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Microbes and Microbial Products as Herbicides

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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 Advisory Board and are selected to maintain the integrity of the symposia. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation. However, verbatim reproductions of previously published papers are not accepted.

Preface

BIOHERBICIDES ARE PLANT PATHOGENS AND PHYTOTOXINS derived from pathogens and other microorganisms that can be useful for weed control. Weeds still cause enormous losses of crops worldwide, even though synthetic herbicides have revolutionized food and fiber production during the past 50 years. Recently there has been increased interest in the control of weeds with bioherbicides and, although this research area is relatively new, some successes have been realized. These natural products and living microorganisms provide alternatives to synthetic herbicides to which some weeds have developed resistance, and whose mobility or persistence in soils has resulted in their detection in run-off and ground water.

Direct use of pathogens in inundative applications may provide effective weed control. Many pathogens with potential as bioherbicides have been isolated and identified from various weed hosts. Although some plant pathogens can attack a variety of host species, pathogens generally have a narrow host range. This narrow range has advantages or disadvantages, depending on the crop and weed-control objectives. From a commercial standpoint, however, it would be advantageous to market a pathogen product with broad-spectrum weed-control activity. Strain selection and genetic engineering offer methods to achieve this end, but another alternative is chemical (agrochemical or other plant metabolic regulator) manipulation or regulation of host plant defense systems, thereby altering host range. This latter strategy could be useful in integrated weed control, wherein biological control and synthetic herbicides are used simultaneously, and especially if synergistic interactions occur between the bioherbicide and herbicide.

Phytotoxins from nonpathogenic organisms also have potential as weed-control agents. Numerous phytotoxic compounds have been isolated and bioassayed for phytotoxic activity, and their chemical structures have been determined. Generally, these naturally occurring compounds have unique chemistries that differ from those of commercial synthetic herbicides. Some of these compounds—or others yet to be discovered—may be used directly as herbicides or provide templates for the development of new, efficacious weed control agents that are less persistent and more environmentally compatible than some compounds currently being used.

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The purpose of the symposium on bioherbicides and of this book was to bring together scientists from a variety of disciplines to present, summarize, review, and update information related to a number of topics: pathogens with potential as bioherbicides, phylloplane–pathogen interactions, aspects of soil microorganisms, formulation and commercialization of microbes as bioherbicides, genetic aspects of pathogens for weed control, and chemistry and biochemistry of microbial–plant interactions. It is hoped that this book will serve as a valuable source of information for researchers involved in both biological weed control and herbicide development. It is particularly hoped the book will serve to motivate chemists to become involved in these new areas of plant research because the answers to many of the questions currently being raised can be found in chemistry.

I am deeply indebted to all the contributors to the symposium and the book for excellent presentations and summations of their research areas. I also express my gratitude to my many colleagues who provided advice and cogent reviews of manuscript drafts. Louis Clarke is thanked for providing high-quality computer drawings for my chapters. I wish to acknowledge the generous support of the Division of Agrochemicals of the American Chemical Society for sponsoring the symposium upon which this book is based. The publication and production guidance and the cooperation of Cheryl Shanks of the ACS Books Department are highly valued. Finally, I thank my wife, Rebecca, for countless hours of assistance, encouragement, patience, and understanding—without which this project would not have been initiated or completed.

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Chapter 1

Microbes and Microbial Products as Herbicides

An Overview

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Microbes (plant pathogens) and microbial products (phytotoxins) have been shown to have potential as weed control agents. Growth in the interest in these alternative weed control methods has been brought about by a need for less persistent, more selective, and more environmentally safe herbicides. Broad-spectrum and selective activity are concepts important to the development of a weed control agent. Generally, pathogens are host-specific while phytotoxins have a wider range of activity. Over the past several years, new pathogens and microbial phytotoxins from pathogens and other microorganisms have been discovered which can be added to the arsenal of biological weed control weapons. Pathogens, and in some cases phytotoxins, may be used directly on the target species; alternatively, phytotoxins or compounds from non-pathogenic organisms may provide template chemistries for new synthetic herbicide designs. Pathogens also have potential for use in integrated approaches to weed management programs, where the organisms can tolerate the presence of other agricultural chemicals and where they may interact synergistically with herbicides. Genetic engineering and microbial strain selection to increase pathogen virulence, alter host range, and enhance interactions with other chemical regulators or synergists may promote infectivity and weed control efficacy. This chapter reviews some recent advances in the area of isolation and identification of novel microbial chemistries with phytotoxic properties and presents perspectives on other aspects of the use of microbes and natrually occuring microbial compounds for weed control.

The developed nations of the world are generally able to produce more food than is necessary to feed their populace. This high

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productivity and these surpluses are largely the result of the use of fertilizers and other agrochemicals to control weeds, insects, and diseases in crops. Although many of the world's nations produce more foodstuffs than are necessary to feed their own people, global food production lies in precarious balance. The difference between agricultural surplus and shortage depends on only a 5% change in worldwide agricultural acreage (1). Estimates are that agricultural output will need to increase 30% by the year 2000 to support the projected world population of 6.6 billion (2). Modern agricultural practices rely heavily on chemical methods of pest control, although a few biological control endeavors have also been successful.

Economics of Weed Problems. Of the nearly 300,000 species of higher plants in the world, only about 10% are weeds; about 1,800 weeds are responsible for economic losses in agricultural production (3). About 300 weed species cause serious economic losses in cultivated world crops. However, only about 200 weed species are responsible for 95% of the weed problems faced by those involved in the production of food and fiber (4). Weeds are costly. Weeds accounted for nearly 42% of the losses of crops to pests in the U.S. in 1971 (Table I). In the U.S. alone, weeds were estimated to cause

Table I. Annual Costs of Plant Pests of Crops

Pest	Losses (x 1,000)	Control (x 1,000)	Total (x 1,000)	Total (%)
Diseases	$3,152,815	$ 115,000	$ 3,267,815	27.1
Insects	2,965,344	425,000	3,390,344	28.1
Nematodes	372,335	16,000	388,335	3.2
Weeds	2,459,630	2,551,050	5,010,680	41.6
Total	8,950,124	3,107,050	12,057,174	100.0

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reductions in productivity resulting in monetary losses for field crops and vegetables of over $7 billion per year in 1975-79 (Table II). Beyond the losses in productivity are actual costs of pesticides and their application. From the late 1960's through 1987, the use of herbicides in the U.S. exceeded the use of insecticides and fungicides combined (Figure 1). On a global scale, other data shows that herbicides accounted for 44% of total pesticide sales in 1985 (Figure 2). Combined global pesticide sales in 1985 were equivalent to about $16 billion U.S. Although advances in weed control have been achieved, weeds continue to cause agricultural losses. Thus, new weed control strategies are needed to augment and/or replace less-than-successful strategies.

Pesticides are crucial for maintanence of our modern food production. However, current patterns of use and misuse of chemical pesticides have resulted in some environmental problems. Ground water contamination by herbicides and other pesticides may threaten

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Table II. Estimated Average Annual Losses Due to Weeds in Several Commodity Groups in the United States, 1975-1979

Commodity group	Average annual monetary losses
	($ x 1,000)
Field crops	6,408,183
Vegetables	619,072
Fruits and nuts	441,449
Forage seed crops	37,400

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public health. Generally, there has been increased public concern about the safety of agricultural chemicals, including herbicides (9), in some cases resulting in public referenda to reduce the use of these compounds. If this trend continues, total mechanical and/or biological weed control measures will have to be implemented to assure adequate food and fiber production. The quality and quantity of food and fiber products will no doubt decrease and their costs will soar if adequate alternative pest control measures have not been established and implemented. Pesticides have been an integral part of commercial agricultural production for so long that agroecosystems themselves have been influenced and, thus, extensive losses due to pests will occur if their use is curtailed (10) without new alternatives.

Growth of the interest in alternative weed control methods has been brought about by the need for less persistent, more selective, and more environmentally safe herbicides. In my view, it becomes obvious that improved weed control measures with chemicals are, and will continue to be an integral part of meeting the increasing world demand for food and fiber. Furthermore, biological control of agricultural pests, particularly weed control with pathogens and microbial products, can play an increasing role in meeting this challenge. Research efforts in academic, governmental, and industrial labortories must be expanded to meet this challenge.

Weed Control - Some Historical Perspectives. Weed control has gone through many stages since man gave up his nomadic hunter-gatherer lifestyle to form agrarian communities. Few new developments in weed control were made from those early days of hand-weeding and cultivation until the development of synthetic herbicides in the mid 1940's. There are currently about 150 herbicides comprising about 15 major chemical classes registered for use in the United States (11). Brian (12) has developed a review of the history of chemical herbicides. A chronology of some weed control developments, with particular emphasis on biological weed control, is presented in Table III. While it cannot totally replace chemicals, biological control has long been recognized as a feasible method of weed control. However, it has had a rather low profile since the coming

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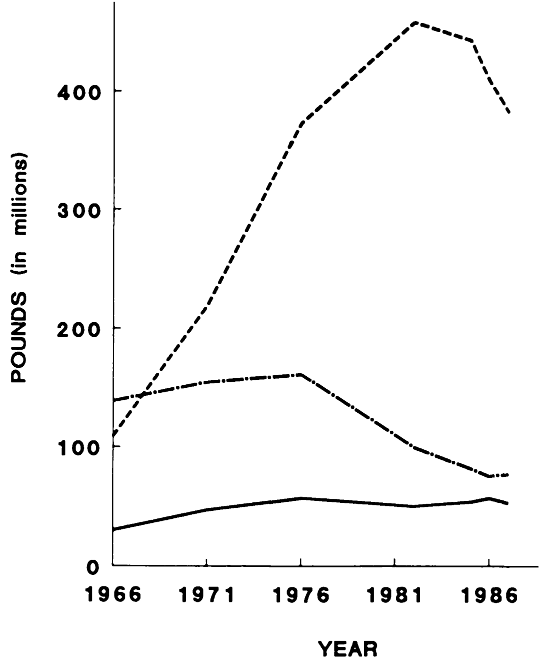

Figure 1. Estimates of pesticide usage in the United States, 1966–1987. (Redrawn from Ref. 7. Copyright 1989 National Academy Press.)

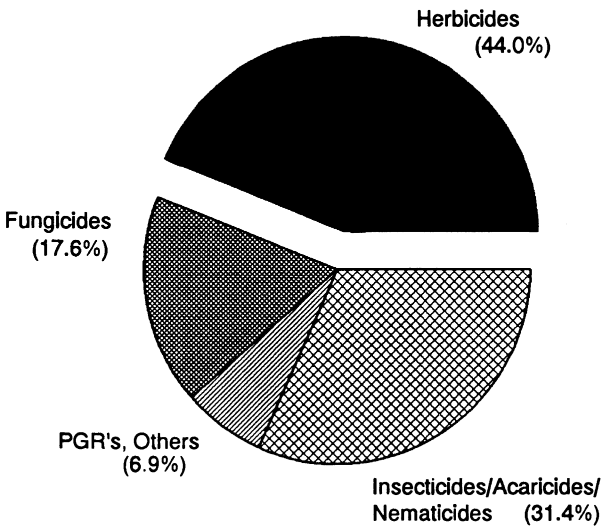

Figure 2. Relative percentage of global pesticide sales for 1985. (Redrawn from Ref. 8. Copyright 1988 Royal Soc. London.)

Table III. Chronology of Some Important Events Related to Weed Control With Herbicides, Biocontrol Agents, and Microbial Products

1859-87:	Julius Sachs studied "chemical messengers" or growth regulators in plants
1896:	Bonnet observed a Bordeaux mixture killed charlock (Sinapsis arvensis L.) (12)
1900:	Charles Darwin published "The Power of Movement in Plants"; studied plant movement relative to light and growth regulating substances in plants.
1901:	H.L. Bolley (U.S.) demonstrated weed control using inorganic salts. (13)
1902:	Introduction of various insects into Hawaii for control of Lantana camara L.
1925:	Eggs of Cactoblastis cactorum imported into Australia from Argentina for control of prickly pear cactus (Opuntia inermis and O. stricta). (14)
1931:	Control of prickly pear cactus in Australia achieved by Cactoblastis cactorum.
1941:	R. Pokorny (U.S.) reported chemical synthesis of 2,4-D (15)
1942:	P.W. Zimmerman and A.E. Hitchcock (U.S.) reported 2,4-D to be a PGR (16)
1944:	P.C. Marth and J.W. Mitchell (U.S.) established selectivity of 2,4-D (17)
	C.L. Hamner and H.B. Tukey (U.S.) used 2,4-D in field weed control (18)
1945:	W.G. Templeman (U.K.) established principle of preemergence soil herbicides
1951:	Weeds, journal of the Assoc. of Regional Weed Contr. Conferences, established
1954:	Role of diseases in weed control recognized (19)
1956:	Weed Science Society of America organized; assumed publication of Weeds renamed Weed Science in 1968
1961:	Weed Research, journal of the European Weed Research Council, published
1968:	Principles of Plant and Animal Pest Control, Vol. 2: Weed Control (publication 1597) published by the National Academy of Sciences, confirming the importance of weed science, including biological control, as a discipline
1969:	Discovery of endemic disease, Colletotrichum gloeosporioides (Penz.) Sacc. f. sp. aeschynomeme on the weed jointvetch [Aeschynomene virginica (L.) B.S.P.] (20)
1970:	International Conference of Weed Control organized by the FAO of the United Nations; showed international concern for world weed problems
1971:	Successful establishment of Puccinia chondrilla against rush skeletonweed (Chondrilla juncea) and skeletonweed [Lygodesmia juncea (Pursh.) D. Don] (21)
1973:	First successful use of augmentative biocontrol of northern jointvetch (22)
1976:	International Weed Science Society formed
1979:	First microbial herbicide (bialaphos) patented (23)
1981:	DEVINE, a formulation of Phytophthora palmivora (Butler) Butler, registered for use against stranglervine (Morrenia odorata Lindl.) in citrus (24)
1982:	Colletotrichum gloeosporioides (Penz.) Sacc. f. sp. aeschynomene registered as COLLEGO, a formulation for selective control of northern jointvetch (24)
1989:	Microbial Strain selection for bialaphos production resulted in 500-fold increase above wild type (25)

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of synthetic chemical herbicides beginning in the 1940's. Most weed biocontrol successes have involved the use of introduced phytophagous insects. The use of plant pathogens and their phytotoxins is a rather new approach. Although plant diseases have been recognized and have intrigued farmers and researchers for years, there have been few attempts to formally apply the concept of the selective use of diseases for weed control. A landmark example of the use of the classical biological control approach (i.e., introduction of an exotic organism to control an introduced pest) for a weed problem was the establishment of *Puccinnia chondrilla* Bubak. and Syd. for control of rush skeletonweed (*Chondrilla juncea*) and skeletonweed [*Lygodesmia juncea* (Pursh.) D. Don] in Australia in 1971 (21). The first weed control success using the augmentative biocontrol approach (i.e., inundative releases of large numbers of indigenous biological control agents to achieve control of a pest) was the use of *Colletotrichum gloeosporiodes* (Penz.) Sacc. f. sp. *aeschynomene* against northern jointvetch [*Aeschynomene virginica* (L.) B.S.P.] in 1973 (22).

