Pectinophora gossypiella) larvae (16). Figure 7 is an electron micrograph of P. gossypiella fed 2 ppm ponasterone A in artificial diet. The insect possessed three head capsules because it underwent two failed molting cycles before death. Even though feeding became impossible after the first inhibited ecdysis (because the adhering second head capsule covered the mouth parts) the larva produced a third head capsule before its death.

While the above observation is interesting and could possibly have some implications for the control of the pink bollworm, the complexity of the steroid nucleus of ponasterone A and other molting hormone analogs and their weak insecticidal effect when applied topically or administered orally to most species of economically important insects may preclude their commercialization. The only commercial use of the molting hormone analogs thus far has been in the sericultural industry for the synchronization of cocoon spinning of silkworm colonies (17).

Juvenile hormone antagonists were first isolated from the bedding plant Ageratum houstonianum (Asteraceae) (18). The active constituents, termed precocenes I and II, were shown to be chromenes (Figure 8). Although these compounds are highly active against several species of insects (e.g., the large milkweed bug, Oncopeltus fasciatus), the precocenes have been found to be effective on relatively few economically important species of insects, and their potential for commercialization may therefore be limited.

Other chromenes, which differ structurally only in the moieties attached to C-6 and C-7 (Figure 8), have been isolated from other species in the Asteraceae, including xerophytic Encelia species (19) and Hemizonia fitchii (20). The H. fitchii chromenes, including enecalinal, eupatoriochromene, and 6-vinyl-7-methoxy-2,2-dimethylchromene (Figure 8), were moderately toxic to house mosquito (Culex pipiens) larvae, with 6-vinyl-7-methoxy-2,2-dimethylchromene being the most active, and to large milkweed bug (O. fasciatus) nymphs, with enecalinal being the most active (Tables I and II, respectively). Although these compounds are insecticidal, they showed no antijuvenile hormone activity. Apparently, the presence of a vinyl or a methyl ketone moiety, such as found in the Hemizonia chromenes, rather than a methoxy substituent, such as found in the



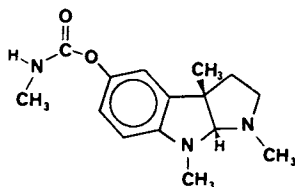


Figure 4. Structure of Physostigmine, an Insecticidal Alkaloid Isolated from *Physostigma venenosum* as a Model Compound for the Synthetic Carbamate Insecticides

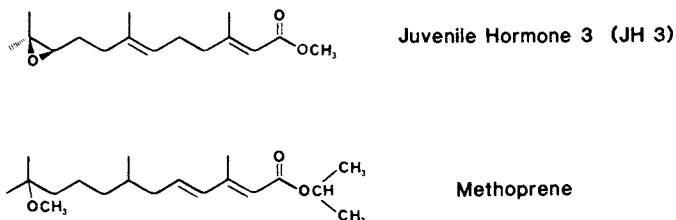


Figure 5. Structures of the Insect Juvenile Hormone 3 and a Commercially Available Analog, Methoprene

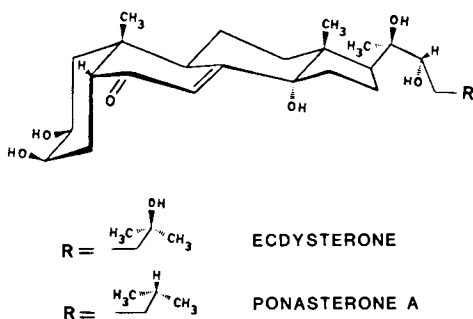


Figure 6. Stereostructures of the Insect Molting Hormone, Ecdysterone, and a Plant Analog, Ponasterone A

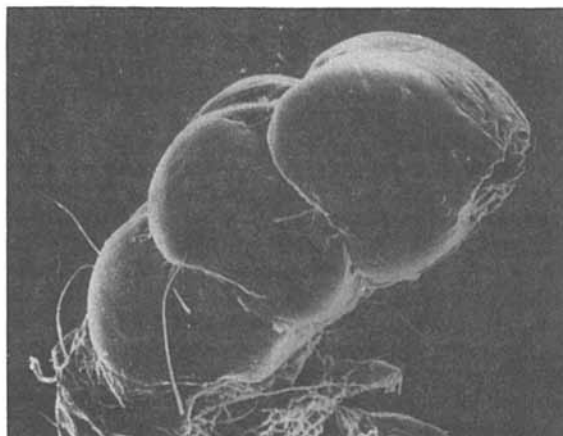
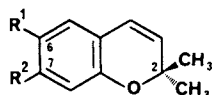


Figure 7. Electron Micrograph Illustrating Ecdysis Inhibition of a Larva of the Pink Bollworm, *Pectinophora gossypiella* after Ingestion of 2 ppm Ponasterone A in an Artificial Diet (Magnification x 100)



	$\underline{R^1}$	$\underline{R^2}$
Precocene I	H	OCH <sub>3</sub>
Precocene II	OCH <sub>3</sub>	OCH <sub>3</sub>
Desmethoxyencecalin	COCH <sub>3</sub>	H
Eupatoriochromene	COCH <sub>3</sub>	OH
Encecalin	COCH <sub>3</sub>	OCH <sub>3</sub>
6-Vinyl-7-methoxy- 2,2-dimethylchromene	CH=CH <sub>2</sub>	OCH <sub>3</sub>

Figure 8. Structures of some Insecticidal 2,2-Dimethylchromenes Isolated from Plant Species in the Asteraceae

precocenes, results in a loss of antijuvenile hormone activity. In fact, Bowers (21-22) found that alkoxy substitution of the chromene aromatic ring in the C-6 and especially the C-7 positions was necessary for antijuvenile hormone activity.

Although assays with other insects should be conducted, it does not seem from an economic standpoint that the Encelia and Hemizonia chromenes themselves are of sufficient potency to warrant adaptation into pest management strategies. However, the relative ease of extraction of the H. fitchii chromenes, as exemplified by their abundance (63%) in the volatile oil (steam distillate) fraction (Table III), coupled with the availability of the xerophytic Hemizonia plant material, make these compounds useful as models for new synthetic or semisynthetic insecticides.

The availability and biological activity of xerophytic plants, such as H. fitchii, make them a promising area of research for the discovery of new insecticides. Xerophytic plants are available on a large scale from marginal regions that generally cannot be used economically for food production (23-24). The foliage of many xerophytic plants has been found to be biologically active, both as insecticides (19, 25) and as insect growth inhibitors (26).

Ellagic acid, a phenolic dilactone (Figure 9), was isolated and spectrally identified as an insect growth inhibitor from the methanolic extracts of five species of xerophytic plants, including Geranium viscosissimum var. viscosissimum, Erodium cicutarium, Tamarix chinensis, Quercus gambelii, and Cistus villosus (26). Against the polyphagous herbivore, Heliothis virescens (tobacco budworm), the EC<sub>50</sub> (the effective concentration for 50% growth inhibition) was found to be 147 ppm or 0.49 mmol/kg diet wet weight (Table IV), well below the 0.77%-1.50% unbound ellagic acid (dry weight basis) found in the hot methanolic extracts of the aerial parts of the five plant species (26). Although the large amounts of unbound ellagic acid could explain, for the most part, the activity of the methanol extracts against H. virescens (EC<sub>50</sub> = 0.0147%), in the fresh plant very little ellagic acid is found in the unbound form, but is instead bound into more complex molecules such as the ellagitannins (27-28). For instance, in a number of species of Geranium, ellagic acid is predominantly bound in an ellagitannin, geraniin (and allied compounds) (Figure 10) (29-30). A milder extraction methodology, that is, one excluding heat, revealed this also to be the case for G. viscosissimum var. viscosissimum. Geraniin was easily detected in tissue which had been extracted at room temperature, while no geraniin was detected in heated samples of similar tissue. Apparently, the large amounts of free (unbound) ellagic acid detected, at least for G. viscosissimum var. viscosissimum, were artifacts of the extraction methodology employed.

Since ellagic acid is probably not encountered free in large amounts in fresh plant tissues, but rather as one or more bound forms, we isolated and bioassayed geraniin. On a mmol/kg diet basis, geraniin was almost twice as active as a growth inhibitor of H. virescens larvae than was ellagic acid (EC<sub>50</sub> = 0.26 and 0.49, respectively) (Table IV). An increased activity of geraniin might be expected since complete hydrolysis of the compound would yield, in addition to ellagic acid, equimolar amounts of brevifolin (or other phenolic lactones), and gallic acid (Figure 11) (31-32).

Table I. Bioassay of Hemizonia fitchii Chromenes with Larvae of Culex pipens

Compound Tested	Instar Tested	LC <sub>50</sub> (ppm)
6-Vinyl-7-methoxy- 2,2-dimethylchromene	1st	1.8
	3rd	3.8
Encecalin	1st	3.0
	3rd	6.6
Eupatoriochromene	1st	6.4
	3rd	13.0

Table II. Topical Bioassay of Hemizonia fitchii Chromenes with Nymphs of Oncopeltus fasciatus<sup>a</sup>

Compound Tested	Instar Tested	LD <sub>50</sub> ( $\mu$ g)
Encecalin	2nd	10
	3rd	11
6-Vinyl-7-methoxy- 2,2-dimethylchromene	2nd	23
	3rd	35
Eupatoriochromene	2nd	b
	3rd	c

<sup>a</sup> Assay period was 8-10 days, sufficient time for control insects to undergo two molts.

<sup>b</sup> No effect was observed with 100  $\mu$ g.

<sup>c</sup> No effect was observed with 200  $\mu$ g.

Table III. Constituents of Volatile Oil of Hemizonia fitchii

Compound Class	Peak Area (% of total)
Volatile fatty acids	0.56
Monoterpenes	29.04 <sup>a</sup>
Sesquiterpenes	2.50
Chromenes	62.70 <sup>b</sup>
Miscellaneous constituents	3.10
Alkanes	1.70

<sup>a</sup> The monoterpene fraction was predominantly made up of 1,8-cineole.

<sup>b</sup> The chromene fraction was predominantly made up of encecalin and eupatoriochromene.

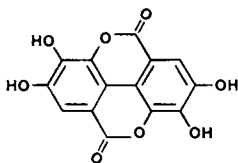


Figure 9. Structure of Ellagic Acid, an Insect Growth Inhibitor Isolated from the Extracts of a Number of Xerophytic Plant Species

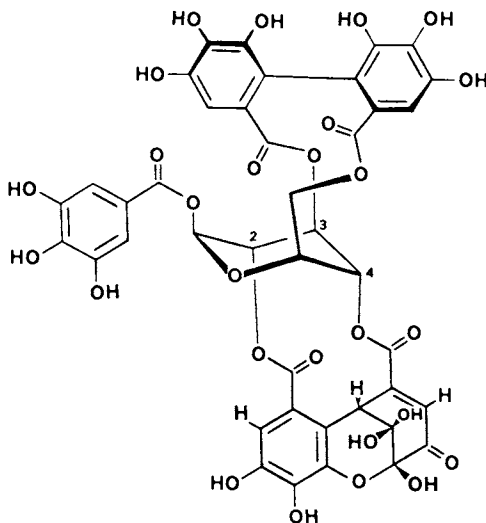


Figure 10. Structure of Geraniin, an Insect Growth Inhibitor Isolated from Geranium Species

Table IV. Growth-Inhibitory Activity of Some Bioactive Constituents Derived from Geranium viscosissimum var. viscosissimum Fed in an Artificial Diet to First-Instar Larvae of Heliothis virescens

Test Compound	EC <sub>50</sub> <sup>a</sup> (mmol/kg diet)	EC <sub>50</sub> <sup>a</sup> (ppm)	Confidence <sup>b</sup> limits	Slope <sup>b</sup>
Ellagic acid	0.49	147	103- 140	1.78
Geraniin	0.26	250	195- 320	1.68
Gallic acid	7.40	1262	931-3843	2.03

<sup>a</sup>EC<sub>50</sub> is the effective concentration of additive necessary to reduce larval growth to 50% of the control values. Units are given in both mmol/kg diet wet weight and ppm of diet wet weight.

<sup>b</sup>Confidence limits and slope were determined using the method of Litchfield and Wilcoxon (66).

Although the biological activity of brevifolin as an insect growth inhibitor is unknown, gallic acid exhibited some growth-inhibitory activity against *H. virescens* larvae ( $EC_{50} = 7.40$  mmol/kg diet) (Table IV).

In light of the susceptibility of at least one species of insect (i.e., *H. virescens*) to ingestion of the free and bound forms of ellagic acid, coupled with the large amounts of such forms found in a number of plant species, ellagic acid may be a constituent of the chemical defenses of certain plants against some insects. As such, it could become a candidate model for compound design, i.e., for the synthesis and semisynthesis of new insecticides, especially if its water solubility and potency can be enhanced. Fortunately, ellagic acid is neither mutagenic (33) nor acutely toxic in tests with experimental animals (34-35) and humans (36). Of course, more active compounds modelled after ellagic acid would also have to be tested for mutagenicity, mammalian toxicity, etc.

Perhaps the most promising of the plant species yielding insecticidal compounds are two species of trees in the Meliaceae, *Azadirachta indica* (neem) and *Melia azedarach* (chinaberry). Both species yield the potent insecticide azadirachtin, a highly derivatized tetranortriterpenoid of the limonoid type. Although the skeletal structure and stereochemistry of azadirachtin have not been totally resolved (Figure 12) (37), the potent and specific effects of this natural product have warranted its evaluation as a source of (38) and a model for (39) new commercial insect control agents. Azadirachtin has a very promising potential because of its potency (comparable to the most potent conventional synthetic insecticides), specificity (affecting behavioral and biochemical and developmental processes peculiar to insects), non-mutagenicity (in the Ames test) (40), and systemic activity in plants (being translocated throughout the plant following absorption through the root system). However, the accumulated information on azadirachtin, while promising, is presently much less than that needed for insecticidal product commercialization (41). The mode of action, structure-activity relationships (SAR's), formulation, and metabolism of azadirachtin are not yet well understood. Furthermore, formulation studies are required prior to product development and commercialization. Consequently, further investigations are needed before the full potential of azadirachtin as an insect control agent or insecticide can be realized.

In order to conduct such investigations, large amounts of purified azadirachtin are needed. Isolation schemes previously reported for azadirachtin have included solvent extraction and partition followed by chromatography, especially open column and thin layer chromatography (TLC) (42-44) and, more recently, high performance liquid chromatography (HPLC) (45-46). While preparative HPLC is an excellent technique for generating azadirachtin of high purity, a general method utilizing the rapid and inexpensive technique of flash chromatography (48) for the efficient preliminary purification of samples that will not readily contaminate expensive HPLC columns was developed (47). Further purification (greater than 99%) was accomplished with preparative HPLC.

Azadirachtin has several effects on a number of economically important species of insect pests, including feeding deterrence,

growth inhibition, and ecdysis inhibition (49-50). Azadirachtin is a particularly potent feeding deterrent (or antifeedant) against the fall armyworm, *Spodoptera frugiperda*, having a  $PC_{95}$  (the concentration resulting in 95% "protection" of treated disks) in a cotton leaf disk assay of  $0.1 \mu\text{g}/1.0 \text{ cm}^2$  leaf disk (Table V). Against the corn earworm, *Heliothis zea*, the  $PC_{95}$  was  $6.2 \mu\text{g}/1.0 \text{ cm}^2$ , which was still a potency greater than that found with other biologically active natural plant compounds such as quassinoids (51) and other limonoids (52-53).

The growth-inhibitory activity of azadirachtin fed in artificial diet to three species of agricultural pests, *P. gossypiella*, *H. zea*, and *S. frugiperda*, was compared to the activity of a number of limonoids isolated from plants in the Meliaceae and the Rutaceae (Table VI). After azadirachtin, the most active limonoid was cedrelone (Figure 13). Cedrelone was unique among the compounds tested in Table VI since it was the only limonoid, besides azadirachtin, to cause an inhibition in ecdysis ( $LC_{50} = 150 \text{ ppm}$ ) when fed to pink bollworm larvae (54).

Nakatani (55) observed that acetoxylation or ketonization of the 7-OH group rendered some Meliaceae limonoids (i.e., trichilins) inactive as feeding deterrents. A similar phenomenon was observed when the growth-inhibitory activities of 7-deacetylgedunin, gedunin, and 7-ketogedunin, but not when the activities of azadiradione and deacetylazadiradione (Table VI and Figure 13), were compared.

When comparing the activities of several Rutaceae limonoids, the most potent seem to have an  $\alpha$ ,  $\beta$ -unsaturated 7-membered lactone (as in obacunone) or at least have the potential to form it (as in nomilin (Figure 14). Apparently, at least *in vitro*, nomilin eliminates its A-ring acetyl group (forming obacunone) much more readily than deacetylnomilin eliminates its A-ring hydroxyl group (56). This fact might explain the much lowered growth inhibitory activity of deacetylnomilin when compared to nomilin or obacunone (Table VI).

Little is yet known concerning the SAR's of biologically active limonoids. However, many of the most potent of the growth-inhibitory limonoids from the Meliaceae and Rutaceae have one or more alkylating centers, including one almost invariably in the A-ring (e.g.,  $\alpha$ ,  $\beta$ -unsaturated ketone or lactone). Cedrelone has 2 alkylating centers, one in the A-ring ( $\alpha$ ,  $\beta$ -unsaturated ketone) and one in the B-ring (diosphenol) (Figure 13). Another example is 6-O-acetylnimbandiol, which has an  $\alpha$ ,  $\beta$ -unsaturated ketone in the A-ring (Figure 13). 6-O-Acetylnimbandiol was insecticidal ( $EI_{50} = 21 \text{ ppm}$ ) when fed to the larvae of *H. virescens*, while the structurally related salannin, which lacks the A-ring ketone (Figure 13), was not (Table VII) (57). Nakanishi (58) has pointed out that natural products with electrophilic moieties tend to be cytotoxic and insect antifeedant. Possibly the growth-inhibitory activity of the limonoids may also be attributed to a nonspecific electrophilic effect.

In addition to its activities of feeding deterrence and growth inhibition, azadirachtin disrupts the molting process of insects by inhibiting ecdysis or the shedding of the "old" skin. The affected insects then die in the pharate condition, unable to feed or locomote. The ecdysis-inhibitory activity of azadirachtin on three species of agricultural pests is shown in Table VIII. Although it is not known

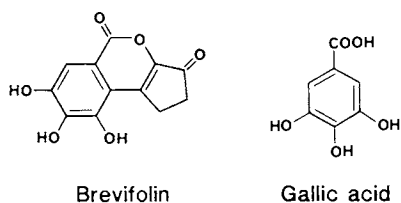


Figure 11. Structures of Two Hydrolysis Products of Geraniin: Brevifolin and Gallic Acid

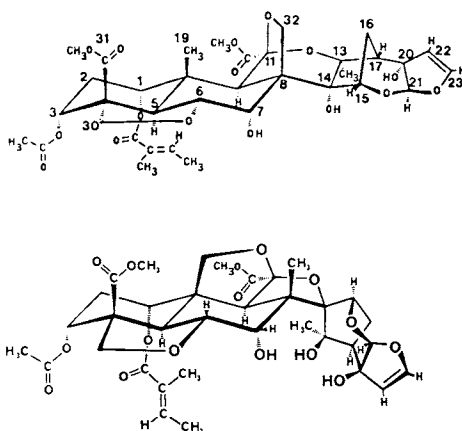


Figure 12. Upper: Previously Accepted Stereostructure of Azadirachtin (43). Lower: Recently Proposed Stereostructure of Azadirachtin (37)

Table V. Cotton Leaf Disk "Choice" Bioassay of Azadirachtin with Third-Instar Larvae of Two Species of Agricultural Insect Pests

Species	PC <sub>95</sub> * ( $\mu\text{g}/\text{leaf disk}$ )
<u>Spodoptera frugiperda</u>	0.1
<u>Heliothis zea</u>	6.2

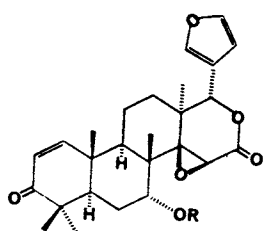
\*PC<sub>95</sub> values are concentrations of azadirachtin resulting in 95% "protection" of treated disks when compared to untreated disks.



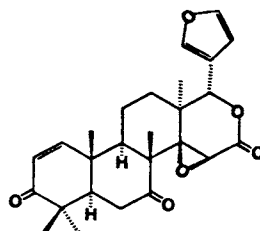
Table VI. Insect Growth-Inhibitory Activity of Some Meliaceae and Rutaceae Limonoids. Values are the Dietary Concentrations for 50% Growth Inhibition (EC<sub>50</sub>)

Limonoid	Insect Species		
	<u>Pectinophora</u> <u>gossypiella</u>	<u>Spodoptera</u> <u>frugiperda</u>	<u>Heliothis</u> <u>zea</u>
Azadirachtin	0.4	0.4	0.7
Cedrelone	3	2	8
Anthotheol	8	3	24
Sendanin	9	11	45
Methyl angolensate	15	40	60
Nkolbisonin	20	65	71
7-Deacetylgedunin	22	60	165
Gedunin	32	47	50
Azadiradione	42	130	250
7-Ketogedunin	51	800	900
Nomilin	-	72	95
Obactnone	-	70	97
Evodoulone	96	120	80
7-Deacetylproceranone	175	350	740
Tecleanine	210	320	-
Limonin	-	756	900
7-Deacetylazadiradione	290	5000	3500
Deacetylnomilin	950	*	*

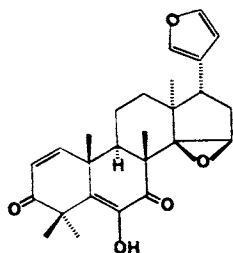
\*No activity was found with 2000 ppm.



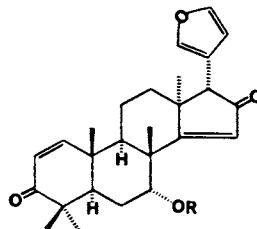
R=Ac GEDUNIN  
R=H 7-DEACETYLGEDUNIN



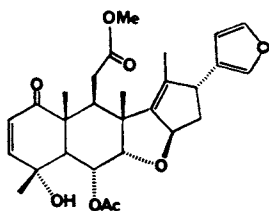
7-KETOGEDUNIN



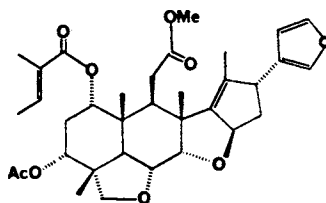
CEDRELONE



R=Ac AZADIRADIONE  
R=H 7-DEACETYLAZADIRADIONE



6-O-ACETYLNIMBANDIOL



SALANNIN

Figure 13. Structures of Some Insect Growth Inhibitory Limonoids Isolated from Plant Species in the Meliaceae

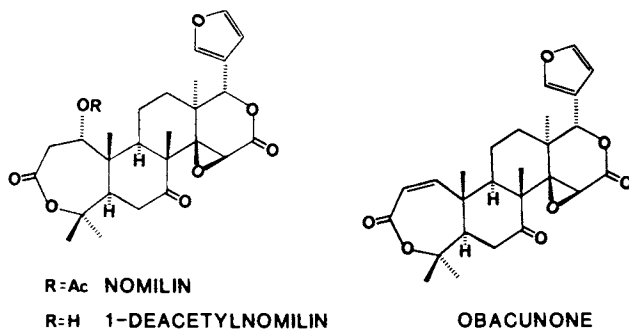


Figure 14. Structures of Some Insect Growth Inhibitory Limonoids Isolated from Plant Species in the Rutaceae

Table VII. Ecdysis- and Growth-Inhibitory Activities of Some Neem Oil Limonoids Fed in an Artificial Diet to First-Instar Larvae of *Heliothis virescens*

Test Compound	EI <sub>50</sub> <sup>a</sup> (ppm)	EC <sub>50</sub> <sup>b</sup> (ppm)	95% Confidence Limits
Deacetylazadirachtinol	0.80		0.66- 0.97
Azadirachtin	0.80	0.17	0.12- 0.23
6-O-Acetylnimbandiol	21.0	0.07	0.05- 0.10
Salannin	c	4.4	15.2 - 29.0
		170	2.8 - 7.0
			138 - 210

<sup>a</sup>EI<sub>50</sub> values are the concentrations resulting in 50% ecdysis inhibition.

<sup>b</sup>EC<sub>50</sub> values are the effective concentrations resulting in 50% growth inhibition.

<sup>c</sup>No mortality was observed at 400 ppm.

how azadirachtin inhibits ecdysis, it apparently does not do so by inhibiting chitin synthetase (49). Possibly the disruption of the molting hormone titre in several species of insects to which azadirachtin was administered (59-61) prevented ecdysis. Ultimately, the disrupted hormone titre and the inhibited ecdysis may have been caused by an interference with the neuroendocrine system, involving the prothoracicotropic and allatotrophic hormones which control the blood titres of molting hormone and juvenile hormone, respectively (45).

Another limonoid isolated from neem seeds and determined to be as potent as azadirachtin as an ecdysis inhibitor has been identified as 3-deacetylazadirachtinol (Figure 15) (57). Both compounds were lethal to 50% of the treated *H. virescens* larvae (EI<sub>50</sub>) at 0.8 ppm in artificial diet (Table VII). Structurally, there are two differences between the compounds. In 3-deacetylazadirachtinol, the C-11-O-C-13 ether linkage of azadirachtin is reductively cleaved at the 11 position and the acetoxyl group at C-3 is hydrolyzed to a hydroxyl group.

The identification of the natural chemical defense mechanisms of plants can be exploited by utilizing the biologically active plant chemical constituents as sources and/or models of new insect control agents. Examples here include pyrethrins from *Chrysanthemum* spp., chromenes from *Encelia* and *H. fitchii*, ellagic acid and geraniin from *G. viscosissimum* var. *viscosissimum*, and limonoids from *A. indica*. Alternatively, a natural chemical defense can be enhanced in a plant, such as in an economically important crop plant, in order to afford endogenous protection from herbivory. Such protection is known as host plant resistance. An example is the wild tomato species *Lycopersicon hirsutum* f. *glabratum*, which has been reported to be resistant to a wide range of arthropod pests of the cultivated tomato, *L. esculentum*. The toxic factor has been identified as 2-tridecanone (*n*-tridecan-2-one) (Figure 16) (62) and related methyl ketones (63) which accumulate in glandular trichomes on the leaf surface. In order to enhance the low levels of 2-tridecanone in *L. esculentum*, explant tissue derived from segregating F-2 populations of crosses between *L. esculentum* and *L. hirsutum* f. *glabratum* were cultured and evaluated for high levels and accumulation of methyl ketones. Crosses were evaluated by a leaf disk bioassay with the tobacco hornworm, *Manduca sexta*, and by gas chromatographic and spectrophotometric analysis (64). Several crosses have been identified thus far which accumulate high levels of methyl ketones (especially 2-tridecanone). The correlation between the amount of methyl ketones and the resistance to herbivory by *M. sexta* in these crosses was -0.56 (65). The goal of this research is to generate a commercial cultivar of tomato which is naturally resistant to insect attack.

In summary, natural plant compounds have been exploited commercially as sources (e.g., pyrethrins, rotenoids, alkaloids) and models (e.g., pyrethrins, physostigmine) of insecticides. Other plant compounds are currently being evaluated for similar uses (e.g., chromenes, limonoids). Still others are being evaluated for use in host plant resistance (e.g., long-chain methyl ketones). Such novel chemicals with potent and often unique biological activities will continue to be discovered and exploited through bioassay and

Table VIII. Ecdysis-Inhibitory Activity of Azadirachtin Fed in an Artificial Diet to First-Instar Larvae of Three Species of Agricultural Insect Pests

Species	EI <sub>95</sub> (ppm) <sup>a</sup>	EC <sub>50</sub> (ppm) <sup>b</sup>
<u>Heliothis zea</u>	2	0.7
<u>Spodoptera frugiperda</u>	1	0.4
<u>Pectinophora gossypiella</u>	10	0.4

<sup>a</sup>EI<sub>95</sub> values are the concentrations resulting in 95% ecdysis inhibition.

<sup>b</sup>EC<sub>50</sub> values are the effective concentrations resulting in 50% growth inhibition.

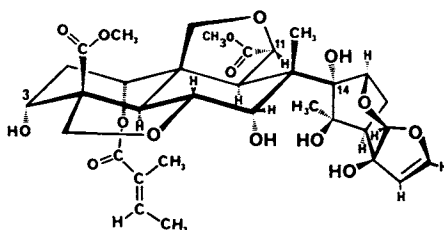
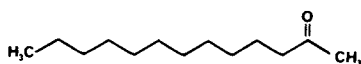


Figure 15. 3-Deacetylazadirachtinol, a Potent Insect Ecdysis Inhibitor Isolated from the Seeds of Azadirachta indica (57)



( $n$ -Tridecan-2-one)

Figure 16. Structure of the Methyl Ketone, 2-Tridecanone, an Insecticide Found in the Wild Tomato Species Lycopersicon hirsutum f. glabratum

through recent advances in isolation chemistry (chromatography) and structural analysis (spectroscopy). The benefits of isolating and identifying these plant chemicals have been proven in the past and plants will continue to be invaluable as renewable resources or chemical progenitors of new insecticides.

#### Acknowledgments

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## Chapter 37

# Interactions Among Allelochemicals and Insect Resistance in Crop Plants

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Many crop plants may have the genetic potential to cut production costs, not only of their own allelochemical defense systems against insect enemies, but also of the synthetic insecticide defense systems humans have devised for plant protection. Common among crop plants are allelochemicals which act as synergists, or chemicals that themselves lack toxicity to insects but can enhance the toxicity of a co-occurring chemical. Most widespread among these synergists are inhibitors of mixed-function oxidases, the membrane-bound metabolic enzymes responsible for the detoxification of a wide variety of xenobiotics. These inhibitors include methylenedioxyphenyl (MDP) compounds. Myristicin, a common constituent of many umbelliferous crops, is as effective a synergist for carbaryl as is piperonyl butoxide, a commercial synergist of synthetic insecticides. Also ubiquitous are synergists that inhibit glutathione-S-transferases, soluble enzymes involved in a number of xenobiotic transformations. These endogenous allelochemicals can synergize both co-occurring toxicants and exogenous synthetic organic insecticides. Such synergists in leaf tissue can significantly increase the toxicity of an insecticide; carbaryl toxicity is thus greatly influenced by the chemistry of the plant on which it is applied. While there are possible complications involved in the use of endogenous synergists to potentiate chemical control of insects, there is great potential for using these chemicals to reduce the impact of synthetic insecticides on nontarget organisms and on the environment without compromising insect control.

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The term "insect-plant interaction" generally refers to plants of the botanical variety, as opposed to plants of the industrial variety; however, weeds, wildflowers, and crop plants face many of the same problems that owners of chemical factories do in the process of manufacturing insecticidal chemicals. One major consideration of insecticide manufacturers is to minimize costs of production; higher costs of manufacture are reflected by higher prices and reduced competitive abilities in the marketplace. Costs are similarly important to plants that synthesize allelochemicals, substances with no known physiological functions which nonetheless possess ecological functions with respect to predators and pathogens of the plant. In the case of plants, "costs" represent biosynthetic costs--energy and material diverted into the formation of defensive chemicals that could otherwise be directed to the production of vegetative and reproductive tissues. That such costs exist and represent a major drain on plant energy and nutrient budgets has been long suspected (1), although direct measurements of these costs are infrequent. One case in which these costs have been estimated involves the wild parsnip, *Pastinaca sativa*, and its major insect enemy, the parsnip webworm, *Depressaria pastinacella* (2). Resistance to the parsnip webworm is largely attributable to the measurable amounts and proportions of furanocoumarins, allelochemicals typical of many plants in the family Umbelliferae. Almost 75% of the variance in susceptibility to the parsnip webworm can be attributed to the relative concentrations of bergapten and sphondin (two furanocoumarins) in the seeds and leaves; the proportion of bergapten in the seeds alone accounts for 36% of the variance. Each of these traits is significantly heritable -- that is, a significant amount of the phenotypic variance is due to additive genetic factors that can respond to selection. Selection, however is a two-edged sword; a response to selection in one trait can, by virtue of linkage or pleiotropy, be correlated with an opposite response in a different trait. A genetic correlation is the correlation between the additive genetic variance for two traits measured on a single individual; as such, it gives an indication of the response of that individual to selection on one trait (3). Three of the four resistance traits in parsnip are negatively genetically correlated with the number of secondary rays produced by the plant; the secondary rays each bear two seeds and thus reflect the genetic potential for seed production, the classic fitness measure in a biennial plant such as wild parsnip. A negative genetic correlation between a resistance factor and secondary ray number indicates that those individuals that are most resistant to their major insect enemy are also less

competitive when the insect is absent (2). Reduced fitness could well reflect the metabolic cost of producing the furanocoumarin resistance factors.

#### Synergists That Inhibit Mixed-function Oxidases

Insecticide manufacturers have responded to pressures to reduce costs in several ways that have direct in the plant world. One method is to use synergists, compounds which may themselves lack toxicity to insects but which enhance the toxicity of co-occurring toxicants (4). Most synthetic synergists act by interfering with the ability of the insect to metabolize the insecticide; synergists thus have the additional benefit of restoring potency of insecticides to insects resistant because of enhanced metabolic capabilities (5). The most widely used commercial synergists are methylenedioxyphenyl (MDP) compounds such as sesamin; these chemicals interfere with the metabolism of pyrethroid and other insecticides by competitive inhibition of microsomal mixed-function oxidases (MFOs), the suite of enzymes responsible for a number of oxidative reactions that convert toxic lipophilic materials into excretable hydrophilic materials (6). The major commercial source of plant-produced pyrethrins, Tanacetum cinerariaefolium, also contains sesamin (7); the plant therefore produces both an insecticide and a synergist.

The widespread occurrence of MDPs and other MFO inhibitors in plants grown commercially for food (Table I) raises the possibility that resistant cultivars may owe their resistance not to variation in the levels of toxicant but rather to variation in the levels of synergists. Again, the parsnip plant provides a good example. In at least two species of Lepidoptera (one, Spodoptera eridania, a noctuid generalist, and the other, Papilio polyxenes, a papilionid specialist pest on parsnip and related umbellifers), furanocoumarins are metabolized by midgut MFOs (27). However, parsnip plants vary considerably in their content of myristicin, an MDP that is a potent synergist of furanocoumarins in the polyphagous Heliothis zea (Lepidoptera: Noctuidae) (28 (Table II). Inasmuch as only 100 mg/g myristicin can increase the toxicity of xanthotoxin (a co-occurring furanocoumarin) fivefold, the 100-fold variation in myristicin content in the leaves of parsnip cultivars (Table III) may make a substantial difference in resistance not only to generalized feeders but also to an adapted pest species such as P. polyxenes.

Table I. Economically Important Plants Containing Methylenedioxyphenyl Compounds

Family	Scientific name	Common name	Ref.
Ericaceae	<u>Vaccinium</u> spp.	blueberry	<u>8</u>
Myristicaceae	<u>Myristica fragrans</u>	nutmeg	<u>9</u>
Pedaliaceae	<u>Sesamum indicum</u>	sesame	<u>10</u>
Piperaceae	<u>Piper betel</u>	betel	<u>11</u>
	<u>Piper nigrum</u>	black pepper	<u>12</u>
Umbelliferae	<u>Anethum graveolens</u>	dill	<u>9,13,14</u>
	<u>Anthriscus sylvestris</u>	chervil	<u>15</u>
	<u>Apium graveolens</u>	celery	<u>9</u>
	<u>Daucus carota</u>	carrot	<u>9,16</u>
	<u>Foeniculum vulgare</u>	fennel	<u>9</u>
	<u>Levisticum officinale</u>	lovage	<u>9</u>
	<u>Pastinaca sativa</u>	parsnip	<u>17,18</u>
	<u>Petroselinum crispum</u>	parsley	<u>9,19</u>
	<u>Pimpinella anisum</u>	anise	<u>20</u>
	<u>Oenanthe japonica</u>	water celery	<u>21</u>

Other MFO synergists in economically important plants:

Benzothiazoles (22): watercress (23), coconut (24),  
tomato (25), soybean (26).

Benzimidazoles (22): coffee (10), tea (10), cocoa (10).

Table II. Mortality (%) of First-instar *Heliothis zea* on Artificial Diets (28) Modified with Additives.

Xantho- toxin (% wet wt.)	Myristicin (% wet weight)			
	0	0.01	0.03	0.10
0.000	1.1	0.0	0.0	0.0
0.100	0.0	6.7	0.0	6.7
0.250	20.0	13.3	43.3	86.7
0.375	23.3	40.0	38.5	93.3
0.500	16.7	30.0	60.0	86.7
1.500	50.0	100.0	96.7	
2.000	86.7			
LC <sub>50</sub>	0.96	0.57	0.38	0.19
(±95% confidence)	(0.79-1.17)	(0.48-0.67)	(0.32-0.46)	(0.17-0.20)
Synergistic ratio	1.00	1.85	2.54	4.97

### Synergists That Inhibit Glutathione-S-transferases

The mixed-function oxidases are ubiquitous in plant-feeding insects and are capable of a wide variety of metabolic conversions (4, 29); they are by no means, however, the only metabolic system involved in the detoxication of xenobiotics. Glutathione-S-transferases (GST) are soluble enzymes that are known to be involved in some forms of organophosphate resistance (30, 31). There is increasing evidence that these enzymes are also involved in the metabolism of plant allelochemicals. Yu (32) demonstrated that several allelochemicals from plants induce *de novo* production of GST and suggested that GST is involved in their metabolism. Yu (33) also demonstrated that many ubiquitous plant chemicals can inhibit the activity of GST and are thus potential synergists. Quercetin (3,3',4',5,7-pentahydroxyflavone) is one such inhibitor (Table IV). Like the MDPs, quercetin and other oxygenated aromatics are widely distributed among crop plants (Table V) and vary within species. Resistant cultivars may owe their resistance to variation in the levels of these synergists. A preliminary study (35) demonstrated that, in Heliothis zea quercetin significantly increased the toxicity of sinigrin, a co-occurring constituent of many crucifer crops.

### Synergists of Synthetic Organic Insecticides

In addition to improving the effect of endogenous toxicants, there is increasing evidence that naturally occurring synergists in plants can enhance the toxicity of synthetic organic insecticides. Marcus and Lichtenstein (34) demonstrated that many essential oil constituents can activate insecticides when applied topically to Drosophila melanogaster; anisaldehyde and myristicin, both MDPs, increase the toxicity of parathion. As mentioned previously, myristicin is comparable to piperonyl butoxide, a commercial synergist, in increasing the toxicity of carbaryl to Heliothis zea (35) (Table VI).

That myristicin can synergize ingested carbaryl gives rise to the intriguing possibility that endogenous synergists in crop plants can be used to enhance the efficacy of externally applied insecticides. If internal synergists can increase the effective dose of an insecticide, then lower amounts of insecticide need be applied to effect equivalent control. Reduced applications are desirable not only in terms of economic savings in control costs but also in terms of ecological impact; reduced applications mean reduced environmental contamination to affect nontarget species. Moreover, decreased environmental residues of insecticides may well

Table III. Myristicin Content of Seeds of Cultivars of  
Pastinaca sativa L. (35)

Cultivar	Myristicin (ppm)
Harris Early Model	3.04
Harris Model	4.96
Avon Resistor	6.89
Offenham	10.78
Tender and True	24.37
All American	41.00
Gladiator	63.50
Hollow Crown Improved	84.02
Fullback Short Thick	149.30

Leaf samples for myristicin analysis were weighed into 10-ml screw-top vials. Myristicin was extracted in ca. 4 ml hexane for 24 h. Fifteen micrograms of octadecane was added as an internal standard. The hexane was decanted and the volume reduced to ca. 0.3 ml. Myristicin was quantified by GLC-FID (3% OV-17, 2 m x 4 mm ID, operated isothermally at 125° C) with a Varian 2700 instrument and a Hewlett-Packard 3390A integrator.

Table IV. Effect of Plant Substances on Detoxifying  
Enzymes in Fall Armyworm Larvae (34)

Additive (0.27%) to Artificial Diet	Glutathione <u>S</u> -transferase (nmol DCNB conjugated/min/mg protein)
None	30.3 ± 1.0
Indole-3-carbinol	117.7 ± 4.9
Quercetin	18.7 ± 0.6
Sinigrin	103.7 ± 3.4

<sup>a</sup>Newly molted sixth-instar larvae were fed meridic diets containing the compounds for two days prior to enzyme assays.

Table V. Distribution of Quercetin Glycosides in Edible vs Nonedible Parts of Fruits and Vegetables (mg of aglycone/kg fresh weight) (47).

Species	Edible part	Quercetin (mg/kg)		
		Other parts of the same plant		
Small radish	Root	40.1	Leaves	0-30
Radish	Root	0	Leaves	35
Rutabaga	Root	ca.0.1	Leaves	40
Horseradish	Root	0	Leaves	50
Scorzonera	Root	< 1	Leaves	230
Potato	Tuber	2	Leaves	770-1000
Tomato	Fruit	2.5-7	Leaves	155-420
Pea	Pod w/o seeds	125-130	Leaves	140-150
	seeds	< 1		
Broadbean	Pod w/o seeds	36	Leaves	1340
	pod	19		
Apple	Fruit	< .01-2	Skin, peel	58-263
Pear	Fruit	< 0.1	Skin, peel	28
Quince	Fruit	< 0.01	Skin, peel	180
Bellpepper	Fruit	< 1	Skin, peel	63
Cucumber	-----		Skin, peel	trace



Table VI. Mortality (%) of *Heliothis zea* on Artificial Diet Modified with Carbaryl and Myristicin (35)

Carbaryl (mg/100 g)	Myristicin (mg/100 g diet)			
	0	10	33	100
1.0	0	3	7	13
1.4	3	27	30	37
2.1	7	60	37	60
3.0	43	-- <sup>a</sup>	70	-- <sup>a</sup>
3.3	90	67	-- <sup>a</sup>	-- <sup>a</sup>

Mortality (%) of *Heliothis zea* on Artificial Diet Modified with Carbaryl and Piperonyl Butoxide (35)

Carbaryl (mg/100 g)	Piperonyl butoxide (mg/100 g diet)			
	0	10	33	100
1.0	0	13	10	7
1.4	3	27	23	33
2.1	7	67	50	70
3.0	43	80	-- <sup>a</sup>	-- <sup>a</sup>

<sup>a</sup> not measured

prolong the useful lifetime of insecticides by delaying the acquisition of resistance (36-38). In addition, resistance to insecticide + synergist is much slower to develop than is resistance to insecticide without synergist (e.g., 39).