Much research has been done in the area of biological control of weeds with plant pathogens since these initial successes. However, what is needed to identify and establish a new individual successful biocontrol candidate remains largely unknown. In my view, a rational and environmentally beneficial approach to the weed control problem is integrated control; i.e., using synthetic chemicals, phytophagous insects, and plant pathogens (8, 21, 26-27) and/or chemicals derived from plants and microbes (28-30). The purpose of this symposium was to assess recent progress and prospects in various areas relating to plant pathogens that attack weeds and novel phytotoxic chemistries from microorganisms. The scope of this book is the control of weeds using microbes and microbial products. This overview chapter touches on some of these areas, with the major focus on the chemistries of phytotoxins produced by plant pathogens and other microorganisms.

Discovery and Screening of Synthetic Herbicides, Microbial Herbicides, and Microbial Phytotoxins

Synthetic Herbicides. Most commercial phytotoxins (herbicides) have been discovered through random screening programs, followed by derivative synthesis and activity studies when a potential chemical candidate is discovered. Seldom has a biorational approach for herbicide discovery been achieved or used. Over the past 45-50 years, perhaps close to a million compounds have been screened for herbicidal activity. The number of compounds screened has continually increased and estimates for the 1980's suggest that 12 to 15 thousand compounds per year were synthesized and screened for each compound reaching the market (28). In the 1990's, the ratio may increase 5-fold.

Such screening of synthetic compounds typically involves greenhouse testing of up to a dozen or so important crop and weed species for both pre-emergence and post-emergence activity. Strategy may be changing somewhat in that inhibition of specific targets in plants is also being considered in order to improve chances of discovering potent herbicides that have minimal effect to non-target sites, especially those in mammals and other vertebrate

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species. Simple model species, cell free assays, and specific enzyme assays may provide additional advantages in such screening programs (31).

Pathogens. The discovery of living organisms for use as bioherbicides is considerably more complex than the discovery of synthetic phytotoxins. "Bioherbicide," as used here, refers to microorganisms (fungi, bacteria, viruses, algae, etc.) or their products that have been used or have potential for use in the augmentative control of weedy species. "Mycoherbicide" has requently been used in the literature to describe the direct use of pathogenic fungal organisms for weed control. Pathogens for weed control represent unique microbe:plant associations. Generally, diseased plants are discovered in the field or greenhouse and the process of isolating and identifying the causal agent begins. The first step is to isolate the suspect pathogen and grow it in pure culture, as described by specific plant pathological techniques (32, 33). Organism identification is an important second step. A reference which is the usual starting point for information on plant diseases in the U.S. is The Index of Plant Diseases in the United States, which gives information on pathogen host-range and distribution (34-35). Several other specific state and foreign indices have been compiled, as summarized by Templeton et al. (37). Listings of fungal diseases, encompassing the entire world literature, are also available (38-41). The next process is essentially proving that the isolated and identified organism is indeed the disease-causing agent. This is accomplished utilizing Koch's Postulates, i.e., the pathogen must always be: found associated with a particular disease symptom of the weed; isolated in pure culture; used to inoculate and induce disease characteristics in healthy weed tissue; reisolated from the inoculated weed and the disease symptoms and recovered pathogen compared positively with the original disease symptoms and microorganism. Several cycles of Koch's Postulates provide an indication of factors such as virulence, stability, and environmental requirements for growth, sporulation, infections, and disease develoment, which are all important aspects for assessing the potential of the organism as a bioherbicide.

As with synthetic herbicides, it is important that bioherbicides have phytotoxic specificity with minimal impact on non-target organisms. However, a broader host range would be very beneficial and make a particular organism more attractive commercially. With pathogens, host-range tests of the organism are conducted prior to field testing (42, 43) using optimal disease-development environments and both susceptible and resistant plants, including all known biotypes of the host and other plants closely related botanically to the known host (43, 44). Even if preliminary tests show a pathogen to have a desirable host range, scrutiny of the epidemiology is a necessary/critical factor in determining the potential for further development as a bioherbicide. Other criteria are important for successful commercialization of the bioherbicide candidates. These include inoculum production, packaging and shelf-life, and various aspects of application technology. A final important criterion for the commercial development of a pathogen is its interaction and compatability with other agrochemicals.

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Phytotoxins from Microbes as Herbicides. The concept here is to use phytotoxins either as herbicides themselves or to broaden the host specificity beyond that inherent in the organism, thus ulitizing non-selective toxins on other non-host weeds. Co-evolution of toxin-producing pathogens with their specific plant host species has resulted in the production of host-specific toxins, i.e., toxins that only affect the species infected by the pathogen. Most known host-specific phytotoxins have been characterized from crop pathogens, while studies on host-specific phytotoxins from weed pathogens have been neglected. Isolation and chemical characterization of host-specific and non-specific phytotoxins from pathogens and non-pathogens may provide templates for analog synthesis to develop herbicides with even more desirable characteristics than some synthetic compounds presently marketed.

Although pathogenic organisms may be the first to come to mind when discussing phytotoxin-producing agents as bioherbicides, many non-pathogenic organisms, including bacteria and fungi, also possess/produce phytotoxic compounds. Indeed, some of the antibiotics isolated from non-plant pathogens have been later shown to possess highly potent phytotoxic properties. Some progress has been made in the areas of isolation and screening of phytotoxins as herbicides, as evidenced in several recent reviews (30, 45-51). Screening of compounds produced by soil microorganisms has also resulted in the discovery of bioherbicides (52-54). Generally, most of these compounds have not been extensively tested as to weed specificity.

It is obvious that these organisms produce many yet unidentified compounds that also may have potential for development as bioherbicides. Several factors determine the extent of the development process. First is the need for a means to produce adequate amounts of the microorganisms for testing. Second is an adequate and appropriate plant bioassay system. Cultural conditions can influence the metabolites produced by a given organism, either pathogenic or non-pathogenic. With a pathogen, the host(s) is known and tests for virulence can be conducted. Indeed, the organism can and should be produced utilizing extracts or plant parts of the natural host(s) in order to maintain virulence. The host(s) should be among the bioassay test plants.

Bioassay. The ability to follow phytotoxic symptoms caused by organisms or isolated phytotoxins throughout purification and production processes is of paramount importance. A problem common to testing of phytotoxins from both pathogens and non-pathogens is the limited amount of material available, which severly restricts the number, size, etc. of plant species that can be used in bioassay or screening tests. Synthetic herbicide compounds are not subject to this limitation. The problem can be compounded for non-pathogenic species. For example, since there is a variety of synthetic media available, it is impossible to easily optimize for phytotoxin production without testing a range of media and various culture conditions.

Several bioassy techniques have been developed which allow testing of biological materials produced in small quantities. The excised wheat coleoptile test (55) has versatility in detecting plant growth promoters and and growth inhibitors. The assay is

simple and quick. The duckweed (Lemna sp.) bioassay system (56, 57) has some additional advantages. An intact plant system is used; growth and chlorophyll content can be measured as well as processes like respiration and photosynthesis. Assays that do not detect light-dependent processes are limiting. A lettuce seed/seedling bioassay (30) has been useful in detecting the effects of relatively small amounts of toxin on growth and germination. Another bioassay for these purposes uses excised oat mesocotyls (58). Other reviews have surveyed screening procedures and bioassays of phytotoxins from microbes (50, 59). Bioassays that have been used for specific herbicides, the detection of photosynthesis inhibitors, and the use of microbes as bioassay subjects have been summarized (60). Various plant growth regulator bioassays have been reviewed (61), but assays that detect only PGR activity may not be useful for bioherbicide screening. Generally, all of the above assays are species-specific and, thus, many compounds with potential phytotoxicities are missed. Cell free assays and/or specific enzyme testing (31) coupled with intact tissue assays should have utility for screening the relatively small amounts of phytotoxins produced by microbes.

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Agrochemical Interactions with Pathogens. Currently there is only sparse information on interactions of bioherbicides with synthetic pesticides (fungicides, insecticides, and herbicides). Such interactions can be either antagonistic or synergistic with the biological activity of the bioherbicide. Some herbicides have been shown to act synergistically with various pathogens to increase various soil-borne root diseases (62). Such interactions, coupled with a bioherbicide, may offer unique weed control potentials. Another aspect of such integrated control is the application of more than one bioherbicide to control more than one weed, especially in cases where an individual organism is relatively specific. An example of this is the use of COLLEGO and Colletotrichum gloeosporiodes (Penz.) Sacc. f. jussiae for control of winged waterprimrose [Ludwigia decurrens (Walt.)] and northern jointvetch in rice (63). COLLEGO has perhaps been more rigorously tested for interactions with various pesticides than have other bioherbicides or potential bioherbicides . The synthetic herbicides propanil [N-(3,4-dichlorophenyl)propanamide] and 2,4,5-T [2-(2,4,5-trichlorophenoxy) acetic acid], applied after COLLEGO treatment in rice, did not inhibit disease infection and development. Some insecticides and the herbicides acifluorfen {5-[2-chloro-4-(trifluoromethyl) phenoxy]-2-nitrobenzoic acid} and bentazon [3-(1-methylethyl)-(1H)-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide], applied in tank mixture or sequential treatments with COLLEGO, did not inhibit disease infection or development on northern jointvetch (64). Another positive interaction has been demonstrated using tank mix combinations of the fungus Colletotrichum coccodes and the plant growth regulator thidiazuron (N-phenyl-N'-1,2,3-thidiazol-5-yl-urea) to increase velvetleaf (Abutilon theophrasti) mortality and increase soybean (Glycine max) yields (65). Important synergistic interactions of herbicides or plant growth regulators could reduce the amount of both synthetic agrochemical and biological inoculum needed to control specific weed problems. More research input is needed in these areas.

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Phytotoxic Compounds Produced by Microorganisms

Many new microbial compounds with potential biocherbicidal activity have been isolated, chemically characterized, and a portion of their biological activities determined using various bioassay techniques or by direct plant screening. Some of these compounds are virulence factors of plant pathogens, thus information on these phytotoxins can benefit the development of microbes as herbicides and potentially provide new chemical herbicides. Data on some of these compounds and their structures are presented. Information on source, biological activity, and possible mode of action (when available) is briefly summarized.

Phytotoxic Compounds Produced by Fungal Weed Pathogens

Curvulins. Curvulin [1], a cyclic polyketide, and O-methylcurvulinic acid [2] were first identified in the late 1960's. Although curvulin is produced by many fungi, its biological activity was then unknown (66). Both compounds were recently isolated from Dreschslera indica, a pathogen of two weed species, spiny amaranth (Amaranthus spinosus L.) and common purslane (Portulaca oleracea L.), and its structure confirmed using x-ray crystallography (67). The compounds were phytotoxic to detached leaves of these host plants, but some other plants were not injured. Drechslera siccans is a fungal pathogen on perennial ryegrass (Lolium perenne L.), Italian ryegrass (L. multiflorium Lam.), and oat (68). Recently de-O-methyldiaporthin [3] was isolated from this fungus, structurally characterized, and shown to be phytotoxic. Host plants, however, showed little or no sensitivity to this compound while soybean, corn, crabgrass were sensitive to 4 nmol applied in 3 µl droplets. Spiny amaranth and barnyardgrass were more tolerant and required 12 and 8 nmol/3 µl droplet, repectively, to cause injury.

Eremophilanes. Many eremophilanes have been isolated from fungi. The first eremophilane compounds that exhibited phytotoxic activity were isolated from Bipolaris cynodontis, a pathogen of bermudagrass [Cynodon dactylon (L.) Pers.] (69). This fungal pathogen produces two eremophilanes, bipolaroxin [4] and dihydrobipolaroxin (reduced analog of bipolaroxin). This reduced analog, lacking the aldehyde moiety, is not phytotoxic, but bipolaroxin causes lesions on bermudagrass leaves at concentrations of 38 µM. Higher concentrations are necessary to injure wild oats (Avena fatua L.), corn, and sugarcane (Saccharum officinarum L.).

Organic extraction and analysis of culture filtrates of Drechslera gigantea, a pathogen of quackgrass [Agropyron repens (L.) Beauv.] and bermudagrass, showed over a dozen eremophilanes were produced (54). Phomenone, petasol [5], and gigantenone [6] caused necrotic symptoms on dicotyledonous plant tissue, but on monocotyledons caused localized retention of chlorophyll ("green islands") (54). Cucumber (Cucumis sativus L.) was an exception, since "green islands" also formed in this dicotyledon. Various pathogens (70, 71) had been known to cause "green islands", an effect also caused by localized application of cytokinins to yellowing (senescing) leaves (72). Zinniol [13] (73) and some

Curvulin [1]

Mono-O-methyl-curvulinic acid [2]

De-O-methyldiaporthin [3]

Bipolaroxin [4]

Petasol [5]

Gigantenone [6]

Maculosin [7]

Ophiobolin A [8]

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

polyhydroxamates (74) are other compounds produced by plant pathogens that cause the "green island" effect.

PR-toxin, another eremophiline, is produced by Penicillium roqueforti Thom. (75). Phomenone has been isolated from Phoma destructiva Plowr., a fungus causing wilt disease in tomato (Lycopersicon esculentum Mill.) (76). Phomenone, PR-toxin, and four derivaties of phomenone were assayed for phytotoxic activity in tomato seedlings and cuttings (77). Phomenone, acetylphomenone, and PR-toxin at 10^{-4} M caused wilting and necrosis and halved the growth rate of shoots and completely inhibited the growth of roots. Phomenone is also produced by the fungus Phoma exigua (78).

Gigantenone and petasol also stimulate auxin-like activity by promoting root formation in mung bean [Vigna radiata (L.) Wilczek] cuttings and rooting of calli in tissue culture of sunflower [Helianthus annuus L.] (54). This effect was absent in the monocot asparagus (Asparagus officinalis L.), but these eremophilanes caused larger and more branched asparagus shoots.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Maculosins. These compounds were isolated from an Alternaria alternata infection on spotted knapweed (Centaurea maculosa Lam.) (79). Although many maculosins were identified, only one compound, maculosin-1 [7] or cyclo l-proline-L-tyrosine, was found to be a host-specific phytotoxin of spotted knapweed at 10 μM. Nineteen other grasses and broadleaved plants were not injured by maculosin-1, even at concentrations of 1 mM. This compound, retaining host specificity of the naturally occurring product, has recently been synthesized from a methyl ester of L-tyrosine and t-BOC-L-proline (80).