There is some experimental evidence that combining endogenous synergists with external synthetic organic insecticides may be an effective alternative form of pest control. Carbaryl toxicity to newly hatched *Heliothis zea* is strongly influenced by the host plant on which the caterpillars are feeding. A concentration of carbaryl that is not toxic to earworms when applied to soybean (*Glycine max*) kills from 50 to 90% of the caterpillars when it is applied to foliage of the umbelliferous plants parsley (*Petroselinum sativum*), celery (*Apium graveolens*), and parsnip (*Pastinaca sativa*) (Table VII). These and other umbelliferous plants are reported to have high levels of MDPs (20), whereas soybean is not known to contain any MDPs or comparable MFO synergists. Given the variation within plant species in MDP content, it may be feasible to design a control program by combining reduced pesticide application with selection of high-synergist cultivars.

#### Possible Difficulties in Applications

There are admittedly problems that can be anticipated in the use of endogenous synergists for insecticide potentiation. First, not all interactions among allelochemicals in crop plants may result in increased insect resistance. Sorghum, for example, produces as a major resistance factor dhurrian, a cyanogenic glycoside; when leaf tissue damage brings dhurrian, normally localized in subcellular organs, into contact with beta-glucosidase, a compartmentalized enzyme, the sugar moiety is hydrolyzed and toxic cyanide is released (40). Goldstein and Spencer (41) recently demonstrated that tannins, complex polyphenol plant constituents that are present in some sorghum cultivars, are able to inhibit beta-glucosidases. The intraplant distribution of tannin and enzyme may thus determine the toxicity of foliage to insects and other herbivores. In addition, many plant chemicals induce MFO activity and reduce the efficiency of endogenous or externally applied insecticides (42, 43). Moreover, many synergists are themselves biologically active. Myristicin, for example, is a hallucinogen (9), and quercetin is highly mutagenic (7). However, many of these endogenous chemicals are concentrated in plant parts that are consumed by insects but discarded by humans. Quercetin is present in highest concentrations in the outer leaves of lettuce, cabbage, and onions and in the peels of pears, apples, and tomatoes; levels inside the fruits or vegetables are vanishingly low (Table V) (44) and,

Table VII. % Mortality (24 hour) of Neonate Heliothis zea on Different Plants with or without Insecticide<sup>a</sup> (35)

Food plant	Control	Carbaryl
Soybean	11.1	20.0
Celery	11.1	90.0
Parsley	0.0	90.0
Parsnip	0.0	50.0

<sup>a</sup>Leaf discs (10 per treatment) were punched out with a 18-mm-diameter cork borer; insecticide-treated discs were in a 0.008% (w/v) solution of carbaryl in acetone, and control discs were dipped in acetone alone and allowed to dry. One caterpillar was placed on each disc and held for 24 hr in a 1-oz creamer cup placed in a controlled-environment chamber (25°C, 16/8 hr light/dark)

Table VIII. Synergistic Ratio<sup>a</sup> for Carbaryl with Piperonyl Butoxide Applied Topically to Insects (46)

	Natural enemies (n)	Plant feeders (n)
Predaceous Neuroptera (1)	6.4 ± 1.1	Folivorous Orthoptera (1) 24.4
Predaceous Coccinellidae (1)	1.5	Sap-feeding Hemiptera (4) 20.3 ± 11.4
Parasitic Diptera (1)	4.0	Folivorous Lepidoptera (8) 4.4 ± 1.4
Parasitic Hymenoptera (7)	8.7 ± 1.8	Herbivorous Coleoptera (11) 6.8 ± 3.6
Overall average (10)	5.2	Overall average (24) 14.0

<sup>a</sup>SR = LD<sub>50</sub> carbaryl/LD<sub>50</sub> carbaryl + synergist

<sup>b</sup>Carbaryl and piperonyl butoxide were administered topically to all species in the ratio of 1:5

particularly for processed food, risks of adverse effects could be lowered considerably simply by selective processing.

Another problem, one that besets virtually any control program, is that synergists are likely to affect nontarget organisms. However, with the synergists localized in the target plant tissue, only those predators and parasitoids that are directly associated with the insect on the target plant are likely to be affected. The principal problems to be expected are that the natural enemies will encounter a higher insecticide dose in prey tissues, since herbivorous insects will be less able to metabolize insecticides, and that the natural enemies will thus ingest synergists directly via their prey and suffer greater mortality from the insecticide. Again, there are data to suggest that predators and parasitoids are not differentially more sensitive to the effects of synergists than are herbivorous insects; this stands in contrast to their considerably greater sensitivity to insecticides (36). Brattsten and Metcalf (46) compared the responses of a variety of insects to carbaryl plus piperonyl butoxide and found no consistent pattern of sensitivity with dietary habits. Closer inspection of their data (Table VIII) reveals that, if anything, natural enemies are less sensitive to the effects of piperonyl butoxide as a synergist. Presumably, reduced sensitivity can be attributed to overall decreased metabolic efficiency in processing xenobiotics in the first place. The LD<sub>50</sub> values for insecticide + synergist vary less than 2%, whereas LD<sub>50</sub> values for carbaryl alone differ by more than 100%.

#### Future Prospects

Crop plants, then, may have the genetic wherewithal to reduce the expense of synthesizing their own defensive chemicals against insect enemies. Moreover, genetic systems exist to potentiate the chemical defense systems humans have designed to protect crop plants. Generally, plants are not screened for the presence of synergists--bioassays for insecticidal activity are generally performed with only one compound at a time (47). Synergists may be commonplace among crop plants, a legacy of generations of evolutionary interactions with insect and other enemies. Once identified, these synergists can be put to use to combat pests of economic importance without enhanced environmental hazard.

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## Chapter 38

# Recent Developments in Chemical Attractants for Tephritid Fruit Flies

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Some problems presented by Tephritid fruit flies and the potential damage which these fruit flies could do to our fruit and vegetable crops on the mainland of the USA are briefly mentioned. The role of attractants in population monitoring and control is also briefly discussed. Two potential Mediterranean fruit fly attractants are presented: (+) -  $\alpha$ -copaene, from angelica seed oil studied both by Fornasiero and coworkers and by Jacobson and coworkers, and 2-methylnaphthalene, from our kerosene studies. Orange oil is presented as a possible commercial source of (+) -  $\alpha$ -copaene. Cost-effective studies must be made before these attractants can be established as being economical and practical. At the present time, trimedlure is still the only lure used for population monitoring of Medflies, and protein hydrolysates are used for baits in control methods.

Fruit flies of the Diptera order, Tephritid family, damage fruit and vegetables all over this earth. Of the many species found, we are concerned here with only five species. The Mediterranean fruit fly, or Medfly, Ceratitis capitata (Wiedmann), the melon fly, Dacus curcurbitae (Coquillett), and the Oriental fruit fly, Dacus dorsalis, are found in the Hawaiian Islands. Efforts are being made to keep these flies from infesting the mainland of the USA. The Caribbean fruit fly, or Caribfly, Anastrepha suspensa (Leow), is now found in Florida, and efforts are being made to eradicate it and to keep it from spreading to other states. From Texas to California, the Mexican fruit fly, or Mexfly, Anastrepha ludens (Leow), presents a potential threat. Recently, a few Mexflies were found in the Los Angeles area.

Very recently, another fruit fly has been added to this list. Dacus latifrons (Hendel), previously found in Taiwan, Malay Peninsula, Thailand, and Laos, has been discovered on the island of Oahu, Hawaii. It is sometimes referred to as the Malaysian fruit

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fly, and at present there is a movement to give the common name of solanaceous fruit fly to this species.

Unlike the small *Drosophila* flies, commonly called vinegar or pomace flies, the Tephritid fruit flies are about the size of the common house flies. Eggs are deposited under the skin of fruits and vegetables, and when the eggs hatch and the insects develop to the larva stage, much damage is done to fruits and vegetables. Such damaged fruits and vegetables have very little commercial value. Fruits grown in certain areas are prohibited in the mainland of the USA to prevent the spreading of these fruit flies. For example, mangoes grown in Hawaii are not allowed to be shipped to the mainland.

The Medfly and the Oriental fruit flies have migrated around the world and have adapted to many fruits and vegetables. As the name implies, the melon fly predominantly attacks fruits in the melon family. *D. latifrons* attacks fruits in the solanaceous family, such as eggplants, sweet and hot peppers, tomatoes, etc. However, as this species adapts itself to new surroundings, it is anticipated that the *D. latifrons* will start attacking other fruits and vegetables as other species have, particularly when urgency for food develops. Another point of interest is that as such insects are transferred to new surroundings, their natural enemies usually do not accompany them. Therefore, the insects in the new environment proliferate and do much more damage in the new environment because they are without the natural enemies to restrict their activities.

The economic impact of an infestation of the Medfly in California in 1981 serves as an example of how serious a problem these flies present. The costs of eradicating the Medfly in California during the 1981 infestation were large. Burk and Calkins (1) reported a conservative estimate of 59 million dollars for chemical controls, 38 million dollars for quarantine and fumigation, and 260 million dollars in crop losses. However these figures are small compared to the estimated 15 billion dollars of fruits and vegetables grown per year in California alone. Now that the importance of controlling these fruit flies has been shown, some control methods and efforts to keep these flies from spreading are presented. Some attractants, such as hydrocarbons from kerosene and essential oils, are discussed.

Insects often use chemical signals for finding shelter, oviposition sites, mates, and food. Until recently, most of chemical insect research has been focussed on how flying insects find their mates. Excellent work has been done in the area of pheromones in the last twenty years or more. Now activity is expanding to allelochemicals, chemicals which mediate interspecific interactions. One of these important studies is how insects find oviposition sites. Another is how they find food. Although chemists focus attention on volatile chemical attractants, it is known that visual and auditory clues also play important roles in the behavior of some insects. In order for chemists to keep a proper perspective, close cooperative research with entomologists is necessary.

In species whose larvae are specialist feeders, finding suitable plants for oviposition is of great importance. Corn earworm moths, *Heliothis* species, will oviposition on twine

impregnated with the extract of corn silk (2). It does not take much imagination to see how such oviposition attractants could be used in control of crop damage. With species which are not specialist feeders, like the Medfly which feeds on many fruits and vegetables, it is much more difficult to find oviposition stimulants.

In the 1981 Medfly infestation in California, an insecticide was mixed with protein hydrolysates (1). Medflies are attracted to protein hydrolysates and feed on this material; hence, this is an efficient way to administer an insecticide.

#### Materials and Methods

Sesquiterpenes were isolated from essential oils purchased or provided gratis by individuals or organizations. Kerosene was provided by the Chevron Chemical Corporation. Reference compounds were purchased or made by conventional synthetic methods.

Isolation, separation, purification, and identification methods which were developed in flavor chemistry were applied in these experiments. Since such methods are described in detail in various places (3, 4), experimental details will not be described here.

#### Discussion

Insect attractants are predominantly used for population monitoring and for control. It is necessary to know the degree of infestation to initiate the most effective control methods. There are two ways to prevent insecticides from entering the food chain by utilizing effective insect attractants. The first method is to attract the destructive insects themselves into a trap by an effective lure, and the second is by attracting their natural enemies so that the insects can be annihilated before they can cause much damage.

The Animal and Plant Health Inspection Service (APHIS) is very much aware of the tremendous potential damage which the Tephritid fruit flies can inflict to crops. This concern has caused APHIS to establish an active program of placing inspection stations at critical airports to monitor shipping of fresh fruits and vegetables to the mainland of the USA. APHIS also has a program of setting out traps for spotting infestations of various insects, including the fruit flies. The California State Department of Food and Agriculture also has a program of trapping fruit flies to detect infestations to halt the spread of these flies into California. For effective monitoring programs, potent attractants are needed. For the Caribflies, Mexflies, and the newly found *D. latifrons*, no good chemical lures are available.

Dr. Chambers has written an excellent review article on "Attractants for Fruit Fly Survey and Control" (5). In this review, Chambers discusses the development of parapheromones and food baits and the implementation of these attractants. Thousands of compounds were screened in Hawaii and in Mexico during the period of 1950 to 1955 (6), and this screening process yielded some promising candidates. Methyl eugenol, proposed by Steiner in 1952 (7), remains the most effective lure for the Oriental fruit fly. Cue lure, found by Beroza in 1960 (8), is a good lure for the melon

fly. Trimedlure, found by McGovern, Beroza and coworkers in 1961 (9), is the lure presently used for the Medfly. Angelica seed oil was indicated in the 1950-1955 survey in Hawaii as an attractant for the Medfly, and Fornasiero and coworkers in 1969 (10) found that  $\alpha$ -copaene is the active ingredient in this oil which attracts the Medfly. Jacobson and coworkers (11) recently found that it is the (+)-rotating chiral isomer of  $\alpha$ -copaene that is effective and that the (-)-rotating isomer is much less effective.

It is interesting to note that these compounds attract mainly the males of the respective species. Therefore, these compounds might be pheromone mimics. Protein hydrolysates, on the other hand, attract both sexes of most Tephritids and are widely used as food baits. However, volatiles from protein hydrolysates may not be simply food attractants. The cyclic imine, 3,4-dihydro-2H-pyrrole, has been identified in protein hydrolysate volatiles (12), and this compound has been identified by Baker and coworkers (13) as the sex pheromone which attracts virgin females to the sexually mature male Medflies. Why the protein hydrolysate volatiles attract the males and other species remains unanswered.

Figure 1 shows the chemical structures of methyl eugenol, cue lure, trimedlure, and  $\alpha$ -copaene. Since these attractants are effective with only the respective species mentioned, it is difficult to compare them quantitatively. However, it seems that the most effective of these is methyl eugenol. It is interesting that if the acetate moiety is taken away from cue lure, the molecule remaining is commonly referred to as raspberry ketone, a compound that has the aroma of raspberry. As mentioned previously, trimedlure is the material that is currently being used as the Medfly attractant in population monitoring. McGovern and Beroza (14) have elucidated the relationship of the various isomers possible in the structure of trimedlure to attractancy to the Medfly. Of the eight structures possible, only four need be considered because the *trans* configuration, with respect to the methyl and the carbobutoxy groups, is necessary for high activity. The *cis* isomers are not very effective as lures. The chloro group can be in the 4- and 5-positions and can be axial or equatorial, hence, the four possible isomers studied. These four isomers are found in the commercial product called trimedlure. Leonhardt and coworkers (15) have developed analytical methods for these isomers so that accurate specifications can be written for trimedlure.

(+)- $\alpha$ -Copaene seems to be several times more potent than trimedlure; that is, if equal amounts of each are set out in traps, more Medflies are caught in traps containing  $\alpha$ -copaene. There is not a sufficient supply of angelica seed oil to provide enough material for monitoring purposes.  $\alpha$ -Copaene was synthesized by Heathcock (16), but since this is a tricyclic sesquiterpene, the synthesis is far too complicated to be economically feasible. Therefore, if  $\alpha$ -copaene is to be a practical lure, a plentiful natural product supply must be found.

Table I shows a survey of some essential oils containing  $\alpha$ -copaene and  $\alpha$ -ylangene. Unfortunately the rotation is not reported in most of the articles, but we have some evidence as to which are attractive to the Medfly. The material from the synthesis by Heathcock (16) would be racemic because the synthesis was not a

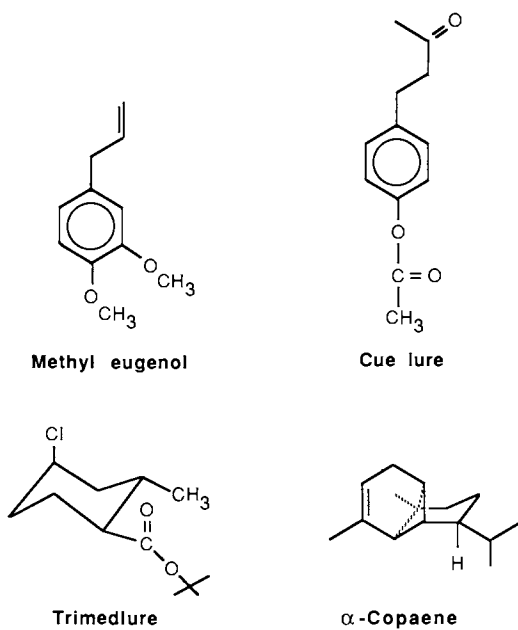


Figure 1. Chemical structures of methyl eugenol, cue lure, trimedlure, and  $\alpha$ -copaene.

Table I. Sources of  $\alpha$ -Copaene

$\alpha$ -Copaene	$\alpha$ -Ylangene	Source
Synthetic	Synthetic	Heathcock
Angelica	--	Fornasiero
Copaiba	--	Fritzsche
Cubeb	--	Fritzsche
Grapefruit	--	Sunkist
Hop	(20% of $\alpha$ -copaene)	Buttery
Orange	--	Sunkist
--	<u>Schizandra chinensis</u>	Sorm
Ylang ylang	--	Fritzsche

stereospecific one. The  $\alpha$ -copaene from angelica seed oil has been shown to be attractive by Fornasiero and coworkers (10) and by Jacobson and coworkers (11). Jacobson (11) has reported that the  $\alpha$ -copaene from copaiba oil has a (-) rotation and has been shown to be not very effective as a Medfly attractant. Our work has shown that  $\alpha$ -copaene from cubeb oil has a (-) rotation and is not very effective. Sufficient supplies of  $\alpha$ -copaene from grapefruit, hop, and ylang ylang oils were not available to take rotations or to test with Medflies. Of the essential oils listed in Table I, it seems that only orange oil is promising as a practical source. Even though the  $\alpha$ -copaene is present at about 0.01% of the total oil, orange oil is produced in such large quantities that it may be a practical source.

Orange oil was investigated because the Medfly is attracted to oranges. When a sesquiterpene cut from orange oil was tested, it was indeed active. It was hoped that valencene would be the active material because it is the major sesquiterpene in orange oil, and it is commercially available. However, valencene is not active. It was found that (+)- $\alpha$ -copaene, a contaminant in the valencene cut, is the active material.

Because  $\alpha$ -copaene is a sesquiterpene, a hydrocarbon, and because of very early reports that kerosene is a Medfly attractant (17), hydrocarbons in kerosene were re-investigated. Also, in the Hawaiian 1950-1955 survey (6), 2-methylnaphthalene was indicated as active. This compound was found in kerosene and was tested for activity again. The positional isomer, 1-methylnaphthalene, has very little activity, but 2-methylnaphthalene did exhibit activity in olfactometer tests by Gothilf and in field trials by Cunningham. Although the activity of 2-methylnaphthalene is somewhat less than

that of trimedlure, because the naphthalene compound is cheaper, it may be cost-effective to use the naphthalene compound. Some alkylbenzene fractions from kerosene showed some activity. Gas chromatographic-mass spectral analyses showed a considerable amount of alkylated toluene compounds. Dreiding models were made, and 2-methyl-3-(3-methylphenyl)heptane was selected to be synthesized on the basis that the shape of this molecule is similar to that of  $\alpha$ -copaene. Unfortunately, this compound showed no activity. Also, no fraction for this alkylbenzene cut from kerosene had much higher activity than the starting material. Therefore, it was assumed that only low-activity compounds are in this fraction. So far, 2-methylnaphthalene is the only compound isolated from kerosene that has shown any promise as a practical Medfly attractant.

Dreiding models of trimedlure and  $\alpha$ -copaene were compared, and the models of these compounds looked surprisingly similar, as well as the 2-methyl-3-(3-methylphenyl)heptane compound. If the dotted bonds of the cyclobutane ring of  $\alpha$ -copaene are broken, and the cyclohexane ring containing the double bond is made aromatic, the phenylheptane compound is obtained. However, it has been stated already that this compound has no activity. Thus, it seems that similar shape may be necessary, but shape alone does not seem to be sufficient. It must be kept in mind that (+)- $\alpha$ -copaene is active, whereas (-)- $\alpha$ -copaene is not. Fornasiero (10) has stated that  $\alpha$ -ylangene is active, but because copaene and ylangene are so difficult to separate, the activity of the ylangene cut may be due to some copaene contamination. However, in view of trimedlure, with chloro and carbutoxy moieties, and  $\alpha$ -copaene, a hydrocarbon with one double bond, being active, it may be possible that there are other compounds which will satisfy shape and polarity requirements to be Medfly attractants. Also, good attractants for Caribflies, Mexflies, and *Dacus latifrons* must be found for effective population monitoring and control of these fruit flies.

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## Chapter 39

# Allelochemicals as Determinants of Insect Damage Across the North American Continent Biotypes and Biogeography

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Insects feeding on plants have varying degrees of specificity which are related to the chemistry of the leaves (or other plant part) fed upon and also to the behavioral, physiological, and ecological adaptations of the insect itself. To permit understanding of the ecological significance of allelochemicals in agriculture and forestry, our research efforts must deal with the interactions of insects and their food plants at all of these levels of biological organization. As an agricultural example, we describe how a change in foodplant preferences has resulted in an obscure and virtually unknown insect (the hop vine borer, Hydraecia immanis, a Noctuid moth) becoming a major pest of corn in Wisconsin and adjacent states. As an example of tree-feeding insects, we describe our recent research with tiger swallowtail butterflies (Papilionidae). We have directed particular attention upon tulip poplar (Liriodendron tulipifera) and quaking aspen (Populus tremuloides) because of their dramatically different underlying allelochemical effects on related insect herbivores. We emphasize the ecological implications of the differences in leaf chemistry between these plant species because the temporal, spatial, qualitative, and quantitative aspects of phytochemistry are so poorly documented. Nonetheless we are currently attempting to identify the specific chemical(s) involved and their mode of action against "unadapted" species, subspecies, and geographic populations.

Subtle differences in allelochemically based host preference and/or the ability to survive on various hosts may have very significant implications for the population dynamics and geographical ecology of insects (1-4). Differential adaptations

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of phytophagous insect species to the various allelochemicals in their hosts will generally be accompanied by a number of other behavioral, physiological, morphological, and ecological adaptations.

A recent review by Diehl and Bush (4) proposes the following classification to help standardize terminology regarding the term "biotype"; (1) nongenetic polyphenisms (also called ecomorphs or phenocopies) in which the same genotype produces various phenotypes in different environments, (2) polymorphic and/or polygenic variants within populations (due to discontinuous or continuous variation within a freely breeding population with a genetic basis), (3) geographic races, which are geographically isolated biotypes (e.g. semispecies or subspecies), (4) host races as "a population of a species that is partially reproductively isolated from other conspecific populations as a direct consequence of adaptation to a specific host" (whether due to isolation by host preference, host-associated allochronic isolation, or some other form of assortative mating arising as a direct result of different host use), and (5) species as "natural populations that are reproductively isolated from one another and that follow distinct and independent evolutionary paths" (with sibling species morphologically so similar that recognition requires additional careful studies of biochemical, cytological, or behavioral traits). These five categories are not necessarily mutually exclusive, and we do find biotypes at several stages of evolutionary divergence amid various processes of speciation (5). While allelochemicals are fundamentally important considerations in the development of host shifts, host races, and speciation, they are only part of the ecological/evolutionary story, and by themselves fall far short of explaining insect/plant interactions, even at the chemical level (6, 7). Variation in agricultural/silvicultural systems for herbivory intensity must consider insect genetics as a major future research effort.

Our first example concerns a new midwestern corn pest, the hop vine borer (HVB), Hydraecia immanis Guenee, and its introduced congener the potato stem borer (PSB), H. micacea Esper. A major shift in host use from hops to corn has occurred suddenly with the HVB, and this has resulted in significant economic losses for growers. This danger is complicated by the arrival in Wisconsin of the more polyphagous relative (the PSB) and the fact that the expanding geographic distributions with economic damage to corn for both species has brought them into natural contact.

Our second example concerns the North American tree-feeding swallowtail butterflies of the Papilio genus (Papilionidae: Lepidoptera). In this species complex, we observe reciprocal inabilities of certain taxa to utilize the favorite foodplant families of other taxa, implying phytochemically based negative genetic correlations (8). Geographic variation in the abilities of various populations of a given subspecies or species to accept/consume/grow and reproduce on plants of these families is also significant (2,3).

#### A Recent Example from Midwest Agriculture

The hop-vine borer (HVB), Hydraecia immanis Guenee, is a stem-feeding caterpillar (Lepidoptera: Noctuidae) which has only very recently been causing severe localized damage to corn in large portions (more than 50 different counties) of Wisconsin,

Minnesota, Iowa, and Illinois (9). As indicated by its common name, the hop-vine borer had been found in close association with hop (*Humulus lupulus*) plants. Wild hops, found from the east coast of the U.S. to the Rocky Mountains, presumably served as the primary host for the insect in North America (10) for the last 100-150 years or longer.

In Wisconsin, hop production during the last century was concentrated in several southern counties where the first reports of serious larval damage (HVB) to corn also originated. Although there has been essentially no commercial hop production east of the Rocky Mountains since the 1930's, small patches of wild or escaped hop along roadsides and drainage ditches near heavily damaged cornfields have been located and *H. immanis* larvae were found feeding on below-ground portions of these plants (9). Thus it appears that isolated endemic populations have continued to exist even in the absence of the hop industry and that the HVB has been able to make the transition from its grass/hops feeding habit to a grass/corn feeding pattern in these areas (9,11). Since hop is a perennial and corn is an annual, this transition in larval feeding has certainly been favored by continuous corn production since the 1940's that has made corn a dependable HVB resource.

Reasons for the lack of any outbreaks in corn prior to 1975 are unknown at this time, but it is possible that the general use of chlorinated hydrocarbons (e.g., DDT, Aldrin, and Dieldrin) in the two decades previous to 1970 maintained a general suppressive effect upon *H. immanis*. It is also possible that small populations have only recently been forced onto corn with the removal of perennial hop plants or other hosts along field edges as a result of changing agronomic practices and/or increased herbicide use such as 2,4-D and 2,4,5-T. In any case, poor weed control, continuous corn culture, no-till, conservation tillage, and reduced tillage favor increasing insect population densities and increase the potential for further spread in the corn belt.

In Europe, a congeneric potato stem borer (PSB), *H. micacea* Esper, is a problem in potatoes, as it is also in corn and many other crops such as sugar beet, rhubarb, onions, tomatoes, strawberries, and raspberries in Scandanavia, the United Kingdom, Russia, and Canada. The PSB has been well established in Canada since the turn of the century, reaching New York State by the mid-1970's. The PSB has since appeared for the first time as far west as Manitoba (C. Ellis, pers. comm.) and recently (1982) in Manitowoc and Kewaunee counties of Wisconsin. In 1984, economically damaging levels of potato stem borers in corn were detected in several locations of Calumet County, Wisconsin. Because of their similar life cycles, habits, damage to corn, and apparent resistance to conventional corn rootworm insecticides, we could expect both the PSB and HBV to increase their densities and/or range throughout the Midwest much as the PSB has (9,12). These concerns are evident in the 1985 establishment of a multistate regional research effort entitled "Impact of integrated crop management practices on European corn borer and related stalk boring insects".

In summary, undetermined factors have recently led to increased local densities of hop vine borers, *Hydraecia immanis*, in the midwest with intense economic damage on corn. This change in feeding behavior from grass/hops to grass/corn has mediated an apparent geographic range expansion. A simultaneous but even more rapid range expansion of the introduced polyphagous potato stem

borer, Hydraecia micacea, has occurred westward across Canada and into Wisconsin. It is unknown whether or not the wider range of acceptable hosts of the PSB has facilitated the more rapid range expansion than observed for the HVB. These facts illustrate the need for understanding the ecological as well as the allelochemical factors involved in host race or biotype formation in phytophagous insect species. It is important to be able to predict these shifts in host usage or at least understand what factors will delineate new geographic range limits. A cursory perusal of Gibbs (13) has shown no known secondary chemicals in common for Zea mays L. and Humulus lupulus L. although preadaptation to corn might be expected on the basis of the adult oviposition preferences for grasses. This relationship will receive much more attention in the near future, owing to its economic importance.

#### North American Distribution of Tree-feeding Papilio

Within Section III of the Papilionidae it is tempting to assume that evolutionary resource partitioning of foodplants via specialization at the plant family level has occurred (8,14). For example, we generally observe P. troilus L. on spicebush, Lindera benzoin, and P. palamedes Drury on red bay, Persea borbornia (both in the Lauraceae); Papilio eurymedon Lucas on Rhamnaceae; P. multicaudatus Kirby on Rutaceae; the California P. rutulus Lucas on Platanaceae; P. g. canadensis R & J and Rocky Mountain P. rutulus on Betulaceae and Salicaceae; and P. g. australis Maynard on only sweetbay, Magnolia virginiana, of the Magnoliaceae. Such patterns of feeding specialization in Papilio have been the basis of many discussions regarding the so-called "chemical arms race" and "coevolution" (15). Since these insects do not migrate or move great distances, the geographic distributions of insect taxa (Figure 1) are of course closely allied with the current distribution of their host plants.

In fact, the primary factor that has enabled P. g. canadensis to inhabit Canada (Figure 1) may be the ability to utilize foodplants of the Salicaceae (e.g., Populus tremuloides Michx., P. balsamifera L., P. grandidentata Michx., and various Salix spp.) and Betulaceae (e.g., Betula papyrifera Marsh and Alnus spp.). These are essentially the only suitable foodplants for P. glaucus available at latitudes north of 50° (16). The southern subspecies P. g. glaucus and P. g. australis favor the Magnoliaceae and do not survive well on plants of the Salicaceae and Betulaceae (2).

In the last two years we have investigated the phytochemical basis of these differential utilization abilities for Papilio larvae on selected plant species of the Salicaceae and Magnoliaceae. Several compounds have been isolated from leaves of both quaking aspen and tulip tree that exhibit biological activity against larvae of the highly polyphagous southern armyworm, Spodoptera eridania Cram. (Lepidoptera: Noctuidae) (17,18). While salicin was extracted in high concentrations from quaking aspen and is known to have multitrophic level effects (19), the most active armyworm antifeedant extracted, isolated, and identified in our studies to date was 1,2-benzenediol (pyrocatechol).

Similar feeding preference tests have been conducted with penultimate and final instars of Papilio g. glaucus and suggest



Figure 1. The geographic distribution and favorite foodplant families of the tiger swallowtails of North America.

that pyrocatechol may represent one of the important compounds involved in the non-usage of Salicaceae by larvae of this subspecies. For example, several different leaf surface concentrations from 24 g/cm<sup>2</sup> to 196 g/cm<sup>2</sup> all significantly reduced preference and feeding rates on painted black cherry leaves (data of J. Hainze and P. Sunarjo in Scriber; 20). Also, we have recently discovered that even the Salicaceae-adapted individuals of the northern subspecies, *P. g. canadensis*, are adversely affected by the quaking aspen constituent pyrocatechol, salicin, and isoniazid. These compounds significantly increased metabolic costs and/or suppressed growth rates for penultimate instar larvae of *P. g. canadensis* (21). Similarly, sesquiterpene lactones from tulip trees have been found to be most likely responsible for deterrent and toxic effects against even the "adapted" *P. g. glaucus* subspecies (22).

Presently, phenolics and terpenoids are the only major class of secondary plant compounds reported to occur in the Salicaceae (Table I) (aos 36-39). Among the array of potential secondary compounds in *Populus tremuloides* that may be involved in our observed antixenosis/antibiosis to the *P. g. glaucus* and *P. g. australis* subspecies are those listed in Table I. Only recently has isoniazid (a pyridine alkaloid) been isolated from quaking aspen leaves (Sunarjo, 40). By contrast, the composition of secondary compounds in tulip trees (Table II) and other Magnoliaceae is highly diverse, including sesquiterpene lactones (42, 48, 50, 52, 54), benzylisoquinoline alkaloids, cyanogenic glycosides, and various essential oils (13,55,56).

Table I. Secondary Compounds Identified In Leaves and Bark of Quaking Aspen (*Populus tremuloides* Michx)

Compounds	Leaves, References	Bark, References
Chrysin	23	
<i>p</i> -Coumaric acid		24
1- <i>p</i> -Coumaroylglucose		24
Gentisyl alcohol		24
Grandidentatin		13,25,26
Pinocembrin	13,23	
Populin	27-29	27
Pyrocatechol (catechol)	29,30	
Quercetin	23	
Quercetin-3-galactoside	29	
Quercetin-3-glucoside	29	
Quercetin-3-rutinoside	29	
Rhamnetin	26,29	26
Salicin	13,23-25,31	13,23,31-33
Salicortin	13,23,31	13,23,25,26,31
Salicyl alcohol		24
Salicyltremuloidin	13,31	
Salireposide	13,24,25,29-31	26,32,33
Succinic acid	26,29	
Tremulacin	13,23,25,26,31, 34	13,23,25,26,31
Tremuloidin	13,24,25,28-30	13,31,33,35
Triploside	30	

Table II. Secondary Compounds Identified from Parts of Tulip Tree (*Liriodendron tulipifera* L.)

Compounds	Tissue of Origin <sup>a</sup>	Biological activity	Reference(s)
(-)-N-Acetylanonanine	HW		41
(-)-N-Acetylnornuciferine	HW		41-4
(-)-N-Acetylasimilobine	HW		41,43,44
(+)-N-Acetylnornantenine	HW		41
Asimilobine	HW		43,44
-Cyclolipiferolide	L		45
Costunolide	RB	cytotoxic	46
Dehydroglaucine	HW		43,44
11,13-Dehydrolanuginolide	L		45
Dihydrochrysanolide	L		45
Epitulipinolide	RB	cytotoxic	42,47,48
Epitulipinolide diepoxide	L	cytotoxic	42
Epitulipdienolide	RB		42,48
Glaucine	HW		13,43,44
N-(2-Hydroxy-2-phenylethyl)-benzamide	B		49
Laurenobiolide	L		45
Lipiferolide	L	cytotoxic	42,50
-Liriodenolide	RB		45
-Liriodenolide	RB		45
-Liriodenolide	L	cytotoxic	42,50
Lirionol	B		49
Liriodendritol			13
Liriodendronine	SW		51
Liriodenine	HW		13,43,44, 50
(+)-3-Methoxy-N-acetylnornantenine	HW		41
O-Methyl-N-norlirinine	B		49
O-Methylatheroline	HW		43,44
Norushinsunine	HW		43,44
Peroxyferolide	L	antifeedant	52
(+)-Pinoresinol	B		49
Saponins	L		13
Syringic acid methyl ester	B		49
(+)-Syringaresinol	HW		43
Syringaresinol di- $\beta$ -glucoside	B		53
(+)-Syringaresinol dimethyl ether	HW		43
Syringaldehyde	HW		43
(+)-Syringaresinol	B		49
Tulipinolide	RB	cytotoxic	46-8
Tulirinol	L	antifeedant	54
(-)-Tuliferoline	HW		41

<sup>a</sup>B = Bark; RB = Root bark; HW = Heartwood; L = Leaves; SW = Sapwood

In addition to the unique and quite dramatic differences in feeding/survival of larvae of the *P. glaucus* complex and their hybrids (20) (Table III), tulip tree and quaking aspen represent phytochemically intriguing foodplants for several other North

American insects (see 18). For example, both plants are unacceptable/unsuitable as food for two notoriously polyphagous insects, the cecropia silkworm, *Hyalophora cecropia* (57), and the southern armyworm, *Spodoptera eridania* (Cram.) (3).

Table III. Survival of *Papilio glaucus* Subspecies 1st instar (neonate) on Three Foodplant Species (1978-1985; Madison, WI). (after Scriber; 8,20)

Eastern Tiger swallowtail subspecies	Survival percentage of newly hatched caterpillars on potential foodplants		
	tulip tree	quaking aspen	black cherry
<i>P. g. canadensis</i> (n)	7% (420)	80% (358)	74% (2872)
<i>P. g. glaucus</i> (n)	81% (2117)	7% (2091)	81% (5514)

### Conclusions

Ecological interactions with insect herbivores in agriculture and forestry depend to a significant extent upon the various allelochemicals and nutrients in the plant tissues. Superimposed upon this dynamic phytochemical foundation (with daily, seasonal, taxonomic, and stress-induced variations), the behavioral, physiological, and ecological variation in the herbivore populations render an understanding of the specific mechanisms of chemical adaptation and counter-adaptation difficult indeed. Even with a single chemically defined allelochemical, the mode of action is variable (dose-dependent, and situation-dependent; 3,58) and the insect response is not easily categorized as to whether the effect is primarily behavioral (e.g. deterrence, or suppressant) or physiological (e.g. toxic; 59).

### Acknowledgments

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## Chapter 40

# Potential Industrial Application of Allelochemicals and Their Problems

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Agrochemical research, by both industrial and academic scientists, has provided compounds of progressively increased activity, with recently discovered insecticides, fungicides, nematocides, herbicides, and plant growth regulators showing outstanding potency. Most of these chemicals are targeted at visible pests, such as weeds and insects. The chemical effect is observable and can be quantified. Such effective pesticides and other agrochemicals are not yet available for several other targets of interest, such as soil-borne pests and diseases (viruses, bacteria). However, it is becoming more difficult to find new chemical entities by using the traditional approaches. Allelochemical research is an area of interest to agrochemical companies, because it provides a new way to control pests, as well as sources of new chemicals to address other problem areas in agriculture.

Use of chemicals in food, feed, and fiber production is an important element in modern agricultural practice. Chemical companies have been providing effective crop protection chemicals, as well as cheap fertilizers, through cooperative research and development programs for the last four decades. These companies are still continuing their attempt to develop new products that will act only on the target pests and have a minimum risk of side effects. However, the probability of finding a product having all the desired characteristics is decreasing. Today, only about one out of every 10,000 chemicals screened enters the second phase of testing, which means the stage of extensive field evaluation. Among about five extensively field tested chemicals, one may become a marketable product. Allelochemicals provide a possible new approach to control pests, as well as sources of new chemicals that may lead us to discover more biologically active compounds and new ways to grow high-yielding and better quality crops. Some of these approaches are discussed by others in this book.

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### Discovery and Review of New Agricultural Chemicals

In order to illustrate the potential of the application of an allelochemical in agrobusiness, let us look at the historical profile of agrobusiness. We have accumulated vast scientific knowledge of how to make new molecules and how the chemicals work. We have better equipment, facilities, and tools to help us study these molecules and yet we have to accept the fact that innovation in this field is becoming more and more difficult. We expect the rate of introduction of new agricultural chemicals to diminish markedly in the future. Table I certainly demonstrates these points.

**Table I. Rate of Discovery and Development of Major Pesticides from 1931 to Date**

Decade	Major Pesticides Introduced
1931 - 1940	1
1941 - 1950	9
1951 - 1960	18
1961 - 1970	19
1971 - 1980	8
1981 -	3

The introduction of products for use in minor crops has become almost impossible because of the low economic incentive. The frequency of introduction of major money-making compounds has been decreasing sharply, although chemical companies have been increasing their total R and D budget. Keep in mind that most of this budget is being spent on defense of existing products rather than in discovery programs. For example, in the 1960's and 1970's, chemical companies enjoyed vast growth by developing good economic returns (DDT, organophosphates, carbamates, pyrethroids, triazines, acetanilides, glyphosate, etc.). In the 1980's, that kind of growth has not been seen yet.

Another reason for decreasing introduction of new products is illustrated in Figure 1. The work required to demonstrate the safety of candidate products and to satisfy registration and other regulatory bodies has become one of the largest portions of budgets and a time-consuming process. Let me make it quite clear that chemical companies don't question the need for careful regulations. It is a fact that we need a careful registration process to assure the product is safe and has minimal impact on the environment and minimal effects on nontargeted organisms.

Since the cost of developing a new agricultural product is so high, we have to consider several factors before the compound is evaluated. The factors are outlined in Table II.

Please keep in mind that these factors are also things we have to consider on allelochemicals for commercialization. These factors are: (1) Biological Efficacy. In the 1960's and 1970's, one to two kg active ingredient/ha rate was an acceptable rate of application;

**Table II. Factors Affecting New Agricultural Chemical/Biological Control Agents**

- 
1. Biological Efficacy (Rate, Resistance)
  2. Cost (Manufacture; Treatment)
  3. Health, Toxicology
  4. Environmental Impact
  5. Method of Application
- 

now we consider 1 kg/ha rate too high. Most of today's new products are used at a rate below 0.5 kg/ha. Resistance problems are another concern, especially for insecticides and fungicides and, more recently, certain herbicides. If cross resistance to a new compound is found, then further development may be cancelled. (2) Cost. The manufacture and treatment per acre should be cheaper than for existing products or offer some other advantage over those on the market today. If the compound is for a minor use or for use only in minor crops, the project could be suspended. For example, if a compound is only to be used in onion or cabbage as a herbicide, the market is not large enough to permit recovering the cost of the R and D; therefore, most companies will put that compound back on the shelf and it will never reach the market. (3) Health and Toxicology. It need not be emphasized how important toxicological information is to any agricultural product; just look at the money spent on toxicology. In general, if acute oral LD<sub>50</sub> values are below 50 mg/kg, a company will mark the program with a "yellow flag", meaning extreme caution. (4) Environmental Impact. Again, I hardly have to mention the importance of environmental data; look at the requirements for environmental impact data for registration of agricultural products. (5) Method of Application. This is an important research area which ties into development of formulations, especially for soil pesticides or relatively volatile products.

Now let us examine some properties of recently introduced agricultural chemicals such as Glean, Arsenal, and Tilt. It is of interest that all of them have the following common characteristics: (1) low application rate: effective rates of these compounds are only several oz./acre or grams/ha; (2) all of them have very low mammalian toxicity; in other words, they are very safe compounds as to acute oral toxicity; (3) all of them are targeted to special uses; (4) some of them were originally found in biological materials and the major mode of action was thoroughly studied.