Ophiobolins. Ophiobolin A [8] was the first member of this group to be identified and is also the group member most studied. Over 20 analogs have been identified. Most of these compounds have been derived from several pathogens of the Drechslera spp. Ophiobolin A was first isolated from Drechslera oryzae, a pathogenic organism responsible for a major epidemic in rice (54). This compound has numerous effects on rice (81). D. maydis was the pathogen that caused widespread corn leaf blight (82) and this organism also produces ophiobolin A and at least five other similar analogs, including 6-epiophiobolin A and 3-anhydro-6-epiophiobolin. These three compounds, plus 3-anhydroophiobolin, have recently been isolated from D. sorghicola = Bipolaris sorghicola, a fungal pathogen of johnsongrass [Sorghum hapepense (L.) Pers.] (83). Four ophiobolins were tested in a leaf-spot assay in sorghum, sicklepod (Cassia obtusifolia L.), morning glory [Ipomoea purpurea (L.) Roth], maize, and bentgrass (Agrostis alba L.) Ophiobolin A and 6-epi-ophiobolin A were more phytotoxic than their anhydro- counterparts against sorghum, sicklepod, and maize (83). Ophiobolin A at low concentrations (5×10^{-5} to 7.5×10^{-6} M) disrupted membrane potential and caused leakage and potasium ion efflux. Calmodulin function is disrupted by this compound (84). D. maydis race T produced 6-epiophiobolin A and 3-anhydro-6-epiophiobolin A, which specifically inhibited malate oxidation in a mitochondrial electron transport assay from Texas-male-sterile (TMS) corn (85). D. maydis race O did not produce these 2 phytotoxins. However, it was phytotoxic to corn and not specific to TMS corn. Ophiobolin G and H have been isolated from Aspergillus ustus (86).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Perylenequinones. Alternaria eichorniae, a pathogen of waterhyacinth [Eichornia crassipes (Mart.) Solms], produces a phytotoxin, alteichin [9], which causes necrotic lesions analogous to the pathogen infection when the compound is applied at 1-10 µg/10 µl droplet (87). The pathogen Stemphylium botyosum var. lactucum is the causal agent of leaf spot on lettuce. It produces stemphyperylenol [10] and stemphyltoxins [11] I (R_1 = H, R_2 and R_3 = OH, R_4 = H_2), II (R_1 = H, R_2 = OH, R_3 and R_4 = H_2), III (R_1 = H, R_2 = OH, R_3 and R_4 = H), and IV (R_1 = H, R_2 = OH, carbons at R_3 and R_4 joined in epoxide linkage) which are analogs of alteichin (88).

Tryptophol. Tryptophol [12] is a natural product in plants and microorganisms and is involved in indoleacetic acid metabolism. A major metabolite from Drechslera nodulosum, tryptophol was isolated and identified from extracts of culture filtrates of this fungal pathogen (89). This report was the first to show that this compound was phytotoxic and caused necrotic lesions on goosegrass (Eleusine indica L.), the weed host of this fungus. Necrotic injury in goosegrass occurred after application of tryptophol at 6.2×10^{-4} M. Furthermore, tryptophol was found in goosegrass infected with D. nodulosum, but not in uninfected grass tissue (89). Phytotoxic activity was found on several other mono- and dicotyledonous plants, but generally higher concentrations were necessary. Tryptophol and o-acetyltryptophol have also been identified as tryptophan metabolites of Ceratocystis fagscearum, a fungal pathogen causing oak wilt (90).

Zinniol. Zinniol [13] was first described as a phytotoxic metabolite of Alternaria zinniae (91). Zinniol is now known to be produced by many Alternaria spp., including fungal pathogens of weeds and crop plants (73). This compound causes necrosis in plants (73), 92) and "green island" formation, as discussed earlier.

Phytotoxic Compounds Produced by Fungal Pathogens of Crops and/or Non-Pathogenic Fungi

AAL-Toxin. Stem canker of tomato is caused by the fungal pathogen Alternaria alternata f. sp. lycopersici (93). Concentrations of less that 10 ng/ml of AAL-toxin [14], a host-specific toxin, can produce disease symptoms. Two phytotoxic fractions have been isolated from fungal culture filtrates that reproduce disease symptoms in susceptible plants. These fractions are termed T_A and T_B and each fraction has 2 chemical components. Collectively these are the AAL-toxins. Structurally in T_{A1}, R_1 and R_3 = OH, R_2 = $O_2C\text{-}CH_2\text{-}CH(COOH)\text{-}CH_2\text{-}COOH$; T_{A2}, R_1 = $O_2C\text{-}CH_2\text{-}CH(COOH)\text{-}CH_2\text{-}COOH$, R_2 and R_3 = OH; T_{B1}, R_1 = OH, R_2 = $O_2C\text{-}CH_2\text{-}CH(COOH)\text{-}CH_2\text{-}COOH$, R_3 = H; and T_{B2}, R_1 = $O_2C\text{-}CH_2\text{-}CH(COOH)\text{-}CH_2\text{-}COOH$, R_2 = OH, and R_3 = H (94, 95). The compound disrupts pyrimidine synthesis by inhibition of aspartate carbamoyl transferase (ACT) (96). Concentrations of AAL-toxin of 10-50 ng/ml cause potent ACT inhibition. A microtechnique for the separation of these structurally related toxins has been achieved using HPLC (97).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Acetylaranotin. A soil fungus, Aspergillus terreus strain C-520, produces several plant growth regulators (98). Three of these phytotoxic compounds have been structurally charaterized as acetylaranotin [15], bis-dithiodi(methylthio)-acetylaranotin, and terrein. Compared to acetylaranotin, these other compounds were only weakly inhibitory in lettuce and rice bioassays. Acetylaranotin reduced lettuce root elongation by 80% at 10 ppm, and rice root length by 50% at 50 ppm and 80% at 100 ppm. Results suggested that the disulfide bridge is required for higher phytotoxicity.

AK-Toxin. The causal agent of black spot disease of Japanese pear, Alternaria kikuchiana, produces two host-selective toxins: AK-toxin [16] I and II (99). The toxins induced necrosis on susceptible pear cultivar leaves at 65 ng/ml, but no response was noted at concentrations up to 6.5 μg/ml in a resistant cultivar.

Altersolanol A. Pigment analysis and testing of compounds from Alternaria porri (Ellis) Ciferri indicated that three reduced anthraquinones were phytotoxins (100). Altersolanol A [17] gave complete inhibition to root growth at 100 ppm in a lettuce bioassay, and 85% inhibition in a stone-leek bioassay. This compound was weakly inhibitory to lettuce at low concentrations (12.5 and 25 ppm), but these solutions caused appreciable inhibition to stone-leek. Analogs altersolanol B and dactylariol were less toxic to these species. Other fungal species such as A. solani and Dactyleria lutea Routien also produce these pigments (100).

AM-Toxin. The pathogen Alternaria mali causes leaf spot disease of apple and produces necrotic spots on fruits, leaves, and shoots of susceptible cultivars. AM-toxin [18], a host-specific toxin, and several related toxins are responsible for these symptoms and their structures have been elucidated and confirmed by total synthesis (101-103).

Anisomycin and Methoxyphenone. Anisomycin [19], which displays herbicidal activity, was first reported by Yamata et al. (104) and was isolated from a soil Streptomyces sp. Barnyardgrass [Echinocloa crusgalli (L.) Beauv.] and crabgrass (Digitaria sp.) were sensitive, but no herbicidal injury was found on turnip (Brassica rapa L.) or tomato (Lycopersicon esculentus L.). Thus, the compound exhibited some selectivity and was the first microbial product used for the development of a synthetic herbicide (methoxyphenone [20], or 3,3'-dimethyl-4-methoxy-benzophenone) (105).

Bialaphos, Phosalacine, and Phosphinothricin. These microbial compounds are the most energetically researched bioherbicides yet discovered. These studies encompass elucidation of the biosynthetic pathways (bialaphos and phosphinothricin), chemical synthesis (phosphinothricin), bacterial cloning and overexpression of the gene for phosphinothricin production, determination of the molecular mode of action of phosphinothricin, and genetic transformation of the phosphinothricin resistance gene into crop plants. Bialaphos {L-2-amino-4-[(hydroxy)(methyl)phosphinoyl]-butyryl-L-alanyl-L-

Alteichin [9]

Stemphyperylenol [10]

Stemphyltoxin [11]

Tryptophol [12]

Zinniol [13]

AAL toxin [14]

Acetylaranotin [15]

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

AK-toxin [16]

Altersolanol A [17]

AM-toxin [18]

Anisomycin [19]

Methoxyphenone [20]

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

alanine} [21] is a metabolite of the soil microbe Streptomyces viridochromogenes. It is the first microbial product produced by fermentation to achieve commercial herbicide status. This compound is marketed in Japan as HERBIACE (106). Bialaphos is a broad-spectrum herbicide of various weeds, including perennials (107). Phytotoxicity symptoms are expressed at a rate intermediate to those of paraquat (1,1′-dimethyl-4,4′-bipyridinium ion) and glyphosate [N-(phosphonomethyl)glycine]. Metabolism by soil microbes results in a half-life of the compound of 20-30 days (108). A peptide analog of bialaphos called phosalacine [22] was isolated from Kitasatosporia phosalacinea, a soil Actinomycete and its fermentation, herbicidal activity, and mechanism of action have been reported (109). This compound differs from bialaphos only by a having a C-terminal leucine moiety rather than alanine. More recently, phosalacine has been patented as a defoliant for hops (Humulus lupulus) (110, 111). Metabolism by peptidases in plants releases the active ingredient of the molecule, phosphinothricin [23]. Phosphinothricin has been isolated from S. viridochromogens and from S. hygroscopicus as the peptide phosphinothricylalanylalanine (bialaphos) (112). Phosphinothricin, a phosphonic acid analog of glutamate, was first synthesized in 1972 (113). Herbicidal properties of this compound as a commercially synthesized ammonium salt (glufosinate or HOE 39866) were first reported in 1977 (114). This herbicide is presently marketed as BASTA by Hoechst AG. Some phosphonic and phosphinic acid analogs of glutamic acid have been known since 1959 to be inhibitors of glutamine synthetase (GS) (115), but not until 1972 was phosphinothricin found to be a potent inhibitor of GS activity (113)(Figure 3). Presently the mechanism of action of phosphinothrcin is well-studied and understood to be GS inhibition (Figure 4).

GS in plants is the key enzyme of the GS/GOGAT (glutamine synthetase/glutamine:2-oxyglutarate aminotransferase) pathway and thus plays a crucial role in ammonia assimilation/reassimilation (117, 118). GS inhibition by phosphinothricin causes accumulation of toxic levels of ammonia. Since ammonia production is increased by photorespiration and conversion of nitrite to ammonia (also light-dependent), nitrogen fertilizers and light act to promote phosphinothricin efficacy (119).

Glutamine depletion caused by phosphinothricin is a critical factor in the mechanism of action of phosphinothricin since glutamine was found to partially ameliorate this herbicidal effect (Figure 4) (116). Glutamine depletion can cause photosynthesis inhibition, and other scenerios may be operative. First, inhibition of protein synthesis, i.e., lack of regeneration of Q_B protein required in light-dependent electron transport would block photosynthetic electron transport, and an amino donor would not be present for glyoxylate transamination. Second, toxic glyoxylate accumulation, i.e., glyoxylate inhibits RuDP carboxylase/oxygenase (120). Third, Calvin cycle intermediate depletion, i.e., lack of GS and/or other enzymes that prevent carbon flow into photorespiration by the oxygenase reaction causes insufficient RuDP for the Calvin cycle.

Very recently a phosphinothricin analog, DL-γ-hydroxyphosphinothricin [24], has been synthesized and shown to be a new potent GS inhibitor (121). This compound and several other

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

O O H O H
CH_3—P—CH_2—CH_2—CH—C—N—CH—C—N—CH—COOH
OH NH_2 CH_3 CH_3

Bialaphos [21]

O O H O H
CH_3—P—CH_2—CH_2—CH—C—N—CH—C—N—CH—COOH
OH NH_2 CH_3 CH_2—CH—$(CH_3)_2$

Phosalacine [22]

O
CH_3—P—CH_2—CH_2—CH—COOH
OH NH_2

Phosphinothricin [23]

O
CH_3—P—CH_2—CH_2—CH—COOH
OH NH_2

γ-Hydroxyphosphinothricin [24]

O
CH_3—S—CH_2—CH_2—CH—COOH
NH NH_2

L-Methionine sulfoximine [25]

O
CH_3—S—CH_2—CH_2—CH—COOH
N—PO_2H_3 NH_2

L-(N^5-Phosphono)-methionine-S-sulfoximine [26]

O
HN—C
H_2C—C—CH_2—CH_2—CH—COOH
OH NH_2

Tabtoxinine-β-lactam [27]

O—CH—COOH
H_2C—CH—NH_2

Oxetin [28]

Figure 3. Comparison of structures of several compounds with potent *in vitro* glutamine synthetase-inhibiting properties.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

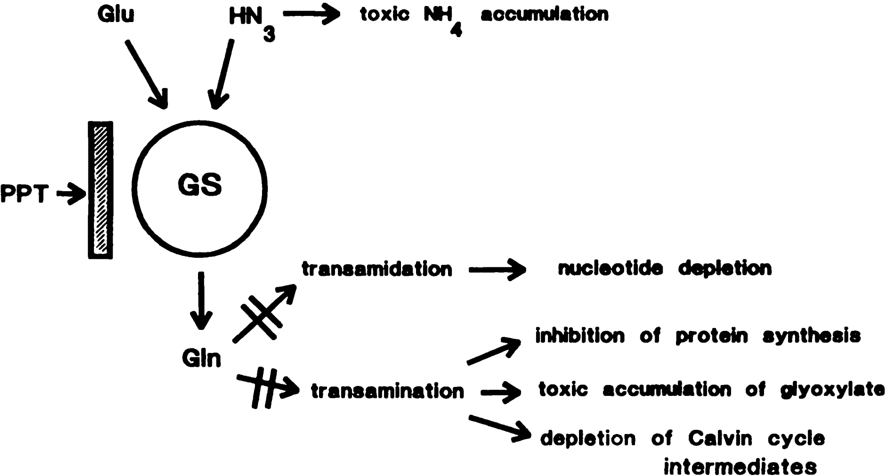

Figure 4. Sites of molecular action of phosphinothricin. (Redrawn from Ref. 116. Copyright 1987 Verlag de Zeitschrift für Naturforschung).

γ-oxygenated phosphinothricins displayed GS-inhibitory properties and in vivo phytotoxicity (122). These compounds should be useful tools to determine the nature of molecular binding of phosphinothricin analogs.

Biotechnology and Biochemistry of Bialaphos Production and of Plants Resistant to Bialaphos. The biosynthetic pathway of bialaphos production has been elucidated using C^{13}-labeled presursors, metabolic inhibitors, blocked mutants, and identification of products accumulated and metabolized by a series of non-producing S. hydroscopicus mutants (Figure 5) (123). Bialaphos is synthesized from three carbon precursors in a series of at least 13 conversions. One step involves an acetyl-coenzyme A-dependent reaction which modifies either demethylphosphinothricin or phosphinothricin. This gene which confers resistance to bialaphos (bar) has been isolated and characterized from S. hygroscopicus (124). This bar gene is both an antibiotic-resistant and biosynthetic gene since it is responsible for self-defense (resistance) of the microbe to bialaphos as well as synthesis of the herbicide (125). The gene encodes a 22kDal polypeptide with acetyl transferase activity (phosphinothricin acetyl transferase) which catylizes phosphinothricin to a non-herbicidal acetylated metabolite.

Researchers have isolated and characterized a transaminase from E. coli K-12, specific for production of L-phosphinothricin (126). Further work has resulted in cloning, characterization, and overexpression of the gene coding for the L-phosphinothricin-specific transaminase from E. coli K-12. These results greatly facilitate biotechnological production of L-phosphinothricin in an enzyme reactor.

Most recently, the bar gene has been introduced and expressed into several plant species using Agrobacterium-mediated Ti plasmid transformation (127). Transgenic plants (tomato, tobacco, potato, alfalfa, oilseed rape, and sugarbeet) were resistant to glufosinate and bialaphos under laboratory and greenhouse conditions at field dose applications. Results of field trials will next assess effects of more rigorous environmental conditions and questions of introduced-gene stability and pleiotrophic effects.

Other Phytotoxins Known to be GS Inhibitors. L-Methionine sulfoximine (MSO) [25] (128), an analog of phosphinothricin, was synthesized some time ago (129). MSO was also patented as a herbicide (130), but phosphinothricin is ca. 100 times more inhibitory to GS than is MSO (131), 132). MSO has been recently shown to occur as a natural product, not in microbes, but in tree bark (Cnestis glabra) (133). However, an analog of MSO, L-(N^5-phosphono) methionine-S-sulfoximine [26], is a potent GS inhibitor and a metabolite of L-(N^5-phosphono)-methionine-S-sulfoximinyl-L-alanyl-L-alanine (134) which is another Streptomyces product (135). Other important GS inhibitors have been reported. Pseudomonas tabaci produces tabtoxinine-β-lactam [27], a GS inhibitor (136). Oxetin [28], derived from Streptomyces sp., is a recently discovered GS inhibitor (137). Refer to Figure 3.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

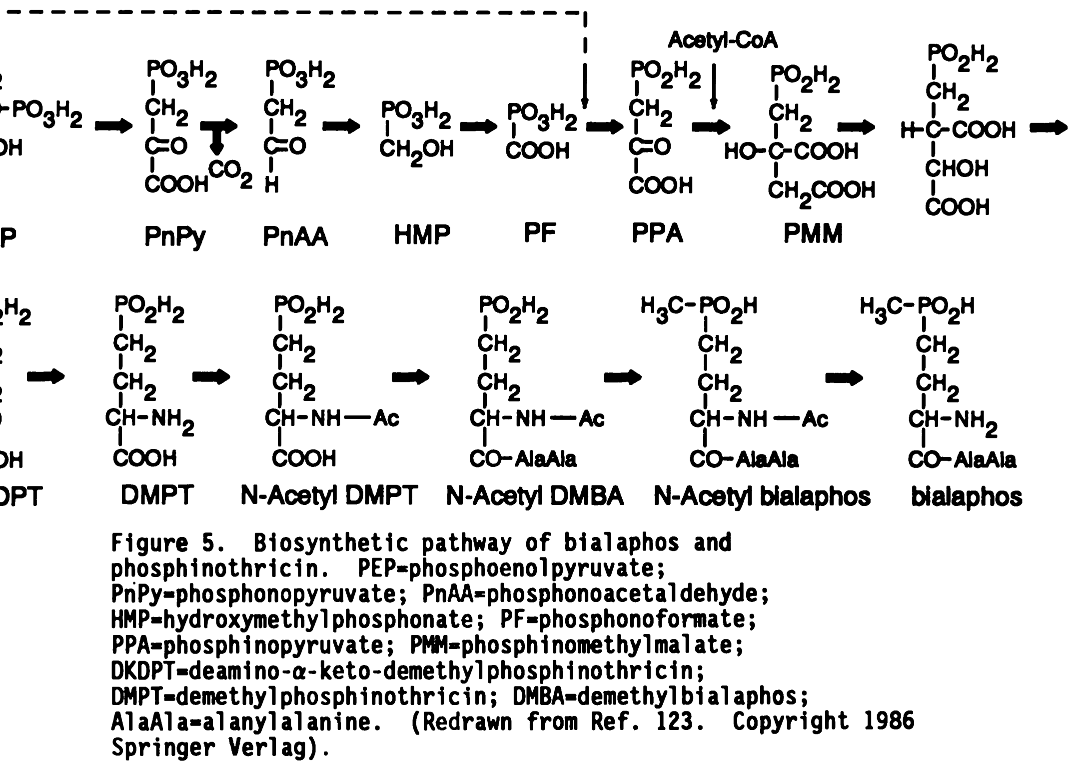

Figure 5. Biosynthetic pathway of bialaphos and phosphinothricin. PEP=phosphoenolpyruvate; PnPy=phosphonopyruvate; PnAA=phosphonoacetaldehyde; HMP=hydroxymethylphosphonate; PF=phosphonoformate; PPA=phosphinopyruvate; PMM=phosphinomethylmalate; DKDPT=deamino-α-keto-demethylphosphinothricin; DMPT=demethylphosphinothricin; DMBA=demethylbialaphos; AlaAla=alanylalanine. (Redrawn from Ref. 123. Copyright 1986 Springer Verlag).

Botrydienal. A new phytotoxin, botrydienal [29], and two phytotoxic metabolites, dehydrobotrydienal and deacetyldihydrobotrydial, have been isolated from a phytopathogenic fungus, Botrytis cinerea (138). The concentration of botrydienal required to give a 50% growth inhibition in a turnip seedling bioassay was 10 µg/ml. Dehyrobotrydienal and deacetyldehydrobotrydial were weaker phytotoxins, requiring 10 and 1000 µg/ml, repsectively, to give the same inhibition.

Cercosporin. Cercosporin [30] is a toxin produced by fungal pathogens of the genus Cercospora. The compound was first isolated in 1957 (139) from the soybean pathogen Cercosporin kikuchii. Its characterization and structure were reported independently (140, 141). The red toxin causes light-dependent lipid peroxidation (142) and membrane disruption in plants (143), by its photosensitizing action that produces singlet oxygen (144).

Chaetoglobosins. Cytochalasins induce selective responses in plants. Cyclchalosin B isolated from Phoma exigua was toxic to periwinkle (Vinca minor L.) and to chicory (Cichorium intybus L.) (145) but caused only moderate growth inhibition in wheat coleoptiles (146).Cytochalsin H [31] did not affect corn plants, but bean plants exhibited epanastic responses and leaf rolling (147). In tobacco, this compound delayed flowering and was active at 10^{-6} M in wheat coleoptile assays (146).

Other effects of cytochalasins on plant growth have been reported (148). Chaetoglobosin K was isolated from Diplodia macrospora and shown to be structurally related to the cytochalasins (146). Phytotoxicity tests indicated inhibition to wheat coleoptiles at 10^{-7} M and may be the most active cytochalasin tested.

Citreoviridin. Citreoviridin [32] has been isolated from several Penicillium species; e.g. P. charlesii (149). The compound exhibited selective and long-term growth inhibition when corn (Zea mays L.), tobacco (Nicotiana tabacum L.), and wheat (Triticum aestivum L.) were tested at 10^{-2} to 10^{-4} M concentrations. Corn shoot growth was inhibited up to 60 days after treatment with citreoviridin at 10^{-2} M. Wheat coleoptile growth was also inhibited, but tobacco seedlings were not affected at these concentrations.

Cladosporin. Cladosporin [33] (R=H) has been isolated from Cladosporium cladosporioides (Frensenuis) de Vries (150), Aspergillus flavus Link (151), Eurotium sp. (152), and Aspergillus repens (153). Initially it was shown to have antibiotic properties, but later cladosporin and cladosporin diacetate (R=CH_3CO-) (synthetically derived from cladosporin) were shown to have phytotoxic properties. Both compounds were phytotoxic to wheat seedlings, but had no phytotoxic effects on tobacco seedlings. Only cladosporin diacetate caused necrosis, stem collapse, and/or growth inhibition in corn seedlings (153).

Colletotrichin. A pathogenic fungus, Colletotrichum nicotianae, is the causative agent of tobacco anthracnose and this organism is the source of three phytotoxins, including colletotrichin [34] (154,

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Botrydienal [29]

Cercosporin [30]

Cytochalasin H [31]

Citreoviridin [32]

Cladosporin [33]

Colletotrichin [34]

Cyclocarbamide A [35]

Cyclopenin [36]

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

155). Colletotrichin (having two hydroxyl groups) is the most phytotoxic of the three compounds and inhibited lettuce hypocotyl and root growth at concentrations as low as 3 ppm. Colletotrichin B (CHO moiety instead of on ring) inhibited root and hypocotyl growth only at 100 ppm. Colletotrichin C (CHO moiety in place of OH on side chain) was less active than colletotrichin, except when concentrations exceeded 30 ppm, when it was more inhibitory to root growth. Colletotrichin and Colletotrichin C were also slightly more inhibitory in a lettuce germination bioassay. These latter compounds at 1 µg also induced lesions similar to those caused by C. nicotianae in pricked tobacco leaves.

Cyclocarbamide A and B. Cyclocarbamide A [35] (R = CH_2-CH-$(CH_3)_2$) and B (R = CH_2-$(CH_2)_3CH_3$) were first isolated from Streptoverticillium sp. by Isogai et al. (156) and found to have phytotoxic activity, i.e., complete germination inhibition in lettuce (Lactuca sativa L.) at low concentration (30 ppm). These compounds had no effect on growth of emerged seedlings, even at 100 ppm.

Cyclopenin. Cyclopenin [36] and cyclopenol isolated from Penicillium cyclopium (NRRL 6233) (157) are phytotoxic compounds causing significant growth inhibition in etiolated wheat (Triticum aestivium) coleoptiles at 10^{-3} and 10^{-4} M. Cyclopenin was more potent and caused necrosis and stunting in corn (Zea mays L.) and malformations in trifoliate leaves of bean (Phaseolus vulgaris L.) at 10^{-2} M.

Desmethyoxyviridiol. A new toxin from Nodulisporium hinnuleum was identified as desmethoxyviridiol [37] and shown to have phytotoxic properties (158). Wheat coleoptiles were inhibited at 10^{-3} to 10^{-6} M and first internodes in oats were plasmolyzed at 10^{-3} M and growth significantly inhibited at 10^{-4} to 10^{-7} M. Corn treated with the toxin at 10^{-2} and 10^{-3} M showed necrotic lesions and stunting at 10^{-4} M. Effects on beans and tobacco plants were essentially nil. Viridiol [38] is a similar microbial phototoxin. A similar phytotoxin is produced by the non-pathogenic fungus, Gliocladium virens (159).

Dihydropergillin. Aspergillus ustus, a fungus growing on pea (Pisum sativum) seeds was found to produce a phytotoxic compound, pergillin (160). Another metabolite from this organism was later isolated and identified as dihydropergillin [39] (161). This structural analog of pergillin was more toxic than pergillin in wheat coleoptile bioassays, i.e., 10^{-3} to 10^{-4} M.

Fusicoccin. Fusicoccin [40] is a major metaoblite of the fungus Fusicoccum amygdali Del., which causes canker in almond (Prunus amygdalus Batsch) and peach [P. persica (L.) Batsch)]. The compound plays a definite role in the disease and is a diterpenoid glucoside (162) with high phytotoxicity causing wilt and necrosis in many plants (163). The chemistry and plant growth regulating properties of fusicoccin and some derivatives and analogs have been summarized and discussed (164).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Desmethoxyviridiol [37]

Viridiol [38]

Dihydropergillin [39]

Fusicoccin [40]

Gabaculine [41]

Geldanamycin [42]

Gostatin [43]

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Gabaculine. This compound is a natural metabolite first isolated from Streptomyces toyacaenis and shown to be a neurotoxin (165). It is a powerful inhibitor of several pyridoxal phosphate-requiring enzymes (166). Gabaculine [41] was originally reported as an inhibitor of gamma-aminobutyrate aminotransferase and strongly inhibits chlorophyll synthesis in plants (167, 168). It inhibits tetrapyrrole synthesis (169) by blocking 5-aminolevulinate formation in plants via inhibition of 5-aminolevulinate dehydratase (170).

Geldanamycin. Geldanamycin [42] was first reported as a product of Streptomyces hygroscopicus var. geldanus (171, 172). This compound (an ansamycin antibiotic, is very similar to herbimycin B, differing only in the presence of a methoxyl group at carbon number 17 (173). Herbimycins, also ansamycin antibiotics produced by a strain of S. hygroscopicus, are herbicidal and active against various dicotyledonous and monocotyledonous plants (173-175). Geldanamycin caused a 50% reduction in garden cress (Lepidium sativum L.) radicle growth at 1-2 ppm and nearly 100% inhibition at 3-4 ppm. At high concentrations, geldanamycin caused severe necrosis and disintegration of radicles.

Gostatin. Gostatin [43] inhibits aspartate aminotransferase in wheat germ (176). There is a similarity in the mode of action of gostatin and gabaculine [41] (177).

HC-Toxin. Early attempts to isolate and characterize this toxin from a corn pathogen, Helminthosporium carbonum race 1 were performed in 1967-71 (178, 179). Many other reports of structure determinations have been summarized (180). Purified HC-toxin [44], a host-specific toxin, causes substantial root growth inhibition at ca 0.5 µg/ml (181). A very similar compound, chlamydocin [45], has been isolated from Diheterospora chlamydospora (182, 183).