Next we consider the process of developing a new agricultural product. Figure 2 is just a simplified chart. It may vary from company to company, from compound to compound. This whole process normally takes 7 to 10 years and more than \$40 million.

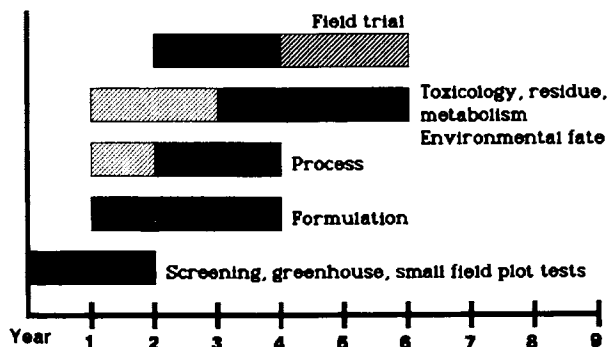


Figure 1. Simplified diagram for registration of new pesticide.

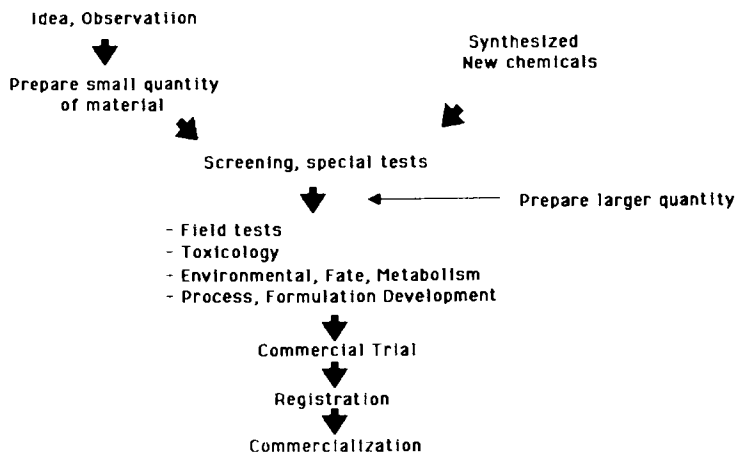


Figure 2. Chemical industry approach to develop new product.

Methods by which to find a biologically active compound may be classified as: (a) random selection/screening; (b) directed synthesis; (c) natural product models; and (d) biorational design. Allelochemical research can be considered as a natural product model. Generally, natural pesticides such as allelochemicals can be the biological compound(s) itself or products or parts of plant tissues.

Let us review again concerns about new agricultural chemicals. Among these factors, toxicology is the most important, given biological efficacy. Most of the common allelochemicals are relatively safe as far as acute oral toxicity is concerned. However, their metabolites may be a problem.

Now I present some examples of possible uses of allelochemicals and their problems for commercialization. Before I give these examples, please note that when chemical companies try to develop an allelochemical for commercialization, it has to fulfill the following requirements: (1) the chemistry has to be well characterized; (2) biological effects have to be quantifiable; (3) toxicology has to be understood; (4) metabolism of the compound has to be well known; and (4) the manufacturing process has to be defined.

Example 1: Citrus oil or citrus root products can indeed kill slugs if they are applied as a contact agent or act as a slug repellent. Although some patents have been issued on the use of citrus oil as a slug control agent, such control with a contact agent is difficult and the chemical compositions of citrus oil and root exudate are ill-defined. Other obstacles for commercialization of citrus oil are: (1) market size is too small to justify the development cost, and (2) an effective delivery method has yet to be developed.

Example 2: Certain plant root exudates contain factors stimulating soybean root growth as well as nodule formation. These stimulating factors contain protein as well as several other chemical components. This research is still at a very early stage, and it will take additional effort to characterize these factors and enormous developmental work before they can be commercialized. It may become too expensive for a company to develop this type of minor special-use product.

Example 3: In an interesting cultural practice, farmers grow special weeds in grape fields in the spring; and after the weeds are killed by flooding the fields, allelochemical(s) are released into the soil to control nematodes. So far, this has only been practiced in certain grape production areas. If it can be demonstrated on other crops, it would certainly be interesting research to follow up. This cultural procedure does indeed produce good quality grapes.

Example 4: In pineapple fields where the intercrop of the rubber plant and sweet potato were used, it was observed that these fields were less weedy than the pineapple-rubber intercrop fields. It was also noted that in the pineapple-sweet potato intercrop fields, there were more sweet potatoes harvested. Whether the sweet potatoes produced the allelochemicals to suppress the gaseous weeds in the pineapple fields or the pineapples produced the allelochemicals to stimulate the growth of the sweet potatoes or the rubber plants produced the allelochemicals to suppress the weeds has yet to be studied.

Examples 3 and 4 illustrate a potential application of allelopathic chemicals in today's agricultural practices, as illustrated in Figure 3: to plant allelopathic plants as a cover crop in the field, especially for tree crops such as peach and citrus. Such plants produce allelopathic chemicals to control soil pests such as nematodes. This approach will be appreciated today, because there is no effective agent to control soil pests on a field scale and, as has been noted, there is no economic incentive for a chemical company to develop a minor-crop pesticide.

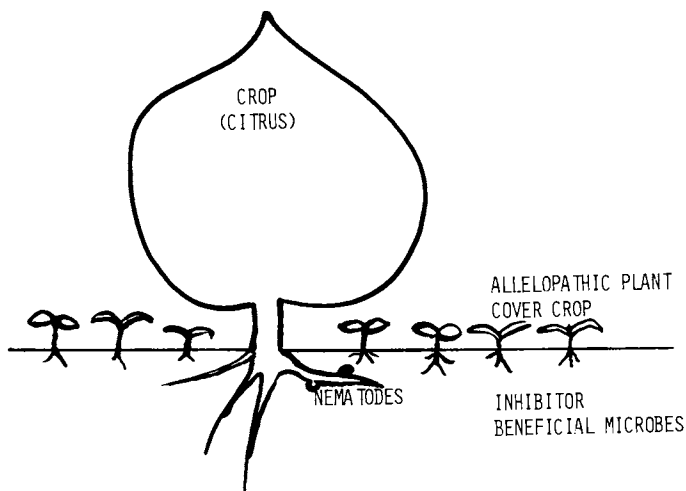


Figure 3. Example of application of allelochemical in agriculture.

The study of allelopathic chemicals is providing an alternative source for new types of chemicals, and yet many chemical companies are reluctant to discuss their research effort in the allelopathic chemical area.

In summary, allelochemical research has generated enormous excitement for organic chemists, natural product chemists, biochemists, and industrial researchers. Many allelopathic compounds remain to be isolated and characterized. In order to commercialize the allelopathic products, there is a lot of hard and tedious work ahead of us. But I believe it will be a rewarding and exciting job. Maybe in eight to ten years, we will hear of many allelopathic products from the farmers and researchers.

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## Chapter 41

# Metabolic Detoxification of Linear and Angular Furanocoumarins by Caterpillars of the Black Swallowtail Butterfly

## Implications in Host Plant Selection Phenomena

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Midgut and body tissues of caterpillars of the black swallowtail butterfly (Papilio polyxenes Fabr.) possess high enzymatic activity that catalyzes the detoxification of linear furanocoumarins, thus explaining the tolerance of P. polyxenes to these phototoxins. Observations from nature indicate that P. polyxenes caterpillars are less tolerant toward the presence of angular furanocoumarins in potential host plants, and our studies with a commonly occurring angular furanocoumarin suggest that metabolic detoxification of such compounds by P. polyxenes occurs at a relatively slower rate than with the linear analogs. The capacity to detoxify dietary furanocoumarins is a major determinant of host plant acceptability by P. polyxenes; furthermore, this phenomenon represents a clear example of herbivore circumvention of a normally effective host-plant-resistance mechanism.

Furanocoumarins are present as secondary constituents in hundreds of plant species from at least eight families, but appear to be most predominant in species of Umbelliferae and Rutaceae (1,2). Furanocoumarins occur as linear or angular analogs, which differ in the angle of furan ring fusion to the coumarin moiety (Figure 1). Well over 100 such compounds have thus far been isolated from plant sources; most of these arise by alkyl or alkoxy substitution at the available aromatic positions, at either of the two olefinic carbons of the furan ring, or, less frequently, at either of the two olefinic carbons of the coumarin lactone ring.

Many if not most furanocoumarin derivatives possess potent photoactive properties. It is generally accepted that the biological actions of furanocoumarins are attributable to the fact that these compounds easily intercalate into the double helix of DNA, where they produce cyclobutane adducts with

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pyrimidine bases upon activation by long-wavelength ultraviolet light (3-5). Active sites are the furan and coumarin lactone ring double bonds; linear furanocoumarins produce both monoadducted and crosslinked DNA, but the configuration of the angular furanocoumarins is such that only monoadducts can be generated. Superoxide radicals and/or singlet oxygen may also be involved in some of the biological actions of these chemicals (6-8).

Furanocoumarins have a number of scientifically interesting and even economically and medicinally important actions. They are effectively used in human medicine in the treatment of vitiligo (skin pigmentation, leukoderma) (9) and psoriasis (10,11), and have shown promise against certain other human maladies (12-15). Plants that contain furanocoumarins are known to cause acute photosensitization (phytophotodermatitis) in man (2,16).

It seems likely that the biosynthesis of furanocoumarins by plants serves as a defense mechanism against plant pathogens and herbivorous animals, and to improve competitiveness against other plant species. Furanocoumarins are in fact known to be active as phototoxins against livestock (17) and as phytoalexins (16,18). Furanocoumarins are also seed germination (19) and plant growth (20) inhibitors, although light activation is presumably not required for these interactions because such activities are expressed in the soil environment.

Most insect herbivores appear to be rather effectively repelled by furanocoumarin-containing plants (21-24). A notable exception to this generalization occurs among some butterflies of the family Papilionidae, whose caterpillars are adapted to feed successfully and in fact preferentially on plants that contain linear, but not angular, furanocoumarins (22). These circumstances prompted us to undertake studies with the black swallowtail butterfly (Papilio polyxenes) and radiolabeled furanocoumarins in attempts to elucidate the nature of the insect/furanocoumarin interactions involved.

#### Metabolic Basis for Papilio polyxenes Resistance to Linear Furanocoumarins

Our initial studies (25,26) determined the comparative fate of a radiocarbon-labeled preparation of the commonly occurring linear furanocoumarin, xanthotoxin (8-methoxypsoralen) in black swallowtail caterpillars and in fall armyworm (Spodoptera frugiperda J. E. Smith) larvae. Black swallowtail caterpillars are known not to be adversely affected by linear furanocoumarins (22), while Spodoptera spp. avoid such plants as food sources (21). Equivalent doses of [<sup>14</sup>C]xanthotoxin (5 µg/g) administered orally to last-stage larvae of either species was followed by dramatically different disposition patterns. Elimination of radiocarbon in the excreta was much more rapid in P. polyxenes (Table I), and [<sup>14</sup>C] residues in body tissues of S. frugiperda accumulated to much higher levels and persisted much longer than in P. polyxenes (Table II). Studies of the nature of the radiocarbon residues in body tissues showed that levels of unmetabolized xanthotoxin in body tissues of

Table I. Radiocarbon Excretion After Oral Treatment of Last-Stage Larvae of *P. polyxenes* and *S. frugiperda* with [ $^{14}\text{C}$ ]Xanthotoxin at 5  $\mu\text{g}/\text{g}^{\text{a}}$

Hours After Treatment	% of Administered Radiocarbon in Excreta ( $\bar{X} \pm \text{S.D.}$ )	
	<i>P. polyxenes</i>	<i>S. frugiperda</i>
1.5	50.3 $\pm$ 6.8	0.9 $\pm$ 1.1
3	62.4 $\pm$ 4.2	18.3 $\pm$ 2.7
6	77.6 $\pm$ 0.8	41.6 $\pm$ 16.5
12	77.6 $\pm$ 6.0	59.7 $\pm$ 8.0
24	100.9 $\pm$ 5.6	87.8 $\pm$ 6.7

<sup>a</sup>Data adapted from Ivie et al. (1983) (25) and Bull et al. (1984) (26).

Table II. Radiocarbon Residues in Body Tissues (Exclusive of Gut and Contents) After Oral Treatment of Last-Stage Larvae of *P. polyxenes* and *S. frugiperda* with [ $^{14}\text{C}$ ]Xanthotoxin at 5  $\mu\text{g}/\text{g}^{\text{a}}$

Hours After Treatment	% of Administered Radiocarbon in Body Tissues ( $\bar{X} \pm \text{S.D.}$ )	
	<i>P. polyxenes</i>	<i>S. frugiperda</i>
1.5	3.6 $\pm$ 1.0	54.6 $\pm$ 4.1
3	3.3 $\pm$ 1.8	36.7 $\pm$ 4.7
6	0.2 $\pm$ 0.1	15.0 $\pm$ 4.6
12	0.1 $\pm$ 0.1	4.3 $\pm$ 2.2
24	0.2 $\pm$ 0.2	2.4 $\pm$ 0.9

<sup>a</sup>Data adapted from Ivie et al. (1983) (25) and Bull et al. (1984) (26).

*S. frugiperda* were at every sampling interval >50 times as high as in body tissues of *P. polyxenes*. These data are highly supportive of the hypothesis that rapid metabolic detoxification of linear furanocoumarins, coupled with rapid excretion of the metabolites, account for the insensitivity of such insects to the adverse effects of these potent phototoxins (25,26). Subsequent studies by us (27) have shown that high titers of mixed function oxidase enzymes in midgut and body tissues of *P. polyxenes* account for the rapid detoxification phenomena observed, which result primarily in cleavage of the furan ring (Figure 2).

Comparative Fate of Linear and Angular Furanocoumarins in *P. polyxenes*

Because the resistance of *P. polyxenes* to the toxic effects of linear furanocoumarins apparently does not extend to the angular furanocoumarins (22), we have undertaken comparative metabolic fate studies with a representative of each of these furanocoumarin classes. Tritiated psoralen or isopsoralen (Figure 1) was administered as before to last stage *P. polyxenes* caterpillars, and the distribution, elimination, and biochemical fate of the compounds determined (28).

Table III. Excretion of Radioactivity After Oral Treatment of Last-Stage Caterpillars of *P. polyxenes* with Either [<sup>3</sup>H]Psoralen or [<sup>3</sup>H]Isopsoralen at 5 µg/g<sup>a</sup>

Hours After Treatment	% of Administered Radioactivity in Excreta ( $\bar{X} \pm$ S.D.)	
	Psoralen-treated	Isopsoralen-treated
0.75	28.5 + 5.7	19.4 + 2.9
1.5	44.8 + 8.8	40.9 + 10.7
3	71.1 + 4.3	67.6 + 8.9
6	83.2 + 6.8	77.7 + 5.9
12	93.0 + 0.9	90.8 + 1.5

<sup>a</sup>Data adapted from Ivie et al. (28).

The disposition patterns of psoralen and isopsoralen in *P. polyxenes* under the parameters studied were not dramatically different. As indicated in Table III, there were no appreciable differences in the rate of excretion of radioactivity by caterpillars treated with the two compounds. In body tissues, however, levels of total radioactivity in isopsoralen-treated caterpillars were consistently about twice those observed in psoralen-treated insects (Table IV). Further, levels of unmetabolized parent compounds retained in body tissues (where toxic effects would be expressed) were on the order of 3 times as high in caterpillars treated with the angular furanocoumarin, isopsoralen (Table V).

Analysis of excreta samples indicated that both psoralen and isopsoralen are metabolized extensively by *P. polyxenes* caterpillars, primarily by the same furan ring cleavage reactions observed in our earlier studies with xanthotoxin (Figure 3).

### Discussion

Our studies with *P. polyxenes* caterpillars and radiolabeled furanocoumarins have provided what appears to be a definitive explanation of the apparent total insensitivity of these insects to the toxic effects of linear furanocoumarins present in their



Figure 1. Structures of a linear furanocoumarin (psoralen) and an angular furanocoumarin (isopsoralen).

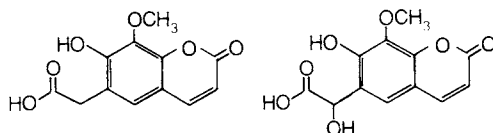


Figure 2. Major metabolites of xanthotoxin (8-methoxypsoralen) in last-stage larvae of the black swallowtail butterfly (*Papilio polyxenes*) and the fall armyworm (*Spodoptera frugiperda*).

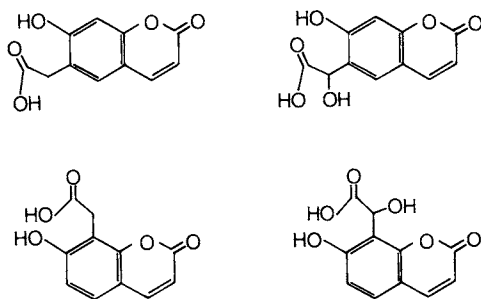


Figure 3. Major metabolites of psoralen and isopsoralen in last-stage caterpillars of the black swallowtail butterfly (*Papilio polyxenes*).

Table IV. Tritium Residues in Body Tissues (Exclusive of Gut and Contents) After Oral Treatment of Last-Stage Caterpillars of P. polyxenes with Either [<sup>3</sup>H]Psoralen or [<sup>3</sup>H]Isopsoralen at 5 µg/g<sup>a</sup>

Hours After Treatment	% of Administered Radioactivity in Body Tissues ( $\bar{X} \pm$ S.D.)	
	Psoralen-treated	Isopsoralen-treated
0.75	9.4 $\pm$ 1.5	19.1 $\pm$ 3.0
1.5	8.8 $\pm$ 1.2	18.0 $\pm$ 5.1
3	5.5 $\pm$ 1.2	9.4 $\pm$ 1.7
6	3.1 $\pm$ 0.4	5.8 $\pm$ 1.4
12	3.0 $\pm$ 0.2	4.5 $\pm$ 0.6

<sup>a</sup>Data adapted from Ivie et al. (28).

Table V. Unmetabolized Psoralen or Isopsoralen in Body Tissues (Exclusive of Gut and Contents) of Last Stage P. polyxenes Caterpillars Treated Orally with Either [<sup>3</sup>H]Psoralen or [<sup>3</sup>H]Isopsoralen at 5 µg/g<sup>a</sup>

Hours After Treatment	% of Administered Radioactivity as Unmetabolized Parent Compound in Body Tissues	
	Psoralen-treated	Isopsoralen-treated
0.75	2.8 $\pm$ 1.0	8.8 $\pm$ 2.0
1.5	1.5 $\pm$ 0.4	6.0 $\pm$ 4.7
3	0.9 $\pm$ 0.6	2.4 $\pm$ 2.0
6	0.4 $\pm$ 0.1	1.0 $\pm$ 0.3
12	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1

<sup>a</sup>Data adapted from Ivie et al. (28).

normal host plants. P. polyxenes has evolved highly active detoxification mechanisms against linear furanocoumarins such that levels of the intact photosensitizers do not accumulate in body tissues (where detrimental light-induced reactions would be expected to occur). On the other hand, at least one furanocoumarin-susceptible insect (S. frugiperda) metabolizes these compounds at a much lower rate, with the result that under constant dietary exposure, toxic levels of the intact phototoxins would presumably accumulate and be retained in the general body circulation.

Differences in metabolic detoxification rates between linear and angular furanocoumarins observed in our studies were not great. However, the fact that the angular furanocoumarin under study accumulated to appreciably higher levels in body tissues of P. polyxenes caterpillars than did the linear analog is

supportive of the hypothesis that a reduced detoxification rate accounts, at least in part, for the susceptibility of P. polyxenes caterpillars to the detrimental effects of angular furanocoumarins. This conclusion differs from our earlier interpretation of data obtained from limited studies on the interaction of unlabeled psoralen and isopsoralen with P. polyxenes (26).

Plant furanocoumarins occur widely in nature and provide formidable obstacles to grazing by herbivorous animals. Some insect species have nevertheless adapted to circumvent this powerful host-plant-resistance mechanism. It has been proposed that the leaf-rolling habit of some insect species may be an evolutionary adaptation to avoid light and thus avoid the toxic effects of furanocoumarins (21). Also, evidence has recently been obtained that the capacity of at least one leaf-mining insect species to detoxify furanocoumarins allows the utilization of furanocoumarin-containing plants as hosts (29).

Our studies indicate that rapid metabolic detoxification of linear furanocoumarins is an effective resistance mechanism for P. polyxenes against the toxic effects of these compounds. It has been postulated that the adaptation of some plants to produce angular furanocoumarins was in response to the reduced effectiveness of the linear furanocoumarins as deterrents for herbivores such as P. polyxenes (22). Such may indeed be true, but our studies on the comparative detoxification of linear and angular furanocoumarins suggest that, at best, the presence of angular furanocoumarins in plants confers only a tenuous margin of relative "safety" against P. polyxenes.

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## Chapter 42

# Physiological Interactions Between Phytophagous Insects and Their Hosts

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External excitatory and inhibitory stimuli for acceptance or rejection of food are modified by the internal condition of the insect (e.g., hunger). This modification can cause a given stimulus to produce different responses, depending upon the internal condition of the insect. After host acceptance, there are still obstacles that the insect must overcome in order to survive, grow, and reproduce. These hurdles include allelochemicals that block nutrient availability, defenses of susceptible plants, poor balance of essential nutrients, and less than optimal water content. Neonate larvae may be far more sensitive to some of these obstacles than older ones.

Numerous models for the strictly behavioral aspects of host plant acceptance have been proposed, but a very intriguing recent one is the "rolling fulcrum" model described by Miller and Strickler in 1984 (1). It is a mechanical analog of Dethier's (2) model for the influence of external and internal factors on insect acceptance or rejection of a candidate host plant. The uniqueness of this model lies in a movable fulcrum that represents the changes in sensitivity to external excitatory and inhibitory stimuli based on the internal condition of the organism (e.g., hunger vs. satiation). As the fulcrum moves, the "leverage" exerted by a given stimulus changes owing to the change in the physiological state of the insect. We have modified the rolling fulcrum model to include postingestive as well as preingestive behavior, and have used it as a conceptual framework to show the relationships between our experiments and certain physiological aspects of plant insect interactions (Figure 1).

The first component of the model is the rolling fulcrum described by Miller and Strickler (1). External stimuli include such things as pH of plant tissues perceived by an insect seeking the phloem, as well as factors closer to the surface, such as volatiles or cuticular waxes. Having accepted the plant (we recognize that this is not as clear-cut an event as the model would

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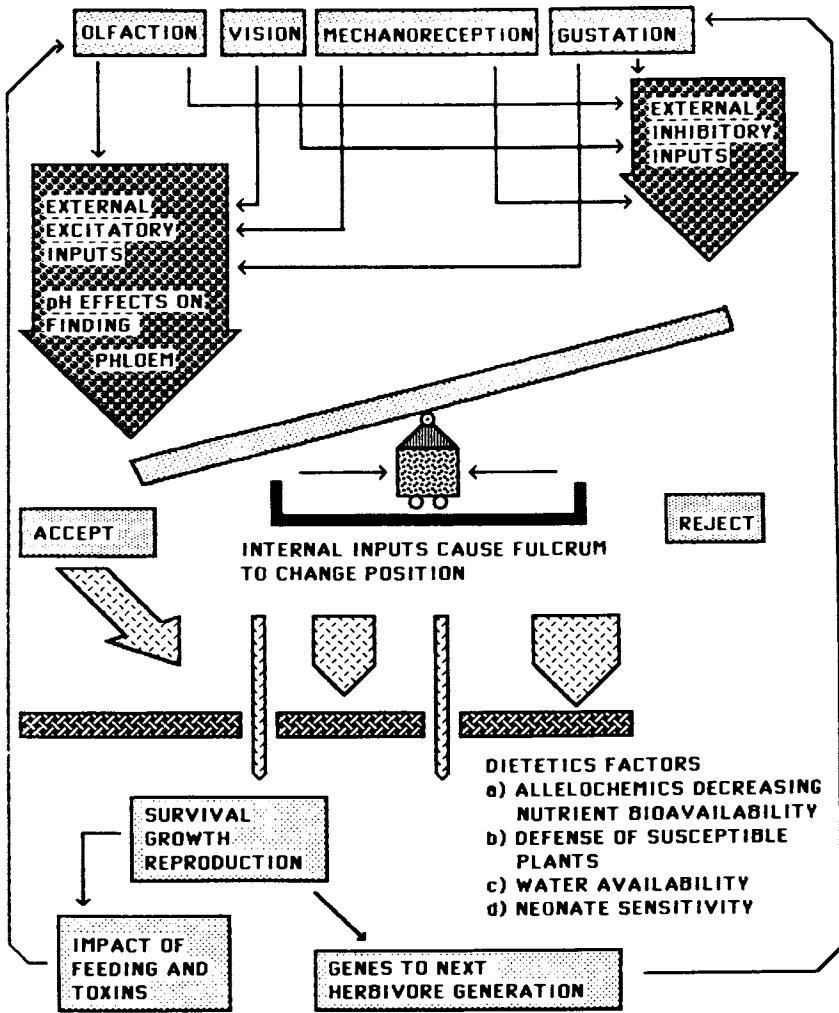


Figure 1. Model for the influence of external and internal factors on insect investment behaviors and the influence of postingestive dietetics factors on survival, growth, and reproduction. Modified from Miller and Strickler (1).

indicate), the insect must then be able to ingest and assimilate essential nutrients and be capable of converting these materials to the energy and structural materials necessary for survival, growth, and reproduction. Allelochemicals blocking the bioavailability of nutrients, various other defenses of even the most susceptible plants, lack of enough moisture, and neonate sensitivity of the insect all form sieves through which not all individuals pass. Those that survive pass on their genes to future generations.

### External Stimuli

The greenbug [*Schizaphis graminum* (Rondani)] is a major pest of cereals and small grains in North America, and is ideally suited to feeding behavior studies. Greenbug (biotype E) feeding behavior involves intercellular penetration of plant tissues by the stylets composed of solidified salivary secretion. By following the stylet sheaths in properly stained and sectioned plant leaves, one can determine not only the number of probes made by the insects, but the depth and success of these efforts. On an artificial liquid diet (3), where the greenbugs have fed through a Parafilm membrane, the intact sheaths are easily counted.

For the aphid, finding phloem must be a formidable task. As the stylets progress forward, they travel for short distances between the cell wall and the cell membrane and may occasionally enter the protoplasm (4). The stylets may bring back small amounts of solubilized material to gustatory sensilla in the epipharynx (5, 6). Forbes (7) and McLean and Kinsey (8) reported finding gustatory chemoreceptors in the stylets of the green peach aphid and in the precibarium of the pea aphid. The greenbug may use a similar method to locate the phloem.

The apparent orientation of insect stylets to the vascular bundles caused by hydrogen ion concentrations was first reported by Fife and Frampton (9). The leafhoppers fed mainly in the phloem tissue, which had a substantially higher pH than the surrounding plant cells. Other discriminating criteria for phloem-feeding insects have been indicated, including carbohydrate concentration (10) and positive hydrostatic pressure (11).

On an artificial diet, biotype C greenbugs (3) responded to pH like the beet leafhopper. Biotype E greenbugs also preferred a slightly alkaline diet (Figure 2).

To determine the pH of plant tissues, young sorghum plants were sectioned horizontally, washed quickly with distilled water, and examined with a binocular microscope. Microelectrodes for pH measurement (Microelectrodes, Inc., Londonderry, NH 03053) were held in micromanipulator arms and placed into individual plant cells. The pH of the phloem was found to be higher than that of the surrounding cells and, not surprisingly, similar to the pH of the optimal artificial diets. These data suggest that greenbugs locate phloem, at least in part, through their sensitivity to pH.

On a susceptible sorghum plant, it may take the insects as long as 30 min to reach the phloem and begin feeding (12). Once the greenbugs locate the phloem and begin feeding, they apparently become less sensitive to the pH of their food source. On artificial diet, this process occurs within seconds of puncturing the Parafilm membrane and fully inserting the stylets. Large groups of greenbugs

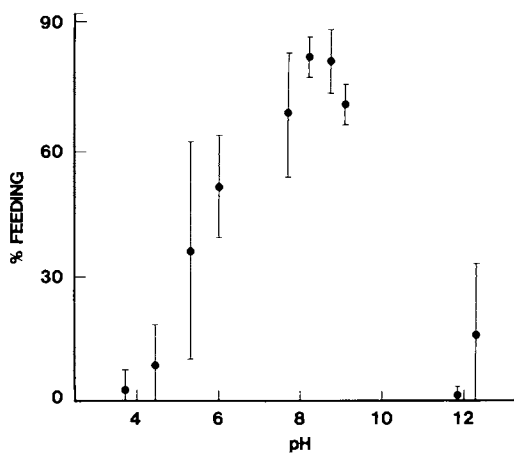


Figure 2. Greenbug preference for diets of different pH in no-choice tests.

were placed on the diet and were allowed to feed undisturbed. At selected intervals, following their introduction to the feeding cages, 40% formic acid was injected into the diet containers. The result of this action was a drop in the pH of the diet from 8.5 to approximately 1.8. In cages where the insects had been given ample time to insert their stylets and begin feeding, few or no behavioral changes were observed. The insects continued to feed and produce honeydew for several hours, until they fell to the floor of the cage and died. This suggests that there is a window of sensitivity or behavioral program that allows them to accurately discriminate dietary pH only while they are probing; they are no longer sensitive to pH once they have become "committed" to phloem ingestion.

The chinch bug, Blissus leucopterus leucopterus (Say), is another serious pest of cereals, threatening grain sorghum production primarily in the tallgrass prairie regions of the Midwest.

In another experiment, chinch bugs were initially placed into sealed J-cups containing a Parafilm-covered diet (greenbug diet) cap identical to those used for aphids. Chinch bugs readily located and fed upon the liquid through the membrane. This is the first example of chinch bug subsistence in the laboratory on an artificial diet.

A major difficulty with chinch bugs not experienced with greenbugs is their nonsedentary feeding behavior. Easily disturbed, chinch bug nymphs and adults are extremely active, particularly if the host (plant or diet) somehow deteriorates from an ideal condition. In addition, chinch bugs must be offered the diet in a manner that allows close contact of the venter with another surface, satisfying a thigmotaxis possibly associated with their cryptic behavior of ovipositing and feeding behind plant leaf sheaths. This requirement is usually accomplished by placing the diet cap upside down in the J-cup and providing a slight space for the insects to crawl underneath by resting one side on the raised center of the cup bottom.

Other experiments have examined more specific feeding requirements and inducements for the chinch bug through the use of feeding arenas offering an array of diets differing in pH. Preliminary results show a marked preference for the 7.0 - 8.7 pH range, with lowest feeding responses at the extremes. Counts of stained stylet sheaths on the Parafilm membranes and weights of fecal material deposited under the diet caps indicate that the bugs were actively probing and feeding preferentially on the more attractive diets.

Studies involving the caging of neonate nymphs or eggs for measurements of rate of development have resulted in the successful rearing of some individuals on diet to the adult stage; however, high mortality was common.

### Nutritional Requirements

For a species of insect to survive (to pass through the sieve of Figure 1), its food must contain the nutrients essential for that species. Nutrition used in this narrow sense (as opposed to the broader term insect dietetics) delineates the minimal nutritional requirements for successful growth, development, and reproduction (13). Serving as a nutrient is not an inherent quality of a

particular compound, but depends upon the physiological capabilities of the ingesting organism. Therefore, a compound that is a nutrient for a fast-growing, immature organism may not be one for a mature individual; a compound that is required at one concentration may be toxic at a tenfold greater concentration (14). However, some compounds are required by most insects. Extensive reviews of these requirements have been edited or written by Rodriguez (15), House (16), Dadd (17, 18, 19, 20), and Hagen et al. (13), and a brief summary of much of this work is contained in a recent review by Reese and Schmidt (21).

Nitrogen. In addition to experiments dealing with specific amino acid requirements, much work has been done in recent years on total nitrogen. Nitrogen appears to impose upper limits to insect growth, with a wide range of growth rates occurring below these limits because of many other factors (22, 23, 24, 25, 26, 27, 28, 29, 30, and Scriber's chapter in this volume).

Water. Although often omitted from lists of essential nutrients, water is the universal biological solvent in which the biochemical reactions of each cell occur. Most living organisms contain far more water than any other compound or group of compounds. Although most insects are 70-80% water, their food may vary from 1 to over 90% water. Stored-product insects have remarkable abilities to conserve water, whereas phytophagous insects may suffer deleterious effects from low dietary moisture.

The effects of water on digestive efficiency are particularly interesting. Soo Hoo and Fraenkel (31) suggested that water content of the diet was important to efficiency of conversion in Prodenia eridania. Hoekstra and Beenackers (32) thought that differences in moisture content of various host plants accounted for differences in efficiencies of conversion for Locusta migratoria. Similarly, Scriber (22) found that lower moisture levels decreased the efficiency of food conversion in Hyalophora cecropia larvae. Reese and Beck (33) noted that dilution of nutrients by water was not compensated for by increased ingestion in Agrotis ipsilon, and that the efficiency of conversion of assimilated food and of ingested food were negatively correlated with percent dry matter of the artificial diet. However, the optimal percent dry matter of the diet was not the same for both efficiency and growth. Growth decreased somewhat on the low-percent-dry-matter diets (high efficiency but less dry matter ingested because of dilution), and decreased a great deal as percent dry matter increased above the control diet level (low efficiency and less dry material ingested). Therefore, optimal growth occurred at a percent dry matter very close to that of the control diet level, but at a percent dry matter below that for maximal dry weight eaten, owing to the relationship between dry weight eaten and the efficiency of conversion of ingested food (33).

A great deal of research has been done on the effects of water content of insect food. Water, like nitrogen, appears to impose upper limits to insect growth, below which a variety of growth rates occur due to the many other possible factors (22, 28, 29 and Scriber's chapter in this volume).

### Allelochemicals Decreasing Nutrient Bioavailability

A second key component of insect dietetics is the effect of defensive allelochemicals on insect growth and development. Many amino acid analogs, phenylpropane derivatives, gossypol-like compounds, alkaloids, tannins, lignans, flavonoids, benzoxazolones, phenolics, benzoic acid derivatives, and benzyl alcohol derivatives have been isolated from plants and have been shown to have deleterious effects on insect growth, development, and reproduction (34). Although allelochemicals are by definition non-nutrients, they have been shown to interact with nutrients. Many of the deleterious metabolic or chronic effects of plant allelochemicals may be due to these interactions. Many of the types of interactions discussed in vertebrate nutrition literature may also be important in insect dietetics (14). Certain allelochemicals structurally resemble essential nutrients closely enough to compete metabolically (35, 36, 37, 38, 39, 40). Allelochemicals may block the bioavailability of nutrients by reducing assimilation, efficiency of conversion of assimilated food, or the efficiency of conversion of ingested food (41, 42, 43, 44, 45, 46).

### Defense of "Susceptible" Plants

We have tested the hypothesis that even susceptible host plants have defenses against insect attack in contrast to an artificial diet containing low concentrations of defensive allelochemicals and having no morphological means of defense. A few species of insects have been observed to have increased fecundity and growth on artificial diets compared to preferred plants (47, 48, 49, 50, 51).

In our experiments, black cutworm (*Agrotis ipsilon*) (BCW) larvae were fed corn seedlings and artificial diet. While there appear to be species of plants that are even more susceptible to BCW larvae than corn, corn was not statistically different from the most susceptible species tested by Busching and Turpin (52), and certainly BCW can occasionally be extremely damaging to corn. Further, Wilson et al. (53), Tseng et al. (54), and Jarvis et al. (55) have screened thousands of maize introductions, inbred lines, and hybrids, and have found only a very few sources of even moderate resistance.

In our experiments, larvae grown on Pioneer 3368A corn seedlings for 8 days weighed only 14-15% as much as larvae fed artificial diet. Funk's G4507A seedlings also inhibited growth compared to artificial diet. By the sixth day, weight of the larvae on plants was 8.1% of that of larvae on diet (56). We are currently conducting experiments with several of the apparently still more susceptible species discussed by Busching and Turpin (52), such as bluegrass (*Poa pratensis* L.), curled dock (*Rumex crispus* L.), lambsquarters (*Chenopodium album* L.), yellow rocket (*Barbarea vulgaris* R. Br.), and rough pigweed (*Amaranthus retroflexus* L.). Preliminary results indicate that all these species support reduced growth compared to artificial diet.

### Sources of Error in Nutritional Indices

In many studies of insect dietetics, nutritional indices have been

used to estimate the insect's nutritional efficiency and energy budget. Unfortunately, experimental errors are often made in collecting the data. Small gravimetric and round-off errors can be magnified algebraically by the equations used to calculate the nutritional indices. Seemingly insignificant errors in calculating the initial dry matter of the food, amount of food eaten, and weight of feces produced can substantially alter the assimilation (AD), efficiency of conversion of assimilated food (ECD), and efficiency of all ingested food (ECI). An electronic spreadsheet computer program can be used to simulate the effect of purposely introduced errors in a nutritional index study.

Schmidt and Reese (57) derived a hypothetical set of nutritional index data for the BCW on artificial diet. Using the electronic spreadsheet to alter the experimental inputs, they systematically identified a number of error parameters. They concluded that controlling the percentage of food eaten (% FE) was a major factor in obtaining consistent results. Coupled with an accurate estimation of the initial dry matter of the diet, this reduced spurious recordings to negligible levels. If the insects ate a large portion of the food given to them, better results were obtained than when they consumed only a small fraction of the diet available. At approximately 80% food eaten (FE), there was less potential for errors in the calculation of percent dry matter of the food than at 20% FE. As the percentage of food eaten decreases, the ECD, AD, and ECI become more prone to error.

Since insect fecal pellets contain both undigested food and nitrogenous waste products, Bhattacharya and Waldbauer (58) subtracted the urine content of the feces from the total weight of the fecal pellet. This provided better estimates of assimilation and conversion of assimilated food. Schmidt and Reese (57) noted that BCW larvae will feed upon their fecal pellets if no other food is available. Growth on fecal pellets is nearly as rapid as growth on diet, suggesting that much of the nutrient content of the diet is not assimilated. The result of fecal feeding on nutritional parameters is an overestimation of the AD and ECI and an underestimation of the ECD.

### Neonate Sensitivity

Sensitivity to deleterious effects of plants. In our effort to develop sensitive bioassays for investigating the effects of plants on insect growth and development, we placed BCW larvae on artificial diet and corn seedlings. Neonate larvae on corn seedlings weighed 1.7 mg 7 days later; neonate larvae placed on artificial diet for 2 days, then fed corn seedlings for 5 days, weighed 10.2 mg. Thus, even though the larvae started on diet spent 71% of the experimental period on corn seedlings, they weighed 6 times as much as those kept on plants from the start. To demonstrate that neonate larvae are more sensitive to the deleterious effects of plants than older larvae, a 48-h "boost" on artificial diet was inserted at various points during the experiment. The data from this experiment (Figure 3) clearly show that a boost during the first 48 h is far more effective at relieving the deleterious effects of the plant than one later in the experiment.

Sensitivity to physiological stress. BCW larvae are sensitive not

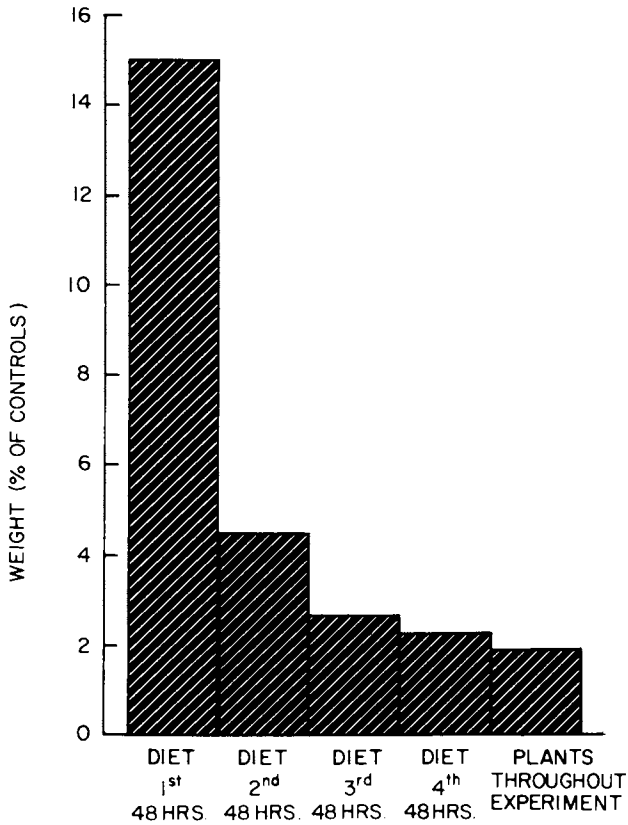


Figure 3. Mean weights of black cutworm larvae fed Funk's G4507A corn seedlings and given a 48-h "boost" on artificial diet at various times during the experiment. Control larvae were fed artificial diet for the entire experiment. Each bar represents the mean of 20 larvae.



only to the growth-inhibiting effects of plants, but also to environmental and dietetic stress. Nutritional index experiments have shown that neonate BCW larvae are affected by physical disturbance, temperature, percent dry matter of their food source, and hydrogen ion concentration (pH) of the artificial diet. Table 1 illustrates the results of experiments where BCW neonates were exposed to variations in these conditions.

The physical handling of insect larvae is a common practice in rearing programs. In Table 1, neonates that were picked up with a paint brush shortly after eclosion grew more slowly and weighed less than undisturbed larvae 10 days later. This sensitivity decreases with age but still can be demonstrated many hours following emergence.

Physical handling not only reduced ingestion (DWE) but altered the insects' ability to assimilate (AD) and convert food (ECI) to body matter. Conversion of assimilated food (ECD) was the only nutritional parameter unaffected by handling (e.e., a larger percentage of digested food was not metabolized for energy). Reduced weight gain, then, cannot be attributed solely to an interruption of feeding. Handling may diminish the insect's oxidative metabolism or cause internal damage to the digestive apparatus and should be avoided when working with neonate larvae.