Herbicidins. Herbicidins A [46] (R_1 and R_3 = CH_3, R_2 = $CO(CH_2OH)C{=}CHCH_3$) and B (R_1 and R_3 = CH_3, R_2 = H) have been produced by Streptomyces saganonensis and have been examined for herbicidal activity in many mono- and dicotyledenous plants. Other analogs, herbicidins E, F, and G, have also been identified, but not examined for herbicidal activity (184-188).

Irpexil and Benzadox. A wood-degrading basidiomycete, Irpex pachyodon, produces irpexil [47], which had antibacterial activity (189). Later, the compound was found to have phytotoxic properties and to be structurally related to several synthetic herbicides, including benzadox (benzamido-oxyacetic acid) [48] (28). Benzadox (and presumably, irpexil) acts via metabolic conversion within plants to form amino-oxyacetic acid, which is a potent pyridoxyl phosphate-requiring enzyme inhibitor (190) and also a non-commercial synthetic herbicide (191, 192).

Macrolides. Recifeiolide [49], a fungal product from Cephalosporium recifei Area Leao et Lobo was a weak growth inhibitor when tested at 100 ppm on germinating lettuce (193). Other macrolides such as cladospolide A & B from Cladosporium

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

HC-toxin [44]

Chlamydocin [45]

Herbicidin A [46]

$CH_3-C(=O)-NH-O-CH_2-COO-CH_3$

Irpexil [47]

Benzadox [48]

Recifeiolide [49]

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

cladosporioides (193) were active at 100 ppm; i.e., cladospolide A inhibited root growth ca 60%, but cladospolide B showed growth promotion potential.

Cis- and trans-resorcylide at 100 ppm inhibited growth of lettuce, rice (Oryza sativa L.), and chinese cabbage (Brassica pekinensis) roots. Light catalyzed interconversion of cis and trans isomers in methanoic solutions (194).

Dehydrocurvularin [50] from Alternaria alternata (Fries) Keissler and various synthetically derived analogs showed varying degrees of growth inhibition in an etiolated wheat coleoptile test (195).

Mevinolin. Mevinolin [51] is a metabolite of the ascomycete Aspergillus terreus. It is a highly specific inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase in a wide variety of eukaryotes and prokaryotes (196-199). The compound causes root growth inhibition in light- or dark-grown seedings at concentrations as low as ca 10^{-7} to 10^{-8} M (199). It also lowers phytosterol production (200).

Moniliformin. The fungi Fusarium moniliforme and F. fusariodes, pathogens of corn and millet, respectively, produce the phytotoxin moniliformin (3-hydroxycyclobut-3-ene-1,2-dione) [52]. This compound induces chlorosis and necrosis in corn, wheat and barley and inhibits growth in tomato, soybeans, and tobacco (201, 202). Because of the phytotoxic activity of moniliformin and the relatively simple structure, numerous syntheses of this compound and analogs have been carried out to obtain herbicidal compounds as discussed by Fischer and Bellus (28). Although some compounds were very effective herbicides, no crop selectivity was obtained. Furthermore, moniliformin was found to be toxic to vertebrates and mammals (201-203).

Nigerazine A and B. Nigerazine B (N-methyl-trans-2,5-dimethyl-N'-cinnamoyl piperazine) [53] (R_1 = H, R_2 = CH_3) was reported (204) from Aspergillus niger about two years prior to identification of Nigerazine A (R_1 = CH_3, R_2 = H) (205). In tests with lettuce seedlings, both compounds showed significant root growth inhibition (ca 40-80% at 100-400 ppm).

Nigericin. Nigericin [54] was first reported in 1949 (206) and later found identical to polyetherin A, a metabolite of Streptomyces hygroscopicus (207). The compound is an ionophore and affects potassium ion transport in mitochondrial, chloroplast, and microsomal membranes (208-212) and inhibits photophosphorylation (208). It also has phytotoxic properties as demonstrated in an intact garden cress seedling bioassay (213). Although nigericin caused a 50% reduction in garden cress radicle growth at 1-2 ppm, no visible necrosis was evident.

Oosporein. A strain of Chaetomium trilaterale was found to produce a red crystalline metabolite with growth-inhibiting and phytotoxic properties when tested on oat, tobacco, and bean (214). This compound [55] is also produced by several other fungi and fungi imperfecti (215, 216) ascomycetes (217) and basidiomycetes (218). This compound was also toxic to vertebrates (214).

Dehydrocurvularin [50]

Mevinolin [51]

Moniliformin [52]

Nigerazine [53]

Nigericin [54]

Oosporein [55]

Orlandin [56]

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Orlandin. A new metabolite, named orlandin [56], was isolated from *Aspergillus niger* on citrus leaves and chemically characterized as bis[8,8'-(7-hydroxy-4-methoxy-5-methylcourmarin]. The compound was phytotoxic at 10^{-3} to 10^{-5} M in a wheat coleoptile growth assay (219). Kotanin, a very similar compound, was isolated from *A. glaucus* (220) and later identified as *A. clavatus* (221). However, kotanin was not phytotoxic to wheat (219) and methylation of the 2 hydroxy groups of orlandin caused loss of phytotoxicity.

Prehelminthosporal. Several plant growth regulator compounds have been isolated and identified from *Helminthosporium sativum* [*Dreschlera sorokiana*]. Helminthosporal was found to be a growth promoter in rice seedlings, but inhibitory to wheat (222, 223). Synthetic analogs had differential effects on lettuce and rice growth (224). Prehelminthosporal [57] from *D. sorokiana* was isolated and the acetate derivative synthesized (225). Both compounds inhibited wheat coleoptile growth at 10^{-3} and 10^{-4} M and both promoted growth at 10^{-5} M. Both compounds caused stunting and chlorosis in corn and no reponse in tobacco plants, but only the acetate derivative caused necrosis in bean.

Radicinin, Deoxyradicinin, 3-Epideoxyradicinol, and Radianthin. *Alternaria helianthi*, a major pathogen of sunflower (*Helianthus annuus*), produces several phytotoxins. Radicinin [58], deoxyradicinin, and 3-epideoxyradicinol [59] were reported early (226, 227). Further work has isolated deoxyradicinol and radianthin [60] (228). Studies on radicinin biosynthesis have indicated the compound is derived from polyketides (229, 230). Deoxyradicinin is also of polyketide origin (227). Recently the mechanism of polyketide-derived biosynthesis of deoxyradicinin, deoxyradicinol, and 3-epideoxyradicinol has been shown to occur via condensation of two polyketide chains rather than from cyclyation and ring-cleavage of a single hexaketide unit (231).

Tentoxin. *Alternaria alternata* produces tentoxin [61], a cyclic tetrapeptide. This phytotoxin causes injury to many monocotyledonous and dicotyledonous weed species, but does not injure maize (*Zea mays* L.) or soybean (*Glycine max* L. Merr.) (29). Tentoxin has been synthesized in the lab (232). Sites of action of this toxin are disruption of energy transfer by inhibition of CF_1 ATPase (233) and alteration of nuclear-coded protein uptake (234).

Tenuazonic Acid. Tenuazonic acid [62] is a fungal metabolite that has a broad phytotoxicity spectrum (soybean, rice, *Datura innoxia*, and lettuce) (235-237). Toxicity to plants has been attributed to reduced protein synthesis via ribosome inhibition (235, 238).

Terphenyllin and Hydroxyterphenyllin. Hydroxyterphenyllin [63] and terphenyllin are produced by *Aspergillus candidus* (239). Terphenyllin (2',5'-dimethyoxy-4,3',4"-trihydroxy-*p*-terphenyl) inhibited wheat coleoptile growth at 10^{-3} M while the hydroxy-analog was inhibitory at 10^{-3} to 10^{-5} M. The tetraacetate of hyrdroxyterphenyllin was not phytotoxic to wheat coleoptiles.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Prehelminthosporol [57]

Radicinin [58]

Epideoxyradicinol [59]

Radianthin [60]

Tenuazonic acid [62]

Tentoxin [61]

Hydroxyterphenyllin [63]

Thiolactomycin [64]

Toyocamycin [65]

Sangivamycin [66]

Tubercidin [67]

Herbiplanin [68]

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Thiolactomycin. Thiolactomycin [64] from Norcardia sp. No. 2-200 is a specific inhibitor of type II fatty acid synthetase in higher plants and E. coli (240). The acetyl-CoA:ACP S-acetyl-transferase is the apparent specific site of inhibition (241). Another antibiotic, cerulenin (structure not shown) inhbits β-ketococyl-ACP synthetase I in bacteria, fungi, and plants, but also is inhibitory to other sites such as polyketide and sterol biosynthesis (242-244). Cerulenin and thiolactomycin inhibited CQ14W-acetate incorporation into fatty acids at I_{50} values of 50 and 4 uM, respectively (245). Recently cyclohexanedione herbicides have been shown to inhibit lipid biosynthesis by inhibition of acetyl-CoA carboxylase (246).

Toyocamycin. Streptomyces toyacaensis produces a phytotoxin, toyacamycin [65] (247). A Streptomyces sp. also produced toyacamycin which was inhibitory to growth of rice, barnyardgrass, crabgrass (Digitaria sanguiralis), lucerne, turnip, and tomato (104). Other structurally related phytotoxic analogs are sangivamycin [66], tubercidin [67], and herbiplanin [68] (248).

Trichothecenes. Macrocyclic Trichothecens. These trichothecens possess a wide range of phytotoxic specificity. Verrucarin A, J, and trichoverrin B are inhibitory in the etiolated wheat coleoptile test at very low concentrations (10^{-7} M) (249). Roridin A [69] was very toxic to tobacco, corn, and bean (Phaseolus vulgare L.) seedlings (249). Trichothecenes also are found in the soil microorganisms Myothecium verrucaria (Albertini et Schweinitz) Ditmar ex Fries and M. roridum Tode ex Fries (250).

A Brazilian shrub, Baccharis megapotamica, contains high levels of trichothecens (baccharinoids) which are closely related to the macrocyclic trichothecens such as roridin A and the verrucarins (250). These compounds are absorbed, metabolized, translocated, and accumulated in the shrubs after being produced by soil microorganisms. The baccherinoids are highly phototoxic except to B. megapotamica and may be important adopted allelochemicals in plant ecosystems completing with these shrubs (251).

Simple Trichothecens. Fusarium tricinctum, isolated from peanuts remaining in soil after harvest, produced a trichothecene compound, neosoliniol monoacetate [70], which was a very potent growth inhibitor in wheat coleoptile bioassays at concentrations as low at 10^{-6} M. In contrast, the compound did not cause complete growth inhibition at 10^{-3} M. A similar compound, diacetoxy-scirpenol, caused slight growth inhibition in pea seedlings at 2.7 x 10^{-6} M and severe inhibition at 2.7 x 10^{-5} M, but was not toxic to wheat seedlings (252).

Triticones. Phytotoxic triticones have been found in Drechslera tritici-repentis, a plant pathogen that causes tan spot on wheat, and in Curvularia clevata, a pathogen of several grasses (253). At least eight triticones have been identified, but only triticones A [71] and B are phytotoxic. These compounds possess an exocyclic double bond adjacent to a ketone moiety.

Roridin A [69]

Neosolaniol monoacetate [70]

Triticone A [71]

Coronatine [72]

Cyanobacterin [73]

Phaseolotoxin [74]

Rhizobitoxine [75]

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Bacterial Phytoxins from Crop Pathogens and Weed Pathogens

Coronatine. Coronatine [72] was first detected in isolates of liquid cultures of Pseudomonas syringae pv. atropurpurea, a pathogen of ryegrass (254). This pathovar also produces another phytotoxic structural analog of coronatine, N-coronafacoylvaline (255). Coronatine applied to Italian ryegrass leaves caused chlorsis and browning (254), it was also phytotoxic to soybean (256). Coronatine is produced by several P. syringae pathogens such as pv glycinea (257), pv. tomato (258, 259), pv. morsprunorum (260), pathogens of soybean, tomato, and Prunus spp., respectively. Recently a plasmid (pPT23A) was shown to mediate production of coronatine in P. syringae pv. tomato (261).

Cyanobacterin. A diaryl-substituted γ-lactone, cyanobacterin [73], was isolated from the cyanobacterium, Scytonemoa hofmanni, a blue-green algae, and is a potent inhibitor of photosynthesis in higher plants (Lemna gibba, corn, peas) and algae (262). The concentration required to inhibit the Hill reaction in PS II was generally lower than that for the classic herbicidal Hill inhibitor diuron (DCMU; N'-(3,4-dichlorophenyl)- N,N-dimethylurea). The compound did not affect electron transport in PS I. The binding site of inhibition may be the D-1 quinone-binding protein (263). Altering substituents of the phenyl ring in this molecule produced less phytotoxic analogs (264).

Phaseolotoxin. Pseudomonas syringae pv. phaseolicola is the only bacterial species known to produce phaseolotoxin [74]. However, the pathogen has many hosts: bean, pigeon pea, Glycine wightii, Macroptillium atropurpurem (sirato), Dolichos spp., (yam bean) Pachyrrhizus prosus, and (mungbean) Vigna radiata (265), which demonstrates a broad phytotoxicity range. This toxin is translocated within the plant and moves to apical meristematic tissue where it retards growth and alters apical dominance (266, 267). The site of action of this compound is ornithine carbamoyl transferase, which regulates arginine synthesis (268).

Rhizobitoxine. Various Rhizobium japonicum strains produce rhizobitoxine [75], whose structure was reported in 1972 (269). Although the host of this bacterium is soybean, this compound is also phytotoxic to many other plant species. This phytotoxin is an analog of cystathionine and acts as an irreversible inhibitor of β-cystathionase which catalyzes production of homoserine from cystathionine (270). Rhizobitoxine also inhibits ethylene production from methionine (271), as does a similar phytotoxin, 2-amino-4-methoxy-3-enoic acid (28).

Stigmatellin. A myxobacteriales species, Stigmatella aurontiaca, produces an antibiotic stigmatellin [76] which also has herbicidal properties (272). This dimethoxychromone alters photosynthetic electron transport at the herbicide-binding protein and the cytochrome b_6/f complex.

Tabtoxin. The phytotoxic compound tabtoxin [77] is excreted by Pseudomonas syringae sp. tabaci, as well as other strains, and is

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Stigmatellin [76]

Tabtoxin [77]

Tagetitoxin [78]

the causal agent of chlorosis and necrosis associated with wild fire disease (273). This phytotoxin has been known for some time to inhibit GS activity (274) which was supported by the fact that supplemental glutamine reversed induction of leaf chlorosis. Later studies, however, indicated that hydrolysis of tabtoxin to tabtoxinime-β-lactam occurred and that the latter compound acted as the GS inhibitor (275).