Another important factor in BCW growth is temperature. In our laboratory, BCW larvae were reared at 25°C on a 16:8 (L:D) photoperiod. Larvae maintained at 22°C, as might be expected, consumed very little food and developed much more slowly than the control larvae. Growth reduction, though, was a function of reduced intake (DWE) and reduced conversion (ECD, ECI) offset by a slight increase in digestibility (AD). The insect retained the food in the gut for a longer period of time and digested a larger percentage of it, but converted less of the digested food to body matter. At higher temperatures the insect's ingestion rate and efficiency of conversion approach an upper limit probably around 30°C.

Altering the dry matter of the diet (DMD) had a significant effect on the dry matter of the larva (DML) and the dry matter of the feces (DMF). This finding supports assumptions made by Reese and Beck (33) that BCW larvae have a limited ability to regulate their bodily dry matter and that the DMF is poorly maintained in comparison to that of other insects. With the exception of the AD, the nutritional index parameters showed a tendency towards an optimal DMD level around 25%. DWE and DWG were positively correlated with this DMD optimum and with each other, as they are in other insects (59).

The final section of Table 1 illustrates experiments in which 0.5 g of KOH or NaOH were added to 150 g of stock diet to make it more alkaline. Both diets had mildly deleterious effects on the larvae, but growth reduction occurred for different reasons. On the diet containing KOH, reduced DWG was a result of diminished food conversion, whereas the NaOH diet primarily reduced assimilation. As expected the insect's overall ability to utilize the alkaline diet (ECI) decreased.

Fecal pH levels were also significantly different from the controls and from the pH of the diet. This finding indicates that BCW larvae can regulate the pH of the hind gut, but only to a limited degree. Whether growth reduction in this experiment was

Table 1. The Effects of Physiological Stress on Black Cutworm Food Utilization. (\* P<.05, \*\* P<.01 Values have been normalized to the controls.)

	DWE	DML	DWG	DMF	AD	ECD	ECI	Fecal pH
Physical disturbance	67**		57**		84**	98**	83	
Temperature °C								
22	8**		5**		134*	56**	71**	
25	100		100		100	100	100	
27	113		117		102	101	103	
Dietary dry matter (%)								
23.37	65**	85	57**	87	92	96	89	
24.61	77**	91	77**	101	99	100	100	
25.84	100	100	100	100	100	100	100	
26.96	78*	96	79*	119	100	99	100	
28.64	49**	121*	43**	148**	93	88	84*	
34.04	39**	148**	28**	122**	94	77*	58**	
pH								
KOH trt.	6.9 pH							116*
control	4.9 pH	103	88*		97	89**	86**	
NaOH trt.	7.2 pH	87	78*		89*	101	89*	112**
control	4.8 pH							

actually mediated by the dietary pH change or by other changes in the diet making it less available to the insect is unknown.

### Summary

External excitatory and inhibitory stimuli for host acceptance or rejection of food are modified by the internal condition of the insect (e.g., hunger). This modification can cause a given stimulus to have a greater or lesser amount of "leverage." After acceptance, there is still a "sieve" through which the insect must pass in order to survive, grow, and reproduce. Some of these obstacles include allelochemicals that block nutrient availability, poor balance of essential nutrients, defenses of susceptible plants, and less than optimal water content. Neonate larvae may be far more sensitive to some of these obstacles than older larvae.

### Acknowledgment

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## Chapter 43

# Structural and Genetic Variation of Natural Pesticides in Pigment Glands of Cotton (*Gossypium*)

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Cotton (*Gossypium*) and other plants in the tribe Gossypieae contain lysigenous pigment glands in leaves and stems. The glands contain high concentrations of biologically active terpenes, including volatile terpenes known to repel or attract insects and nonvolatile terpenoid aldehydes that have a wide range of antibiotic activity. We surveyed over 30 *Gossypium* species for structural and quantitative variations in these terpenes, and made interspecific crosses to analyze genetically the structural variations of terpenes. The results of these experiments and their implications for future research are discussed.

The genus *Gossypium* includes four species cultivated for cotton fiber and over 30 wild species. The major cultivated species, *G. hirsutum*, is referred to as Upland cotton and is grown throughout the world. *G. barbadense* is grown mainly for thread and high quality textiles. In different countries or regions it is known as Egyptian, Pima, Tanguis, or Sea Island cotton. *G. arboreum* and *G. herbaceum* are grown mostly in Asia on small acreages in marginal production areas with minimal use of agrochemicals; these species are referred to as Asiatic or Old World cottons.

*Gossypium* and the other seven genera of the tribe Gossypieae are distinguished from other species of the Malvaceae by the production of lysigenous pigment glands (1). The spherical glands consist of a central cavity filled with yellow oil surrounded by a single layer of flattened epithelial cells. In green tissues the epithelial cells contain chrysanthemins and other anthocyanin pigments giving them a dark appearance (2). In leaves and young stems the glands are located below the palisade and hypodermal cells, respectively. In old stems and roots the glands also are scattered throughout the bark.

The importance of pigment glands as sources of toxins in cottonseed was suspected early in the twentieth century. In 1915 Withers and Carruth (3) showed that the toxicity of cottonseed to animals was due to the compound gossypol localized in pigment glands.

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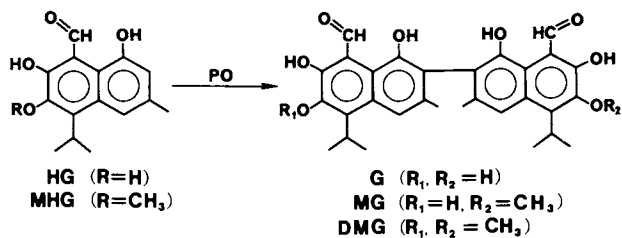
The structure of gossypol (G; Scheme 1), however, was not determined until the 1940's and was confirmed by total synthesis in 1958 (4). Gossypol is a dimeric product formed from hemigossypol (HG; Scheme 1) by the action of peroxidase (5). All species of Gossypieae produce gossypol in pigment glands of either the seed or foliage.

The importance of pigment glands in cotton-pest interactions became apparent with the discovery of glandless cottons in the late 1950's. These cottons subsequently were developed with the aim of increasing the use of cottonseed in animal feeds and in human food. In field trials, however, glandless plants were damaged more severely than glanded plants by several cotton pests, and they were attacked by several herbivores (beetles, cutworms, pill bugs, rodents, and pheasants) that did not normally feed on glanded plants (6). Based on these discoveries, several programs were initiated to develop highly glanded (and presumably high-gossypol) cottons with increased resistance to insects. The toxicity of flower buds from certain cottons, however, was greater than could be accounted for based on their gossypol contents (7), which indicated that other compounds were involved. Consequently, in the 1970's we began a series of studies to identify the allelochemicals in pigment glands. In general, two groups of chemicals, terpenoid aldehydes and volatile terpenes, were found. Detailed descriptions of the structures, biological activity, biosynthesis, and genetic control of these compounds are given in the following sections.

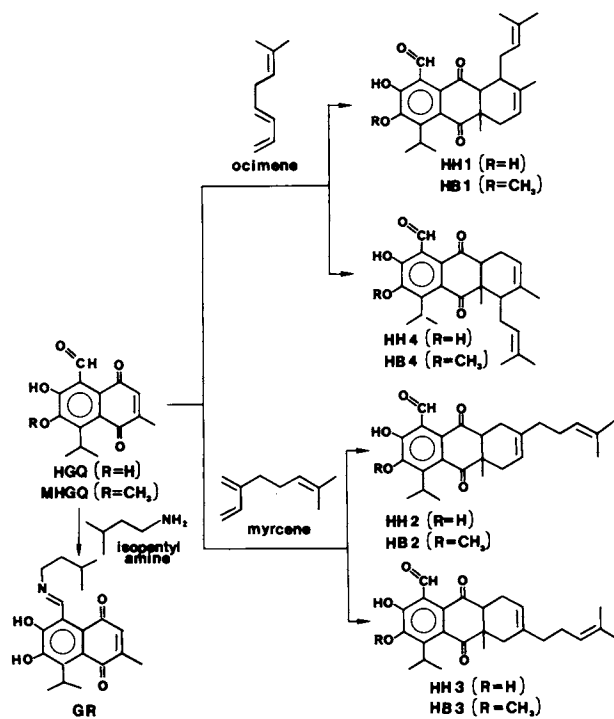
#### Terpenoid Aldehydes (TA) and Derivatives in Pigment Glands

TA in Upland and Asiatic cotton. When extracts from glanded and glandless cottons were compared by thin-layer chromatography, we found six yellow pigments and one red pigment uniquely associated with glands. Each yellow pigment gave a typical aldehyde reaction with acidic phloroglucinol or 2,4-dinitrophenylhydrazine. One of the yellow pigments was identified as gossypol, while the other five were identified as hemigossypolone (HGQ; Scheme 2) and the derived TA, heliocides H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub>, and H<sub>4</sub> (HH<sub>1</sub>, HH<sub>2</sub>, HH<sub>3</sub>, HH<sub>4</sub>; Scheme 2) (8 - 11). Helicoides H<sub>1</sub> and H<sub>4</sub> are the isomers derived from the Diels-Alder reaction of hemigossypolone with *trans*- $\beta$ -ocimene, and heliocides H<sub>2</sub> and H<sub>3</sub> are similarly formed from hemigossypolone and myrcene (Scheme 2).<sup>3</sup> Neither reaction requires an enzymatic catalyst, because they occur spontaneously at room temperature. The concentrations of H<sub>1</sub> and H<sub>2</sub> are always two to three times those of H<sub>4</sub> and H<sub>3</sub>, respectively, apparently because steric hindrance of the methyl group on hemigossypolone favors the former products. The red pigment, named gossyrubilone (GR; Scheme 2), is the condensation product of hemigossypolone and isopentylamine (12).

Hemigossypolone along with its derivatives occur only in tissues with differentiated chloroplasts (12). Nongreen tissues such as seed embryos, roots, flower petals, staminal tissue, and inner stem bark contain only gossypol as the prominent TA. Thus, the key enzyme involved in diverting terpenoid biosynthesis from hemigossypol to hemigossypolone may be formed during differentiation of chloroplasts.



Scheme 1



Scheme 2



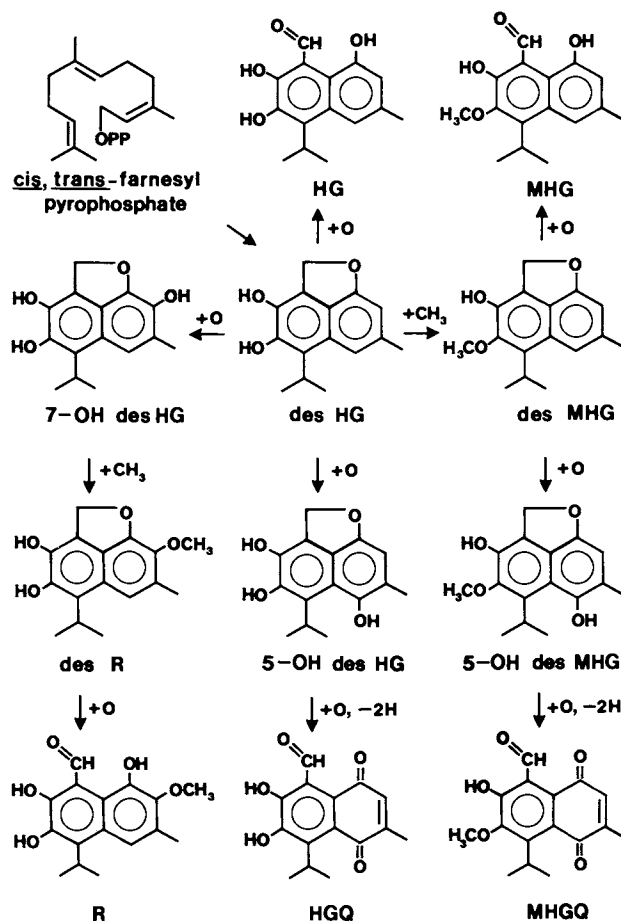
TA in *G. barbadense* cotton. Analyses of TA in *G. barbadense* cultivars revealed five compounds not found in Upland or Asiatic cottons (12). These compounds were identified as the 6-methyl and 6,6'-dimethyl ethers of gossypol (Scheme 1; MG, DMG) and the 6-methyl ethers of hemigossypolone (Scheme 2; MHGQ) and of heliocides H<sub>1</sub> and H<sub>4</sub>. The latter two compounds, which are formed from ocimene and MHGQ, were given the trivial names heliocide B<sub>1</sub> and B<sub>4</sub> (Scheme 2; HB<sub>1</sub>, HB<sub>4</sub>); the B symbols were used for the methylated heliocides to indicate their discovery in *G. barbadense*. No heliocide derivatives of myrcene (i.e., HH<sub>2</sub>, HH<sub>3</sub>, HB<sub>2</sub>, and HB<sub>3</sub>) were found, indicating that *G. barbadense* does not synthesize appreciable myrcene. Apparently methylation is introduced early in the terpenoid biosynthesis pathway of *G. barbadense*, because similar percentages of methylation (50-70%) are found in all of the different types of TA found in pigment glands of leaves. High levels of gossypol methyl ethers also are found in nongreen tissues, indicating that the methylating enzyme is not uniquely associated with chloroplasts.

TA in wild cottons. Many wild cottons have TA contents similar to those of *G. barbadense*. Thus, 30-80% methylation of terpenoids and a predominance of ocimene-derived heliocides also occurs in *G. anomalum*, *G. bickii*, *G. capitis-viridis*, *G. darwinii*, *G. longicalyx*, *G. tomentosum* and *G. sturtianum* (12). In the latter two species, the TA quinone concentrations are much greater than the heliocide concentrations, indicating that very little ocimene or myrcene is formed in these species.

The wild New World diploid cottons contain two TA patterns not found in other species. Ten of these 12 species contain only gossypol or gossypol and its methyl ethers in pigment glands of all tissues (12). Thus, the enzyme necessary for formation of TA quinones apparently is absent from green tissues in these species. *G. raimondii* uniquely produces the TA raimondal (R; Scheme 3) as the only major TA in pigment glands (13). This structural variation, like TA quinone formation, is limited to green tissue; gossypol is the predominant TA in nongreen tissue. *G. gossypioides* is the only New World diploid species that produces abundant hemigossypolone and heliocides H<sub>1</sub>-H<sub>4</sub> similar to Upland cotton.

#### Volatile Terpenes in Pigment Glands

Terpenes in cultivated Upland cottons. Elzen et al. (14) compared the volatile terpene content of five pairs of genetically similar glanded and glandless cotton cultivars and strains. In all pairs, the volatile terpene content of leaves from the glanded plants was more than 100 times that from the glandless ones. Glandular localization of volatile terpene content was confirmed by studies which showed that over 3% of the oil collected from glands consisted of volatile terpenes in approximately the same ratios as found in total plant extracts, while fluids from surrounding cells were devoid of these compounds. The total composition of volatile terpenes collected directly from pigment glands of calyxes of 'Acala SJ-1' is shown in Table I; structures of volatile terpenes emitted by cotton are shown in Fig. 1.



Scheme 3

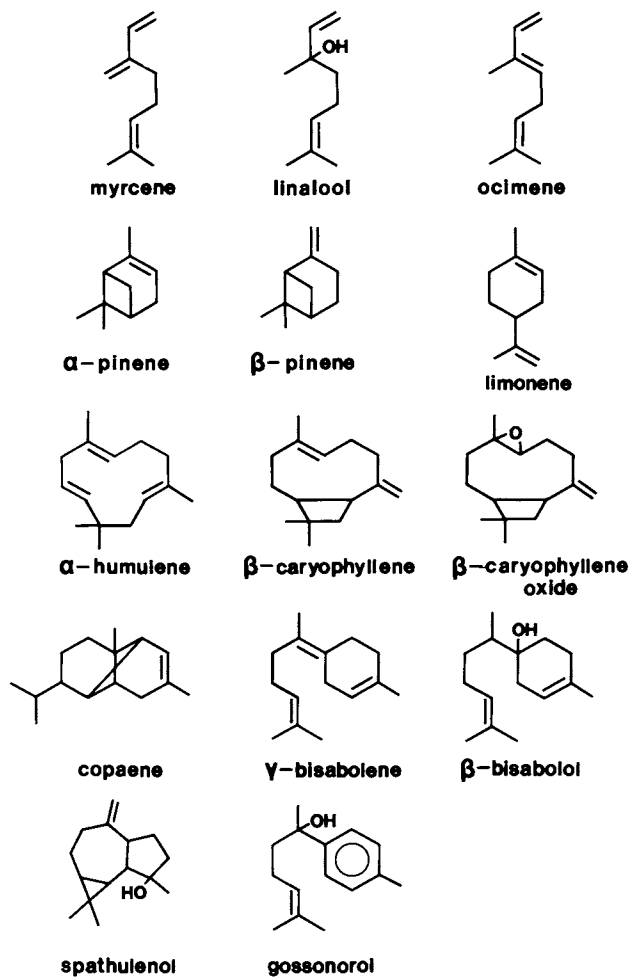


Figure 1. Volatile Terpenes Emitted from Cotton.

Table I. Volatile Terpene Concentrations in Oil Collected from Pigment Glands of Calyxes of *G. hirsutum* 'Acala SJ-1'

Compound	ppm	Compound	ppm
$\alpha$ -Pinene	11580	$\beta$ -Caryophyllene	3215
$\beta$ -Pinene	1929	$\beta$ -Caryophyllene oxide	129
Myrcene	2572	$\gamma$ -Bisabolene	643
Ocimene	252	$\beta$ -Bisabolol	7074
Limonene	643	Spathulenol	386
$\alpha$ -Copaene	955	Gossonorol	386
$\alpha$ -Humulene	1286	TOTAL	31050

Terpenes in *G. barbadense* and Upland race stocks. Different cultivars of Upland cotton have similar profiles of volatile terpenes, but *G. barbadense* and certain insect-resistant Texas race stocks of *G. hirsutum* (15 - 17) have profiles different from the cultivars. *G. barbadense* lacks myrcene,  $\gamma$ -bisabolene and  $\beta$ -bisabolol, which are major components in cultivated Upland cotton, but it has greater concentrations of  $\alpha$ -copaene (18; Table II).

Table II. Percentage Distribution of Volatile Terpenes in Pigment Glands of Leaves of Cultivars and Texas Race Stocks of *G. hirsutum* and of *G. barbadense* 'Seabrook Sea Island 12B2'

Compound	Cultivars <sup>a</sup>	Texas Race Stock						SBSI 12B2
		254	695	810	953	1055	1123	
		(% of total volatile terpenes) <sup>b</sup>						
Pinenes	5-8	4	1	<1	29	4	<1	17
Myrcene	8-12	3	1	6	14	5	<1	0
Ocimene	5-8	2	<1	4	3	5	<1	<1
$\alpha$ -Copaene	1-2	<1	2	6	1	6	6	4
$\alpha$ -Humulene	9-12	17	11	10	2	11	4	15
$\beta$ -Caryophyllene	26-34	71	40	35	1	37	10	63
$\beta$ -Caryophyllene oxide	<1	1	1	35	<1	17	45	0
$\gamma$ -Bisabolene	6-9	0	18	3	17	11	9	0
$\beta$ -Bisabolol	11-15	0	17	0	13	<1	24	0
All others	6-9	2	9	1	20	4	2	1

<sup>a</sup>Data for five cultivars adapted from Elzen *et al.* (14) and given as range.

<sup>b</sup>Determined by the methods of Elzen *et al.* (14).

Resistant *G. hirsutum* race stocks Texas No. 254 and 810 also are deficient of bisabolene and  $\beta$ -bisabolol; stocks 810, 1055, and 1123 have greatly increased caryophyllene oxide contents; and stock 953 has greatly reduced concentrations of caryophyllene, caryophyllene oxide, and humulene (Table II). It is tempting to attribute the known increased resistance to insects in the Texas race stocks to the changes in volatile profiles, but this hypothesis remains to be proven.

Terpenes in Asiatic and wild cottons. Volatile terpene profiles also were measured in two collections of the African wild cotton, *G. anomalum*, and in two collections of the Asiatic cotton, *G. arboreum*. Both species lacked or had very low levels of  $\gamma$ -bisabolene and  $\beta$ -bisabolol (Table III). The Asiatic cottons also contained large percentages (7.5-33.0%) of caryophyllene oxide. We recently began to study the volatile terpenes in New World wild species. These preliminary studies indicate that species that lack hemigossypolone also are devoid of all volatile terpenes. We have no explanation for this interesting relationship.

Table III. Percentage Distribution of Volatile Terpenes in Pigment Glands of *G. arboreum*, *G. anomalum*, *G. hirsutum* and their Hybrids

Compound	Species or Hybrid <sup>a</sup>				
	<i>G. ar</i>	<i>G. an</i>	<i>G. hi</i>	<i>G. ar</i> x <i>G. an</i>	<i>G. ar</i> x <i>G. an</i> x <i>G. hi</i>
	(% of total volatile terpenes) <sup>b</sup>				
Pinenes	0-1	24-27	11	0-3	1
Myrcene	<1-4	0	9	0-12	1
Ocimene	<1-7	0	5	0-14	1
$\alpha$ -Humulene	15-20	15-17	12	6-14	17
$\beta$ -Caryophyllene	44-60	58-59	34	12-54	55
$\beta$ -Caryophyllene oxide	7-33	0	<1	1-61	17
$\gamma$ -Bisabolene	0-3	0	<6	0-<1	2
$\beta$ -Bisabolol	0-<1	0	14	0-2	5

<sup>a</sup>Four geographical collections of *G. arboreum* (*G. ar*), two collections of *G. anomalum* (*G. an*), *G. hirsutum* cultivar 'CAMD-E' (*G. hi*), two *G. arboreum* x *G. anomalum* hybrids and one [(*G. arboreum* x *G. anomalum*) x 'CAMD-E'] hybrid; data given as ranges for more than one collection or hybrid.

<sup>b</sup>Determined by methods of Elzen *et al.* (14).

### Biological Activity of Cotton Allelochemicals

The TA and volatile terpenes in pigment glands of cotton show a variety of allelochemical effects which have been reviewed (6,19,20). Gossypol, the most thoroughly studied TA, is toxic or antibiotic to many monogastric animals, insects, nematodes, fungi, gram-positive bacteria and enveloped viruses. At sublethal doses it also causes male sterility in various animals, including man (21). The heliocides were named for their antibiotic activity toward Heliothis spp., and are also antibiotic to other insects (19,22) and toxic to animal cells (23). Two insect specialists on cotton (the Alabama leafworm, Alabama argillacea, and the cotton bollweevil, Anthonomus grandis grandis) are not adversely affected by gossypol at the concentrations found in most commercial cultivars (6,24). The bollweevil appears to actually prefer glanded plants over glandless ones.

Only a few efforts have been made to evaluate the effects of TA structural variations on toxicity. Methylation decreases toxicity of hemigossypol to Verticillium dahliae (25), of gossypol to rat mast cells (23), and of various TA to Heliothis spp. (22). In contrast, methylation increases the toxicity of various TA to nematodes (26) and the toxicity of heliocides to rat mast cells (23). The order of toxicity of different TA to rat mast cells is: quinones > heliocides > naphthols > binaphthols (23). Gossypol generally is somewhat more toxic to insects than heliocides, and quinones are least toxic (19,22).

At low concentrations, gossypol stimulates feeding by some insects even though it is inhibitory at higher concentrations. A model to describe both the stimulatory and inhibitory effects of gossypol on Heliothis larval growth rate as a function of concentration has been proposed (19). It is not known whether other TA also show this dual allelochemical effect.

Volatile terpenes from pigment glands may also be beneficial or detrimental to herbivores which feed on cotton. Several volatile terpenes, especially in mixtures, attract boll weevils (27), cotton leaf worms (18), and beneficial parasitoid wasps that attack the tobacco budworm, a pest of cotton (28). Caryophyllene oxide slightly stimulates or inhibits growth of Heliothis virescens depending on concentration (19), and has a strong synergistic interaction with the TA gossypol. Both the feeding stimulation and antibiotic activities are amplified by mixing appropriate concentrations of the two chemicals. A human hypersensitive reaction to cotton fields and processing plants also has been attributed to volatile terpenes from pigment glands (29). Volatile terpenes probably act as deterrents to feeding by some herbivores, but this remains to be determined.

### Biochemical and Genetic Regulation of Terpenoids

Terpenoid aldehydes. The structural variations of TA in Gossypium probably originate from three variations in the oxidation and methylation of desoxyhemigossypol (desHG) as proposed in Scheme 3. The basic (or primitive) pathway probably is the one in which uncontrolled, partial methylation of desHG occurs, and both desHG and desMHG are converted first to HG and MHG, respectively, (Scheme 3)

and then to G, MG and DMG (Scheme 1). In this case 30-80% of the TA are methylated and the availability of methyl donors is the only restriction on methylation.

The two most common variations of the proposed basic pathway involve: 1) the introduction of oxygen into the position para to the furan oxygen of desoxyhemigossypol (desHG) and desoxyhemigossypol 6-methyl ether (desMHG) to form sequentially 5-hydroxy desHG and 5-hydroxy desMHG, HGQ and MHGQ, and heliocides H and B, respectively, in green tissues, and 2) the regulation (inhibition) of methylation to give mostly the 6,7-dihydroxy TA in all tissues. The first variation is found in all except 13 Gossypium species and the second variation occurs in all except 12 species. Several species, such as Upland and Asiatic cottons, show both variations. The third variation of desHG metabolism probably involves oxidation at the position adjacent to the furan oxygen to form sequentially 7-hydroxy desHG, 7-methoxy desHG, and eventually raimondal in green tissue. This variation occurs only in G. raimondii, and may have given a survival advantage to this otherwise most primitive Gossypium species (1).

The fact that all cotton species possess a structural gene for methylation can be demonstrated by inducing TA synthesis in juvenile cells. For example, 2- to 7-day-old radicles of Upland and Asiatic cottons, especially if chilled, will form considerable (>30%) quantities of methylated TA (30, 31) even though methylation is not expressed in pigment glands (12). Likewise, TA formed by suspension-cultured cells of these species in response to heat-killed fungal cells have >30% methylation (31, 32). When G. hirsutum with restricted methylation is crossed with species having abundant methylation, the F<sub>1</sub> hybrids, except those from G. sturtianum, do not show methylation (Table IV) and the F<sub>2</sub> progeny from G. barbadense hybrids segregated approximately 1/2 methylated to three nonmethylated (6).

Table IV. Percentages of Methylation of Total Terpenoid Aldehydes in Pigment Glands of Various Gossypium Species and Hybrids with G. hirsutum

Species	Methylated TA <sup>a</sup>	
	Parent species	<u>G. hirsutum</u> hybrid
	%	
<u>G. hirsutum</u>	<5	---
<u>G. barbadense</u>	>60	<5
<u>G. tomentosum</u>	>70	<5
<u>G. longicalyx</u>	>50	<5
<u>G. robinsonii</u>	>40	<5
<u>G. sturtianum</u>	>50	>50

<sup>a</sup>Estimated visually on TLC plates by published methods (12).

These observations indicate that all cottons possess a structural gene for methylation, but many species have evolved dominant regulatory genes that restrict or prevent this reaction especially in pigment glands and in differentiated tissue. A modification that prevents regulation by the G. hirsutum regulator gene apparently has evolved in the structural gene for methylation in G. sturtianum, because interspecific F<sub>1</sub> hybrids from this species always showed >50% methylation.

The production of quinones (HGQ and MHGQ) or raimondal (R) in green tissues appears to have resulted from the evolution of dominant structural genes, because both characters are dominant in interspecific hybrids. Data for raimondal are shown in Table V. Similar dominance of quinone formation in hybrids occurred when G. arboreum (a quinone-producer) was crossed with G. thurberi, G. trilobum, or G. raimondii, which do not produce quinones. In F<sub>2</sub> progeny, raimondal production segregates as a single dominant gene.<sup>2</sup>

Table V. Percentages of Raimondal<sup>a</sup> in the Total Terpenoid Aldehydes from Pigment Glands of Gossypium Species and Their Hybrids with G. raimondii

Species	Raimondal <sup>a</sup>	
	Parent species	<u>G. raimondii</u> hybrid
		%
<u>G. raimondii</u>	>90	---
<u>G. arboreum</u>	<1	>50
<u>G. thurberi</u>	<1	>50
<u>G. harknessii</u>	<1	>50
<u>G. gossypoides</u>	<1	>50
<u>G. hirsutum</u>	<1	>50
<u>G. barbadense</u>	<1	>50

<sup>a</sup>Estimated visually on TLC plates by published methods (12).

Other variations of TA composition in pigment glands depend on the production of ocimene or myrcene to produce the heliocides. Ocimene and myrcene probably are formed from linalool (Figure 1) by alternative dehydration reactions. Changes in structure of the dehydratase could affect the relative percentages of the two products. In agreement with this concept, some quinone-producing species produce a predominance of ocimene adducts, others a predominance of myrcene adducts, and some produce similar amounts of each. The quantities of free ocimene and myrcene have not been determined. Three species, G. darwinii, G. tomentosum, and G. sturtianum, produce very low concentrations of heliocides but very



high concentrations of TA quinones. Thus, linalool production or dehydration is probably low in these species. In crosses between high-ocimene and high-myrcene species the genes controlling these characters behaved as separate dominant genes (6). Further studies are needed to determine whether these genes are allelic.

Volatile terpenes. In an initial study of regulation of volatile terpene synthesis we made the triple-species tetraploid hybrid [(G. arboreum X G. anomalum) X G. hirsutum]. The chromosome number of the G. arboreum X G. anomalum diploid hybrid was doubled with colchicine to restore fertility before crossing with G. hirsutum to give the final tetraploid hybrid. Volatile terpene profiles were measured at each stage of hybridization and are shown in Table III. These studies indicate that the production of large percentages of caryophyllene oxide in G. arboreum is due to a dominant gene, probably for an epoxigenase enzyme, that can be transferred into Upland cottons. Likewise, the production of  $\gamma$ -bisabolene and  $\beta$ -bisabolol in G. hirsutum apparently is due to a partially dominant gene that is missing in G. arboreum and in G. anomalum. The Texas race stocks that have markedly different volatile terpene profiles (Table II) should allow additional studies of the genetic regulation of volatile terpene biosynthesis. Studies of the volatile terpenes in other wild Gossypium species will determine whether other structural variations exist.

#### Genetic Manipulation of TA's

On the basis of our studies of regulation of synthesis we have developed Upland breeding stocks that have high percentages of methylation in the TA. Two approaches were used to obtain high percentages of methylation. In the first approach, the recessive non-regulating genes from G. barbadense or G. tomentosum were substituted into G. hirsutum in place of the normal regulator gene. Because all three species are tetraploids, this was accomplished by simply hybridizing the species, self-fertilizing the  $F_1$  progeny, selecting for methylation in the  $F_2$  progeny, and repeating this cycle by backcrossing selected progeny to G. hirsutum. Methylation was inherited as a recessive character. During six backcrosses into Upland cotton, this approach has been complicated by inconsistent expression of the regulator genes and undesirable associations between the methylating genes and poor agronomic characteristics. In the second approach we transferred the dominant structural gene for methylation from G. sturtianum into G. hirsutum. The two species were hybridized, the hybrid was doubled with colchicine to a hexaploid, and G. hirsutum was backcrossed five times, first into the hexaploid, then into the pentaploid, and finally into three successive aneuploid generations. At each step, plants were selected for methylation before backcrossing. After five backcrosses, the dominant methylating gene was expressed in 34% of the progeny. There has been continuous progress in improving the agronomic characters of these plants and in the percentage of plants showing methylation, indicating that this is the more effective method of manipulating methylation.

We have also developed stocks that produce mostly raimondal in place of the normal TA in pigment glands. This was accomplished by making the triple-species hybrid [G. hirsutum X (G. arboreum X G.

raimondii]]. The chromosome number of the *G. arboreum* X *G. raimondii* diploid hybrid was doubled with colchicine before crossing with *G. hirsutum*. The three-species hybrid and subsequent progeny containing *raimondii* have been backcrossed for five generations into *G. hirsutum* with continuous improvement of agronomic characters and the continued production of *raimondii* as a dominant character controlled by a single gene.

We have only recently begun to transfer genes that control the structure of volatile terpenes. Our targets are genes that control ratios of ocimene/myrcene, caryophyllene/caryophyllene oxide, and  $\gamma$ -bisabolene and  $\beta$ -bisabolol/total terpenes. Results to date suggest that it is possible to change the ocimene/myrcene (and thus heliocide H<sub>1</sub>, H<sub>4</sub>/heliocide H<sub>2</sub>, H<sub>3</sub>) ratios, to greatly increase caryophyllene oxide, and to eliminate  $\gamma$ -bisabolene and/or  $\beta$ -bisabolol from cultivated cottons.

### Applications

The development of near-isogenic stocks of Upland cotton, that contain the different genes controlling TA and volatile terpene synthesis in the *Gossypium* species, should allow us to determine the evolutionary significance of the changes in synthesis that have occurred. Some stocks undoubtedly will be more susceptible to certain pests, possibly because of the primitive characters substituted into them. Hopefully, other stocks with altered TA and volatile terpene contents may show improved resistance to pests, because most of these compounds have allelochemical effects (19, 20). These stocks will allow us to map several of the key genes involved in terpene synthesis and possibly to determine the evolutionary origin of these genes. These stocks should be ideally suited for working out the mechanics of transferring genes with genetic engineering techniques, because single genes cause specific changes in measurable natural products. Finally, these stocks should be valuable for isolating gene products, such as enzymes that control oxidation and methylation of terpenes.

Detailed understanding of TA and volatile terpene biosynthesis may eventually allow us to manipulate these pathways to produce allelochemicals normally not present in *Gossypium*. For example, specific enzymes might be transferred from the Hibisceae tribe (Malvaceae family), the Bombacaceae, or Ulmaceae to redirect the flow of terpene biosynthesis. Each of these groups of plants contains allelochemical terpenoids different from those in *Gossypium* but based on the cadalene ring structure (33). Transfer of one or at most a few genes from these species may allow redirection of the pathways in cotton toward compounds found in these other plants. The rapid progress in biotechnology could soon make the possibility of such transfers a reality.

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## Chapter 44

# Toxicants from Mangrove Plants

## Ichthyotoxins from the Philippine Plant *Heritiera littoralis*

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The mangrove plant *Heritiera littoralis* (Sterculiaceae) has been utilized by natives of the Philippines as a fish poison. The hexane extract of this plant has shown toxicity to fish (*Tilapia nilotica*). A novel sesquiterpene which was assigned the name heritianin has been identified as one of the compounds with ichthyotoxicity. The structure elucidation of heritianin and other compounds in the hexane extract of *Heritiera littoralis* is presented with bioassay data.

Mangroves are widespread in tropical and subtropical regions. They are considered to be the trees and bushes growing in the saline intertidal zones of sheltered coastlines. Their root systems are thus regularly inundated with saline water; however, dilution by flooding may occur once or twice annually (1). Four vascular plants associated with tidal mangrove swamps in Southeast Asia have been utilized by local inhabitants for their toxic properties (2). The natural sap or aqueous extract from the leaves and bark is used to stupefy fish, as poison for spear and arrow tips, and applied to tooth cavities and skin to deaden aches and pain.

Plant toxins have been known for many centuries. Rotenoids (rotenone), alkaloids (nicotine, coniine, strychnine), terpenoids (ovabin and hymenovin) are among the classes of natural products which provide numerous toxins (3-12). Toxins in plants often have the role of feeding repellents. They appear to be synthesized by plants as a defense against insects and other animals.

A remarkable number of insecticidal plants seem to have been recognized first as fish poisons. This group has received special scientific attention; for example, the already mentioned rotenone and other rotenoids of *Derris* and *Lonchocarpus* species (3-6), the lupine alkaloids of *Sophora* species (13), and a saponin (medicagenic acid) from *Medicago sativa* leaves (8).

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It can be imagined as only a short step from the aboriginal discovery of fish-killing properties to the practical test for insecticidal activity. Knowledge of the toxins of higher plants has led to a variety of useful drugs, an understanding of certain aspects of intermediary metabolism and metabolic diseases, and a useful body of knowledge of the prevention of human and animal poisoning by plants.

Mangrove plants provide a source of compounds having physiological activity. The objective in this research has been the isolation and structure determination of the toxic compounds obtainable from the hexane extract of the dried roots of Heritiera littoralis Dryland.

H. littoralis belongs to the Sterculiaceae family; it is a low, evergreen, seashore tree up to 15 m. tall. Prior to this study, little was known of the chemistry of H. littoralis roots. Only a few reports have appeared in the literature in regard to the chemical constituents of the leaves of this plant. It has been reported (14) that young and old leaves from 22 mangrove species of Northern Queensland (Australia) were investigated for inorganic ions and organic acids. The contribution of low molecular weight carbohydrates (glucose, fructose, and sucrose) and nitrogen-containing compounds (proline, total methylated onium compounds) to osmoregulation in mangrove was also determined (15). H. littoralis stores low molecular weight carbohydrates as organic solutes in high concentration. The contribution of total methylated onium compounds and amino acids to the amount of total nitrogen was very low in H. littoralis as compared with the other mangrove species investigated.

#### Materials and Methods

Sample collection. Various plant parts (bark, leaves, roots, and twigs) of H. littoralis were collected (16) from the Mangrove Forest Reserve in Pagbilao, Quezon in the Philippines. The plant materials were chopped into smaller pieces before extraction. A re-collection of 21 kg of air-dried ground roots was made for bulk extraction.

Extraction. Each dried plant part was Soxhlet-extracted with petroleum ether. The solvent was evaporated in vacuo to give a residue labeled Fraction A (Scheme I). The marc was air-dried and extracted with ethanol in a Soxhlet apparatus. Evaporation of the solvent in vacuo yielded Fraction B. The aqueous phase was removed by freeze-drying and the water-soluble residue was labeled Fraction C.

The petroleum ether residue (Fraction A) was redissolved in petroleum ether to give an experimental concentration of 0.1 part per thousand (0.1 ppt). The residue from the chloroform fraction (Fraction B) was redissolved in 95% ethanol. Aqueous and chloroform fractions were tested at final concentrations of 0.1 ppt.

Bioassay. A bioassay procedure which was referred to as "quick screening test" involving toxicity to fish was selected as a guide to the toxic compounds (16, 17). On this basis, the

aqueous fraction and Fractions A, B, and C were bioassayed to determine which plant parts and fractions were toxic to fish.

Tilapia fish (25-35 mm length) were selected as the test organisms because of their ability to live in adverse conditions. Five juvenile fish were acclimated in a glass tank containing 2 liters of clean, conditioned water. The extract was mixed with the water in the experimental medium. The tanks were not aerated nor the fish fed during the 24-hr test. Bioassays of the organic solvent extracts were performed at a concentration of 0.1 ppt.

### Results and Discussion

The extracts of H. littoralis that were obtained as illustrated in Scheme I were bioassayed with the quick screening test. A 100% mortality during the first 24 hr was used as the criterion for activity. The results of the preliminary bioassay are given in Table I. These data show that petroleum ether extracts of root, twig, and leaves were toxic to fish (Tilapia nilotica) within 20-45 min. On the other hand, the chloroform and aqueous fractions from the ethanol extract of only the roots showed activity within the 32-90 min of the "quick screening test." On the basis of the preliminary bioassay, the roots of H. littoralis were selected as the part of the plant to be examined. No mortality was observed in control exposures.

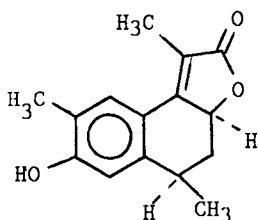
Table I. Toxicity of Mangrove Plant Heritiera littoralis on Juvenile Tilapia nilotica

Plant Parts	<u>Petroleum Ether Extract</u>			<u>Ethanol Extract</u>					
	Yield (%)	Conc (ppt)	Time (min)	<u>CHCl<sub>3</sub> Fraction</u>			<u>H<sub>2</sub>O Fraction</u>		
				Yield (%)	Conc (ppt)	Time (min)	Yield (%)	Conc (ppt)	Time (min)
Root	0.87	0.1	20	0.40	0.1	32	3.45	0.1	90
Bark	2.78	0.1	NM	0.67	0.1	NM	6.56	0.1	NM
Twig	0.46	0.1	20	0.29	0.1	NM	2.61	0.1	NM
Leaves	6.83	0.1	45	3.14	0.1	NM	0.45	0.1	NM

NM = No mortality

Time (min) = Maximum length for the death of all fish.

We have previously reported (18, 19) the isolation of heritol (I) from the methylene chloride soluble portion of the chloroform-soluble Fraction B (Scheme I).



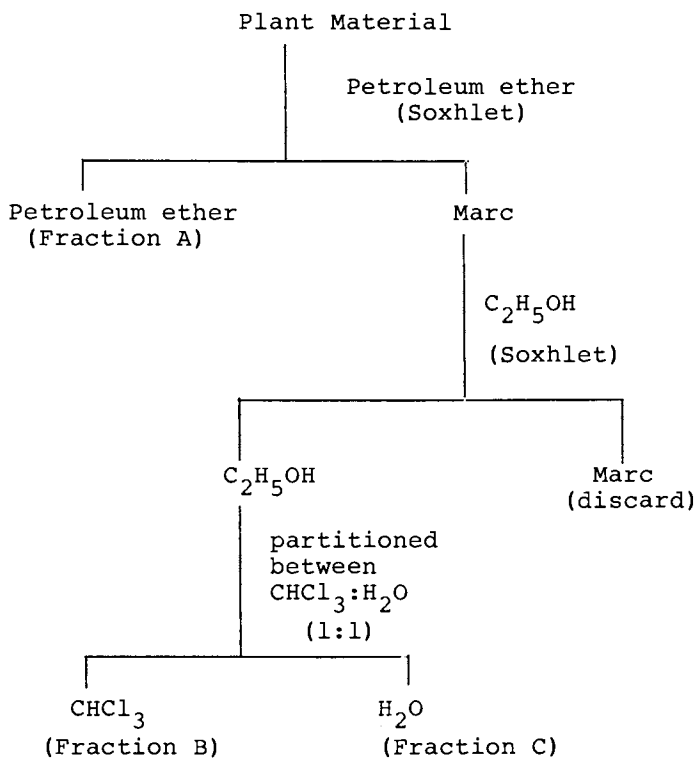
Heritol (I)

The structure was assigned on the basis of spectral evidence, chemical analysis, and consideration of the isoprene rule. Structure I was confirmed for heritol by x-ray diffraction experiments. Heritol (I) has demonstrated ichthyotoxicity (90 min) to *Tilapia nilotica* fingerlings (25–35 mm length: 0.05–0.25 g dry weight) at a concentration of 20 ppm. Heritol is particularly interesting since it occurs in a mangrove plant and possesses toxic properties as well as a novel sesquiterpene skeleton containing an  $\alpha,\beta$ -unsaturated  $\gamma$ -lactone moiety.

In this paper we report upon a detailed examination of the hexane extract (Fraction A) of 21 kg of the roots of *H. littoralis*. The dry hexane extract was subjected to chromatography on a silica gel column and eluted with a hexane/ethyl ether gradient. From the different fractions eluted, three crystalline compounds were isolated; two of which are new natural products. The compounds described herein are tentatively named compounds II and III. The additional compound will be discussed in a future paper.

Compound II, m.p. 260°–261°C, was isolated as colorless needle-shaped crystals and was identified as friedelin by IR, NMR, and TLC comparison with the corresponding data reported for friedelin in the literature (20).

Compound III was recrystallized from a 1:1 hexane-ether mixture as colorless needles, m.p. 198°–199°C. The molecular formula  $C_{16}H_{18}O_4$  was deduced by high resolution mass spectrometry. This formula indicates an unsaturation number of eight. The bands exhibited by the IR spectrum at  $1750\text{ cm}^{-1}$  and  $1640\text{ cm}^{-1}$  (conjugation of C=C double bond with a carbonyl group) are typical of an  $\alpha,\beta$ -unsaturated- $\gamma$ -lactone. This deduction was confirmed by the UV spectrum, which showed an absorption at 226 nm, and by the  $^{13}\text{C}$  NMR spectrum (Figure 1), which at very low fields displayed a low-intensity signal at 176.8 ppm (line 1), which is characteristic of a quaternary carbon (21). This strongly implied a carbonyl group. Chemical evidence for the presence of a  $\gamma$ -lactone was obtained with the alkaline iron (III) hydroxamate test (22) in which III gave a red-blue color. Compound III gave a negative test for a phenolic group with the



Scheme I. Preliminary Extraction Procedure for Plant Material.

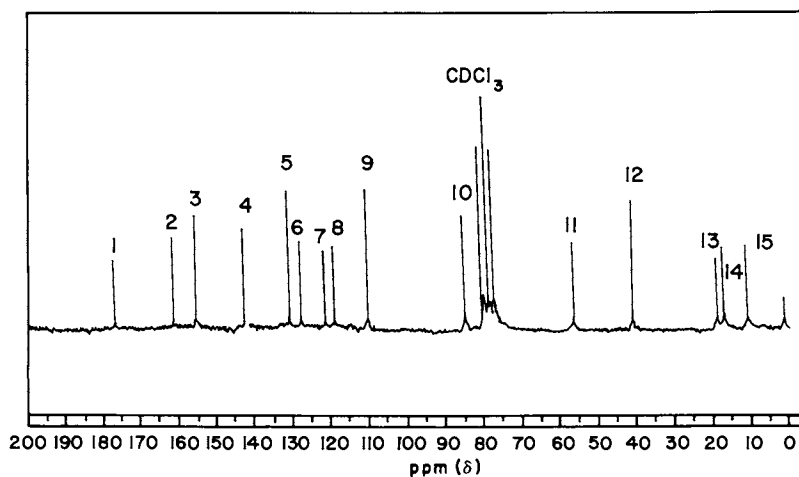


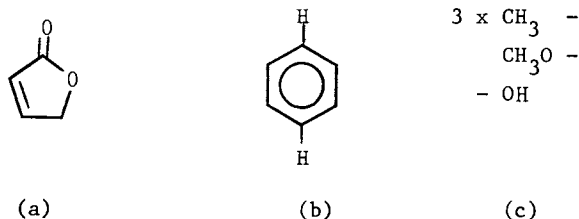
Figure 1.  $^{13}C$  NMR Spectrum of Compound III.



ferric chloride/potassium ferricyanide reagent. A band at  $3430\text{ cm}^{-1}$  however revealed the presence of a hydroxy group.

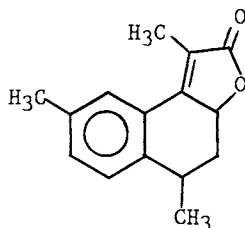
The  $^1\text{H}$  NMR spectrum (Figure 2) gave peaks at 7.40 (1H, s) and 6.86 (1H, s), which indicated the presence of two isolated protons on an aromatic ring. The  $^{13}\text{C}$  spectrum afforded 15 lines indicating symmetry within one portion of the molecule. Two aromatic carbons presented the same chemical shift at 121.4 ppm (line 7). Lines between 160-110 ppm (3,5,6,7, and 9) are typical of a tetrasubstituted benzene, while two lines of the same high intensity at 130.7 and 110.4 ppm indicate no substitution in the para position on the aromatic ring (22). The intensity ratio of these five lines furthermore points to symmetric ortho substitution. A further study of the  $^1\text{H}$  NMR spectrum provides evidence of three nonequivalent methyl resonances at 1.55, 2.15, and 2.25 ppm. Two of the resonances are singlets, which is proof for attachment to quaternary carbons. The third methyl group with double multiplicity should be attached to a methine carbon. The singlet at 3.90 ppm is characteristic of protons on a methoxyl group.

The data therefore suggest that the molecule contains the following partial structures:



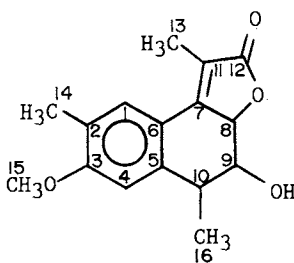
The molecule has an  $\alpha,\beta$ -unsaturated  $\gamma$ -lactone moiety, (a); an aromatic ring with no para substituent, (b); and three different groups of substituents, indicated by (c). The (a) and (b) structures account for seven degrees of unsaturation. These data suggest that an additional ring must be present in order to complete the unsaturation number of eight presented by the molecule. A double bond is excluded because no signal was observed for a vinylic proton in the  $^1\text{H}$  NMR spectrum. This was confirmed by the  $^1\text{H}$  NMR spectrum, which gave a triplet of doublets (1H,  $J = 2$  and 7 Hz) at 3.49 ppm coupled to two protons, and a doublet of doublets (1H,  $J = 1$  and 8 Hz) at 4.80 ppm which indicates a proton coupled to one proton. These two low-field doublet and triplet peaks exhibit further splitting because of a long-range coupling which occurs in zig-zag conformations. These findings point to a conformationally rigid system.

Incorporating partial structures (a) and (b) into the ring and consideration of the isoprene rule leads to the assignment of structure IV as the basic skeleton for compound III.



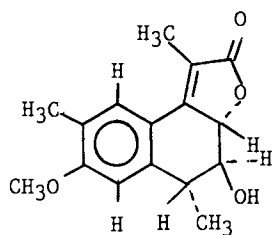
IV

The downfield doublet at  $\delta$  4.80 in the  $^1\text{H}$  NMR spectrum (Figure 2) must arise from an allylic proton situated on carbon bearing oxygen. The  $^{13}\text{C}$  spectrum (Figure 1) shows the resonance of the allenic carbon at 85 ppm (line 10). The hydroxyl group must be attached at C-9 since the  $\text{FeCl}_3/\text{K}_3\text{Fe}(\text{CN})_6$  test indicated the alcoholic character of this group, and finally the position of the methoxyl group must be at C-3. Therefore, the following structure is proposed for III.



III

The  $^1\text{H}$  NMR spectrum gave information about the stereochemistry at the three asymmetric carbons (C-8, C-9, and C-10) of III. The proton H-8, attached to the C-8-bearing oxygen, is split by the vicinal proton H-9 into a doublet with a  $\underline{J} = 8$  Hz, which indicates interaction of two axial hydrogens. The doublets appeared downfield ( $\delta$  4.80) due to the deshielding effect of the oxygen attached to C-8. Each doublet is further split by long-range homoallylic coupling (23) between H-8 and H-13 ("W path") with a  $\underline{J}_{8\ 11} = 1$  Hz. The axial proton H-9 on C-9 is split into a triplet by the vicinal protons H-8 and H-10, which are shifted downfield ( $\delta$  3.80) by the deshielding effect of the oxygen of the geminal hydroxyl group. The coupling constant  $\underline{J} = 7$  Hz indicated axial-axial interaction among these protons. Each peak of the triplet is split into another doublet by an axial-equatorial proton interaction ( $\underline{J} = 2$  Hz). The coupling constant of 7 Hz demonstrates the axial stereochemistry of H-10. The correct structure and stereochemistry of compound III is shown below.



Heritianin (III)

This structure was confirmed by x-ray crystallography (Figure 3) of acetylated III. A crystal of dimensions 1.8 x 0.5 x 0.4 mm was used for data collection on a Enraf-Nonius CAD-4 diffractometer equipped with MoK $\alpha$  radiation and a graphite monochromator. Crystal data: C<sub>18</sub>H<sub>20</sub>O<sub>5</sub>, MW = 316.4, orthorhombic, space group  $P2_12_12_1$ . The unit cell parameters were:  $a = 5.160(5) \text{ \AA}$ ,  $b = 17.397(5) \text{ \AA}$ ,  $c = 18.660(7) \text{ \AA}$ ,  $\rho$  (calcd.) = 1.26,  $Z$  (molecules per unit cell) = 4,  $\mu$  (calcd.) = 0.86 cm<sup>-1</sup>. Data were collected by  $\omega$ - $2\theta$  scans of variable speed (24). As a check on the stability of the instrument and the crystals, two reflections were measured after every 50 reflections; the standards fluctuated within a range of <1%. On independent octant of data was measured out to  $2\theta = 44^\circ$ . A total of 1267 reflections were measured of which 848 had  $I \geq 3$  (I) and were used in the refinement. The intensities were corrected for Lorentz and polarization effects but not for absorption ( $\mu = 0.86 \text{ cm}^{-1}$ ). The structure was solved by direct methods by using the program Multan 78 (25) and refined by full-matrix, weighted least squares method (26) to give discrepancy indexes of  $R = 0.097$  which were calculated by equations 1 and 2.

$$R = [ |F_o| - |F_c| ] / \Sigma |F_o| \quad (1)$$

$$R_w = [ \Sigma_w ( |F_o| - |F_c| )^2 / \Sigma_w (F_o)^2 ]^{1/2} \quad (2)$$

Carbon and oxygen atom positions were refined with anisotropic thermal parameters. Hydrogen atoms were not located. Figure 3 shows the molecular structure and the atom numbering scheme utilized for the x-ray data presented in the supplementary material for heritiana acetate. Coordinates, bond length, and bond angles for compound III acetate are available as supplementary material.

To our knowledge, there is no report of this compound in the chemical literature. Thus, III is a novel fish toxicant which was assigned the name heritianin.

Friedelin (II) and heritianin (III) were bioassayed at a concentration of 100 parts per million using the same experimental procedure as in the preliminary "quick screening test." Friedelin was inactive while heritianin showed 100% mortality in 2 hr. Table II shows the results of the bioassay data for III at concentrations lower than 100 ppm. The LC<sub>50</sub> apparently lies

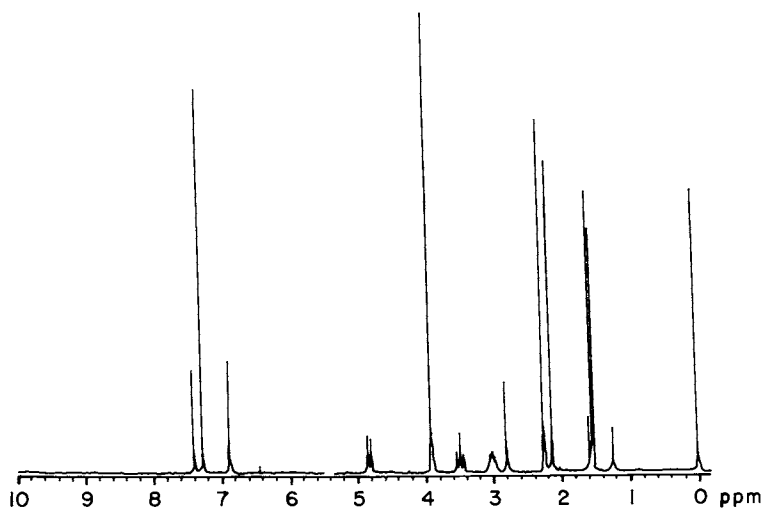
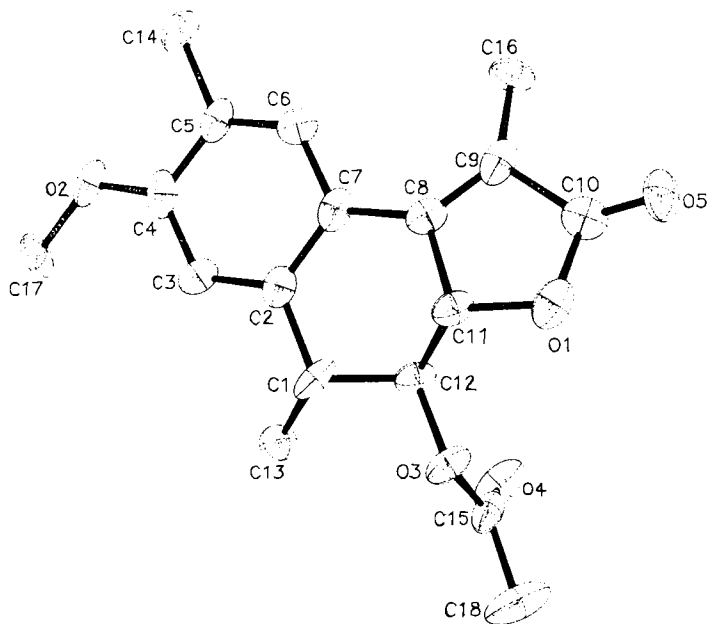
Figure 2. <sup>1</sup>H NMR Spectrum of Compound III.

Figure 3. X-Ray Structure of Acetylated Compound III.

between concentrations 20 ppm and 30 ppm. Further examination of the hexane extract of *H. littoralis* and bioassay studies is in progress.

Table II. Toxicity of Heritianin (III) to Fish at Different Concentrations

Concentration (ppm)	IM (h)	TM (h)
50	1.30	10.00
40	1.40	4.45
30	2.30	7.40
20	NM	NM

NM = No Mortality

IM = Initial Mortality

TM = Total Mortality

#### Conclusion

There is a great need for new biodegradable agrochemicals which could be compatible with the environment. Toxic compounds such as heritol (I) and heritianin (III) have potential as natural pesticides. Plants from tropical regions of the world offer particularly intriguing possibilities in this regard since they are subjected to severe disease and insect pressures. This is especially true for mangrove plants because of their proximity to water. Heritianin is of special interest since it occurs in a mangrove plant and possesses toxic properties as well as a novel sesquiterpene structure containing an  $\alpha,\beta$ -unsaturated  $\gamma$ -lactone moiety.

#### Acknowledgments

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## Chapter 45

# Assessment of Allelopathy Among Microbes and Plants

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Allelopathic effects of microbes on plants, whether due to direct production of toxins by the microbes or to toxic molecules produced during microbial decomposition of organic residues, have been difficult to prove. The problem can be related to difficulties in characterizing and incorporating environmental factors regulating the allelopathic relationship such as soil water, and temperature. The need to follow production of allelopathic compounds and mechanisms of synthesis, fate of the allelochemicals in the soil, their uptake by and specific damage to the plant, and reisolation of the allelochemicals or their breakdown products in the plant makes the task of establishing allelopathic mechanisms difficult. Yet, complete knowledge of all these processes is essential for assessment of allelopathy among microbes and plants. Two cases are presented to examine the allelopathic potential of toxins from decomposing crop residues and the role of microbial root colonizers that produce toxins.

There are numerous reports describing the allelopathic (phytotoxic) effects of microbial products on crop growth, particularly in conjunction with heavy residues from the previous crop (1-5). The cause of the reduced crop growth has been attributed to the production of a variety of toxic compounds such as phenolic acids, short-chain fatty acids, patulin, and many others (6-9). These compounds may be produced directly or indirectly during the microbial decomposition of organic residues under varying environmental conditions, such as when the soil remains wet over an extended period of time. As moisture becomes more limiting to a cropping system, microbial-associated allelopathic problems generally decrease (10).

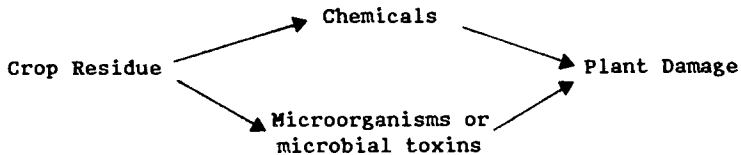
Allelopathic problems have been especially troublesome with conservation tillage systems (6,7,9). An example is the reduced growth of winter wheat when it is direct-drilled into stubble (Figure 1). In the heavy residues (left), the plants grew poorly,

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but where residues were low (right), the plants grew very well. Conservation tillage practices are defined here as direct-drilling of seeds into the surface residues of previous crops (no-till) or as shallow incorporation of crop residue into the soil with a significant portion still remaining on the soil surface (stubble-mulch). The purpose of maintaining residues at or near the soil surface is to protect the soil from water and wind erosion. Conservation tillage cropping practices are essential for protection of erodible croplands, for maintenance of soil productivity, and for reducing off-site damage that leads to deposition of soil on land away from the land where erosion occurred. Unfortunately, common conservation tillage practices are not always compatible with efficient crop production practices and often result in poor crop growth (6,7). In many cases the poor crop growth has been attributed to phytotoxicity (allelopathy) caused by the production of toxic chemicals during microbial decomposition of residues (4,11,12,13). Other problems appear to be associated with microbes that are carried on residues and then colonize plant roots, causing reduction in plant growth (14,15). While some of these organisms appear to be minor pathogens, those mentioned by Elliott and Lynch (15) inhibit winter wheat growth by colonizing the root surface and producing a toxin that adversely affects the plant without readily visible root damage (16,17).

The difficulty in allelopathic studies has been to link the allelochemicals directly with the plant growth problems. Before the role of crop residue, soil management, or other practices in allelopathic problems can be evaluated, a direct link must be established between the source or production of the chemicals and plant damage. Plant damage may be caused by the chemicals produced from residue decomposition or from the microorganisms residing in the residues.



Two cases will be presented to illustrate the difficulty in establishing allelopathy and in linking the agent to poor crop growth. Specifically, the role of phenolic acids and the role of toxin-producing pseudomonads colonizing wheat roots will be examined. Other cases concerning the direct and indirect effects of allelochemicals such as acetic acid produced during crop residue decomposition are presented in this symposium by J. M. Lynch. The emphasis of this discussion will not be a review of existing literature, but on establishing a conceptual framework for a realistic assessment of the allelopathic phenomena and the role and fate of allelochemicals in the soil.

### Phenolic Acids

Phenolic acids have been implicated in allelopathic problems for some time (1,5). There is sufficient evidence in the literature to



build a strong linkage between phenolic acids and plant damage observed under conservation tillage. Evidence for considering the phenolic acids as allelochemicals can be summarized in the following sequence of observations: (a) Plant damage is observed in the presence of heavy crop residues in the field, such as under the conditions of conservation tillage; (b) phenolic acids are produced during the decomposition of crop residues; (c) phenolic acids are found in soil extracts; and (d) phenolic acids can cause plant damage. A number of phenolic acids have been identified in the soil (1,3), including ferulic, *p*-coumaric, vanillic, syringic, and *p*-hydroxybenzoic acids. Inhibition of plant growth by these and other phenolic acids has been demonstrated in nutrient solution studies (e.g. 18,19).

However, when the effects of phenolic acids on seed germination and seedling growth were tested in soil, no inhibition was observed even when the amounts of phenolic acids applied were much greater than the amounts normally detectable in the soil (20). This lack of observed allelopathy in the presence of phenolic acids in soil could have been due to several reasons. Phenolic acids may not be a problem in soil or may only be a problem in soils where they are not inactivated. Inactivation could be chemical or biological. Phenolic acid allelopathy may only be a problem when portions of the seedling or plant are in intimate contact with the decomposing residues and the chemicals are transferred directly from the residue to the plant with limited or no contact with soil. Direct evidence linking phenolic acids and phytotoxicity under field conditions or simulated field conditions has been difficult to obtain. One problem is that much of our information on the effect of phenolic acids is based on extracts from plant material. As Fisher (21) has pointed out: "It seems unlikely that the allelopathic chemicals that may be extracted from plant material are actually those that reach the host plant, yet nearly all our information on allelopathic compounds is derived from extracts that have never been exposed to the soil." To prove a direct linkage between phenolic acids and plant damage, information is needed on the dynamics of phenolic acid production, the processes regulating their presence in soil solution, and the mechanism of their uptake by the plant.

An assessment of the rates and duration of phenolic acid production from a residue is an important first step. Laboratory and field studies for assessing the dynamics of phenolic acid production must include considerations of the nature of the residue, soil properties, nutrient status of the system, microbial biomass interrelationships, temperature, moisture, residue placement in or on the soil, and other factors that relate to the field. Soil properties in the field are especially important when organic residues are incorporated. When soils are wet, such as those with more than -0.02 MPa water potential, oxygen diffusion is impeded and anaerobic conditions prevail, especially in soils that are high in clay content. Under these circumstances, microbial byproducts change dramatically and one result, for example, is an increase in the production of phenolic acids. Phenolic acid production is also affected by temperature (22) and soil fertility status (23). While the C:N ratio of an organic residue may influence the rate of its decomposition and, hence, the rate of phenolic acid production, the

readily available C and N content of the residue may have a more direct bearing on the decomposition rate (24). Most studies have been aimed at the identification of the various phenolic acids produced; however, more attention will now be needed to assess how much and for how long a period these phenolic acids are produced and how changing environmental and field conditions affect production of these compounds.

Once the phenolic acids are produced and present in the environment, physical, chemical, and microbiological interactions will occur. These processes and reaction rates should be defined. The phenolic acids in soil solution can be adsorbed onto soil particles. However, one should examine this adsorption process with care. Adsorption processes have often been characterized by using a batch equilibration method. Many such studies did not examine whether the adsorption process was reversible. The adsorption of many phenolic acids is actually much more complicated than that depicted by the batch equilibration method. A number of binding mechanisms may be involved, including hydrogen bonding, ligand exchange, and complex formation. These compounds can also react chemically with soil constituents. For instance, recent studies (25) have demonstrated that Fe and Mn oxides can readily oxidize phenolic acids and cause their immobilization in soil. This reaction cannot be readily reversed.

In addition to chemical reactions, microorganisms can degrade phenolic acids to CO<sub>2</sub> and H<sub>2</sub>O or partially degrade them to metabolic products that may also be allelopathic (26). More important than the mode of degradation of these compounds are the environmental and soil conditions under which these compounds are degraded. Factors such as soil properties, nutrient status, microbial biomass, temperature, and moisture affect the dynamics of decomposition. In addition to the processes of degradation, processes that transport the compounds from the site of production, such as leaching by water or volatilization into the atmosphere, will also affect the concentration of these phenolic acids in soil solution. The rate of movement and transformation of phenolic acids in the soil will determine what influence these compounds have on the vegetation in the vicinity.

The mere presence of phenolic acids in soil solution under conditions that have resulted in allelopathy does not conclusively prove that these compounds are the causative agents. Commonly, allelopathic studies involving phenolic acids have focused either on isolating the acids from areas of poor plant growth in the field by using rigorous extractants or on observing plant growth inhibition in the presence or absence of phenolic acids added to a growth medium. These approaches do not indicate how the plant is damaged or if the added or extracted compounds are taken up by the plant. It is necessary to provide evidence that these compounds are taken up by the plants and that damage to the plants can be directly associated with the presence of these compounds in plant tissues or organs. Tracer-labeled phenolic acids can best serve these investigations. Several crucial questions must be answered in order to derive a realistic assessment of plant damage and, furthermore, to devise means to alleviate such damage in the field. For instance, does damage occur at or on the root surface, or is the chemical(s)

taken into the plant? What are the uptake kinetics? Are the effects of different phenolic acids, when present together, additive or synergistic? Evidence has shown that the effects of short-chain fatty acids on plants are synergistic (27). Moreover, are the breakdown products of phenolic acids toxic to the plant and, if so, what are the uptake rates, uptake mechanisms, and modes of action of these metabolites? Knowledge of how these chemicals are taken up by the plant and the mechanism of action will help in designing field studies to demonstrate how phenolic acids in soil solution and plant damage are linked.

The requirements for establishing direct linkage between phenolic acids and plant damage can be depicted in a flow diagram (Figure 2). This diagram provides a conceptual framework of the relationships among the input factors and processes affecting the outcome. Before phenolic acids can be linked to plant damage, the whole gamut of processes from production to transformations and uptake must be assessed under proper conditions of temperature, moisture, soil conditions, nutrient status, and microbial activities. When such a mechanistic model is in place, allelopathy can then be predicted by measurement of a few key factors.

#### Allelopathic Wheat Root Colonizers

Bacteria that are not considered true pathogens but do cause detrimental changes in the plant when they colonize the roots are receiving attention. In their work with deleterious rhizobacteria isolated from sugar beets in the field, Suslow and Schroth (14) found that these organisms reduced sugar beet seed germination, caused root distortions and root lesions, reduced root elongation, increased infection by root-colonizing fungi, and significantly decreased root growth. But direct evidence for bacterial antibiosis on plant roots was still lacking (28). More recently, Fredrickson and Elliott (16,17) established that nonfluorescent bacteria of the genus Pseudomonas could significantly reduce winter wheat root and shoot growth through the production of a toxin. These studies showed the relationship was indeed allelopathic, according to Molisch's definition as quoted by Rice (29), where allelopathy is defined as biochemical interactions between all types of plants, including microorganisms.

The inhibitory organisms were isolated from the rhizoplane of winter wheat plants growing in the field. The pseudomonads are aggressive root colonizers that can be found on wheat plant roots in high numbers and appear to be associated with heavy residues from the previous crop (15). Some isolates are so inhibitory that winter wheat root growth is nearly prevented in laboratory bioassay (Figure 3). Other studies showed that the pseudomonads inhibited the growth of Escherichia coli C-1a (Figure 4) (16). However, it was found later that the inhibition of root growth and of E. coli C-1a did not always correlate (30). Presumably, this is because we do not understand the factors affecting toxin production in culture media. The inhibition of winter wheat root growth and of E. coli C-1a could be reversed by l-methionine; however, when root exudates from winter wheat cultivars that showed differential resistance to the toxin were examined, no differences in l-methionine exudation patterns

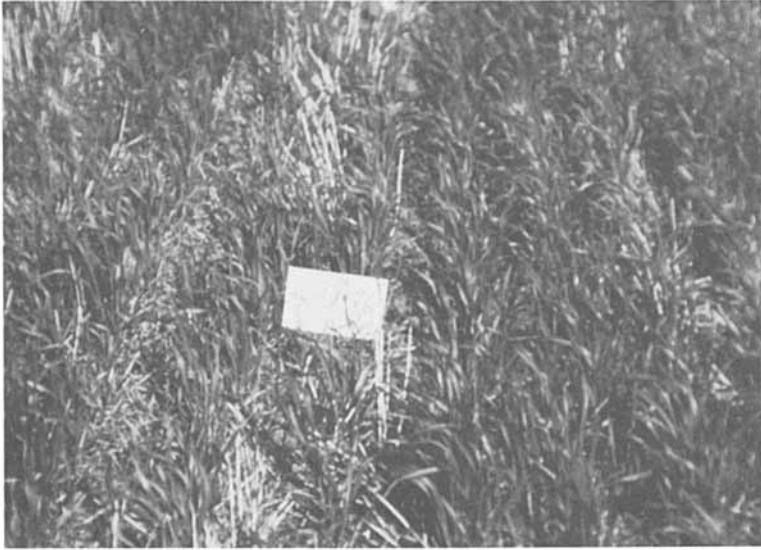


Figure 1. Growth of winter wheat no-till seeded in heavy residues (left) in comparison with that seeded in low residues (right).

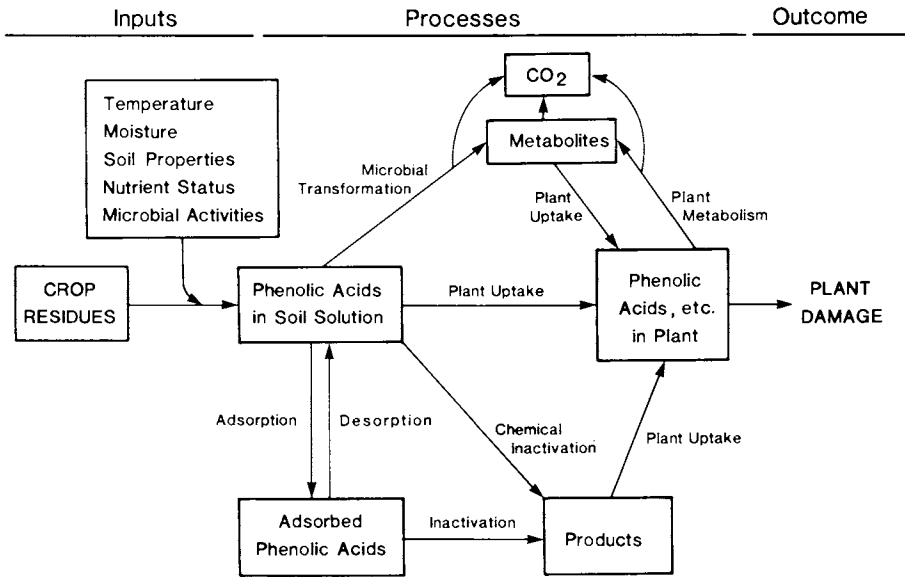


Figure 2. Conceptual model for assessing allelopathic potential of phenolic acids.

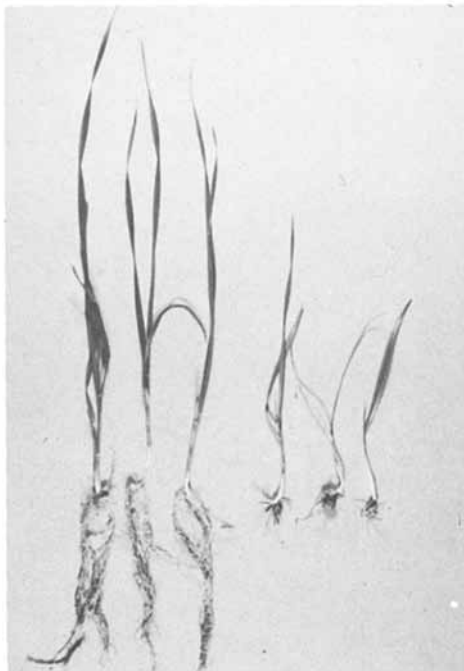


Figure 3. Effect of an inhibitory pseudomonad on the growth of winter wheat seedlings (control, left; treated, right).

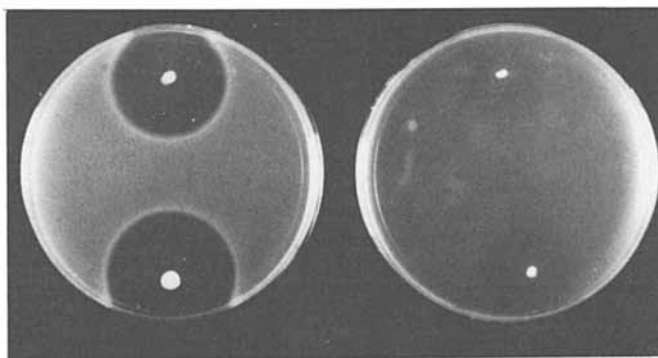


Figure 4. Inhibition of *E. coli* C-1a growth by a pseudomonad inhibitory to winter wheat growth (cured with mitomycin C on the right).

were noted (30). While the inhibitory pseudomonads isolated from winter wheat could colonize the roots of several crops, the inhibition was greatest for winter and spring wheat. Winter barley, lentils, and peas were inhibited to a lesser extent while spring barley and oats were not inhibited by the organism (31). The culture filtrate from the organism did not cause as severe root growth inhibition as the organism itself (31). These examples do show that the organisms and toxin show some specificity. This may not be surprising as Woltz (32) pointed out that secreted toxins are often highly specific to species and cultivars.

In related studies, marked inhibitory pseudomonads that were placed on winter wheat and barley straw in the laboratory and in the field colonized the straw, especially the barley straw, to the virtual exclusion of the resident bacterial population when incubated at 5°C. The laboratory study showed that high numbers of the introduced organisms persisted throughout a 35-day study (31). Figure 5 shows the effect of treating winter wheat seeds with an inhibitory pseudomonad before they were no-till planted into methyl bromide-fumigated Palouse silt loam soil. The plants from the treated seeds (left) were much poorer than those from the nontreated seeds (right) (33).

These studies provide a strong case for allelopathy by the pseudomonads and indicate that Koch's postulates have been satisfied. However, more work remains to be done to determine the agronomic importance of the allelopathic problems associated with microorganisms. Accomplishment of this objective requires that more information be gathered in several areas. The mechanism of toxin production by the organisms and the effect of the toxin on the plant must be determined. We must know why and how the organisms colonize roots and residues so aggressively. Root exudation by the plant may have a profound influence on root colonization and toxin production. The effects of tillage, residue management practices, and crop rotations on the presence of these organisms and their colonization of winter wheat roots must be determined in order to provide direction for alleviation of the problem. Approaches may be the development of TOX-negative inocula, resistant varieties, and better cropping systems.

First, a quick bioassay must be developed to determine the presence of the inhibitory pseudomonads. While the *E. coli* C-1a assay shows promise (16), it frequently does not work well; at present, the slow and laborious agar tube-plant bioassay (15) must still be used for best results. The apparent problem is that we are unable to control the toxin or TOX<sup>+</sup> expression, because it appears that TOX<sup>+</sup> expression can be low on artificial media. The toxin must be identified and genetic mechanisms controlling TOX<sup>+</sup> must be defined. Then cultural techniques can be devised to control TOX<sup>+</sup> expression. This should provide a tool whereby a rapid inexpensive assay, such as the *E. coli* C-1a assay, will be more accurate so that it can be used to rapidly survey for the presence of these organisms on residues and on winter wheat roots in the field. This would provide evidence for the presence of these organisms and the effect of crop rotation, residue management, and soil management. It would also more easily allow the testing of TOX-negative inocula.



**Figure 5. Effect of inhibitory pseudomonads on winter wheat growth in the field (treated seed, left; untreated seed, right).**

Research must be conducted to determine how the toxin is taken up by the plant and what its effects are on the plant. Labeled toxin may be required initially to determine when and if the plant has taken up the toxin in the field in order to separate the allelopathic effect from that caused by plant pathogens. This knowledge would assist identification of the problem in the field as the presence of the inhibitory pseudomonads on roots is innocuous, because little noticeable effect occurs on the root except the stunting and occasional root deformation. Plant color is not normally affected.

Spontaneous antibiotic-resistant mutants of these organisms are suitable for initial root colonization studies; however, transposon mutants will be more suitable for detailed laboratory and field studies. This procedure is useful for sorting out the genetic relationships of these organisms and for determining the mechanisms controlling toxin production.

The effect of plant root exudation and exudation patterns on root colonization and expression of toxin production must be considered. For example, it may be important to determine the effect of root exudates from cold-stressed plants on these organisms, since the exudates apparently first appear just after the plants break winter dormancy (34). These data should provide information on root colonization potential, possible stimulation or reduction of toxin production, and mechanisms of plant resistance to the organisms.

Ecological studies are desirable to determine the effect of temperature and moisture on the organisms, particularly under field conditions. These organisms appear associated with conservation tillage systems where residues remain on or near the soil surface. This situation is paradoxical since residues on the surface generally remain drier than when incorporated into the soil and bacteria normally thrive better under high moisture conditions. The effect of water and temperature on survival of these organisms should be tested with various crop residues and various anatomical parts of the residue. These studies should provide clues for residue management to alleviate the problem.

After these steps are accomplished, it should be possible to survey for the organisms, to relate their abundance to management practices such as tillage and rotation, and to develop management alternatives to reduce their impacts. Importantly, more trials of bacteria-treated seed are necessary to determine the precise agronomic importance of these organisms.

Earlier studies indicated that wheat cultivars responded differently to these organisms (15). If necessary, it should be possible to develop resistant varieties, especially when we know the mechanisms of plant uptake and mode of action of the toxin within the plant. One biological control may be the development of a TOX-negative inoculum for seed treatment. These bacteria may act like other rhizobacteria, which are known to increase plant growth, apparently by displacing nonbeneficial bacteria in the plant rhizosphere (14).

### Conclusion

Allelopathic interactions caused by chemicals produced during the decomposition of crop residues and by toxins produced by bacteria



growing on plant roots appear to create serious agronomic problems. However, direct proof and assessment of the importance of these problems have been difficult. For both cases presented here, i.e., phenolic acids and phytotoxins produced by pseudomonads colonizing the surface of winter wheat roots, more detailed information is still needed.

For the phenolic acids, rates of production, reactions in the soil, effects on the plant, and regulation by the environment must be determined and modeled. For the inhibitory pseudomonads, mechanisms controlling degree and persistence of root colonization by these organisms should be ascertained. In addition, data on genetic mechanisms controlling toxin production and the effects on the plant are needed. Use of models will be helpful in describing these systems. Assessments of crop damage by allelopathy can be accomplished and controls can be developed only after such information is known.

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## Chapter 46

# Phytotoxins from Plant Pathogens of Weedy Plants

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Weeds are host plants to an array of plant pathogenic fungi and bacteria and there is current interest in the use of these organisms for weed control. Commonly, associated with pathogens are one or more phytotoxins involved in causing disease. Thus, one alternative approach in weed control is the use of phytotoxins or their derivatives for direct application to the noxious plant. A necessary prelude to this approach is the isolation, characterization and biological testing of phytotoxic metabolites. Recently, we have done such experiments by isolating alteichin from Alternaria eichorniae, a pathogen of water hyacinth; and bipolaroxin, a host-selective phytotoxin from Bipolaris cynodontis, a pathogen of Bermuda grass. X-ray crystallography was used to determine the structures of these phytotoxins.

In the past few decades food production has increased through plant breeding, fertilizer use, mechanized farming, and chemical pest control. The most recent U.N. study, the FAO World Food Report, indicated that 1984 food production was 4% greater than 1983. But the report noted that further improvements will be required if food production is to keep pace with population growth. One area for potential improvement is the control of weeds. We have been working on a novel approach to weed control, and find the preliminary results encouraging.

The approach was quite simple conceptually and can be briefly summarized. Many pathogens of crop plants produce symptoms on their hosts through the production of phytotoxins (1). It seemed logical to assume that weed pathogens would also produce compounds acting as phytotoxins, and that these phytotoxins could serve as novel agents for the control of weeds. We began with a target list of the world's worst weeds (2), and searched for their fungal or bacterial

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pathogens. Searching involved direct field observation, examination of the relevant literature, and discussions with plant pathologists. Once promising pathogens were located, they were cultured in the laboratory, and their extracts examined for phytotoxic activity. Then standard isolation and structure determination techniques were used to identify the compound(s) responsible for the phytotoxic activity.

There was precedent for this approach. Plant pathogens obtained from specific weedy plants have recently been used as weed control agents (3), and very recently metabolites of soil borne microorganisms have been used for the same purpose (4). We hoped that by focusing on weed pathogens, we would find novel and more selective chemical agents. At the very least the chemical agents will suggest structural types and arrangements of functional groups that will serve as starting points for the development of improved herbicides. Until now, such an approach has not been seriously considered, and weed pathogens are not well represented in the world's culture collections. Many described earlier have suffered the fate of desiccation and death on the shelves of plant pathologists whose work may not have been appreciated at an earlier time.

#### The Weed Host-Pathogen System

Several factors of the host-pathogen system must be considered in selecting specific cases to study. The pathogen should be expected to produce a phytotoxin. For several plant pathogens -- the rusts, mildews, viruses, and nematodes -- there is little or no record of phytotoxins being associated with the diseases that they cause. The most notable group of phytotoxic producers are the Fungi imperfecti and some plant pathogenic bacteria (5). Our initial studies have focused on the fungal pathogens. Geography is also expected to play an important role in selecting pathogens. The most effective pathogens are likely to be found near the center of origin of the host plant since the pathogen will have had the longest time to specialize in attacking the host.

Culturing and toxin production. Once a pathogen is chosen for further study, it should be placed in a permanent culture collection to ensure its continued availability. A liquid culture medium must support growth and production of toxic or damaging secondary metabolites. Ideally, it would be a defined medium (contain no extracts of plants, yeast extract, etc.). A defined medium simplifies the isolation procedure and ensures that the extract is not contaminated with added metabolites. Unfortunately this is not always possible. The mechanisms that regulate toxin production in pathogens are poorly defined, but many organisms require the presence of certain plant metabolites to maximize toxin production (6). These plant metabolites are not precursors of the toxins, but somehow activate the toxins' biosynthetic pathways (6). An efficient way to culture pathogens is to use a standard defined medium with the addition of the crude aqueous extract of one or two young host plants (6).