Tagetitoxin. The bacterial phytotoxin tagetitoxin [78] is produced by Pseudomonas syringae pv. tagetis. It causes chlorosis in marigold (Tagetis patulc L.) and zinnia (Heliopsis sp.) at ng levels and has been isolated, purified (276), and structurally characterized (277). Chlorosis occurs in developing but not mature leaves (276, 278). Ultrastructural and physiological evidence indicates that the phytotoxic effects may be relatively plastid-specific (279, 280). Wheat seedlings treated with the toxin produce pigment-deficient leaves, lacking both large and small subunits of ribose 1,5,-disphosphate carboxylase (RuBPCase), and their respective plastid-encoded and nuclear encoded mRNA's (281). Furthermore, plasmid (70S) ribosomes and cytosolic (80S) ribosomes are depleted by toxin action. Tagetitoxin inhibits RNA polymerase in chloroplast extracts from pea; however, wheat germ RNA polymerase II is insensitive to the phytotoxin (282). Tagetetoxin also inhibits eukaryotic RNA polymerase III (283).

Many other phytotoxins not presented here have been structurally characterized and tested in some plant bioassay systems. Numerous other reported phytotoxin studies are in the "active fraction" stage and structure elucidation of the active components has not been achieved. Nevertheless, from the data presented, it can generally be concluded that: diverse microbes produce a broad range of chemistries with phytotoxic activities; chemical and biochemical synthesis of almost all of these compounds is unknown (but derivitization studies to alter phytotoxic activity have been used in a few instances); knowledge of the spectrum of species susceptible to these compounds is incomplete (many weed and crop species have not been tested; some compounds are toxic to organisms other than plants); and the molecular mode of phytotoxin action is unstudied or unknown for most. The major exception is the vast amount of data on bialaphos, phosalacine, and phosphinothricin, which have achieved commercial status.

Herbicide, Bioherbicide, and Microbial Phytotoxin Targets

From the survey of microbial compounds with phytotoxicity, it can be noted that mode of action studies are generally lacking. However, mode of action and molecular target site investigations generally occur only after a particular compounds has reached market status. As pointed out previously, most herbicides have been discovered using random screening programs rather than from an applied rational approach to herbicide design, target, and synthesis. A few attempts to rationally design herbicides chose inhibition of sites of photophosphorylation uncouplers (284), glycolate oxidase (285), oxidation of indoleacetic acid (IAA) by peroxidase (286), and secondary plant metabolism, i.e., phenlyalanine ammonia-lyase (287)

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

and shikimate dehydrogenase (288). Some inhibitors designed for these sites were effective but none of the compounds reached commercial herbicide development. It is very probable that other unpublished rational approaches to herbicide development have ended in a similar manner. A variety of somewhat general targets for herbicidal action have been known for some time (289) and knowledge of herbicide (or inhibitor) action implicates some of these general and specific sites to be altered by certain compounds (290-293) (Figure 6). Changes in the biochemistry at some of these sites, when affected by adequate herbicide (or inhibitor) concentration, can lead to plant death. Natural evolutionary processes in plant pathogens have resulted in a 'rational' or selective design of phytotoxins for certain plants and molecular sites within plants. Thus, these and other as yet uninvestigated sites may be targets for these naturally occurring compounds. Much more data correlating molecular action with chemical structure will be necessary to fully utilize the rational herbicide design approach. Correlations of mode of action data of synthetic herbicides with similiar microbial phytotoxin studies should yield valuble new information that could lead to novel herbicides. Presently the molecular sites of action of many currently marketed herbicides are known and data on their physiological and biochemical interactions and secondary effects in plants is accumulating (31,290-293).

Microbes and Microbial Products as Herbicides: Other Aspects

General Considerations. If microbes (pathogens) and microbial products (from pathogens and non-pathogens) are to be successful in the commercial herbicide arena, several criteria must be met. The pathogenic organism must be highly virulent, economically feasible to produce, have an acceptable shelf-life, and be effective in an range of environmental conditions. Some success with pathogens has been made [ex. Collego and DeVine (23)] and there are many potentially useful pathgens that can control various weeds when applied augmentatively (294-296). Production of bioherbicide organisms (spores, mycelia) can become a problem when scaling-up from laboratory to industrial production. With fungi (mycoherb-icides) problems of production are dependent on the growth habit of individual species (297).

One of the main problems in this area with regard to fungal pathogens is that of providing proper environmental conditions, especially a dew (or water) period which ensures spore germination and infection (or infection if mycelial preparations are applied). With bacterial weed pathogens, the problem is again moisture and wounding. A second problem in pathogen formulation is getting the organism to adhere to plant surfaces. Some successful answers to both these problems of formulation have been made as summarized in reviews (297, 298).Application technology for microbial weed pathogens is somewhat analagous to herbicide surfactant and additive technology (299); not that these technologies are at the same stage, but that herbicide potential can be increased with their use. More chemical research will be needed to provide formulations for application of pathogens for weed control.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

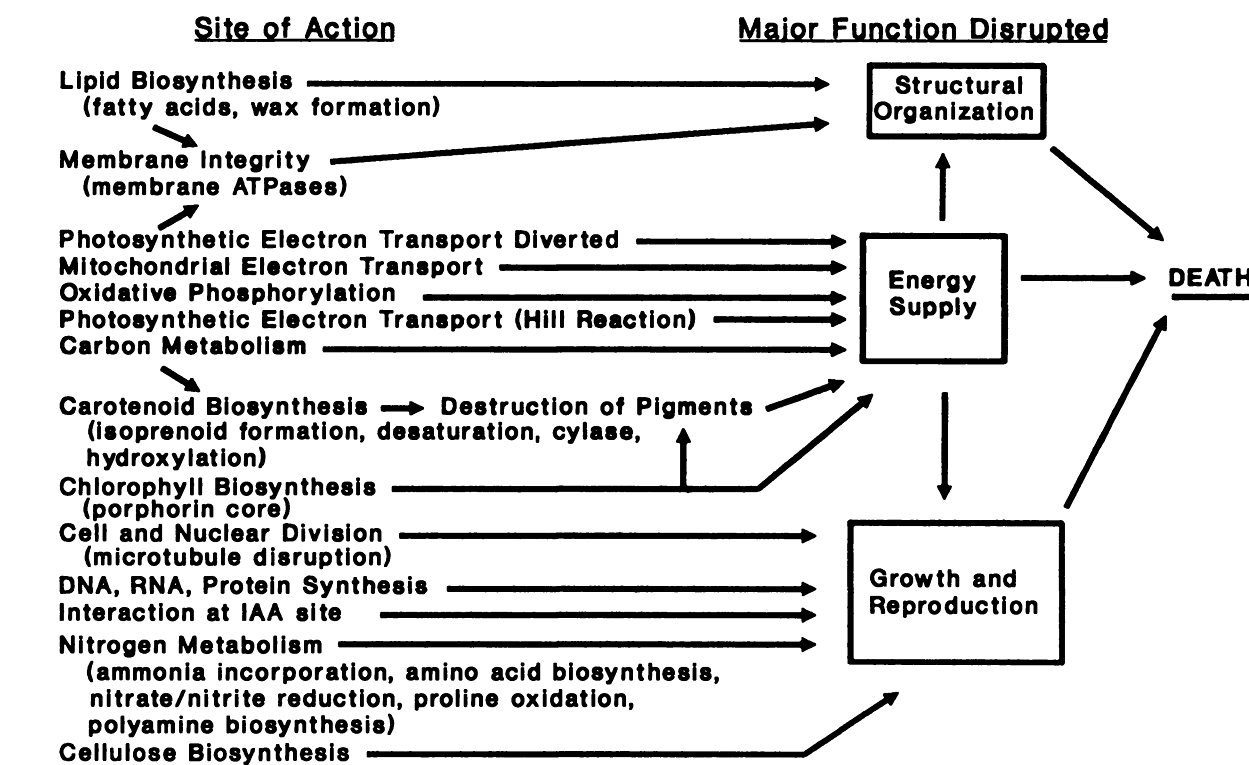

Figure 6. Possible targets for herbicides, microbial phytotoxins, and/or pathogens. (Based on a diagram in Ref. 289. Copyright 1974 Academic Press and supplemented with information in Refs. 290-292).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch001

Genetic and Strain Selection of Microbial Candidates. As previously presented, bialaphos and phosphinothricin strain selection technologies, genetic transformation, and fermentation methodology is unsurpassed in the microbial production of herbicides. These approaches should be applied to some of the more promising microbial pathogens to produce highly efficacious weed pathogens or to microbes for the overproduction of potent toxins for use as bioherbicides. Some aspects regarding this have been presented (300, 301). Success in pathogenicity gene isolation and cloning (302, 303) and transformation methodology (304) of plant pathogens will ensure major advances in microbial bioherbicide technology. Advances in the area of restricting host range of pathogens with broad-specificity have been made and are in progress. The selection of a non-sclerotial mutant of *Sclerotina sclerotiorium* which is a ubiquitous, non-specific pathogen has great utility in bioherbicide advances (305). Strain selection and genetic manipulation can be time-consuming and the pay-off may be uncertain or long-term.

Biochemical/Chemical Considerations. Biochemical analysis of plant defense mechanisms and of pathogen infection processes have been extensively studied for some time, however these studies have almost all been on crop plant-pathogen interactions. Various enzymes are active in these interactions (306) but, these processes have essentially been neglected in weed-pathogen situations. There is a need to examine such mechanisms in weeds and microbial bioherbicides so that chemical regulators (specific enzyme inhibitors, herbicides, PGR's, etc) can be identified and used to augment pathogen or phytotoxin efficacy in weed control. Such studies should obviously be closely correlated with toxin and herbicide mode of action; even secondary herbicide action may play major roles in regulation of pathogen efficacy.

Conclusions

The wide range of chemical classes of phytotoxins from microbes indicates that microbial products from plant pathogens and non-pathogens are unique sources for potential bioherbicides and as templates for new synthetic herbicides. However, several problems hinder their full development into practical weed control weapons. Often, only small amounts of these new chemistries have been isolated, or are isolable, reducing expanded research by cooperators in other academic disciplines and limiting the size of bioassay and screening programs. Furthermore, the unique chemistries involved may not easily fit into commercial synthesis programs; synthesis routes may be difficult, time-consuming and costly. Fermentation processes to achieve large quantities of phytotoxins or organisms may be complex due to the fastidious nature of some organisms. Further research on the molecular mode of action, the activity spectrum, persistence, and non-target effects needs to be accomplished. Cooperative efforts of chemists, biochemists, biologists, plant pathologists, and weed scientists will be required to achieve this goal. Political and social acceptance of this new technology using these complex and dynamic agents (microbes) as agents of weed control will also be necessary (307).

Acknowledgments and Disclaimers

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Chapter 2

Maculosin

A Host-Specific Phytotoxin from *Alternaria alternata* on Spotted Knapweed

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A strain of Alternaria alternata on spotted knapweed produces a family of diketopiperazines including maculosin, which is an L-tyrosine-L-proline analog. Maculosin is a host-specific toxin of Centaurea maculosa L. (spotted knapweed), the only one currently known from a weed pathogen. Current studies are underway to isolate and characterize a cytosol (soluble) protein receptor for maculosin in spotted knapweed. Affinity column chromatography employing maculosin covalently bonded to Sepharose 6-B has been used successfully to isolate an active protein fraction. Another phytotoxin produced by A. alternata, tenuazonic acid, acts in a synergistic manner with maculosin. Phytotoxin production in A. alternata seems to depend on one or more host plant metabolites.

Each weed species is a host to an array of plant pathogenic fungi and bacteria. Many of these organisms induce disease symptoms by virtue of phytotoxin production (1). By focusing on these plant pathogens and on their phytotoxins we hope to find novel, environmentally compatible, host-selective chemical agents for weed control.

In the past several years a number of investigations on the chemistry of phytotoxins from weed pathogens has uncovered novel structures. The biological activity of these compounds varied from non-selective to narrow host range phytotoxicity (2, 3, 4, 5, 6). An ideal phytotoxin from a weed pathogen would have the unique property of expressing host specificity at the species

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or cultivar level. To date, however, phytotoxins with this extreme specificity are known only from crop plant pathogens (1,7). Agroecosystems with a strictly homogeneous genetic base, typical of crop plants, may serve as a huge reservoir of essentially identical plant material. A pathogen attacking such a crop, under the proper environmental conditions, has the potential to produce an epidemic. Such an organism can be easily observed, isolated and examined for phytotoxin production. Common weed species, however, usually exist in a population with a broad, mixed genetic base, and are often intermingled with other plants. This precludes the development of such an epidemic and reduces the probability of discovering a weed pathogen capable of producing host specific toxins.

At the outset of this study we chose to examine Centaurea maculosa L. (spotted knapweed), a species of immense economic importance in the northwestern United States. This plant threatens forage production of millions of acres of rangeland in this region (8). It also has invaded choice recreational lands of Yellowstone and Glacier National Parks. After an intensive search for diseased knapweed plants throughout Western Montana, a severely diseased plant was found in the environs of Butte, Montana (9). Approximately 30% of the leaves were blighted with large black-brown necrotic lesions, and at least 90% of the flowers showed decay and extensive black mycelial growth. Three different fungi were isolated from the lesions and placed into pure culture by the single spore isolation technique (9). Subsequently, each was tested for its ability to reproduce disease symptoms when inoculated onto a spotted knapweed plant (9). Only one of these fungi proved to be pathogenic and satisfied all of the criteria of Koch's postulates (9). It was identified as Alternaria alternata. Although various form species of A. alternata attacking crop plants produce host specific toxins, none has been isolated and identified that selectively attacks weed species (10). Mass liquid cultures of our isolate of A. alternata were analyzed to determine its ability to produce phytotoxins selective to spotted knapweed.

Isolation and Identification of Diketopiperazines

The phytotoxic ethyl acetate soluble extracts of the fungal culture were chromatographed on Bio-Beads S-X4 (hexane-CH_2Cl_2-EtOAc, 4:3:1, v/v). The resulting fractions of this chromatography, and all subsequent chromatographies, were tested by the standard leaf puncture bioassay using spotted knapweed leaves (2). Fractions four, five, and six were phytotoxic. Size exclusion chromatography of fraction five on Sephadex LH-20 (CH_2CL_2-MeOtBuiPrOH, 1:1:1, v/v) yielded maculosin, 1. Similar treatment of fraction six gave a mixture, which was resolved by centrifugal countercurrent chromatography ($CHCl_3$MeOH-H_2O, 25:34:20, v/v, lower phase mobile) to compounds 2 and 3. Size exclusion chromatography of fraction four on Bio-Beads S-X8 (CH_2Cl_2-cyclohexane, 3:2, v/v) yielded diketopiperazine 4.