Bioassay. A prerequisite for phytotoxin isolation is the availability of a suitable bioassay (5,7,8). Each step of the isolation

procedure must be monitored by checking the biological activity. The most common and simplest procedure is to place a few microliters of the test solution over a small puncture wound on a detached leaf. The puncture wound enhances the access of the toxin to the leaf tissue. The leaf is then placed in a petri dish containing a filter paper saturated with water. The top cover of the plate is sealed with parafilm, and the plate is incubated under controlled light and temperature conditions. Toxin activity is usually indicated by chlorotic, necrotic, or colored spots on the leaf. Other methods for bioassay involving CO<sub>2</sub> fixation, or effects on organelles, whole plants, protoplasts, tissue cultures, or plant parts are outlined (5,7).

The bioassay is complicated by the highly variable response of different plants to a given compound, i.e., host specificity. Some toxins are completely nonspecific; they affect all plant species used in the bioassay at virtually all concentrations. At the other extreme are the host-specific toxins; they affect only a certain plant species, and at reduced concentrations will even discriminate among cultivars within a species. We are most interested in finding highly host-specific toxins for common weeds, but this is not an easy task. Such host-specific toxins are not likely to be easily found in the plant pathogens from weed hosts because of the wide genetic diversity of the hosts in nature. Ironically, highly host-specific toxins are well known for crop plants. The large areas of crop plants in monoculture make selection for highly specific pathogens much easier. The most dramatic recent example is the corn blight epidemic of 1971 where large areas of corn plants containing Texas male sterile germ plasm were highly vulnerable to *H. maydis* (9). Fortunately there is an intermediate group of phytotoxins that display some host selectivity. They are completely ineffective at all concentrations against some species, but will affect others. Selectivity with this group of phytotoxins may occur within a range of toxin concentrations, between toxin-sensitive plants, or even within cultivars of a certain species.

#### Phytotoxin Isolation and Characterization

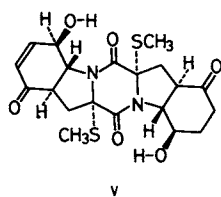
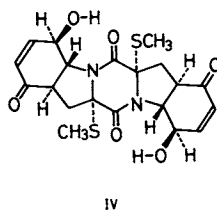
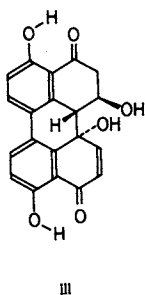
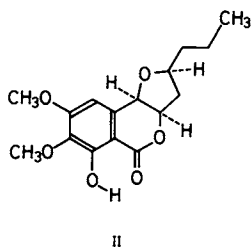
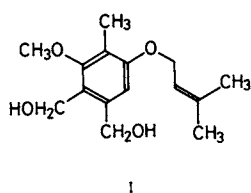
Most characterized phytotoxins are from crop plants and include a large array of chemical families. Proteins, peptides, glycopeptides, glycosides, phenolics, terpenoids, macrolides, and others have been characterized (5,7). Thus, no general isolation scheme is available, and each case must be handled separately. To date the majority of plant pathogens that we have examined has produced relatively small molecular weight compounds -- compounds that easily lend themselves to extraction from culture fluids by organic solvents. This could reflect our current technology and not apply generally to weed pathogens. Further purification is accomplished by techniques such as flash column chromatography, preparative thin layer chromatography, and preparative high performance liquid chromatography. The ultimate goal is to define the precise molecular structure of the phytotoxin(s). Spectroscopic techniques such as mass spectrometry, <sup>1</sup>H NMR and <sup>13</sup>C NMR, and UV have been extremely useful. Since the quantities of the phytotoxins are typically limited and the range of structural types rather large, x-ray

diffraction has proved to be a most powerful tool. Most, but not all, of the phytotoxins that we have investigated have ultimately been characterized by x-ray diffraction. It is not unusual for weed pathogens to produce phytotoxins that are identical or closely related to phytotoxins that attack crop plants. Some examples of this are zinniol (I) produced by a number of *Alternaria* spp. (10), and monocerin (II) from *Exserohilum turcicum*, a pathogen of Johnson grass (11). Thus the investigator should be well acquainted with the phytotoxin literature and have access to reference samples and/or spectra.

Recently, we have found that pathogens with a restricted host range and unrelated to common crop pathogens do produce novel phytotoxins. These novel phytotoxins have both unusual chemical structures and surprising, in some cases unprecedented, biological activities. We will summarize these studies below, beginning with the least selective phytotoxins and progressing to phytotoxins with some host selectivity.

Water hyacinth is an economically significant weed native to the Amazon basin. In the recent past, it has become a widespread pest throughout much of the world. Water hyacinth is a serious problem in paddy crops such as rice and taro, and in some areas it has become so prolific that it blocks formerly navigable waterways, irrigation canals, and drainage ditches. *Alternaria eichorniae* attacks the leaves of water hyacinth, and the resulting lesions sometimes cause leaf death. Because of this form of symptom expression and the relatively narrow host range of the fungus, *A. eichorniae* was examined for its production of phytotoxic metabolites. Bostrycin, a reduced anthraquinone with phytotoxic activity, had previously been isolated from this source (12). Our reinvestigation led to the isolation of alteichin (III), a doubly hydrated form of 4,9-dihydroxyperylene-3,10-quinone. The structure of this rather sensitive molecule was deduced by x-ray crystallographic analysis (13). At least one other phytotoxin, closely related to alteichin, was also present in the culture fluids. The preliminary spectral analysis of this additional component suggests that it is the triply hydrated version of the parent quinone, but additional work will be required to fully elucidate the stereochemistry. Alteichin is, not surprisingly, very sensitive to acid and is readily converted to the water-insoluble parent quinone. The parent quinone may well be the ultimate phytotoxin. Alteichin does not show host selectivity in either the whole leaf or protoplast assays. In all test plants it causes necrotic flecks in leaf puncture wounds (2.7 mM (2% ethanol)) within 12 h of application. The alteichin-induced lesions resemble those caused by *A. eichorniae* on water hyacinth.

Exserohilone, from *Exserohilum holmii*, is a fungal leaf pathogen of *Dactyloctenium aegyptium* (crowfoot grass), a serious weed in all major tropical and semitropical agricultural areas of the world. While a major product was identified as monocerin (II) by spectrometric means, two novel phytotoxins were also isolated from liquid shake cultures of this fungus. These new phytotoxins were shown to be diketopiperazines (IV) and (V) by standard spectroscopic and x-ray crystallographic analyses (14). Both of these compounds cause necrotic lesions at  $10^{-4}$  -  $10^{-5}$  M. The



lesions are usually surrounded by a reddish brown border on a variety of plant species.

Dihydropyrenophorin, from *Drechslera avenae*, is a leaf pathogen of both wild and cultivated oats. It causes reddish brown lesions with a necrotic sunken center. At least one compound isolated from broth cultures of this fungus caused comparable lesions on oats and a variety of other plants at  $3.2 \times 10^{-4}$  M (15). The phytotoxin was characterized by spectrometric analyses and chemical conversion as (-)-dihydropyrenophorin (VI), an important dilactone macrolide (15). However, the major product obtained in our extraction procedure used to isolate (-)-dihydropyrenophorin was the diol VII (16), which was not active in our bioassay tests.

We have not yet assigned the stereochemistry of the secondary alcohol centers in VII or VIII. The simplicity of the NMR spectrum of VIII indicates a symmetrical molecule. This would most plausibly be the molecule with twofold symmetry, but we cannot yet rule out the meso form. Upon oxidation VII could be converted to pyrenophorin (VIII). Pyrenophorin was also active in the biological assays, but was not found in *D. avenae*. It is produced by several other fungi (17).

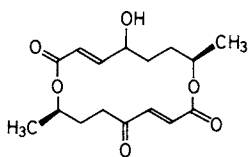
Biologically, the most interesting aspect of (-)-dihydropyrenophorin is that it causes reddish lesions on Johnson grass at  $10^{-6}$ ,  $10^{-7}$ , and  $10^{-8}$  M wherein no other plant species tested shows any sensitivity whatever at these concentrations. Thus, it would appear that VI is host selective. To our knowledge Johnson grass is not a host of *D. avenae*.

Bipolaroxin (IX) is the most host-selective phytotoxin. It was isolated from *Bipolaris cynodontis*, a fungal pathogen on Bermuda grass (18). The toxin produces reddish lesions and "runners" on treated leaves of Bermuda grass and Johnson grass (host plants of the pathogen) at  $10^{-5}$  M, but at these concentrations no other plant species tested is affected. Bipolaroxin is a highly oxygenated member of the eremophilane family. When the aldehyde group is reduced (yielding dihydrobipolaroxin (X)) all phytotoxicity appears to be lost even at concentrations as high as  $10^{-3}$  M (15). Bermuda grass is one of the most notorious weeds in the grass family since it has been listed as a problem in at least 40 different crops. The zonate lesions produced by *B. cynodontis* greatly resemble those caused by bipolaroxin. Mode of action studies on bipolaroxin may be facilitated by the use of the  $^{14}\text{C}$ -labeled compound. Recently, we have learned that [ $^{14}\text{C}$ ] mevalonate administered to cultures of the fungus serves as an excellent precursor to both IX and X.

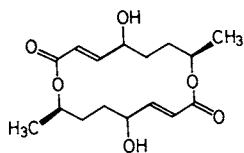
#### Future Work

The majority of the novel phytotoxins known from weed pathogens are summarized in this report. Since only a few pathogens have been studied to date, there are many more organisms yet to be examined. For example, there is no report of a phytotoxin from a bacterial weed pathogen, although such compounds surely exist. It seems unlikely that extremely host-selective (host-specific) phytotoxins will be readily found from weed pathogens since there is relatively little genetic selection pressure on the pathogen. This arises from the highly diverse genetic background of any weed population.

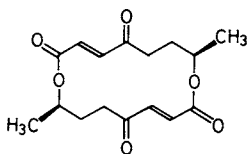




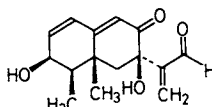
VI



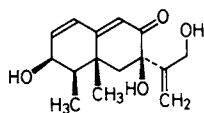
VII



VIII



IX



X

Nevertheless, we are encouraged by the findings on bipolaroxin (18) to believe that host-selective compounds do exist.

Thus far, there have been no biochemical/physiological studies showing the effects or modes of action of any of the phytotoxins from weed pathogens on their hosts. Also, chemical modification (5) of the toxins should shed light on the bioactive portion of the molecule. The intrinsic biological activity of these phytotoxins certainly warrants considerable study, since there is potential for them to serve as herbicides or models for herbicides, and because they possess somewhat unexpected biological activities.

#### Acknowledgments

We thank Dr. E. S. Luttrell of the University of Georgia for supplying cultures of many of the weed pathogens. The work at Bozeman was funded by a grant from Rohm & Haas Co. and the Montana Agricultural Experiment Station. The work at Cornell was partially supported by a grant, NIH CA 24487.

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## Chapter 47

# Chemical Ecology of Quinolizidine Alkaloids

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The biochemistry and physiology of quinolizidine alkaloids is reviewed with respect to their role in lupin metabolism. Minor roles of the alkaloids may be nitrogen transport and nitrogen storage, but their main function is that of chemical defense. Alkaloid concentrations in the plant are in the same order or even higher than the inhibitory concentrations against pathogens that have been established experimentally. Alkaloid-free lupins are highly susceptible to herbivore predation, which shows that the alkaloids are obviously important for the survival of a lupin plant.

Quinolizidine alkaloids (QA) are thought to be typical natural products of many Leguminosae (1-3) but a few isolated occurrences have been reported also in unrelated families, e.g. Chenopodiaceae (1), Berberidaceae (1), Papaveraceae (1), Scrophulariaceae (4), Santalaceae (5), Solanaceae (2), and Ranunculaceae (1). These observations could indicate that the genes for QA biosynthesis are probably not restricted to the Leguminosae but are widely distributed in the plant kingdom; however, they are only rarely expressed in the other families. We could support this belief by recent experiments using plant cell suspension cultures. A short-term and transient QA formation could be detected after induction even in "QA-free" species, such as Daucus, Spinacia, Conium, and Symphytum (6).

In order to understand the biological function of QA we have to analyze their physiology and biochemistry first.

### Biochemistry and Physiology of Quinolizidine Alkaloids

In the first step lysine is decarboxylated to cadaverine. Then three cadaverine units are incorporated into the tetracyclic QA skeleton, such as in lupanine, which serves as a precursor for most of the other QA. Recent tracer experiments have been reviewed (3, 7). In

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our laboratory we have concentrated on the enzymology of QA formation and have been able to characterize more than four distinct new enzymes; a review is available (8).

Site of alkaloid formation, transport, and accumulation. QA are formed in the aerial green parts of legumes, especially in the leaves (9). In lupin leaves we succeeded in localizing the key enzymes of QA biosynthesis in the chloroplast (10, 11), where the formation of the precursor lysine also takes place. Like most of the processes that are located in the chloroplast, QA biosynthesis is regulated by light (8) and QA formation follows a light-dependent diurnal rhythm (12, 13). The alkaloids formed in the leaves are translocated via the phloem (13, 14) all over a lupin plant, so that all plant parts contain alkaloids. QA are accumulated and stored preferentially in epidermal and subepidermal tissues of stems and leaves (15, 16). Especially rich in alkaloids are the seeds, which may contain up to 5% (dry weight) alkaloid (equivalent to 200 mmol/kg).

Turnover of quinolizidine alkaloids. Like many other natural products, especially nitrogen-containing compounds, QA are not inert end products of metabolism, but compounds with a high degree of turnover. This phenomenon becomes especially evident during germination and seedling development. Most of the alkaloids are metabolized and their nitrogen is probably used for seedling growth (17).

#### Role of Quinolizidine Alkaloids in the Metabolism of Lupins

Primary metabolism. Having briefly reviewed the physiology and biochemistry of QA in the sections above, we can consider the question as to why do lupins produce alkaloids. First of all we can ask whether the QA may play a role in the primary metabolism of a lupin plant.

Alkaloids are translocated in the phloem sap like other photosynthates, and QA contribute about 8 % to the overall nitrogen. Since QA are readily metabolized by cells, the alkaloids could thus play a role as a minor means of nitrogen transport.

Species that produce rather few and heavy seeds store up to 5 % alkaloids in addition to ca. 30 % storage protein. We have estimated that QA contribute ca. 10 % of the total nitrogen stored in lupin seeds. Therefore another minor role of QA could be nitrogen storage (17, 18).

We could not find other areas of primary metabolism in which QA might play a part. Since we can assume that all metabolites found in organisms have a definite function (19), we suggest that the nitrogen storage function of QA is of only minor importance and probably not sufficient to explain the complex physiology of QA.

Chemical ecology. It has been generally accepted that many of the so-called "secondary metabolites" play a role in the interrelationship of plant - plants, plant - microbes, and plant - herbivores (20-23). In a series of experiments we have sought to determine whether lupin alkaloids are important in an ecological context.

What should be the requirements of a plant defense chemical?

1. It should display a significant activity under experimental conditions.
2. Its concentration in the intact plant should equal or exceed the experimentally defined inhibitory concentrations.
3. It should be present in the plant at the right time and the right place.
4. It should be ecologically relevant.

We have isolated pure alkaloids and studied their effect on the multiplication of potato-X-virus (Figure 1), on the growth of gram-positive and gram-negative bacteria (24, Table I) and of fungi (24, 25, Table I), the germination of lettuce seeds (18, 26), and the feeding of insects and molluscs (27, 28, Table I). Furthermore, QA are toxic for mammals, as has been reviewed (2, 26, 27). It is remarkable that QA are active, not in one, but in all the interactions studied. The structure of the individual alkaloids determined the effect only to some degree. For example, the esters of 13-hydroxy-lupanine, such as 13-tigloyloxylupanine, seemed to be more toxic and repellent than lupanine (27, 28). Vertebrate toxicity seemed to be highest in the  $\alpha$ -pyridone alkaloids, i.e. in QA in which the  $\alpha$ -ring is oxidized (cytisine, anagryrine).

Table I. Inhibitory Concentration of Quinolizidine Alkaloids on Various Organisms

1 = Lupanine, 2 = sparteine, 3 = cytisine, 4 = tigloyloxylupanine  
- = not determined

Pure alkaloids were added to food or nutrient media.

Organisms	Alkaloids				Reference
	1	2	3	4	
I. Inhibition of seed germination					
<u>Lactuca sativa</u> (ED <sub>50</sub> , mM)	7	50	6	2	26
II. Inhibition of bacterial growth					
Air-borne bacteria (ED <sub>50</sub> , mM)	5	0.5	-	3	24
<u>Streptococcus viridis</u>	-	0.2	-	-	24
<u>Micrococcus luteus</u>	-	1	-	-	24
<u>Mycobacterium phlei</u>	-	7	-	-	24
<u>Bacillus megaterium</u>	-	0.5	-	-	24
<u>Bacillus subtilis</u>	-	0.5	-	-	24
III. Inhibition of fungal growth					
Phytopathogenic species (ED <sub>50</sub> , mM)	-	5-50	-	-	24
<u>Erysiphe graminis</u>	1	1	-	-	25
IV. Insect toxicity (LD <sub>100</sub> , mM)					
	3-12	9-50	-	6	18
V. Mollusc feeding deterrence					
<u>Helix pomatia</u> (ED <sub>50</sub> , mM)	1-7	0.7	2	-	27

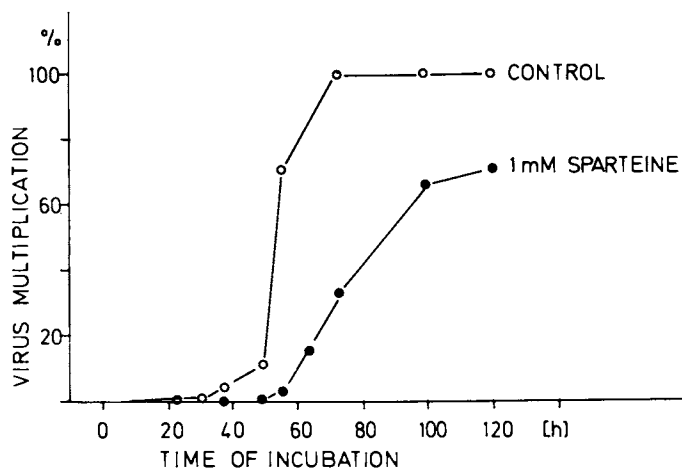


Figure 1. Inhibition of Potato-X-virus Multiplication by Sparteine.

Leaves of *Nicotiana tabacum* were treated with infective potato-X-virus. About 3 h after infection leaf discs were prepared (10 mm diameter) and incubated on water containing 1 mM sparteine. Controls were incubated on water without alkaloid. At the specified intervals samples (50 discs each) were taken and virus concentration was determined by an ELISA assay according to standard procedures (B. Lerch and M. Wink, unpublished data).

The dose-response curves were rather similar for most organisms studied (24-28), showing  $ED_{50}$  values of 0.5-5 mmol/L. At first view this concentration seems to be rather high and of doubtful significance. However, as can be seen in Table II, the alkaloid concentrations in a legume plant are usually 3-20 mmol/kg in somatic tissues and up to 200 mmol/kg in the seeds, which are most important for the survival of a plant species. Thus the *in vivo* concentrations of QA are in the same range as the effective doses or even orders of magnitude higher (Table I, II). Furthermore, epidermal cells are the main storage site of QA in lupin tissues (16), where the local alkaloid concentrations are between 20 and 200 mmol/kg. Thus epidermal cells, which usually are the ones to ward off a microbial or herbivorous attack, can function as a chemical barrier (16).

Table II. Concentrations of Quinolizidine Alkaloids in Parts of Plants

- = not determined

Plant Part	Alkaloid Concentration (mmol/kg fresh weight)	
	<i>Lupinus polyphyllus</i>	<i>Cytisus scoparius</i>
Leaves	4-20	1-5
Stems	4-15	8-20
Epidermis	25	200
Flowers	5-10	5-10
Pollen	8	-
Carpels	5	-
Petals	2	-
Maturing fruits	5-10	1
Mature seeds	100-150	8
Roots	1	0.1

We were also able to show that lupin leaves can increase their alkaloid content by a factor of 2-4 within a few hours after mechanical wounding (which could imitate a herbivorous attack) (29). This means that the defense system is able to respond to environmental stress.

All these data support the idea that QA may function as chemical defense compounds. We also tested whether this chemical defense is relevant for the survival of a lupin plant. Lupins offer a unique chance to explore this question experimentally; plant breeders have selected "sweet" varieties, which have a very low alkaloid content. These varieties can be compared to semi-bitter or bitter ones. We have grown *Lupinus albus* strains that differ in their alkaloid content in our experimental garden and greenhouse and have monitored their susceptibility to attack by plant pests. As can be seen from Figure 2, "sweet" lupins are preferentially eaten by rabbits (*Cuniculus europaeus*) or are infested by aphids (Aphidae) or leaf miners (Agromyzidae). Literature data also support the assumption that alkaloid-rich lupins are much more resistant to plant pests than "sweet" varieties (30-32). We conclude therefore, that QA are indeed important for the fitness of a lupin plant and that they constitute a major part of its chemical defense system, in which

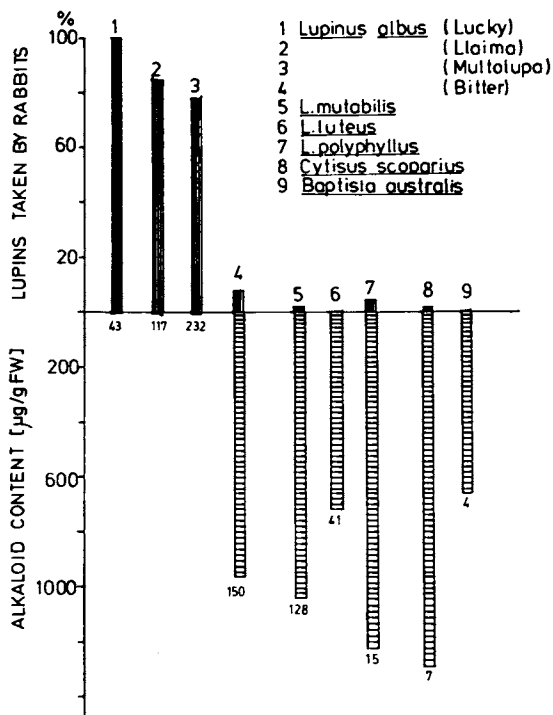


Figure 2. Herbivory in Relation to Alkaloid Content of Plants. 1. Predation by rabbits (*Cuniculus europaeus*). Rabbits were allowed to feed in the experimental garden, in which a variety of legume species were cultivated. The number of plants evaluated is given at the bottom of each column.



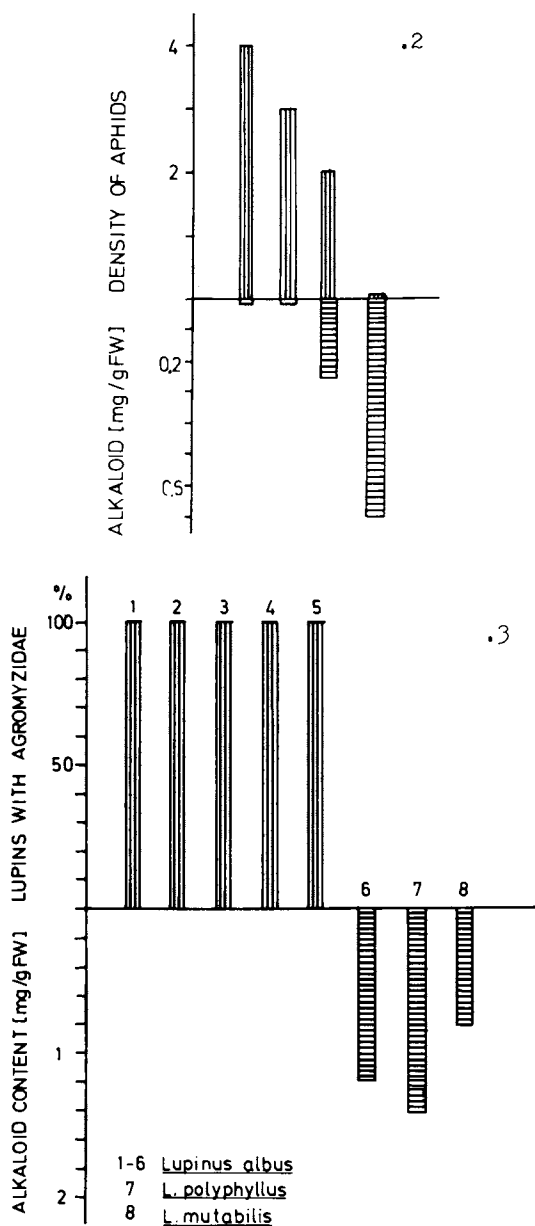


Figure 2. Herbivory in Relation to Alkaloid Content of Plants.  
 2. Infestation by aphids (*Myzus* sp.). *Lupinus luteus* plants were kept in a greenhouse and had reached flowering stage at the onset of infestation. Scale: 4 = very abundant, 3 = abundant, 2 = few, 1 = very few, 0 = no aphids.  
 3. Infestation of young lupin plants (greenhouse experiments) by Agromyzidae. About 50-100 plants each were evaluated.

other compounds, e.g. simple phenolics, flavonoids, and anthocyanins, are also active.

We have to be aware that there are always exceptions to a general biological rule. While QA protect lupins against most pathogens, a few specialized ones have overcome the defense barrier by techniques that are still unknown. We showed recently (33) that this adaptation can be advantageous in two aspects: a) A specialist may find an ecological niche which is not occupied by other species. b) A specialist may accumulate the alkaloids itself and can use the acquired toxicity for its own defense (33).

### Implications

Producing nitrogen-containing compounds is a costly process for plants, since nitrogen usually constitutes a limiting growth factor. This limitation can be overcome if an N-containing toxin is degradable and the nitrogen reusable when needed. Maybe as a consequence of their metabolic instability QA can be thus used as minor N-transport and N-storage compounds. So QA have evolved as rather versatile defense chemicals. Legume species, which do not accumulate QA, generally produce other toxic nitrogen-containing compounds, e.g. non-protein amino acids, lectins, or protease inhibitors (34). Rosenthal (34) came to similar conclusions concerning the role of canavanine in legumes, i.e. that it is a biotoxic, nitrogen-storing defense compound.

Many features of the physiology of QA can be interpreted as adaptation to make them available for chemical defense. QA transport in the phloem sap is highly advantageous since it keeps away phloem-feeding insects, such as aphids. QA storage in epidermal cells is strategically important since this tissue forms the first defense barrier. Since seeds and seedlings contain valuable storage material (protein, lipids) they are highly attractive for herbivores. To protect this developmental stage by extremely high toxin concentrations seems to be important for the survival of a species.

Plant species that produce many small seeds usually do not invest in toxins, whereas species with few nutrient-rich seeds generally do (18, 22). The knowledge about these adaptations is valuable if we think of manipulating plant secondary metabolism, for example in plant cell cultures, to produce economically important natural products.

The structure of QA has not evolved at random but was obviously selected in a way to obtain a degradable and biotoxic compound. As our data show, the activity seems to be of a very broad range and is not specific for one organism, which would be a desirable trait if we think of exploiting natural products as plant-protection substances or therapeutic agents. It should be recalled that QA are useful in medicine as antiarrhythmic and obstetric drugs (2). These observations also hold true for most other natural products. We would stress that natural products form a preselected reservoir of bioactive compounds, useful for further chemical manipulation.

Lupin seeds provide a protein- and lipid-rich diet that is almost equivalent to Soya. In order to avoid the toxic alkaloids, sweet lupin varieties have been selected by plant breeding. But in view of our results it is doubtful whether it is wise to destroy a natural resistance factor. It is thus not surprising that sweet

lupins can only be grown successfully if we use man-made chemistry to substitute for the lost plant-made chemical defense. Alternatives could be a. breeding of a "bitter-sweet" lupin that is alkaloid-rich in the green parts, but sweet in the seeds, or b. cultivation of resistant alkaloid-rich plants, but debittering of the seeds by extraction after harvest.

#### Acknowledgment

Our work was supported by the Deutsche Forschungsgemeinschaft. I thank Mrs. C. Theuring and Ms. S. Schmidt for technical assistance.

#### Legend of Symbols

QA = quinolizidine alkaloids, ED = effective dose, LD = lethal dose

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## Chapter 48

# Diterpenoids as Insect Antifeedants and Growth Inhibitors: Role in *Solidago* Species

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Diterpenoids have a wide range of biological activities. Their role in plant-insect interactions, both as antifeedants and growth inhibitors, is reviewed. Four ent-kauranes, kaur-16-en-19-oic acid, (-)-kauran-16-ol, 15-hydroxy-(-)-kaur-16-en-19-oic acid, and 17-hydroxy-(-)-kaur-15-en-19-oic acid, have recently been isolated from the leaves of *Solidago nemoralis*. These compounds were found to have antifeedant activity against *Trirhabda canadensis*.

Plant chemists show several different approaches to the way they view chemicals in plants. Some are interested in the isolation of the molecule per se, its structure and synthesis (1). Others are searching for interesting and hopefully patentable biologically active plant products or molecules as new drugs (2), as antibiotics (3), or as pesticides (4). A third group seeks to understand the role such compounds (allelochemicals) play in the environment, that is the way in which they may influence or control many of the complex interactions that occur between living organisms in natural plant communities (5, 6). As has been shown in this Symposium these three approaches are not necessarily mutually exclusive.

A study of the latter type is made more difficult since plants, even a single plant, usually contain a large number of different classes of chemical compounds and at the same time may be interacting with a wide variety of different organisms as well as with each other (7, 8). Nevertheless over recent years great progress has been made towards understanding the role of allelochemicals in natural systems particularly with regard to insect-plant interactions (9-12).

Terpenoids are one of the many classes of allelochemicals known to play an important role in such interactions (13). Of particular interest are the diterpenoids. These are widely distributed in plants, and are also present in fungi and marine organisms, and as such provide a ready source for the isolation of new compounds (13-15). They also show a wide range of biological activities (13-15). These include antitumor properties (16), antimicrobial activity (17),

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fertility regulation (18), plant growth regulation (19, 20), and allelopathic action (21). Several groups of diterpenoids are also known to be active as larval growth inhibitors or feeding deterrents against a number of agricultural and forest pests (22-24) (Table I).

#### Biological Activity of Diterpenes Against Insects

Bicyclic diterpenes-clerodanes. An important group of insect antifeedants are the clerodane diterpenoids, which have been isolated from several different plant families (13)(Figure 1). Particularly well studied are the antifeedant activities of caryoptin and clerodin, and their derivatives, from Clerodendron and Caryopteris, Verbenaceae, against the tobacco cutworm Spodoptera litura L. (25-27).

Examining the bitter-tasting leaves of Ajuga remota, an East African medicinal plant, known locally to be resistant to insects, led to the isolation of another group of clerodanes, the ajugarins, active against both the monophagous African armyworm, Spodoptera exempta, and the polyphagous S. littoralis (22, 28). When added to artificial diets ajugarins are also insecticidal to the silkworm, Bombyx mori, but merely inhibit the growth of the pink bollworm, Pectinophora gossypiella (28). Ajugareptansones A and B from A. reptans (29, 30), and ivains I-IV from Ajuga iva also exhibit high antifeedant activity against the African armyworm and other lepidopterous species (31).

Two chlorine-containing clerodanes, tafricanins A and B, with similar antifeedant properties, have been isolated from a South African bush, Teucrium africanum (32). Teucjaponin B, from Teucrium japonicum, is also inhibitory to the feeding of Spodoptera litura (33).

Belles et al. have recently discussed the structure-activity relationships of several natural clerodane diterpenoids and their derivatives, and have compared their activity with that of some synthetic butenolide derivatives (34).

Grindelane diterpenoids. Grindelanes, labdane-type diterpenoids (Figure 2) from Chrysothamnus and Grindelia species, show significant antifeedant activity (35). Two grindelane diterpenes, 18-hydroxygrindelic acid and 18-succinyloxygrindelic acid, present in the bicarbonate-soluble fraction of Chrysothamnus nauseosus (Pall.)Britt, the rabbit bush, were found by Rose (36) to inhibit feeding of third-instar Colorado potato beetles. Additional grindelane diterpenes, 6 $\alpha$ -hydroxy and 6 $\beta$ -hydroxygrindelic acids, also inhibited feeding by the aphid Schizaphis graminum (37).

Cuticular diterpenes-duvanes and labdanes. Cutler et al. have found that the cuticular diterpenes of green tobacco have both allelopathic and insect-deterrent effects (38). Present in the cuticle are duvane and/or labdane diterpenes (Figure 3) The levels of these specific cuticular components are believed to be responsible for the observed resistance of some types of tobacco to green peach aphids Myzus persicae (Sulzer), tobacco budworm Heliothis virescens (F.), and tobacco hornworm Manduca sexta (L.) (39).

Tricyclic diterpenes-resin acids. Wood resin constituents, e.g. abietane derivatives, have also been implicated in pest resistance (Figure 4). Among the conifer-associated sawflies many prefer to feed

Table I. Diterpenes with Known Biological Activity against Insects

Source	Family	Diterpene(s)	Insect affected	Reference
Clerodanes				
<u>Clerodendron tricotomum</u>	Verbenaceae	Clerodendrins A & B, 3-epicaryoptin	<u>Spodoptera litura</u>	(25-27)
<u>C. cryptophyllum</u>	Verbenaceae	Clerodendrin A	<u>Spodoptera litura</u>	(25-27)
<u>Caryopteris divaricata</u>	Verbenaceae	Caryoptin, clerodin, and derivatives	<u>Spodoptera litura</u>	(25-27)
<u>Ajuga remota</u>	Labiatae	Ajugarins I-IV	<u>Spodoptera littoralis</u> , <u>Spodoptera exempta</u> , <u>Schistocerca gregaria</u> , <u>Bombyx mori</u> , <u>Pectinophora gossypiella</u>	(22, 28)
<u>A. reptans</u>	Labiatae	Ajugareptansones A & B	<u>Spodoptera littoralis</u>	(29, 30)
<u>A. iva</u>	Labiatae	Ivains I-IV	<u>Spodoptera littoralis</u>	(31)
<u>Teucrium africanum</u>	Labiatae	Tafricanins A & B	<u>Locusta migratoria</u>	(32)
<u>T. japonicum</u>	Labiatae	Teucjaponin B	<u>Spodoptera litura</u>	(33)
<u>Grindelane diterpenoids</u>				
<u>Chrysothamnus nauseosus</u>	Asteraceae	18-Hydroxygrindelic and 18-succinyloxygrindelic acids 6 <del>6</del> -Hydroxygrindelic and 6 <del>6</del> -hydroxygrindelic acids	<u>Leptinotarsa decemlineata</u>	(36)
Duvenes and labdanes				
<u>Nicotiana</u> spp.	Labiatae	Labda-12,14-dien-8 <del>α</del> -ol, labda-13-en-8 <del>α</del> ,15-diol, <del>α</del> 6-4,8,13- <u>duvatrien-1</u> , 3-diols, <del>α</del> 6-4,8,13- <u>duvatrien-1-ols</u>	<u>Schizaphis graminum</u>	(37)
			<u>Heliothis virescens</u> , <u>Manduca sexta</u> , <u>Myzus persicae</u>	(39)
Abietanes				
<u>Pinus banksiana</u>	Pinaceae	13-oxo-8(14)-podocarpen- 18-oic, dehydroabietic,	<u>Neodiprion swainnei</u> , <u>N. rugifrons</u> , <u>N.</u>	(40-42)

<u>Larix laricina</u>	Pinaceae	palustric, levopimaric, neoabietic acids Abietic, neoabietic, dehydroabietic, isopimaric, sandaracopimaric acids	<u>dubiosus</u> , <u>N. lecontei</u> <u>Pristiphora erichsonii</u> (43, 44) <u>Schizaphis graminum</u> (37)
Norditerpenedilactones <u>Podocarpus nivalis</u> , <u>P. hallii</u> , <u>P. gracillior</u>	Podocarpaceae	Nagilactone C, D, F, podolide	<u>Pectinophora gossypiella</u> , (45-47) <u>Heliopsis zea</u> , <u>Spodoptera frugiperda</u> , <u>Musca domestica</u> , <u>Laspeyresia pomonella</u> , <u>Epiphyas postvittana</u>
Grayanoid diterpenes <u>Kalmia latifolia</u>	Ericaceae	Kalmitoxin-I, kalmitoxin-IV, grayanotoxin-III	<u>Lymantria dispar</u> (48)
Kauranes <u>Helianthus annuus</u>	Asteraceae	Trachyloban-19-oic and kaur-16-en-19-oic acids	<u>Homoesoma electellum</u> , <u>Heliopsis virescens</u> , <u>H. zea</u> , <u>Pectinophora gossypiella</u> (50, 51)
<u>Helianthus</u> spp.	Asteraceae	Ciliaric and angelylgrandifloric acids	<u>Homoesoma electellum</u> (52)
<u>H. occidentalis</u>	Asteraceae	(-)-cis and (-)-trans ozic acids	<u>Lepidopterous larvae</u> (54)
Isodon diterpenes <u>Rhabdosia</u> spp.	Labiatae	Isodons and derivatives	<u>Spodoptera exempta</u> , <u>S. littoralis</u> (55, 56)



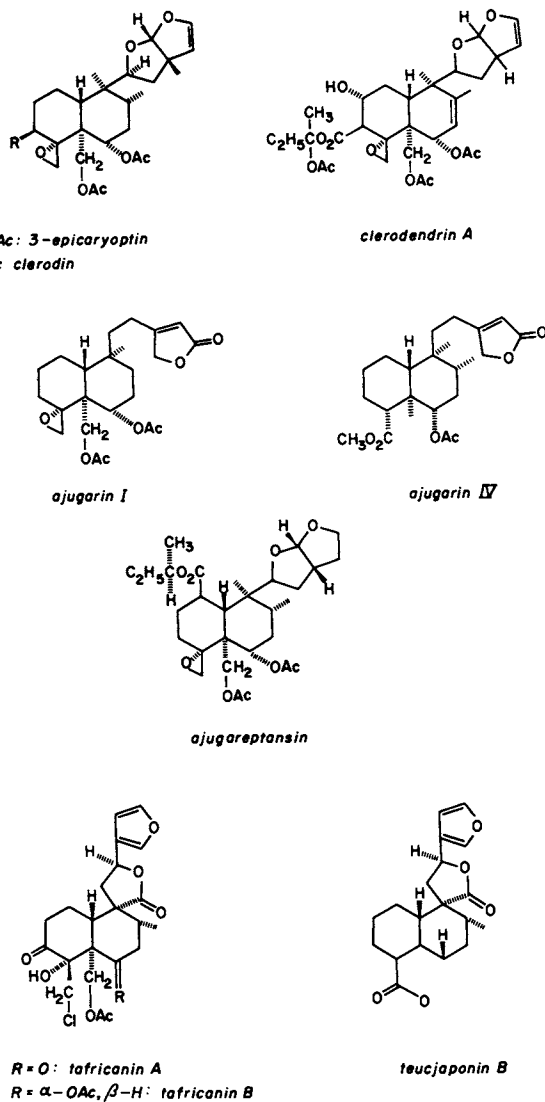


Figure 1. Clerodanes with insect antifeedant activity.

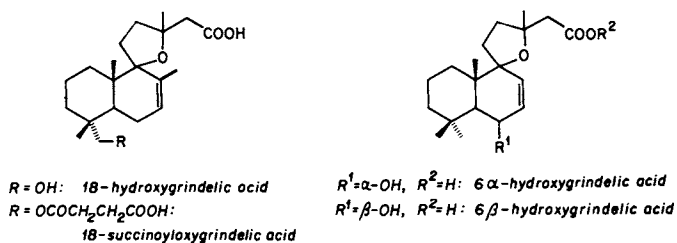


Figure 2. Grindelanes with insect antifeedant activity.

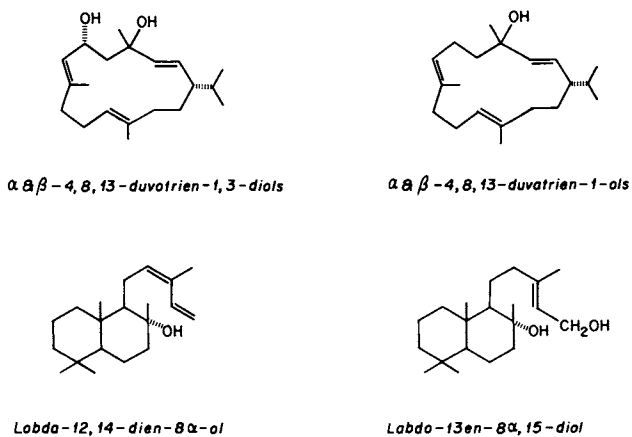


Figure 3. Cuticular Diterpenoids--Duvatrienes and Labdanes important in host-plant resistance to insects.

on mature needles rather than on the current season's needles of their respective hosts. Bioassay studies with needle extracts indicate that diterpene resin acids are important repellents.

All and Benjamin (40) observed that the monophagous sawfly species, Neodiprion swainei Midd. and N. rugifrons Midd., did not feed on juvenile foliage of their host jackpine, Pinus banksiana Lamb. The antifeedants were found by Ikeda et al. (41) to be 13-oxo-8(14)-podocarpin-18-oic acid and dehydroabietic acid. Schuh and Benjamin, working with two additional species of sawfly, N. dubiosus and N. lecontei (Fitch), found that palustric and levopimaric acids and neoabietic and palustric acids respectively, when painted on to one-year-old foliage of jackpine, also acted as feeding deterrents (42).

Diterpene resin acids, abietic, dehydroabietic, 12-methoxyabietic, sandaracopimaric, and isopimaric, are also antifeedants for the larch sawfly, Pristiphora erichsonii (Hartig) (43). Abietic, neoabietic, dehydroabietic, and isopimaric acids significantly reduce consumption rates, feeding efficiencies, and growth rates when topically applied to their natural food plant, tamarack, Larix laricina (Du Roi) K.Koch (44). Pure sandaracopimaric acid and levopimaric acid also act as feeding deterrents to aphids (37).

Norditerpenedilactones. The resistance of Podocarpus nivalis and P. hallii to insect attack is attributed to the high concentration of the norditerpenedilactone, nagilactone C, in the foliage (45, 46). Podocarpus gracilior is also resistant in nature to insect attack. As part of an apparently multichemical defense mechanism, nagilactones C, D, F and podolide (Figure 5) show insecticidal activity against Heliothis zea, Spodoptera frugiperda, and Pectinophora gossypiella (47).

Grayanoid diterpenes. A number of grayanoid diterpenes have been isolated from the mountain laurel, Kalmia latifolia L. (Figure 6) (Ericaceae). Kalmitoxin-I is the major antifeedant to the polyphagous gypsy moth, Lymantria dispar (48). Kalmitoxin-IV and grayanotoxin-III are also significantly deterrent to feeding.

Tetracyclic diterpenes-kauranes. Several diterpene carboxylic acids from Helianthus species have been shown to inhibit insect growth (49) (Figure 7). Kauranes, trachyloban-19-oic and ent-kaur-16-en-19-oic acids, found in the florets of Helianthus annuus, inhibit larval development of the sunflower moth, Homeosoma electellum L. (50), and of several other lepidopterous species (51). These acids are widely distributed in other Helianthus species and may be important in the resistance of sunflowers to insects. Herz et al. (52) have recently shown that two other kauranes from the sunflower, ciliaric acid and angelylgrandifloric acid, when fed to the sunflower moth result in both higher mortality and retardation of growth. However ciliaric acid, from H. argophyllus, shows no insecticidal activity against Spodoptera litura or Culex pipiens (53).

Two diterpenoid acids, (-)-cis- and (-)-trans-ozic acids, may also contribute to host plant resistance to several insect species in Helianthus occidentalis (54).

Isodon diterpenes. Highly oxygenated  $\delta$ -seco-ent-kaurane diterpenoids, the isodons (Figure 7), isolated from species of Isodon (now Rhabdosia)

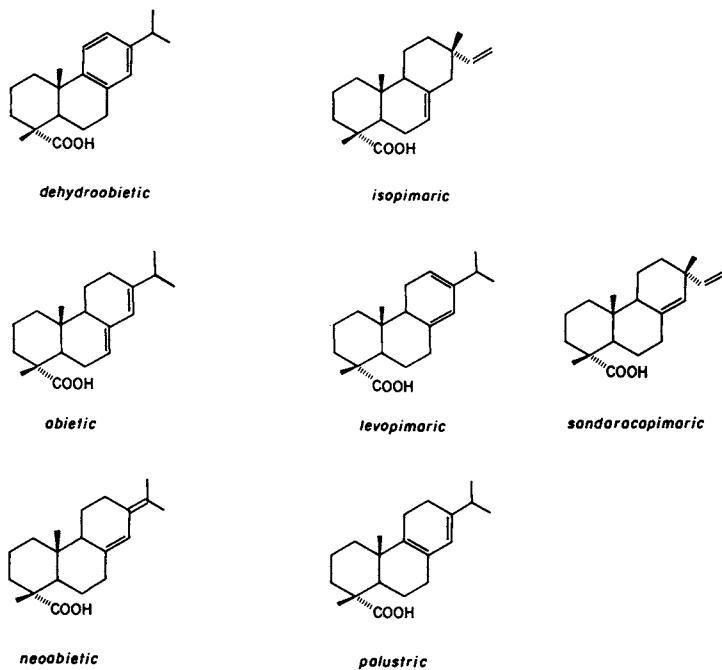


Figure 4. Abietanes with insect antifeedant activity.

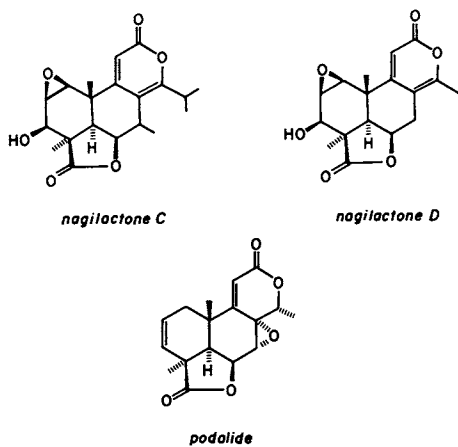


Figure 5. Norditerpenedilactones with insect antifeedant activity.

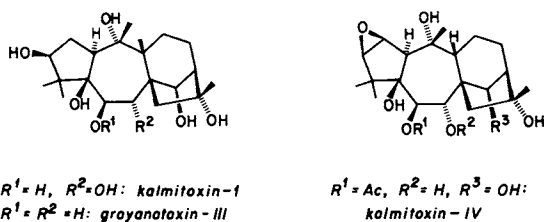


Figure 6. Grayanoid Diterpenes with insect antifeedant activity.

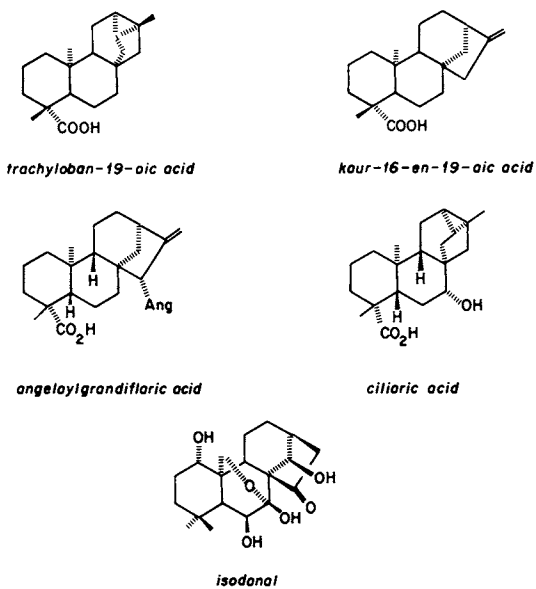


Figure 7. Kauranes that inhibit insect growth and development.

show growth inhibitory activity against both Spodoptera exempta and S. littoralis (55, 56). The growth inhibitory activities appear to be relatively specific for lepidopterous larvae.

### The Genus Solidago

Solidago is a mostly North American genus of about 125 species. It belongs to the Asteraceae (Compositae), a family with a very distinctive chemistry (57, 58). The genus itself contains several different classes of secondary compounds including a number of diterpenes (59). During the 1960's and 1970's Anthonsen in Finland, and McCrindle and his coworkers in Canada, isolated about twenty-five different compounds from the roots of Solidago species, mainly clerodanes and labdanes. But in the last five years attention has turned to the aerial parts of the plant and compounds of several other classes of diterpenes, abietanes and kauranes, have been isolated (60-79) (Table II).

As many of the same types of diterpenes play an active role in the resistance of other plants to insect attack (see above), it is interesting to investigate the role of diterpenes in Solidago and its interactions with insects. Solidago has the advantage that the behavior and the ecology of many of the associated insects are well known (80).

Solidago is subject to colonization by a number of different phytophagous insects (81-87), parasites, and predators (88-90). Many of these insects are highly selective in their choice of food plants (91-93), as are, for example, Trirhabda beetles (Coleoptera, Chrysomelidae), which will feed on certain interspersed species of goldenrods but will avoid others. T. canadensis readily feeds on the leaves of both S. altissima and S. missouriensis but completely rejects either S. nemoralis or S. rigida when all four species are growing in the same garden (92). Messina also found that T. virgata rejected S. nemoralis to a greater extent than other Solidago species under field conditions (93). Thus S. nemoralis appears to be particularly well defended against Trirhabda beetles, (92, 93), suggesting a possible role for the diterpenes in S. nemoralis as feeding deterrents.

### Isolation of Diterpenes from Solidago Species

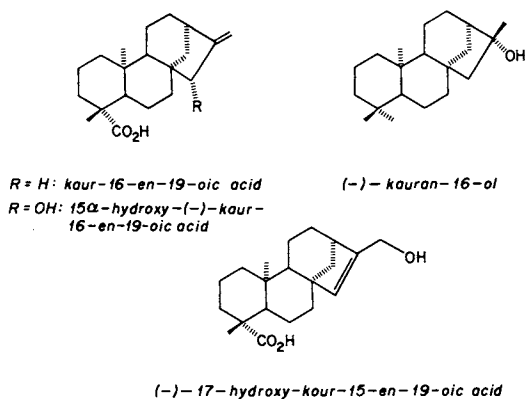
Leaves of S. nemoralis and S. altissima were collected from Professor P. Morrow's experimental gardens located at Cedar Creek, University of Minnesota.

Dry milled plant material was thoroughly extracted with petroleum ether/ether (2:1) (67). The extracts were separated on a kieselgel 60 silica column, and eluted with petroleum ether/ethyl acetate and ethyl acetate/methanol mixtures of increasing polarity. Compounds were further purified by silica TLC using a variety of solvents or on a HPLC, Dynamax semipreparative silica column, with 98% hexane/2% dichloromethane solvent.

Four kauranes present in the petroleum ether/ethyl acetate fractions of S. nemoralis were fully characterized by mass spectrometry, nuclear magnetic resonance spectrometry and infrared as (-)-kaur-16-en-19-oic acid, (-)-kauran-16 $\alpha$ -ol, 15 $\alpha$ -hydroxy-(-)-kaur-16-en-19-oic acid and 17-hydroxy-(-)-kaur-15-en-19-oic acid

Table II. Distribution of Diterpenes in Species of *Solidago*

Species	Plant part	Class	References
<i>Solidago altissima</i> L.	Roots	Clerodanes	(60)
	Leaves	Clerodanes	(61, 62, 63)
<i>S. arguta</i> Ait.	Roots	Clerodanes	(64)
<i>S. canadensis</i> L.	Roots	Labdanes	(65, 66, 67)
	Leaves	Labdanes	(67)
<i>S. elongata</i> Nutt.	Roots	Clerodanes	(68)
<i>S. flexicaulis</i> L.	Roots	Absent	(69)
<i>S. gigantea</i> Ait.	Roots	Clerodanes	(70, 71, 72)
var. <i>serotina</i> Cronq.			
<i>S. juncea</i> Ait.	Roots	Abietanes, clerodanes, ent-kauranes	(72)
<i>S. missouriensis</i> Nutt.	Roots	Abietanes, labdanes	(73, 74)
	Leaves	Kauranes	(75)
<i>S. nemoralis</i> L.	Roots	Clerodanes	(67)
	Leaves	Abietanes	(67)
<i>S. odora</i> Ait.	Roots	Absent	(67)
	Leaves	Absent	(67)
<i>S. rigida</i> L.	Roots	Kauranes	(69)
	Leaves	Kauranes	(75)
<i>S. rugosa</i> Mill.	Roots	Abietanes, clerodanes, kauranes, labdanes	(67)
	Leaves	Kauranes, labdanes	(67)
<i>S. sempervirens</i> L.	Leaves	Labdanes	(76)
<i>S. serotina</i> Ait.	Roots	Clerodanes	(77, 78)
<i>S. shortii</i> Torr. & Gray	Roots	Clerodanes	(69)
<i>S. virgaurea</i> L.	Roots	None	(69)
	Leaves	Clerodanes	(79)

Figure 8. Kauranes isolated from the leaves of Solidago nemoralis.Table III. Effect of Diterpenes from Solidago nemoralis on Feeding by Larvae and Adults of Trirhabda canadensis

Compound	Feeding Inhibition (%) <sup>a</sup>	
	Larvae	Adults
Kaur-16-en-19-oic acid	no significant effect	stimulatory <sup>**b</sup>
<u>ent</u> -Kauran-16-ol	40 <sup>**</sup>	33 <sup>**</sup>
15 $\alpha$ -Hydroxy-(-)-kaur-16-en-19-oic acid	42 <sup>**</sup>	49 <sup>***</sup>
17-Hydroxy-(-)-kaur-15-en-19-oic acid	42 <sup>**</sup>	49 <sup>**</sup>
In combination	42 <sup>*</sup>	32 <sup>*</sup>

a) Concentration 1 mg/mL. Results from 20 replicates of three separate experiments  
 b) Significance levels (Paired T-test)  
 \* 0.05 \*\* 0.01 \*\*\* 0.001



(Figure 8) (94). These four compounds were not present in S. altissima.

#### Antifeedant activity of kauranes isolated from S. nemoralis

A modification of the disc assay method was used to test the isolated kauranes for antifeedant activity. Freshly collected leaves of S. altissima were painted with the pure compound, dissolved in methanol, at concentrations of 1 mg/mL and 0.5 mg/mL in order to approximate the concentration present in the leaves. Control leaves were coated with methanol. Two treated and two untreated leaves were placed in a petri dish containing moistened filter paper. Eight Trirhabda larvae or four adults, collected from the same field site, were starved for several hours, introduced into the dishes and these kept under standard conditions for a period of twenty-four hours. Feeding was determined in two ways : by visually estimating the total amount of damage to each leaf and by weighing the leaves before and after the experiment. There was no significant difference between the results obtained using either of these methods. Antifeedant activity is expressed as the ratio consumed area of treated leaf/ consumed area of control.

Most previous experiments with kauranes and closely related compounds have been concerned with their effects on insect growth and development. However in this case the deterrent effects of the isolated compounds to Trirhabda canadensis were investigated as a first step in understanding the feeding behavior of this insect in the field.

(-)-Kaur-16-en-19-oic acid had no deterrent effect on feeding by larvae of Trirhabda , but surprisingly was stimulatory to feeding by the adults (Table III). The three other kauranes significantly reduced feeding, in the larvae by 40 per cent and by 32-49 per cent in the adults. In combination the four compounds reduced feeding by 32 per cent. Overall this constitutes a substantial reduction in feeding by Trirhabda beetles and confirms the allelopathic role ascribed to diterpenes.

However these compounds either singly or in combination do not account for the total lack of feeding activity observed either in the laboratory or in the field. Resistance of S. nemoralis to insect attack seems to involve, as is expected, not a single class of compounds but a multichemical response. Other compounds isolated from S. nemoralis showing antifeedant activity to Trirhabda canadensis are at present under investigation.

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## Chapter 49

# Plant-Plant Recognition: Chemistry-Mediating Host Identification in the Scrophulariaceae Root Parasites

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As little as a year ago we reviewed our understanding of the chemistry mediating host recognition in the parasitic angiosperms (8). Although a number of haustoria-inducing factors had been found, it was clear that the underlying mechanism for this plant-plant interaction was not understood. This paper reviews the identification of 2,6-dimethoxy-p-benzoquinone (2,6-DMBQ) in Sorghum roots as a haustoria-inducing principle for Striga asiatica (Scrophulariaceae). The unique timing events required for Striga parasitism suggest that haustorial induction is mediated through degradation of host root surface components by the parasite's enzymes. Quinones, such as 2,6-DMBQ, would be released and used as recognition signals. This kind of biological recognition may be common to many of the Scrophulariaceae and, in fact, could be a general mechanism for plant-plant interaction.

There are usually a few plants that tend to predominate in a given habitat. While a number of factors contribute to the ecological success of these plants, including their differing ability to use natural resources such as water, light, and nutrients, another more aggressive, chemically based mechanism was proposed in 1832 by DeCandolle (1). He suggested that plant-produced chemicals may adversely affect neighboring plants. More than a century later, Molisch (2) coined the term allelopathy to include the release of biochemical toxins into the environment by microorganisms and higher plants. A variety of allelochemicals, ranging from simple gases to complex aromatic compounds, have now been identified. Clearly such compounds are playing a significant role in the interaction between plants, and this symposium highlights the potential of allelochemicals in agriculture.

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An example of a specific plant-plant interaction is seen in parasitic angiosperms. These plants have evolved the capability of expropriating the resources of other plants for their own benefit. Many parasites exhibit narrow host ranges. As a prerequisite for their success these parasites must have developed methods to distinguish between host and non-host plants. In 1977, Alsatt *et al.* (3,4) proposed that parasitic plants, like herbivorous insects, may use host defense chemicals as recognition cues. Therefore, a study of this system may not only provide some insight into the mechanism of how one plant is able to identify neighboring plants, but also uncover some aspects of the development and specificity of the host's allelochemistry.

### The Parasitic Angiosperms

Parasitic angiosperms are represented throughout ten families, and because of their agronomic importance much has been written about them (5-8). They can either be facultative or obligate and in many ways are typified by the hemiparasite Agalinis purpurea and the holoparasite Striga asiatica (both Scrophulariaceae). While Agalinis purpurea is capable of parasitizing a wide variety of hosts, Striga asiatica has a host range that is restricted to grasses, such as corn, sorghum, millet, sugar cane, and rice. Because of this parasitism of grain crops, Striga infestations are very devastating and affect the food supply of millions of people in Africa and Asia.

Striga has at least two levels of host recognition, one associated with germination, and another at the level of the development of the haustorium, a specialized attachment organ common to all root parasites. Our laboratory has shown that these processes are mediated by separate and specific chemical cues present in the host (8). Germination, the first commitment to a host, has been found to be induced in Striga by strigol, a sesquiterpenoid exuded by cotton roots (9). However, cotton does not serve as a host for Striga, and, in fact, can be used as a catch crop to reduce Striga infestation. The germination stimulant from a natural host has yet to be identified.

Haustorial development in Striga is very tightly regulated. Riopel and Baird (10) have shown that, regardless of temperature, induction of the haustorium is not possible 5 to 7 days post-germination. This implies that germination must take place at a distance where the growing radicle can reach the host's surface in a period of 5 days, roughly 5 mm. In addition, Striga develops only a terminal haustorium (Figure 1), and once formed, radicle elongation ceases. For successful host attachment, haustorial induction must occur within ca. 50  $\mu\text{m}$  of the host surface. Therefore, the chemistry mediating these processes must not only explain how Striga recognizes a host, but also account for the mechanism of distance regulation.

### Haustoria-Inducing Factors from Gum Tragacanth

Axenic cultures of Agalinis grown in the absence of a host develop few or no haustoria (4,11). Induction of this organ is rapid upon

exposure of Agalinis purpurea to host roots (11). Fully formed haustoria can be observed within 12 hr, and this response can easily be quantitated. By use of haustorial induction in Agalinis as a bioassay, the first haustorial inducers, xenognosin A (1) and B (2), were isolated and characterized from gum tragacanth (12,13), a commercially available plant exudate. An efficient total synthesis of xenognosin A, which allowed sufficient flexibility for the study of structure-activity relationships, pointed toward a strict structural specificity (14). These studies established the meta-methoxyphenol moiety and the propene double bond as critical for biological activity (13). A simple change in the regiochemistry of the methoxy substituent, as in compound 3, drastically reduced the activity, and removal of either the methoxy or the hydroxy groups rendered the compound totally inactive.

This specificity seemed quite remarkable since Agalinis parasitizes a broad spectrum of hosts. Nevertheless, flavanoids and phenolics have frequently been cited as playing a role in allelopathy (15). Xenognosin A has also been found in Pisum as a stress metabolite (16,17), and xenognosin B has been shown to be a biosynthetic precursor of the phytoalexin medicarpin (18). These findings suggested that the xenognosins are constitutive antibiotics necessary for host defenses (8) and supported Atsatt's original proposal that such compounds could serve as recognition cues for a suitable host. The identification of the xenognosins, hence, constituted the first experimental support that parasitic angiosperms mediate host selection through the identification of specific molecules present in, and potentially exuded by, their hosts.

#### Haustorial Inducer in a Natural Host

Attempts to document and quantitate exuded materials from Agalinis host plants gave results that were not readily explained by the simple exudation of specific allelopathic agents (8,19,20). Since Striga requires a more critical commitment to host plants and a more specific host selection, it was reasoned that a study of this obligate parasite may help define the mechanism for host recognition. If recognition is mediated by exuded materials, they must be present in sufficient concentrations in the soil only near the surface of the host root, but may exist in higher concentrations within the root. Therefore, the roots of Sorghum, a grass easily grown in the laboratory and readily parasitized by Striga, were extracted and fractionated as directed by a bioassay for haustoria-inducing activity (21). This procedure allowed for the purification of 100  $\mu\text{g}$  of a single crystalline yellow compound from 600 g of Sorghum roots (22).

Electron impact mass spectroscopy (70 eV, 200°C) of this compound gave a molecular ion at  $m/z$  168.0389, and a chemical composition of  $\text{C}_8\text{H}_8\text{O}_4$  (calc. 168.0422). The UV spectrum [ $(\text{CH}_2\text{Cl}_2)$ :  $\lambda_{\text{max}}$  284 ( $\epsilon = 10,233 \text{ M}^{-1} \text{ cm}^{-1}$ ) and 376 nm ( $\epsilon = 447 \text{ M}^{-1} \text{ cm}^{-1}$ )] of the natural product was characteristic of a quinonoid



nucleus. This data, together with the  $^1\text{H}$  NMR [ $(\text{CDCl}_3)$ :  $\delta$  3.75 (s, 6H); 5.83 (s, 2H)] supported a dimethoxy-substituted *p*-benzoquinone structure.

Three isomeric benzoquinones, differing only in the position of the methoxy substituents, satisfied the data (4, 5 or 6). NMR spectroscopy, instrumental in the characterization of the xenognosins, was of less utility for the structural elucidation of these highly symmetrical quinones. Mass spectrometric analysis, however, gave fragment ions at  $m/z$  112 and 80, indicating a retro-Diels-Alder cleavage of the benzoquinone ring of either quinone 4 or 5. The benzoquinone 6 would be expected to give fragment ions at  $m/z$  142 and 82. Moreover, its quinonoid protons should resonate further downfield ( $\delta$  6.58). These data ruled out 2,3-dimethoxy-*p*-benzoquinone as the haustorial inducer.

The electronic and vibrational spectra of benzoquinones are very diagnostic. Of the two possible isomers remaining, the 2,5-substituted quinone (5) would be expected to give two different electronic transitions of equal intensity around 280 nm, and only one carbonyl stretching band. The Fourier transform infrared spectrum ( $\text{CH}_2\text{Cl}_2$ ) of the haustorial inducer showed strong absorptions at 1698 ( $\nu_{\text{C=O}}$ ), 1646 ( $\nu_{\text{C=O}}$ ) and 1597  $\text{cm}^{-1}$  ( $\nu_{\text{C=C}}$ ). These spectroscopic data established the haustorial inducer as 2,6-dimethoxy-*p*-benzoquinone (2,6-DMBQ, 4).

All three isomeric dimethoxyquinones were synthesized (22-24), and these synthetic compounds confirmed the assignment of 2,6-DMBQ as the haustorial inducer.

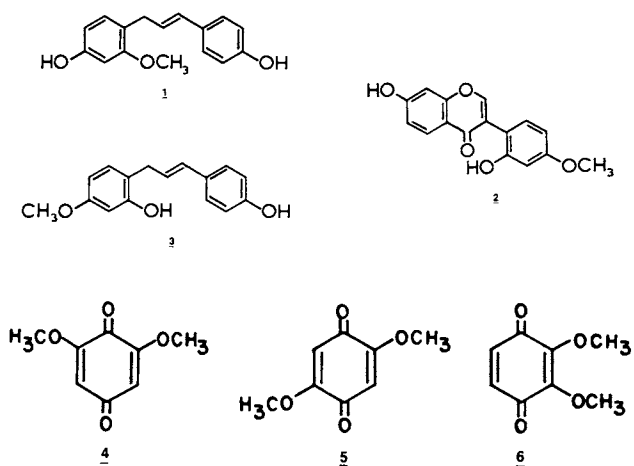
In order to probe the structural specificity of the quinone, several synthetic analogues were prepared (22) and tested for haustoria-inducing activity. These results are presented in Table I. The monomethoxy (9) and ethoxymethoxy quinones (8) were only one-tenth as active as 4. More drastic structural alterations completely abolished activity. The unsubstituted benzoquinone (10), as well as the dimethyl (11) and the dihydroxy (12) derivatives, which lack a methoxy functionality, were totally inactive.

As in the xenognosins, the presence of at least one methoxy functionality appears to be of critical importance. All three isomeric dimethoxyquinones fulfill this requirement, but only 2,3-DMBQ (6) and 2,6-DMBQ (4) induce haustoria. Both compounds have been shown to inhibit mitochondrial electron transport (25,26). In contrast, the 2,5-isomer (5) shows no such inhibition of respiration and does not induce haustoria. A possible connection between the inhibition of electron transport and the haustoria-inducing activity of these quinones has yet to be clarified.

## Discussion

To date, several naturally occurring compounds which are capable of inducing the development of the haustorium have been characterized. The first haustorial inducers identified were the xenognosins, and their connection with the host's constitutive antibiotics suggested that they were specific recognition cues (8). 2,6-Dimethoxy-*p*-benzoquinone, the only active principle

Figure 1. Electron Micrograph of the Developing Haustorium of *Striga asiatica*. (Courtesy of Dr. Vance Baird).



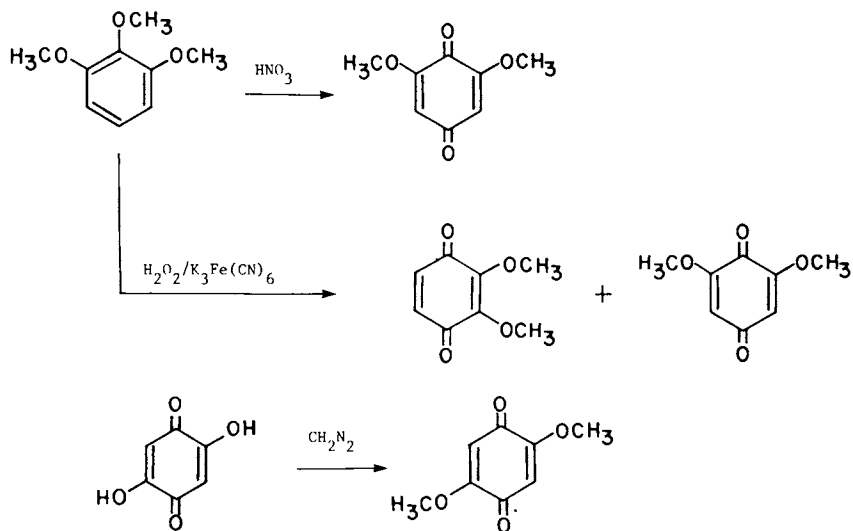


Table I. Biological Activity of Benzoquinone Analogues

	R <sup>2</sup>	R <sup>3</sup>	R <sup>5</sup>	R <sup>6</sup>	ED <sub>50</sub> (μM)
4	OCH <sub>3</sub>	H	H	OCH <sub>3</sub>	0.32
5	OCH <sub>3</sub>	H	OCH <sub>3</sub>	H	inactive
6	OCH <sub>3</sub>	OCH <sub>3</sub>	H	H	1.80
7	OCH <sub>3</sub>	H	H	OCH <sub>2</sub> CO <sub>2</sub> H	0.47
8	OCH <sub>3</sub>	H	H	OCH <sub>2</sub> CH <sub>3</sub>	2.00
9	OCH <sub>3</sub>	H	H	H	5.00
10	H	H	H	H	inactive
11	CH <sub>3</sub>	H	H	CH <sub>3</sub>	inactive
12	OH	H	OH	H	inactive

The ED<sub>50</sub> represents the concentration necessary to induce haustorial development in 50% of the Striga seedlings.

found in Sorghum, has a wide spectrum of biological activity (27-31), including allelopathy (32,33). Therefore the quinone, like the xenognosins, could be tied in with host defenses. Xenognosin A contains the biologically critical meta-methoxy phenol and the quinone possesses the same moiety at a higher oxidation state. Structural modifications altering this relationship eliminate the ability of both compounds to induce haustorial development. While this similarity is striking, 2,6-DMBQ, unlike the xenognosins, is widely distributed among the plant kingdom and has been found in roots, bark, aerial portions, and fruits of higher plants (34). Various substituted quinones are biosynthesized in many plants via the shikimate pathway and formed through a critical oxidative decarboxylation of benzoic acids, such as syringic acid (35,36).

Recognizing this common decarboxylation step in the biosynthesis of quinones sheds new light on MacQueen's (37) demonstration that several phenolic acids are inducers of haustorial development in Striga hermonthica (Table II). The phenolics are as potent as the quinones (Table I), and in both classes of compounds a methoxy substituent is important for biological activity. These findings suggested that the activity of the acids could be the result of their metabolic breakdown to the quinones (22). This connection between the benzoic acids and the quinones provided the first evidence for a unifying mechanism of haustorial induction. However, all attempts to identify the acids, quinones, or any haustoria-inducing activity in Sorghum exudate were unsuccessful.

Studies of pathogenic fungi suggested an explanation for the absence of inducer molecules in host root exudate. Quinones such as 2,6-DMBQ have been shown to be released by white rot fungi as terminal oxidation products of lignin model compounds (38). These findings substantiated previous reports (39-41) that laccases, phenol oxidases using  $O_2$  as the oxidant, are directly involved in lignin degradation (42,43). In fact, the presence of such enzymatic activity has been correlated with virulence in white rot fungi.

A specific chromogenic compound, syringaldazine, has been developed to test for the presence of these enzymes (44). The compound, which is yellow in its reduced form, is oxidized to a red-purple quinone methide in the presence of laccase or peroxidase. It has been used as a fast and simple screening procedure for these enzymes in pathogenic fungi (44). The use of syringaldazine to stain the roots of lettuce, pea, and Sorghum seedlings shows enzymatic activity localized in the root hair zone of these plants (22). Both Agalinis and Striga, which have poorly developed or no root hairs, thus show the presence of phenol oxidase along the surface of the root and on the meristematic tip, a region not stained in the other plants. While this histochemical assay does not prove that these enzymes have the required specificity, it does provide evidence for the presence and differential localization of the necessary oxidative enzymes.

Fungal laccases readily oxidize ortho- and para-substituted phenols (45-48), such as syringic (15) and sinapic (13) acids, to the corresponding quinones. Experiments with Triticum vulgare

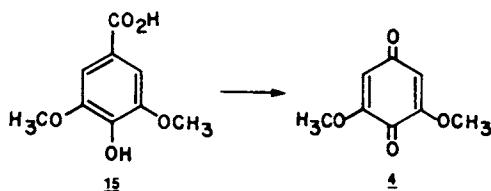


Table II. Biological Activity of Phenolic Compounds

	ED <sub>50</sub>		ED <sub>50</sub>
	1.1 × 10 <sup>-5</sup> M* 2.5 μg/mL		5.2 × 10 <sup>-6</sup> M* 1.0 μg/mL
	5.1 × 10 <sup>-7</sup> M* 0.1 μg/mL		inactive
	inactive		inactive

The ED<sub>50</sub> represents the concentration necessary to induce 50% of the Striga seedlings to form haustoria. \*MacQueen, M. (37).

(wheat) indicate that 2-methoxy- and 2,6-dimethoxy-p-benzoquinone arise from oxidative decarboxylation of vanillin and syringic acid, respectively (44). Thus, it is conceivable that parasitic enzymes could oxidize xenognosin A (1) to a methoxy quinone. Structural modifications such as reduction of the propene double bond (e.g., dehydro-xenognosin A), a change in the methoxy position (e.g., 3), or removal of the hydroxy group would drastically reduce the biological activity (8).

While the presence of oxidative enzymes on the parasite and the structural correlations seen with the active haustorial inducers do not provide unequivocal evidence in favor of host recognition mediated through degradation of host surface components by parasitic enzymes, they present a strong argument for such a mechanism. Preliminary experiments suggest that high molecular weight carbohydrates can be removed from Sorghum root surface, which on incubation with Striga seedlings generate 2,6-DMBQ. A more thorough characterization of the surface components from the roots of Sorghum and of the parasitic enzymes responsible for the release of 2,6-DMBQ need to be completed before this mechanism of host recognition can be further substantiated. However, this process would unify the mechanism through which both Striga and Agalinis control the initiation of haustorial development.

Haustorial formation represents a meristematic differentiation in Striga and radicle elongation terminates with the induction of haustorial development. These observations imply that the distance between the parasite's meristematic root tip and the host root surface is critical. Premature haustorial induction would render the parasite unable to reach its host. With Agalinis, early haustorial induction is not fatal, but successful attachment to a host provides a selective advantage. This mechanism of biological recognition may be common to many of the parasitic Scrophulariaceae. In fact, surface recognition between two eukaryotic organisms is a fundamental feature of allelopathy. An active screening of the environment by specific plant enzymes could provide more specific information about competitors, and even information about the location of those plants. The proof of such a mechanism would go one step further to dispel the belief that plants are passive organisms, only able to utilize the resources of their surrounding environment, and highlights one of the plant kingdom's elaborate and sophisticated uses of chemistry.

#### Acknowledgments

We are indebted to the Research Corporation and the Frasc Foundation for support, the USDA (5901-0410-9-0257 and 58-7B30-3-597) for jointly supporting this laboratory and that of Professor James Riopel at the University of Virginia, and The University of Chicago Cancer Center for instrumentation support. DGL gratefully acknowledges support from the Alfred P. Sloan Foundation and the Camille and Henry Dreyfus Foundation, Inc. and MC is grateful for support from the Aileen S. Andrew Foundation.

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## Chapter 50

# Plant Growth Regulators and Insect Control Agents from Marine Organisms

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In contrast to work in the burgeoning field of plant allelochemicals, very little effort has been directed toward the study of plant growth regulators from the marine biosphere. We have initiated a program to determine whether there are plant growth regulators in marine organisms and, having detected such activity, to isolate and identify the active compounds. A number of extracts have exhibited plant growth promotion or inhibition. Three active compounds have been identified; one is the known auxin indole-3-acetamide. The other two are novel indoles. A number of marine organisms have been shown to avoid predation by the action of toxins or feeding deterrents upon potential predators. Testing of the activity of such compounds against insects has revealed some insecticidal activity in several sesquiterpenes and diterpenes.

Sessile marine organisms, much like their terrestrial counterparts, exist in an environment characterized by intense competition for living space. There is considerable evidence, amid some controversy (1, 2), that many species have developed chemical expedients to insure their survival; survival may take the form of cohabitation with, domination over or eradication of competitors for space, as well as the use of toxins or feeding deterrents to repel predators.

Plant growth regulators seem to play a major role in dominance/eradication situations in the terrestrial environment and might well play an important role in the marine biosphere as well. Periodic reports of the discovery of growth regulators in marine algae have appeared (3-8), but these studies have all been comprised of qualitative analyses for known plant growth regulatory substances. More recently, a few articles have appeared that indicate the presence of growth regulatory substances of unknown structure in some algae (9-11). There are no previous reports of growth promoters or inhibitors in the extracts of sessile marine invertebrates.

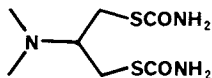
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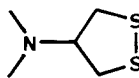
Nearly six years of collecting work in the Bermudian archipelago has afforded us an opportunity for continuing observation of the competition for space on the rocky substrates of the shallow water reef ecosystem. Algae, sponges, tunicates, coelenterates and corals can be seen to participate in an intense competition for the limited space available. To survive, an organism must either avoid predation or grow so rapidly as to offset losses to predators; in addition, the organism must have sufficient space for growth and reproduction. This space can be acquired by rapid growth, overgrowth of other organisms, or toxicity to neighboring organisms; in turn, space can be protected through prevention of overgrowth by other species. Organisms which tolerate overgrowth without apparent deleterious effect can also be observed; such overgrowth might, in fact, be welcomed or encouraged as a means of camouflage protection.

Such observations led us to the hypothesis that there is likely a diversity of growth promoters and inhibitors to be found in marine algae and invertebrates. We thus began an examination of selected extracts for plant growth regulatory behavior.

Our view of the marine biosphere as a source of leads to novel insect control agents evolved from the dramatic developments in marine chemical ecology over the last dozen years. A number of antifeedants and toxins have been identified during studies of sponge-nudibranch (12) and alga-mollusc and alga-fish (13) relationships. Reef communities are comprised of exceedingly complex inter-species relationships and the struggle for space, food and survival is intense; it thus stands to reason that chemical, as well as physical, defenses are essential for survival. At issue is whether or not the deterrence of or toxicity to invertebrates observed in the marine environment could be extended to the terrestrial biosphere. If so, new insect control agents might be available from this unlikely source and marine natural products would then represent a large, almost wholly untapped reservoir of potential insecticides and feeding deterrents. Apart from the development of Padan, 1, from a lead provided by nereistoxin, 2, from the marine worm Lumbriconereis heteropoda (14), little attention has been rendered to secondary metabolites from marine organisms as insect control agents.



1



2

We initiated this line of research by testing isolated compounds for their effects upon two insects, the grasshopper (Melanoplus bivitattus or M. sanguinipes) and the tobacco hornworm, Manduca sexta. We could thereby test for activity against adult

and larval stage crop pests; the tobacco hornworm assay allows us to test for toxicity, feeding deterrence and juvenile hormone effects in a single assay (15). We have recently begun testing crude extracts in order to focus our isolation efforts on compounds with potential insect control activity; the tobacco hornworm assay is well suited to bioassay-guided fractionation.

The selection process for choosing organisms to be screened for plant growth regulation is based primarily on observations made in the natural marine habitat. Organisms which proliferate in competitive environments or which tolerate epiphytic organisms are likely sources of growth promoters, while organisms which survive by avoiding overgrowth or encroachment by other organisms might yield growth inhibitors. Similarly, organisms which exhibit no signs of predation are considered likely candidates for insecticidal screening.

### Materials and Methods

Plant Growth Regulation Assays. Initial screening for activity involves the testing of crude extracts in a simple, quick bioassay utilizing lettuce seeds. Concentrations of 0.5 to 5 mg/mL of the crude extracts are tested; 5 mL of solution at each concentration are pipetted into a small Petri dish. A Teflon ring is placed in the dish for support of a small screen, which is placed atop the ring. Twenty lettuce seeds are placed on the screen and allowed to germinate and grow under white light, usually for 72 hours. The germination rate at each concentration is recorded as a percentage of controls; individual root and coleoptile lengths are determined, averaged for each concentration and recorded as a ratio of average test sample length divided by average control length. Purified compounds are tested in a similar fashion, although the concentration range examined is  $10^{-5}$  to  $10^{-10}$  M.

Insecticidal Assays. The grasshopper assay has been described (16). Eggs of the tobacco hornworm, *Manduca sexta*, are obtained from Carolina Biological Supply and are hatched and reared on an artificial agar-based diet (Black Cutworm) obtained from Bio-Serv Inc. Five to seven days after hatching, the larvae are weighed and transferred to sterile cups containing agar diet impregnated with test compounds or extracts. Dichloromethane, acetone or water solutions of test materials are added to the warm diet and mixed thoroughly. After evaporation of the solvent and setting of the agar, the diet is cut into 25-g wedges and five larvae are placed in a cup with the 25-g of impregnated diet material. Controls consist of diet treated only with the solvents used to dissolve the test materials.

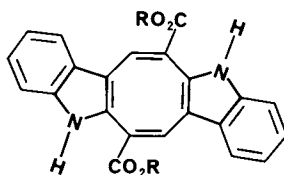
The tobacco hornworms are observed over a seven-day period for signs of toxicity, reduced feeding, relative weight gain (compared to controls) and ability to shed exuvia (ecdysis).

### Results

Plant Growth Regulation. Three plant growth promoters have been identified thus far. Caulerpin, 3, a unique bisindole pigment found in some, but not all species of the green alga *Caulerpa*, was

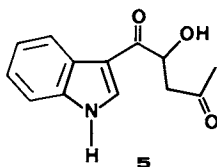
the first compound to demonstrate activity in our studies (17). Both caulerpin and its hydrolysis product **4** exhibit activity similar to that of indole-3-acrylic acid, increasing root growth of lettuce seedlings to 115-118% the length of controls.

Equally intriguing is the case of the sponge *Dysidea etheria*. *D. etheria* is a successful competitor for space on the reef; it is also known to harbor algal communities on its surface and within the sponge mass. Crude extracts of the sponge exhibited plant growth regulatory activity. Our investigations of the polar organic soluble extracts yielded two growth promoters, indole-3-acetamide and the novel indole **5** (18). The previously unknown **5** induced root length extensions of 15% over controls; this unique indole was subsequently found in another sponge, *Ulosa ruetzleri*.



**3** R = CH<sub>3</sub>

**4** R = H



These initial successes led us to undertake a more organized and thorough study of marine invertebrates and algae for plant growth regulatory activity. The lettuce seed assay permits an evaluation of effects upon germination and subsequent seedling growth. Table I provides details of those extracts exhibiting some inhibition of germination.