The dichloromethane soluble extracts were permeated through Sephadex LH-20 (CH_2CL_2-MeOH, 1:1, v/v). Centrifugal countercurrent chromatography as described above yielded compounds 5, 6, and 7. Table I summarizes the isolation scheme for diketopiperazines 1-7.

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1

2

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6

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Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch002

Table I. Summary of Isolation Data, Diketopiperazines 1-7

Compound	Molecular Weight/Formula	Isolation Method[a]	Rt[b]	% Yield[c]
1	260/$C_{14}H_{16}N_2O_3$	Sephadex LH-20[d]	2.10	0.74
2	244/$C_{14}H_{16}N_2O_2$	CCC[e]	0.38	0.39
3	244/$C_{14}H_{16}N_2O_2$	CCC[e]	0.45	0.44
4	224/$C_{12}H_{20}N_2O_2$	Bio-Beads S-X8[f]	1.33	0.23
5	196/$C_{10}H_{16}N_2O_2$	CCC[e]	0.70	0.10
6	210/$C_{11}H_{18}N_2O_2$	CCC[e]	0.63	0.23
7	168/$C_8H_{12}N_2O_2$	CCC[e]	0.56	0.08

[a]Final step in purification
[b]Retention time in hours
[c]% yield = mass of compound obtained/mass of total organic extract x 100
[d]Column 2x135cm; eluant CH_2Cl_2-MeOtBu-iPrOH (1:1:1,v/v); flow rate 1.9 mL/min
[e]Column volume 285 mL; eluant $CHCl_3$-MeOH-H_2O (25:34:20,v/v); flow rate 4 mL/min; lower phase mobile
[f]Column 2.5x140cm; eluant CH_2Cl_2-cyclohexane (3:2,v/v); flow rate 1.5 mL/min

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch002

Only compounds 1 and 2 were phytotoxic to spotted knapweed; 1 was at least an order of magnitude more toxic than 2. The molecular formula of 1 was determined to be $C_{14}H_{16}N_2O_3$ by high resolution mass spectrometry. Phenolic and amide functional groups were indicated by infrared absorptions at 3590, 3380 and 1670 cm^{-1}, respectively. Detailed analyses of the mass spectral fragmentation and ^{1}H-NMR data resulted in the proposal of structure 1. The absolute stereochemistry was established by the synthesis of cyclo(L-Pro-L-Tyr) from the t-BOC derivative of L-proline and the methylester of L-tyrosine following literature procedures (10). This procedure is known to proceed without racemization. The synthetic and natural materials were identical by ^{1}H-NMR, mass spectrometry, optical rotation and phytotoxicity (9).

^{1}H-NMR and mass spectrometry revealed that 2 and 3 were stereoisomers of composition $C_{14}H_{16}N_2O_2$. Infrared spectroscopy indicated an amide moiety and ^{1}H-NMR decoupling provided the gross structure cyclo-Pro-Phe. Comparison of the ^{1}H-NMR spectra and optical rotation with those of synthetic materials led to the identification of 2 as cyclo(L-Pro-L-Phe) and 3 as cyclo(L-Pro-D-Phe) (9).

The remaining four diketopiperazines, 4-7, were identified in the same manner: molecular formulae by mass spectrometry and structural details from IR, mass spectral fragmentations and ^{1}H-NMR decoupling experiments.

Maculosin-Structure-Activity

Once the seven diketopiperazines had been isolated and identified, they were examined for phytotoxicity. The compounds were first applied to knapweed leaves and hypocotyls following the described procedures (2). The most active compound was 1, cyclo(L-Pro-L-Tyr), which produced lesions at 10^{-3}, 10^{-4}, and 10^{-5} M. At 10^{-6} M a slight necrotic fleck appeared in 72 hours, but this was not considered active by our standards. Compounds 2 and 3, cyclo (L-Pro-L-Phe) and cyclo (L-Pro-D-Phe), differed in phytotoxicity: the L, L diastereomer induced necrotic lesions on knapweed leaves at 10^{-4} M, but the L, D isomer was not active, even at 10^{-3} M. Compounds 4, 5, 6 and 7 were not active toward knapweed at any of the test concentrations. These tests were repeated with hypocotyl tissue, and similar results were obtained, although in 47% of these tests, cyclo (L-Pro-L-Tyr) induced lesions at 10^{-6} M.

Comparison of the diketopiperazines isolated and tested in this study suggests that certain functional groups are necessary for activity. Maculosin, the most active substance, possesses a phenolic moiety, not uncommon in phytotoxins. The minor activity of 2, cyclo(L-Pro-L-Phe), compared to the inactivity of its diastereomer, suggests the importance of conformation to bioactivity. Studies on diketopiperazines containing proline clearly showed that L, L compounds assume an extended conformation rendering both the aromatic moiety and its hydroxyl group accessible for interaction with a biological site (11, 12). It is noteworthy that the yields of the various diketopiperazines were quite consistent in repeated fermentations; maculosin was always the most abundant of the dipeptides.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch002

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch002

Maculosin-Bioactivity

At this point, maculosin had been established as a phytotoxin of spotted knapweed, but its host selectivity or specificity needed to be established. A test designed to establish the host-range of the toxin utilized the simple leaf assay with a wide variety of plants. Both monocots and dicots were included in the test range, including several composites. The assays were run at 10^{-3}, 10^{-4}, and 10^{-5} M. Maculosin consistently induced necrotic lesions on knapweed leaves, but in no case was lesion induction observed on any other test plant. The nineteen test plants were chosen at random, although an attempt was made to include several composites and plants known to be hosts to other form species of Alternaria alternata. Of particular interest is the recent observation that other species of Centaurea, including C. diffusa, C. cyanus, and C. repens, were not affected by synthetic maculosin in leaf bioassay tests at 10^{-4}M. These host-range tests, while not exhaustive, clearly establish maculosin as the most host specific weed phytotoxin on record (see Table II).

Throughout this study we used knapweed test plants grown from seeds collected from several geographic locations. Some diversity was seen in the susceptibility response of plant from different locales. Knapweed reproduces, in typical composite fashion, from wind-borne seeds carried from the parent plant. Seeds collected from plants within a twenty foot radius of the original diseased specimen were most affected by maculosin. Indeed, plants grown from seeds of these plants actually exhibited necrotic lesions at 10^{-6} M in leaf bioassay tests. Knapweed seeds were also collected from various locations in Gallatin County and the Bitterroot Valley, Montana. Plants grown from these seeds were used in the assays cited in Table II. One plant was actually somewhat resistant to maculosin: necrosis was only induced at 10^{-3} M. These results were expected, since wide genetic diversities among populations of this plant most likely exist. Such diversities would be expressed in the susceptibility range of the plant to macul sin.

Maculosin - Mode of Action

Understanding the mode of action of a host-specific toxin is of interest. It might provide insight into the development of plant pathogen-host plant dynamics (1). Also, the nature, location, and function of the toxin receptor-site might be discovered. This could eventually lead to a molecular genetics approach to weed control. A critical step in doing such studies is to have available isotopically labelled toxin possessing a high specific radioactivity. Commonly, labelled toxin is prepared via the administration of appropriate precursors to cultures of the pathogenic fungus. Ultimately, some small quantity of the labeled toxin is obtained which has a specific radioactivity one or more orders of magnitude less than that of the precursor (low specific radioactivity). Methods for obtaining toxins of an extremely high specific radioactivity may involve organic synthesis using

Table II. Summary, Host Specificity Screening of Maculosin[a]

Maculosin (1): Concentration (M)[b]	Natural 10^{-3}	10^{-4}	10^{-5}	Synthetic 10^{-3}	10^{-4}	10^{-5}
DICOTS:						
Centaurea maculosa (spotted knapweed)	+++	++	+	+++	++	+
Lactuca sativa (lettuce)	-	-	-	-	-	-
Citrus limon (lemon)	-	-	-	-	-	-
Lycopersicon esculentum (tomato)	-	-	-	-	-	-
Malus sylvestris (apple)	-	-	-	-	-	-
Helianthus annuus (sunflower)	-	-	-	-	-	-
Cucumis sativus (cucumber)	-	-	-	-	-	-
Euphorbia esula (leafy spurge)	-	-	-	-	-	-
Bidens pilosa (marigold)	-	-	-	-	-	-
Taraxacum officinale (dandelion)	-	-	-	-	-	-
Artemisia tridentata (sagebrush)	-	-	-	-	-	-
Cirsium arvense (Canadian thistle)	-	-	-	-	-	-
Euphorbia milii Desmoul. (crown of thorns)	-	-	-	-	-	-
MONOCOTS:						
Sorghum halapense (johnsongrass)	-	-	-	-	-	-
Poa annua (bluegrass)	-	-	-	-	-	-
Avena sativa (park oat)	-	-	-	-	-	-
Agropyron repens (quackgrass)	-	-	-	-	-	-
Digitaria ischaemum (crabgrass)	-	-	-	-	-	-
Zea mays (corn)	-	-	-	-	-	-

[a]+++: weeping necrotic lesion > 4 mm from inoculation site; ++: necrotic lesion 2-4 mm; +: necrotic lesion 0.5-2 mm; -: no lesion present or fleck < 0.5 mm.
[b]in molarity (M)

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precursor compounds possessing high specific radioactivity. ^{14}C-maculosin was synthesized following the same protocol as before, using ^{14}C-L-tyrosine as a precursor (10). ^{14}C-maculosin was purified by preparative TLC on silica gel plates. The final product has identical spectral, chromatographic, and biological properties as that of the natural product (9). Preparations of synthetic maculosin made in this manner have a specific radioactivity of 0.025 μCi/μmole. Much higher specific activities are possible, especially if both ^{14}C-tyrosine and ^{14}C-proline are used as starting materials.

Three to four days after the application of ^{14}C-maculosin to an unwounded leaf surface there is little or no movement of radioactivity from the inoculation site. Even in a previously wounded leaf, there is only slight movement of labelled material from the point of application. However, there was uptake of radiolabel by knapweed roots suspended in a solution of ^{14}C-maculosin. These initial studies have provided useful leads for the practical application of maculosin as a knapweed control agent: derivatization of the compound may be necessary for entry and distribution of maculosin in the plant.

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Maculosin Binding

A phytotoxin may require binding sites within a plant in order to affect it adversely (1, 14). In the case of maculosin, a host specific toxin, a very specific receptor site may exist in spotted knapweed. Such a site may be a protein that has a normal role in the maintenance of cellular function, but coincidentally serves as a phytotoxin receptor (14, 15). Binding of the toxin by the receptor may result in a disruption in the functional role of the protein or result in a cascade effect involving other proteins. Either effect could result in cellular death (1, 11).

In preliminary studies, the binding of ^{14}C-maculosin was noted in the cytosolic (soluble) fraction of a spotted knapweed leaf extract, and little if any in the insoluble fraction. Binding activity was reduced or destroyed by treatment of the soluble fraction with heat or proteases, suggesting that the toxin receptor is a protein. Purification of the receptor has been pursued successfully using size exclusion column chromatography combined with affinity chromatography. The affinity support consists of epoxy activated Sepharose 6B to which synthetic maculosin has been attached. Further work should reveal the role of the receptor in the process of toxin-induced symptom production.

Conversion of Maculosin

Three to five days after treating spotted knapweed leaves with ^{14}C-maculosin, and after necrotic symptoms appear, virtually all of the ^{14}C-maculosin is converted to at least 3 other compounds. These compounds are insoluble in ethylacetate and soluble in methanol. It will be interesting to elucidate these compounds and to learn if any of these compounds bind to the maculosin receptor. Furthermore, it is conceivable that maculosin, itself, is not toxic

to the plant, but that one or more of its derivatives is the actual toxin. If this is true, it would mean that the plant has the capacity to destroy itself via the enzymatic conversion of maculosin to one or more toxins.

Other Phytotoxins of *Alternaria alternata*

Besides diketopiperazines, there are two other classes of phytotoxins produced by *A. alternata* on spotted knapweed: tetramic acids and perylenequinones (16). Both groups of compounds are non-host specific and require relatively large concentrations for biological activity to be expressed, eg. 10^{-3}-10^{-4}M (16). These observations, however, clearly illustrate the fact that plant pathogens can produce multiple phytotoxins of varying specificity. These phytotoxins may also interact. In fact, tenuazonic acid in the presence of maculosin caused a toxic-synergistic reaction at 10^{-5}M (16).

We have also observed that the production of these phytotoxins by *A. alternata* is controlled by metabolites from the host plant (9, 16). The fungus is always grown with an effusion of the host plant added to the culture medium. Elimination of the extract from the broth results in little or no phytotoxin production, in spite of abundant mycelial growth. Other fungi also seem to be dependent upon host plant products to activate phytotoxin production (17, 18).

Future Work

Maculosin is the first compound to be isolated, characterized, and synthesized that has the properties of a host specific toxin from a weed pathogen. Many questions can now be posed relative to its production by the fungus, its receptor(s) in the plant, its derivatization within the plant, its mode of action, and its interaction with other toxins.

It is now the time to seek more earnestly other fungal pathogens of weed species that may also produce host specific phytotoxins. These are likely to be found in the fungal genera *Dreschlera* and *Alternaria*, but others are certainly not to be excluded. More work on the chemistry and mode of action of these compounds will not only allow a deeper understanding of host plant-parasite interactions and fungal phytotoxicology, but also provide potential new chemical leads for environmentally compatible, specific herbicides.

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Chapter 3

Biochemistry of Non-Host-Selective Phytotoxins

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Many plant pathogens produce non host-selection toxins as part of their attack mechanisms. The benefical application of these toxins to agricultural problems requires knowledge about their structure, metabolism, regulation and mechanism of action. Such knowledge is still quite meager except for structural studies and, to a lesser extent, mechanism of action. The majority of bacterial toxins are small, modified peptides, plant growth hormones, or high molecular weight polysaccharide-like molecules. Fungal toxins are structurally more diverse and, correspondingly, involve a wide variety of biogenetic pathways. Some toxins are activated in the host by hydrolytic cleavage; whether this is commonplace or not is unknown. Even though these toxins have no plant selectivity, they are are highly selective in how they act (i. e., inhibit specific enzymes). Since many pathogens also contain these targets, they possess multiple mechanisms for protecting themselves against their own toxins.