**Table I. Plant Growth Regulation Assays - Germination<sup>a,b</sup>**

Organism	Extract Concentration (mg/mL)						
	0 (control)	0.5	0.1	2.0	3.0	4.0	5.0
BA-18 <sup>c</sup>	9/9	9/9	9/9	6/9	7/9	7/9	3/9
BS-2 <sup>d</sup>	19/20	18/20	18/20	15/20	14/20	8/20	8/20
BS-15 <sup>d</sup>	9/9	4/9	7/9	6/9	7/9	5/9	- <sup>g</sup>
BS-24 <sup>d</sup>	9/9	9/9	9/9	9/9	8/9	4/9	- <sup>g</sup>
BS-43 <sup>d</sup>	17/20	10/10	10/10	10/10	10/10	2/10	2/10
BS-70 <sup>d</sup>	20/20	20/20	20/20	20/20	18/20	- <sup>g</sup>	0/20
BT-1 <sup>e</sup>	20/20	18/20	18/20	19/20	10/20	10/20	5/20
BT-11 <sup>e</sup>	9/9	9/9	9/9	9/9	6/9	6/9	- <sup>g</sup>
BT-21 <sup>e</sup>	20/20	20/20	20/20	19/20	17/20	7/20	4/20
BT-9 <sup>f</sup>	9/9	9/9	9/9	9/9	6/9	6/9	- <sup>g</sup>

NOTE: Some of these data appear in ref. 26

<sup>a</sup>reported as # seeds germinated/# seeds tested<sup>b</sup>not active: BA-3<sup>c</sup>, BA-19<sup>c</sup>, BS-9<sup>d</sup>, BS-32<sup>d</sup>, BS-65<sup>d</sup><sup>c</sup>alga<sup>d</sup>sponge<sup>e</sup>tunicate<sup>f</sup>coelenterate<sup>g</sup>no measurement taken**Table II. Plant Growth Regulation Assays - Root Length<sup>a</sup>**

Organism	Extract Concentrations (mg/mL)						
	0 (control)	0.5	1.0	2.0	3.0	4.0	5.0
BT-1 <sup>b</sup>	18.5	21.2	18.1	13.6	4	8	4
BT-11 <sup>b</sup>	17.4	4.5	2.7	2.3	1.6	1.4	- <sup>e</sup>
BT-21 <sup>b</sup>	18.5	16.9	13.6	7.8	9.8	6.5	3.5
BS-2 <sup>c</sup>	16.8	- <sup>e</sup>	5	3.4	3.4	3.1	- <sup>e</sup>
BS-9 <sup>c</sup>	7.7	6.7	3.9	3.2	2.8	1.8	- <sup>e</sup>
BS-15 <sup>c</sup>	21.1	16.5	7.9	7.1	6.2	2.5	- <sup>e</sup>
BS-24 <sup>c</sup>	26.8	18.4	12.2	14.5	14.4	- <sup>e</sup>	- <sup>e</sup>
BS-32 <sup>c</sup>	25	17	15.6	9.3	8.8	7	- <sup>e</sup>
BS-43 <sup>c</sup>	27.8	10	8	3	3	1	1
BS-65 <sup>c</sup>	13.7	12	8	7	6	3	3
BS-70 <sup>c</sup>	13.7	9	7	4	- <sup>e</sup>	0.5	0
BT-9 <sup>d</sup>	20.4	15.5	11.1	11.9	10.3	9.5	10.4
BT-15 <sup>d</sup>	20.4	16.3	17.3	18.7	17	16.8	11.1

NOTE: Some of these data appear in ref. 26

<sup>a</sup>reported in mm; measurements made 72 h after germination<sup>b</sup>tunicate<sup>c</sup>sponge<sup>d</sup>coelenterate<sup>e</sup>no measurement made: fungal contamination or concentration not run

More dramatic data were provided from measurements of root and coleoptile lengths in seedlings treated with test extracts. As is evident from the data presented in Tables II and III, inhibition or growth was observed in many of the extracts tested. These preliminary results suggest that marine organisms, especially sponges, must be considered very likely sources of new growth regulatory substances. Efforts to identify the active constituents in a number of these organisms are now underway.

**Insect Control.** Grasshoppers were chosen as an assay organism because they are a major crop pest in the grain producing plains and plateau states, they are readily available year round from the Agricultural Experiment Station at Montana State, and they are relatively easy to maintain in the laboratory. Two species, *Melanoplus bivitatus* and *Melanoplus sanguinipes*, have been used in these assays.

**Table III. Plant Growth Regulatory Assays - Coleoptile Length<sup>a</sup>**

Organism	Extract Concentrations (mg/mL)						
	0 (control)	0.5	1.0	2.0	3.0	4.0	5.0
BT-1 <sup>b</sup>	3.75	3.7	3.1	3.1	2	4	- <sup>e</sup>
BT-21 <sup>b</sup>	4	3	2.4	2.9	2.5	3.3	- <sup>e</sup>
BS-2 <sup>c</sup>	2.5	- <sup>e</sup>	1	<1	<1	0	- <sup>e</sup>
BS-15 <sup>c</sup>	9.25	- <sup>e</sup>	16.2	- <sup>e</sup>	- <sup>e</sup>	- <sup>e</sup>	- <sup>e</sup>
BS-43 <sup>c</sup>	3	<1	<1	0	0	0	0
BS-65 <sup>c</sup>	3	2.5	2	2	0	0	0
BS-70 <sup>c</sup>	3.2	3	3	1.5	- <sup>e</sup>	1	0
BT-9 <sup>d</sup>	3.2	2.6	3.8	3.3	2.7	3.1	3.2
BT-15 <sup>d</sup>	3	4.2	4.5	4.9	3.6	4.4	3.1

<sup>a</sup> reported in mm; measurements made 72 h after germination

<sup>b</sup> tunicate

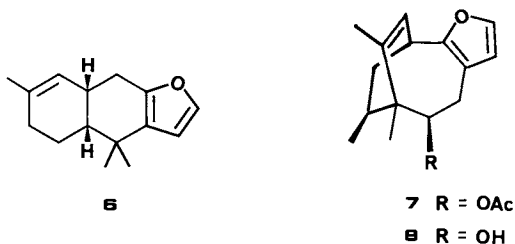
<sup>c</sup> sponge

<sup>d</sup> coelenterate

<sup>e</sup> no measurement made: fungal contamination or concentration not run

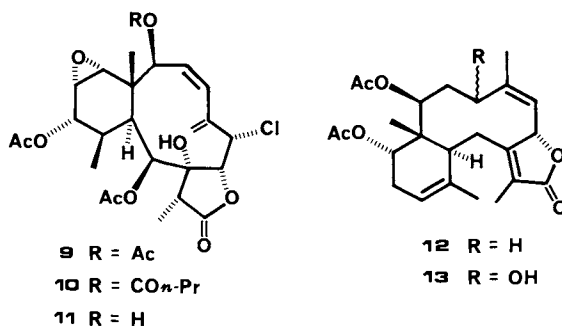
Grasshoppers, however, wreak their damage as adults, while the great majority of agricultural damage is done by larval stage insects. The tobacco hornworm has served as a laboratory model for insecticidal screening (15).

The sponge *Dysidea etheria* and the nudibranch *Hypselodoris zebra* have yielded a number of sesquiterpenes, some of which exhibit feeding deterrent activity against fish (19, 20). The more abundant of these compounds, 6, 7 and 8, have been tested in the insect assays. Furodysin (6) is toxic to grasshoppers at both test doses (3 mg and 1 mg per leaf section) and is the most potent and rapid acting of all the compounds studied thus far. To our surprise, furodysin was not toxic to late larval stage tobacco hornworms at doses of 250 ppm in an artificial diet. Instead, all the test insects suffered some form of difficulty in molting. Most of the insects were unable to shed their exuviae completely and two hornworms developed double head capsules (21). Further tests of the tobacco hornworm's response to 6 are under way.



The other two *Dysidea* metabolites tested, 5-acetoxy- and 5-hydroxy-nakafuran-8, **7** and **8**, exhibited a different activity profile against the grasshopper. The acetate **7** was toxic at the high dose and antifeedant at the lesser dose, while the alcohol **8** was antifeedant at both doses. Surprisingly, the acetate **7** exhibited no activity in the tobacco hornworm assay.

Some time ago, we initiated a study of the coelenterate *Briareum polyanthes* to ascertain whether there was a chemotaxonomic basis for the species distinction between the *Briareum* found in Bermuda and the more common *B. asbestinum* found throughout the Caribbean. We have found a series of new diterpenes, **9-13**, with the briaran skeleton (17,22,23). It has been implied (24,25) that this class of compounds serves as a chemical defense against predation in the soft corals and sea pens which produce them, but this has not yet been demonstrated by bioassay.



Two of these compounds, brianthein Y (10) and brianthein W (12) have, been tested in the grasshopper assay. Brianthein Y proved toxic at the high dose, but inactive at the lower dose; brianthein W produced no deleterious effect at either dose.

These same two diterpenes were subjected to the tobacco hornworm assay. Brianthein Y, 10, was quite toxic at 250 ppm, killing the exposed larvae within thirty hours. There appeared to be no inhibition of feeding. On the other hand, the less functionalized brianthein W, 12, elicited only 50 percent mortality over five days, and the surviving larvae suffered from extremely poor weight gains (only 6% of controls) despite feeding freely on the treated diet.

Ten to twelve diterpenes of this briaran class are available from our extracts of the gorgonian *Briareum polyanthes* and the sea pen *Ptilosarcus gurneyi*. Testing of this whole group will provide some useful structure-activity relationship data. A less functionalized analog of 10 might be a viable synthetic target. Dose response tests of 10 and 12 are planned to establish a baseline LD<sub>50</sub> for the series.

The tobacco hornworm assay is quite amenable to the screening of crude extracts for insect control activity. Table IV summarizes the results obtained to date. Most noteworthy is that the aqueous extracts tend to be inactive; nearly all the activity, observed in the form of toxicity and/or weight gain inhibition, resides in organic extracts. In the limited sampling conducted thus far, sponges would appear to exhibit the best activity in the assay.

Table IV. Insecticidal Screen - Tobacco Hornworm<sup>a</sup>

Organism	Extract <sup>b</sup>	Mortality	Weight (% control)
BT-1 <sup>c</sup>	A	1/10	61
BT-7 <sup>c</sup>	A	0/10	76
BD-15 <sup>d</sup>	A	0/10	54
BS-15 <sup>d</sup>	O	0/10	10
BS-28 <sup>d</sup>	O	3/9	27
BS-43 <sup>d</sup>	O	0/10	82
BS-43 <sup>d</sup>	A	1/10	66
BS-65 <sup>d</sup>	A	4/10	126
BS-65 <sup>d</sup>	O	2/10	24
BS-70 <sup>d</sup>	O	2/10	15
BS-70 <sup>d</sup>	A	1/9	108
BAn-1 <sup>e</sup>	O	5/8	40
BT-9 <sup>e</sup>	A	0/6	67
RH1-1B <sup>e</sup>	A	0/8	90
BA-3 <sup>f</sup>	O	0/8	70
BA-18 <sup>f</sup>	A	1/8	78

<sup>a</sup>crude extracts tested at 3000 ppm

<sup>b</sup>A-aqueous; O-organic

<sup>c</sup>tunicate

<sup>d</sup>sponge

<sup>e</sup>coelenterate

<sup>f</sup>alga



### Conclusions

Three plant growth promoters and five compounds with insecticidal or insect repellent activity have been identified from marine organisms. In addition, a number of extracts have been shown to be active in one or both of the assay systems.

Although this project is still in its formative stages, the results obtained thus far clearly demonstrate that:

- 1) there are novel growth regulators to be found in marine organisms;
- 2) invertebrates are just as likely as algae to contain growth regulators; and,
- 3) marine natural products which play a role in the defense of the producing organisms against predators are likely to be active as insect control agents.

Marine fauna and flora, then, show great promise as a source of leads to new agrochemically useful plant growth regulators or insect control agents.

### Acknowledgments

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## Chapter 51

# Tannins as Antifeedants to Mammalian Herbivores— Still an Open Question?

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Tannins have been widely considered to reduce the digestibility and thus nutritional value of plant material to herbivores. Evidence from *in vivo* studies exploring the potential allelochemical effects of tannins is reviewed together with information regarding the probable mechanisms leading to observed effects. It is concluded that whilst tannins do act as anti-feedants, there is little evidence to support the hypothesis that the underlying cause of this is digestibility reduction. An attempt to resolve the evident contradiction between these findings and the view based on *in vitro* models of digestion, which anticipate the *in vivo* precipitation of proteins by tannins, is made. The tannin-protein interaction at the molecular level is reassessed in the light of recent work concerning bile surfactants, soluble tannin-protein complexes, and the specificities of tannin-protein interactions. New standards are proposed for the satisfactory *in vitro* modelling of digestion in the presence of tannins.

Since the early work of Feeny (1) a considerable number of studies concerning the influence of tannins on the voluntary consumption and digestion of plant material have become available, although hitherto these have not been comprehensively reviewed (2,3). The responses by animals to diets containing tannins are reported to range from a reduction in voluntary food intake leading eventually to starvation and even death (4), to increased consumption of a relatively tannin-rich diet over and above that found with a relatively tannin-poor diet (5,6). Whilst concerned mainly with the effects of tannins on mammalian herbivores, this review has also taken account of studies on other vertebrates. Details of individual studies that

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form the background to this review are given in Table I. Discussion of these is not repeated in the text, which concentrates on examining the general trends that can be construed from this information.

### Experimental Designs of Feeding Studies

The studies described in Table I are concerned with observations on thirty-eight studies of vertebrate feeding interactions. Many of these involve animals fed artificial diets containing tannic acid or quebracho tannin, although tannins from some nine plant families are represented in experimental diets and a wider range still in those studies reporting field observations. Tannins, in the widest sense, are included here (e.g. phlorotannins and protein-complexing phenolic resins), the presence of atypical types being noted in the Table and also in the text where conclusions depend critically on studies concerning them.

The information collated in Table I falls into six categories. (i) Information concerning the individual experimental systems and their interpretation in ecological terms. Thereafter evidence for the following measurable effects of dietary tannin consumption has been sought, (ii) altered food consumption rates, (iii) altered growth rates, (iv) altered digestive efficiencies, (v) altered nitrogen nutrition, and finally (vi) metabolic or pathological effects.

No two groups of workers have used the same methodology in investigating the effects of dietary tannin consumption. Each study listed in Table I concerns itself with a named species of herbivore feeding upon a tannin-containing diet. The observed relationships between tannin content and its presumed effects are based upon correlations. Some investigators have shown that correlation is strongest between tannin content as opposed to other likely and measured variables (Table I, studies 22,36,37) yet by no means all investigations are so thorough.

Many of the diets are artificial and the tannin they contain is thought to be the only source of dietary allelochemicals. Except where stated these represent "no choice" feeding situations. By contrast whole plant diets will normally contain other allelochemicals as well as tannins. Furthermore, whole-plant diets are less easily controlled for variation in other factors such as their fibre, nitrogen, and water content. In these respects results from the consumption of whole-plant material are less easily interpreted. The main advantage in employing whole-plant studies is that they provide an ecologically realistic diet concerning which herbivore preference is usually known. Additionally many of the studies of animals consuming whole plants have been conducted in the field where they are feeding on the plant by choice. In Table I such information as is known about the ecology of the situation is provided in the notes marked "A". Succeeding information in the Table provides data on the measurable effects of tannin consumption; these are discussed here in terms of their probable underlying mechanisms.

The most frequent measurement or observation made is whether animals consume tannin-rich food in preference to tannin-poor (or tannin-free) food. In a "no choice" feeding experiment this may be reflected in reduced or enhanced food consumption when experimental

Table I. Studies Dealing with the Consumption of Tannins by Vertebrate Herbivores

Key: headings indicate the herbivore(s) and source(s) of tannin in the diet (which is either artificial [AD] or whole-plant [P]). The findings of each study are then listed in categories A-E as described in the main text. Abbreviations used below are explained in Table II.

- 1 Giant Tortoise (Testudo gigantea) / "Tortoise Turf" [P]. (7)  
**B:** Rejection limits (<80% consumption) for tannin-containing plants, 2.1 mM and 0.4 mM for CT and HT respectively.
- 2 Quelea (Quelea quelea) and other birds / Sorghum (Sorghum bicolor; Graminae [P]). (8,9)  
**B:** Crop damage i.e. grain consumption, is inversely proportional to CT levels. Bird damage is greater than insect damage. Repellent action of sorghum tannin to the quelea has been demonstrated.
- 3 Muscovy Ducks (Cairana moshata) / Beans (Vicia faba; Leguminosae [P]). (10)  
**B:** CR depressed. **C:** GR depressed. **D:** NUE depressed.
- 4 Egg-laying hens / Beans (Vicia faba; Leguminosae [P]). (10)  
**E:** Egg weight and hatching rate depressed.
- 5 Chickens / Carob pod tannin (Ceratonia siliqua; Leguminosae [AD]). (11)  
**C:** GR depressed. N.B. CT and some HT present.
- 6 Chickens / Sorghum tannin (Sorghum bicolor; Graminae [AD]). (12,13)  
**B:** CR depressed. **C:** GR depressed. **D:** Reduced protein digestion (13). **E:** Slightly elevated liver lipids. Some deleterious effects (not NUE) are methionine-reversible. N.B. CT only (14).
- 7 Chickens / tannic acid [AD]. (14)  
**B:** GR depressed. **C:** GR depressed. **E:** Slight increase in liver lipids, methionine and choline partially correct this, 0-4-methyl-GA found in urine.
- 8 Ptarmigan species (Lagopus lagopus, L. mutus, L. lecurus) / Subarctic browse [P]. (15)  
**B:** Evidence for resin avoidance in relation to protein complexing ability and digestibility ( $p < 0.001$ ) of the phenolic resin, which may thus be acting as a tannin. Resins also deter feeding by other subarctic browsers but the tannin-like action of the resins they consume is less well established.
- 9 Canada Geese (Branta canadensis) / Coastal marsh vegetation [P]. (16)

Table I. Continued

- B:** CR depressed. Phenolics were found to be the major proximate cue rather than nutrient quality. Both quebracho and TA gave similar results on captive animals.
- 10 Koala (Phascolarctos cinereus; Phascolarctidae) / Eucalyptus species (Myrtaceae [P]). (17)  
**A:** Hindgut fermenter, eats only Eucalyptus sp. **D:** High fecal nitrogen levels may be due to tannins.
- 11 Snowshoe Hare (Lepus americanus; Lagomorpha) / Subarctic browse [P]. (18)  
**B:** CR depressed in some circumstances, total phenolics not well correlated to this but protein-complexing phenolics are ( $p < 0.002$ ). N.B. Resins, TP, and protein complexing phenolics separately measured.
- 12 Woodrat (Neotoma fuscipes; Rodentia) / Oak and other phenolic-rich foliage [P]. (19)  
**A:** Oak specialist; foliage contains 16% CT and 40% TP. **B:** CR high despite tannin, indeed tannin-rich foliage preferred to available plant material defended by other allelochemicals. **C:** GR positive in contrast to the response of the non-specialist N. lepida, which loses weight on an oak diet. Nevertheless AD low in contrast to tannin-free diets, although processing rates are fast. **D:** High nitrogen retention on oak compared to that in N. lepida; nitrogen losses attributed to loss of endogenous protein, not dietary protein.
- 13 Stephens Woodrat (Neotoma stephensi; Rodentia) / Juniper (Juniperus communis; Cupressaceae [P]). (20)  
**A:** Specialist on juniper which is never less than 80% of the diet. **B:** Selection for plant parts with low levels of allelochemicals. N.B. Both CT and terpenoids present.
- 14 Arctic microtine rodents (three species) / Labrador Tea extract (Ledum palustre; Ericaceae [AD]). (21)  
**A:** Component of naturally available vegetation. **B:** CR reduced. **E:** Reduced body fat, reduced litter size.
- 15 Prairie Vole (Microtus ochrogaster; Rodentia) / tannic acid [AD]. (5)  
**B:** CR increased for 6% TA over 3% TA where this can allow completion of dietary protein requirements. **E:** Uronic acid excretion in response to TA yet high protein levels in the diet depress this, possibly because TA remains in the gut bound to protein.
- 16 Laboratory rat / tannic acid [AD]. (4,22,23,24,25)  
**B:** CR depressed. **C:** GR depressed. **D:** NEX increased, attributed to endogenous protein loss and not dietary protein loss; increased fecal levels of the mucoprotein component glucosamine reported. **E:** Larger animals less likely to die, yet mortality rate did increase. LD(50) 2.26 g/kg. Fatty liver resulted from GA not TA per se which did not pass through the gut wall unhydrolyzed. GA and its methylated derivatives excreted

Continued on next page

Table I. Continued

- in the urine. Methionine and choline did not alleviate these effects; blood composition unaltered. On a 3% diet intestinal mucus secretion rose but this did not afford complete protection as glandular atrophy and mucosal erosion occurred.
- 17 Laboratory rat / tannic acid [AD]. (6)  
**A:** This study is separated from the above as it involved feeding TA at low levels (27 weeks at 0.64 and 1.128%. **B:** CR slightly increased. **C:** GR depressed, calorific values of feces increased. **D:** Fecal NEX increased, NUE depressed, but counterbalanced by decreased urinary nitrogen.
  - 18 Laboratory rat / Condensed tannin [AD]. (22)  
**C:** GR depressed, but not as much as for TA. **D:** NEX increased, attributed to endogenous protein losses.
  - 19 Laboratory rat / Sericea lespedeza tannin extract (Lespedeza cuneata; Leguminosae [AD]). (26)  
**E:** (i) 2% administered over extended periods gave no effect; phenolics not absorbed into the body. (ii) Acute dose of 3 g/kg body wt., by stomach tube delivery, killed rats within a week. Lumps of tannin-rich material found in stomach after death.
  - 20 Buffalo (Syncerus caffer; Artiodactyla) / Savannah vegetation [P]. (27)  
**A:** Tannins and oils in some grasses may reduce CR but only when the animal is restricted in alternative food sources.
  - 21 Elk (Cervus elaphis; Artiodactyla) / Fireweed (Epilobium angustifolium; Onagraceae) and Maple (Acer saccharum; Aceraceae [P]). (28)  
**A:** Results compared to a tannin-free hay plus alfalfa diet. **D:** NEX increased; relationships between fecal nitrogen (y) and dietary nitrogen (x) were  $y = 0.77 + 0.486x$  with tannin and  $y = 1.02 + 0.443x$  without tannin. For the 0 to 5% range of x employed in the experiments it is the constant factor which determines y, not the term in x, which suggests that nondietary nitrogen loss is increased by tannins.
  - 22 Sheep / Varieties of Sericea lespedeza (Lespedeza cuneata; Leguminosae [P]). (29)  
**A:** The variety with the highest tannin content had the most nitrogen and least fibre. **B:** CR depressed by tannins irrespective of other factors.
  - 23 Sheep / Lucerne extract [AD]. (30). **D:** Increased protein uptake attributed to dietary tannins in certain circumstances.
  - 24 Sheep (Orkney Isles breed) / Seaweeds [P]. (31)  
**A:** The animals are adapted to this (staple) diet by virtue of their specialized rumen biota. **B:** CR depressed by astringent seaweeds, the most astringent types (Ascophyllum nodosum, Fucus vesiculosus) not being consumed when others were available. Phlorotannins are the presumed cause of astringency.

Table I. Continued

- 25 Goats (*Artiodactyla*) / Oak plus alfalfa mixture. [P]. (32)  
**B:** CR depressed. **D:** NEX increased. **E:** No toxic effects revealed through hematological tests.
- 26 Goats / Blackbush (*Coleogyne*; Rosaceae [P]). (33)  
**A:** Goats given either high- or low- tannin-containing foliage. Foliar protein level directly proportional to foliar tannin in this instance. **B:** CR depressed by high tannin levels. **C:** A comparative increase in GR for animals consuming high-tannin diets, i.e. tannins did not adversely affect the digestion and utilization of nutrients in the high tannin-feeds.
- 27 Cattle / *Sericea lespedeza* (*Lespedeza cuneata*; Leguminosae [P]). (34,35)  
**A:** Various high- and low-tannin cultivars fed to cattle. **B:** CR depressed by high CT. **C:** AD depressed. **D:** NEX increased. **N.B.** Contains CT.
- 28 Cattle / tannic acid [AD]. (34)  
**A:** Results compared to those above for CT given at the same level. **B:** CR unaffected. **C:** AD unaffected.
- 29 Vervet Monkeys (*Cercopithecus aethiops*; Primates) / *Acacia tortilis* and *A. xanthophloea* (Leguminosae [P]). (36)  
**B:** Tannins were consumed but nevertheless they were negative feeding cues. **E:** Tannins may well have contributed to nutrient stress at a time when high mortality occurred. Tannins in feces indicated their ability to pass through the gut unaltered.
- 30 Howler Monkey (*Alouatta pallida*) / Tropical rain forest vegetation [P]. (37)  
**B:** Phenolics seem unimportant in food selection compared to protein and fibre.
- 31 Black and White Colobus Monkey (*Colobus guereza*) / Tropical rain forest vegetation [P]. (38)  
**B:** Tannins consumed but there is definite selection against tannin-rich foliage; some consumption may however be beneficial to avoid bloat.
- 32 Black Colobus Monkey (*Colobus satanas*) / Tropical rain forest [P]. (39)  
**B:** Foods low in tannins selected, but fibre is major negative feeding cue.
- 33 Red Colobus Monkey (*Colobus badius*) / Tropical rain forest [P]. (40)  
**B:** No clearly discernible role for tannin as a major determinant of food selection is evident.
- 34 South Indian Leaf Monkey (*Presbytis johnii*) / Tropical rain forest vegetation [P]. (41)  
**B:** Diet low in fibre and tannin although neither is an absolute deterrent.

Continued on next page



Table I. Continued

- 35 Chimpanzee (Pan troglodytes) / Fruits naturally consumed [P]. (42)  
**B:** Suggestive evidence that CT may influence feeding on fruits.
- 36 Yellow Baboon (Papio cynocephalus) / Acacia gums from A. tortilis and A. xanthophloea (Leguminosae [P]). (43)  
**B:** Gum with least tannin and most carbohydrate preferred.
- 37 Western Gorilla (Gorilla g. gorilla) / Tropical lowland rain forest vegetation [P]. (44)  
**B:** More CT eaten than by the mountain gorilla yet it is not a major influence on food selection relative to lignin and fibre; tannins are however helpful in considering why particular plants are rejected.
- 38 Mountain Gorilla (Gorilla g. berengei) / Tropical montane vegetation [P]. (45)  
**B:** Astringent (tannin-containing) plant parts tend not to be eaten.
-

animals are compared to control populations feeding upon a tannin-free diet. It should be noted that these two situations are not identical, as the "no choice" experiment requires the animal to consume tannin to complete its other nutritional requirements, whereas the situation allowing the expression of a preference between food materials provides an escape from this. Tannins are also generally expected to reduce food consumption rate by virtue of the unpleasant (to humans) astringent sensation they produce in the mouth (46). Tannins present in plant tissue are, upon release through mastication, anticipated to complex with salivary mucoproteins as well as those of the mouth surface and so may directly reduce the ease and comfort with which food is swallowed because of effects upon buccal lubrication. Observations regarding consumption rate (CR) or palatability are recorded under notes "B" in Table I. The definitions of CR and other nutritional indices used in Table I are given in Table II.

Table II. Definitions of Terms Used as Abbreviations in Table I and the Text

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Nutritional indices

CR = Consumption Rate, = Food Ingested per unit time.

GR = Growth Rate, = Biomass gained per unit time.

AD = Approximate Digestibility.  
= (Food Ingested - Feces) / Food Ingested.

ECD = Efficiency of Conversion of Digested Food.  
= (Biomass gained) / (Food Ingested - Feces).

ECI = Efficiency of Conversion of Ingested Food.  
= Biomass Gained / Food Ingested (= AD.ECD).

NEX = Nitrogen Loss in Feces per unit time.

NUE = Nitrogen Utilization Efficiency.  
= Nitrogen (biomass) Gained / Nitrogen Ingested.

Phenolics

CT = Condensed Tannin, GA = gallic acid (a component molecule of TA), HT = Hydrolyzable Tannin, TA = Tannic Acid, TP = Total Phenolics.

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Another frequent observation in tannin-related feeding experiments is that of animal growth rate in relation to tannin consumption, as used by Feeny (1). It is important to realise that animals may grow less well on a tannin-containing diet because (i)

they eat less food, (ii) they digest the food they do eat less well, or (iii) they are being poisoned; indeed all three of these may apply. Observations relating to growth rate (GR) are recorded under notes "C" in Table I. Whilst showing an important effect these data yield no information upon the possible mechanisms by which tannins may affect herbivores.

In work on insects, the efficiency with which ingested food is converted to animal biomass (ECI) is normally considered in terms of two components; (i) the approximate digestibility (AD) of the food and (ii) the efficiency with which digested food is then converted into animal biomass (ECD). The latter measurement generally requires changes in animal biomass to be expressed in terms of dry weight or calorific values and thus this measurement and the  $ECI = AD \cdot ECD$  model has seldom been used as a basis for measurement in studies of vertebrates. In this paper we have attempted to apply the basic tenet of  $ECI = AD \cdot ECD$  because we feel it offers the best conceptual framework for considering the effects of tannins on digestion in vertebrates. Of major importance is the distinction between the impact of tannins on AD as opposed to ECD. If AD is affected by tannin (i.e. digestibility is reduced) then tannin is acting on the process of digestion as expected by Rhoades (47); however, if ECD is affected then the tannin is acting on the post-digestive processes involved in the conversion of digested food into animal biomass. Whilst tannins might be envisaged to act in a quantitative manner on either component of ECI, there is no currently accepted mechanism to explain any effect on ECD, and such an effect would be at variance with the conclusions of Rhoades (47) that, "tannins act by forming relatively indigestible complexes thereby reducing the rate of assimilation of dietary nitrogen" and "that tannins and related phenolics act mainly by inhibition of digestive processes, providing protection against all classes of enemies that obtain nutrients by the breakdown of plant tissues". An examination of comparable literature on insects assessed in the same way as in Table I reveals that depression of ECD (not AD) is typical for insect herbivores. Furthermore, it is important to realize that the results for AD and ECD treat the gross features of digestion, and with regard to AD, no distinction is made as to whether tannins affect the digestive enzymes or the substrates, and amongst substrates no differentiation is made between the digestion of protein versus other substances. It might thus be possible for AD to appear not to be greatly affected whilst the specific digestion of protein was.

Measurements of excreted or fecal nitrogen (NEX) are frequently reported. Two difficulties in interpretation arise: first, the measurement of NEX for mammals is generally exclusive of the urinary component and second, all measurements are inclusive of endogenous protein losses. These endogenous losses may include enzymes and mucus, and tannins are likely to increase the loss of these thus making it impossible to measure undigested dietary protein as NEX. This proviso applies to all indices of nitrogen nutrition and needs to be circumvented by tracer experiments. Information relating specifically to nitrogen nutrition, a limiting factor to animal growth (48), is collated under notes "D" in Table I.

Finally there are several reports of metabolic or pathological effects consequent upon the consumption of tannins. These range in severity from unusual metabolites appearing in the urine of animals

consuming tannins to records of death. Needless to say, these reports are not expected for allelochemicals which are only supposed to inhibit digestion. For most of these effects the causal mechanisms point to the activity of tannins or their component phenolics from within the animal's tissues, so requiring their passage from the gut. Information on these aspects of tannin consumption is presented under notes "E" in Table I.

With the nature of the evidence to hand now established, the effects of tannins on vertebrate herbivores will be described. Thereafter a concluding section will compare these and examine the differing effects dependent on the chemical nature of the tannins involved.

#### Evidence for the Allelochemic Effect of Tannins from Feeding Studies

Evidence derives from thirty-eight studies representing work on reptiles, birds, and both marsupial and eutherian mammals. Most reports however concern birds (eight) and mammals (twenty-nine): in the latter the following groups are represented, Phascolarctidae, Lagomorpha, Rodentia, Artiodactyla, and Primates. Many of the reports in Table I (1,2,3,6,7,8,9,11,13,14,16,22,24,25,26,27,29,31,32,34,36,38) indicate low palatability or reduced consumption for high-tannin diets although the effect may be small and only a minor influence on food selection (see 20,28,30,33,37). There are no cases reported for mammals consuming lethal doses of tannin and at the opposite extreme there are no cases of phagostimulation due to tannins in situations where an alternative tannin-free diet is available. Nevertheless there are two reports of increased CR for "no choice" feeding on low levels of tannin (15,17). Given that there are antinutritional effects resulting from tannin consumption, these reports of increased CR could be rationalised in terms of the animals making good their nutritional losses due to tannins by the consumption of additional nutrients (again see 15,17).

There are no reports of enhanced growth rates for mammals consuming dietary tannin. Of the nine reports (3,5,6,7,12,16,17,18,26) where GR has been measured, four (3,6,7,26) attribute a depressed GR to tannin consumption. The nutritional indices available for invertebrates are not generally available for vertebrates, but there are two reports (17,27) that indicate reduced AD or increased fecal calorific values. This has not been observed in all cases (28), a particularly interesting situation (26) being that of goats fed a starvation diet where the diet producing the greatest GR was that in which animals consumed the most tannin. The conclusion drawn from this study was that tannins did not exert a negative influence on digestion, but that they normally acted upon CR (33). Given the evidence that is available, it seems clear that tannins often influence GR but as yet no firm and general conclusions seem possible as to the mechanisms involved.

There is a useful amount of information relating fecal nitrogen to dietary tannin consumption with, in all nine cases (3,6,10,16,17,18,21,25,27), fecal nitrogen increasing with high-tannin diets. Where authors have expressed a view on the cause of this (16,18, see also 21) they have indicated an increased loss of endogenous rather than dietary nitrogen. On this evidence there is no conclusive support for the idea that tannins act to inhibit

protein digestion in vertebrates; indeed this does not seem likely, particularly in view of one report (23) suggesting that tannins may aid protein uptake in some circumstances. Only one report (17) of both fecal and urinary nitrogen measurement is available and this indicates compensatory lowering of urinary loss of nitrogen to balance increased fecal loss.

Measurements of overt toxicity for tannic acid have required the use of stomach tubes to deliver lethal doses to rats and it seems inconceivable that any animal would consume sufficient plant material to receive a toxic dose. However, there is evidence (4) that tannins affect avian egg hatching rates and chick mortality. Less drastic is the incidence of glandular atrophy and mucosal erosion in the gut or changed lipid levels in the liver (see 7,16). There are also several reports (7,15) of glucuronide or methylgallic acid excretion associated with tannic acid consumption. In summary there is evidence that tannins produce deleterious effects on vertebrate herbivores, but these, like the ones found with invertebrates, are not symptomatic of strict digestibility-reducing agents.

#### Vertebrate-Invertebrate and Tannin-Tannin Comparisons

Recent publications on tannins and their effects on herbivores have tended to deal specifically with only vertebrate (2,3) or invertebrate (49,50) herbivores, and have drawn slightly different conclusions for the two groups. Simplistically, these are that tannins do not act as effective digestibility-reducers in insects, and that they have some, but not completely effective, deterrent action against mammals.

A similar analysis to that in Table I of the available data relating to invertebrate herbivores reveals that the evidence for tannin-reduced CR is as strong as for vertebrate herbivores, and that differences between these two classes of herbivore seem to be very much a function of the kinds of experiments which have been performed on them. Particularly lacking (for ethical & practical reasons) are measures of ECI and its two component indices AD and ECD in vertebrate herbivores. Because of the gap this leaves in the available evidence for vertebrate herbivores it is not possible to conclude that they differ from invertebrates in the effects tannins produce on AD versus ECD. With respect to nitrogen nutrition the question is comparatively more open for vertebrates, but nevertheless, given the available evidence for both groups of herbivores, it is likely that tannin-induced losses of endogenous protein are more important than tannin-reduced digestion of dietary protein in both groups. This, coupled with the evidence for damage to the gut by tannins, and their influence on mortality, and their xenobiotic metabolism in both groups of herbivores, clearly suggests that tannins do not necessarily act predominantly upon digestibility.

There is good and repeated evidence that tannins can reduce GR, so it is not in doubt that they can exert an allelochemical action. Many early studies focussed particularly on the negative effects of tannins on nitrogen nutrition in an attempt to explain this reduction in GR. In this respect their promotion of endogenous protein loss, coupled with the potential drain on methionine because of its action as a methylating agent for xenobiotic phenolics (51), does suggest that tannins may exacerbate conditions of limiting nitrogen

nutrition. The mechanism whereby this may occur is not, however, likely to be by direct protein digestibility reduction. With regard to the process of digestion it is worth pointing out some beneficial effects of tannins. The explanation for phagostimulation in the insect *Anacridium melanorhodon* induced by tannic acid (50) was that the animal could possibly use the hydrolytically derived gallic acid in endogenous syntheses, but this does not appear to be general to most insect species. For ruminants, tannins have been suggested to be generally useful in preventing bloat (52), and in protecting protein from digestion before its entry into the small intestine (30).

The information in Table I reveals differences between some of the important types of tannin. Tannic acid is unique for its occurrence in all reports of diet-induced gut lesion and gastrointestinal damage (vertebrate or invertebrate); it is also the only tannin for which metabolism and excretion are reported. By contrast condensed tannins are not thought to leave the gut lumen. Thus, in comparison with tannic acid condensed tannins would not be expected to drain 1-carbon metabolism, notably methionine resources. However, the reports by Elkin (12) and Ford (53) of corrective methionine treatment for chicks fed a condensed-tannin diet, indicate that some condensed tannin may enter the body or that methionine can react with tannin in the gut, so perhaps the position is once again not clear cut on tannin structure and its impact on methionine levels. The conclusion can, however, be made that despite its hydrolyzability, tannic acid does exert an allelochemical effect which is not abolished by hydrolysis.

Very little information exists on the phenolic protein-complexing resins, except for that on creosote bush and some arctic plants. In contrast to condensed tannins and hydrolyzable tannins, these are typically ether-soluble compounds; this may allow their entry into the body across cell membranes, and thus give them the potential for action outside the gut lumen. Reports for animals consuming seaweed indicate that astringent protein-precipitating substances (presumably phlorotannins), are potentially important in marine plant-herbivore interactions.

Despite chemical differences and the possible differences between tannins (*sensu lato*) in their effects on digestion, all share the common feature of astringency, and so almost certainly reduce plant palatability by this property. Given the evidence that tannins seem to exert effects on GR principally by reducing CR, it is pertinent to ask whether their "bark is worse than their bite". In view of a small but significant number of reports where tannins seem to be beneficial to animals consuming them and the paucity of results demonstrating GR reduction due to reduced ECI or toxic effects, it remains to be proved that animals use astringency as a negative feeding cue to avoid nutritionally dire consequences. On the available evidence from feeding trials the effects of tannin consumption would appear to be significant but not acute; such consumption seems to generally reduce fitness, as is seen from a number of physiological effects that appear in animals feeding on levels of tannin above average for their natural diet for prolonged periods (6,7,15,16,17).

Evidence for the Allelochemic Effect of Tannins from In Vitro Studies

The evidence from in vivo experiments and observations, as reviewed above, clearly does not point to any substantial digestibility-reducing effect following the ingestion of tannin-rich food. This leaves us with an apparent paradox because tannins do form insoluble precipitates with proteins (54,55,56) and these do resist the proteolytic action of trypsin, as demonstrated by Feeny (54). However, it must be stressed that these reports all concern in vitro observations; there is no published evidence that insoluble tannin-protein complexes actually form in vivo. Indeed, since Feeny's original work (55) and that of Rhoades (57) we are aware of no further work on the in vitro modelling of proteolysis; research has tended to concentrate on the conditions leading to tannin-protein precipitate formation, and to assume that precipitates once formed are relatively indigestible.

Recent work at our laboratory (58) suggests that this viewpoint is at fault in several respects. For example, we have found that tannins and proteins can be present in soluble systems, at reactant concentrations that might reasonably be expected to occur in the digesta and at pH values common in insect herbivores and approaching those in the mammalian small intestine (pH 7.5-8.5). In these soluble systems we found substantial inhibition of the tryptic degradation of proteins in the presence of high levels of tannin. By contrast, increased levels of proteolysis (similar to those produced by first heat-denaturing the protein) were observed when low levels of tannin were incorporated in a system containing bovine serum albumin (BSA) as the substrate for trypsin. We believe that these similar instances of enhanced proteolysis caused by heat-denaturation and interaction of protein with tannins mean that tannins, as with other polyanions (59), can denature protein structure when they form complexes with them. The resulting denatured protein can then be expected to be more susceptible to proteolytic attack (60).

These results indicate the error in assuming that tannins act only after the formation of a precipitate; tannin-protein interactions in solution are clearly also important. The rate-enhanced tryptic proteolysis observed with BSA and relatively low levels of tannin may also be of in vivo importance, as extensive surveys of tropical vegetation (61,62) indicate that many plants contain the right balance of tannin and protein for this to occur. However, this effect now needs to be demonstrated with a major plant protein. An effect, potentially much more important in vivo, is that caused by the bile constituents cholic acid and glycocholate. Both of these surfactant substances dissolve tannin-protein precipitates at pH 6.2, and experiments with cholic acid show that it can in these conditions substantially diminish the inhibitory effect of tannins on the tryptic proteolysis of several proteins. Martin and Martin (63) have shown comparable effects with experiments using the surfactants present in the guts of insects.

In systems used to follow the esterolytic activity of trypsin in the presence of BSA (64), tannins do not appear to interact directly with trypsin because of their greater affinity for reaction with BSA (64). In the absence of BSA some tryptic activity is lost however even in the presence of glycocholate, which prevents any precipitates from forming. This indicates that bile surfactants may act only to

reverse the aggregation of tannin-protein complexes into precipitates and not to promote the dissociation of soluble tannin-protein complexes (i.e. the tannin-trypsin complexes in this instance). These points concerning surfactants, together with that concerning the apparent denaturing effect of tannins on protein, can be brought together in a model for tannin-protein interactions. In a system containing a tannin (TA) and several proteins, tannin-protein interactions are assumed to only involve one protein (Pr), namely that for which the tannin had greatest binding specificity. Given  $n$  molecules of tannin ( $nTA$ ) and  $n'$  of protein in its native state ( $n'Pr[nat]$ ), small soluble complexes of variable stoichiometry will initially form ( $TAn-Prn'$ )-sol. The existence of these may be only a transitory stage before their aggregation into a precipitate or flocculum i.e. ( $TAn-Prn'$ )-ppt. pH change or surfactants such as bile salts are thought to disrupt the precipitated complexes, releasing the smaller soluble complexes. At some stage in the process it is thought that protein structure will have become appreciably denatured and so these redissolved soluble complexes can be designated as ( $TAn-Prn'$ [denat])-sol.

Drawing on this and the reported specificity of tannin-protein interactions (65) leads to the conclusion that any useful *in vitro* modelling of the impact of tannins on digestion must consider more than pH and the concentrations of the buffer, enzyme, substrate, and tannin. The actual enzyme-substrate system must be nutritionally realistic to control for specificities of the reaction of tannins with proteins (including enzymes); gastrointestinal mucoproteins should perhaps also be included on the same grounds. Besides all this, misleading results may still be obtained if bile surfactants are omitted from the equation.

In summary, the mismatch between expectations based upon the early work of Feeny (1) and Rhoades (57) and the accumulated evidence of work on live animals underlines our basic ignorance of detail in the digestive process. That animals tend to reject astringent food is not in doubt, but what they avoid by doing so is still something of an open question.

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