Practically all phytotoxins are non-selective, that is, although the pathogens that produce them may selectively attack only certain plant species, this is not determined by the toxins. Usually, in fact, preparations of these toxins will affect almost any higher plant species as well as other eukaryotic and prokaryotic organisms. On the other hand, the subcellular sites where these toxins act are in most cases highly specific and are unique for each toxin. In fact, the degree of biochemical specificity they exhibit is every bit as great as that of the host-selective toxins. The only difference between the two groups is that the targets of non host-selective toxins are more widely distributed among plant species (Table I).

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Table I. A Classification Scheme of Phytotoxins Based on Their Proposed Site of Action

Phytotoxin	Proposed site of action[a]
Fumaric acid	OH, NH_2, SH groups
Tabtoxinine-β-lactam	glutamine synthetase
Fusicoccin	plasmalemma ATPase
Tentoxin	coupling factor 1
T-toxins	mitochondrial membrane
AAL-toxins	aspartate carbamoyl-transferase

SOURCE: Reproduced from reference 29.

[a]These examples cover the range in non host-selective toxins from no specificity (fumaric acid) through those that are site specific, but have targets in microbes and animals too (T-β-L), only plants (fusicoccin), or selected plant species (tentoxin). Host-selective toxins (T- and AAL- toxins) have targets biochemically similar to those of advanced non host-selective toxins, but their distribution is more highly restricted.

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The usual symptoms elicited in the host by phytotoxins, to name the most common, are chlorosis, necrosis, wilting, water soaking, stunting, growth abnormalities and root formation. A special word about phytotoxins that induce root formation and growth abnormalities. I mean to include here not only those compounds that are synthesized solely by microorganisms, but also those that are also products of higher plants (e. g., auxins, gibberellins and cytokinins). The critical points here are that their synthesis must be carried out by the pathogen and that it is this production that is responsible for the growth abnormality. Thus, just because a compound is synthesized by a higher plant is no reason a priori to disallow it as a phytotoxin.

Unfortunately, symptom expression can tell us very little about a toxin's mode of action, since each symptom can result from toxins acting at very different locations in the complex physio-chemical events of metabolism. Furthermore, the biochemical means by which any one of these symptoms can be produced are many and complex, and can involve quite different pathways.

We are most aware of symptoms that are visible above ground, hence almost all of our attention has been on toxins of aerial pathogens. It's quite clear though that soil-borne microorganisms also produce toxins. Some of these are now being examined in more detail for their ability to selectively kill or slow the growth of weedy plant species (1 and Chapter 15). I suspect that their study will unveil some quite exciting and unusual structures and mechanisms of action.

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Although many phytopathogenic fungi and bacteria—some would say all—synthesize phytotoxins, we have, overall, only very limited information on their structure, biosynthesis, regulation and mechanism of action. Most of our information comes from a relatively few well-studied cases (2). Hence, we can expect there will be much more information available in the near future, since there is an increasing interest in phytotoxins by scientists from many diverse disciplines.

Most phytotoxins have low molecular weights (<1,000) and are synthesized by soluble enzyme systems. A few though, such as HC-toxin, appear to be synthesized on a template much like peptide antibiotics (3), while others, like cerato-ulmin which contains 128 amino acid residues, require a mRNA template and are synthesized in the same manner as proteins.

Phytotoxins are classical secondary metabolites and as such possess all the characteristics one would expect of such compounds. They are: a) structurally heterogeneous, b) contain a variety of functional groups, c) produced by a restricted number of microorganisms, d) produced at specific phases in the growth cycle, e) highly dependent on nutritional and environmental conditions for synthesis and f) not readily metabolized.

The interrelationships between phytotoxins, as secondary metabolites, and primary metabolism is little understood even at the physiological level. Indeed, most of what we do know has come from mimicking studies previously carried out with classical antibiotics. These suggest that phytotoxin synthesis is regulated similarly to that of antibiotics.

The biosynthetic pathways along which phytotoxins are synthesized, particularly in fungi where they have been most extensively studied, are well known (2). Fungal toxins are much more diverse in this regard, being derived from: a) the shikimic acid pathway, b) the TCA cycle, c) the fatty acid pathway, d) a branch of the acetate-polymalonate pathway, e) the acetate-mevalonate pathway, f) various amino acid pathways branching off from the shikimic acid pathway, glycolysis and the TCA cycle, and g) combinations of two or more of the preceding pathways. Bacterial toxins are synthetically more simple and can be viewed as products of either (f) plus derivatization, or (g).

I'd like to illustrate some of these general points from work done in my laboratory on toxins that cause chlorosis, emphasizing points that relate to their potential use in biological control.

Tabtoxinine-β-Lactam

HN, O, OH, CH_2, CH_2, CH_3, CHOH

H_2N-CH-CO-NH-CH-CO_2H

Figure 1. Tabtoxin.

The first really molecular study of phytotoxins was done at the Rockefeller Institute, starting in the late 1940's by a physiologist-biochemist team, Braun and Woolley, who worked with Pseudomonas syringae pv. tabaci. This bacterium produces the so called wildfire toxin, the name coming from the rapidity with which chlorosis is induced by the pathogen producing toxin in the leaves of the natural host, tobacco. They proposed a structure for this toxin, and because of its presumed structural and physiological resemblances to methionine sulfoximine, a recently discovered compound that interfered with methionine metabolism, they hypothesized that wildfire toxin acted in a similar, if not identical, manner.

I started work some years later on what appeared to be a somewhat similar toxin produced by a related pathovar of Pseudomonas syringae attacking oats. But this toxin seemed to be different; structurally it was a dipeptide composed of threonine and/or serine and a second unknown amino-containing component (4,5). Also, it required light for activity (6), a necessity which would not pertain if the toxin directly affected methionine metabolism. However, additional work eventually led us to the realization that the two toxins were indeed the same, and furthermore that many other closely related bacteria selectively attacking bean, maize, pea, wheat, coffee, soybean, or weedy grasses also produced the toxin. First, Stewart (7) and then our group presented the correct structure of the wildfire toxin (8). We now know that this toxin is really a mixture of two analogs, one containing threonine (Figure 1) and the other serine. They are now properly called tabtoxin, mol. wt. 289, and (2-serine)-tabtoxin, mol. wt. 275, respectively. Furthermore, both really are pretoxins because they themselves have no biological activity. Certain strains of the pathogen and plants as a whole contain peptidases which cleave off the serine/threonine, releasing the true biologically active component, tabtoxinine-β-lactam (T-β-L) 2-amino-4-(3-hydroxy-2-oxoazacyclobutan-3-yl)butanoic acid, mol. wt. 188 (9).

Data from specific carbon-labeling experiments have shown that the 6-carbon backbone of T-β-L arises from the condensation of a modified aspartate and a 2-carbon fragment from carbons 2 and 3 of pyruvate. Synthesis of the backbone constituents occurs via the 6-phosphogluconate or Entner-Doudoroff pathway, probably in much the same way as lysine is formed. The β-lactam ring carbon comes from a C-1 metabolic pool (10); additional work has shown that this carbon is provided by the methyl group of methionine (11). This is quite interesting because it shows that β-lactam ring formation in T-B-L proceeds differently from that of the fused β-lactam rings of the penicillins and cephalosporins. The β-lactam ring is readily converted via intramolecular translactamization at neutral to basic pH's to the δ-lactam form; this molecule has no biological activity. This reaction occurs both in culture and in the infected plant.

Rather that interfering with methionine metabolism, T-β-L is a specific, site-directed inhibitor of glutamine synthetase (GS), a critical enzyme for nitrogen assimilation (12). The widespread

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requirement of this enzyme for living organisms explains the equally broad spectrum of activity this toxin has. Actually, wildfire toxin affected not only all the higher plant species we tried, but was toxic to fungi, bacteria, lower plants and vertebrates as well. Interestingly, pv. tabaci itself is not affected by T-β-L, even though its GS is sensitive in vitro (13). As yet, we do not fully understand the reasons for this except to say that the bacterium possesses several potential mechanisms for protecting itself against the toxin (14). Which ones actually function in vivo we cannot presently say.

Tentoxin

Figure 2. Tentoxin.

There are a variety of mechanisms by which toxins can cause chlorosis besides that of tabtoxinine-β-lactam. This is illustrated by tentoxin, cyclo(-L-MeAla-L-Leu-MePhe[(Z)Δ]-Gly-), mol. wt. 414 (Figure 2), which is produced by the fungus Alternaria alternata. It causes chlorosis via a inhibition of chloroplast coupling factor one (CF_1) (15). This is a particularly interesting case because the toxin shows an apparently unusual host selectivity: depending upon the plant family, it may affect all species, all species within certain genera, or selected species within a genus (16). The exact reason(s) for this are unknown, but we have hypothesized that host selectivity is determined by the form of CF_1 the particular plant contains (17). One form tightly binds tentoxin; species having this form are sensitive to the toxin and become chlorotic. The second form does not bind tentoxin to any degree; species having this form are resistant to the toxin and remain green. In support of this idea, reciprocal crosses were made between sensitive and resistant Nicotiana species. Since CF_1 is maternally inherited, we expected that the reaction of the F_1 progenies to tentoxin would be identical to that of the maternal parent. Such was the case (Table II) (18). Sensitivity also is absolutely correlated with the in vitro response to tentoxin of the maternally inherited form of CF_1; that found in resistant species is unaffected by tentoxin at levels 10,000-fold higher than is required for 50% inhibition of the CF_1 from sensitive species (19). Also, when CF_1-depleted chloroplasts from sensitive species are reconstituted with CF_1's from resistant species, cyclic photophosphorylation is protected against tentoxin. However, when CF_1's from sensitive species are used in the reconstitution, the reaction is sensitive to tentoxin (20).

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Table II. Reaction of Seedlings of Interspecific Nicotiana Hybrids to Tentoxin (20 µg/ml)

Hybrid			Reaction [a]
solanifolia	x	knightiana	+
knightiana	x	solanifolia	–
cordifolia	x	tabacum	+
tabacum	x	cordifolia	–
raimondii	x	knightiana	+
knightiana	x	raimondii	–

[a] A + sign denotes leaf chlorosis; a – sign denotes a normal appearance of the plant.

Presumably, at some point in plant evolution, a mutation occurred within one of the structural genes encoding CF_1 that rendered it sensitive to tentoxin. Subsequently, these two forms of the protein were passively distributed among later-evolving plants. How such plants originating beyond this point have evolved is therefore mirrored in their reaction to tentoxin.

Additional targets of tentoxin have been proposed (21-23). However, whether or not there is a common denominator to all these putative targets, whether one or more of these proposals is in error, or whether there are indeed multiple and distinct targets of this toxin remains to be elucidated.

The [D-MeAla] analog of tentoxin has been shown to exist in multiple conformations in solution, which differ in their ability to inhibit coupled election transport in chloroplasts (24). This finding is the first demonstration that conformers of a molecule can have different biological activities, and points to the idea that this kind of behavior might be usefully considered in the design of biocides.

Tagetitoxin

Figure 3. Tagetitoxin.

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Tagetitoxin represents yet another kind of chlorosis-inducing toxin. Produced by Pseudomonas syringae pv. tagetis in necrotic leaf spots, this toxin is then effectively translocated to the host's apex where it causes a very characteristic chlorosis. The lower leaves and petals appear outwardly unaffected. This is because the apex is rapidly making proteins, whereas the lower leafs have a much lower rate of protein synthesis and thus are less affected. Provisionally, tagetitoxin has been assigned a very unusual structure, a substituted 9-oxa-3-thiabicyclo [3.3.1]nonane, mol. wt. 416 (Figure 3) (25). Its mechanism of action is also unusual. In intact plants, it selectively inhibits the action of chloroplastic RNA polymerase (26), but appears to have no affect on the other RNA polymerases contained in the nucleus and mitochondrium (Mathews, D. and Durbin, R. D. J. Biol Chem., in press.) However, in vitro it inhibits nuclear RNA polymerase III and to a much less extent polymerase II, but at higher concentrations than in the case of in planta chloroplastic RNA polymerase inhibition. Polymerase I though is not affected. This spectrum of activity is unique. Thus, tagetitoxin may prove to be a very valuable metabolic probe as well as a potential therapeutic agent. It also might be useful as an herbicide. Furthermore, if the reason(s) why the toxin can traverse the plasmalemma and chloroplast envelope but not the nuclear membrane were known, it might be very valuable information for the design of organelle-specific biocides.

Interestingly, in culture most isolates of the bacterium do not produce tagetitoxin; however, the addition of specific amino-containing compounds will "turn on" its production. Other such examples are known, which points up the importance of providing the proper nutritional and enviromental conditions (e.g., temperature, micronutrients, pH, C or N source) in screeing programs.

Concluding Remarks

I suspect that the synthesis of many non host-selective toxins by pathogens have been chance events. As Turner (27) has stated, the origins of toxic and non-toxic metabolites, "are often so similar that the possession of antibiotic activity must be regarded as a fortuitous property of the product." It seems this probably comes about because plants contain a multitiude of potential receptors for phytotoxins. It only remains for the pathogen to exploit this by the production of a compound that can interact with and perturb the function or structure of the receptor-containing plant molecule. Given the capability of microorganisms, expecially fungi, to synthesize a myriad of diverse, small molecules and their genetic plasticity, it is not suprising then that they produce toxins.

Once a toxin-target interaction has been established, it likely provides a selective advantage to the pathogen and thus becomes fixed in the population. That said, one may legitimately ask what advantage toxins provide. In the general sense we can argue that most toxins must have some positive survival value because the amount of genetic information required for their synthesis, regulation and for self protection mechanisms is substantial and would not be sustained unless it had positive

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survival value (28). Also, we tend as plant pathologists to believe that the ability to synthesize phytotoxins should make pathogens more successful at causing disease. However, although there are a number of examples in which investigators have shown that toxin production increases the amount of disease, Tox^- mutants still appear to multiply and survive just as well as their wild type counterparts. So one has to be careful in deciding what basis is to be used to define fitness. Perhaps also, in some cases the potential advantage of toxin production to the pathogen has to do more with its saprophytic rather than parasitic phase. As students of plant disease, we may be viewing pathogens in entirely too narrow a view.

Implicit in these thoughts is the notion that phytotoxins increase disease and therefore by definition must be produced *in planta*. One needs to remember though that many toxic metabolites are produced in culture which either are not produced *in planta* or are produced at such a low level that they are not important in the elicitation of a particular disfunction. Genetic manipulation might be used to alter this situation to benefit biolgical control.

In this regard, if one is thinking about the transfer of toxin-synthesizing genes, one also may have to consider the simultaneous transfer of self-protection genes if microrganisms possess the target. Depending upon whether these genes are clustered and/or situated near the synthetic and regulatory genes for the phytotoxins, it may be a much more different proposition to genetically engineer toxin production than it first would appear.

Toxins have evolved to take advantage of very subtle structural features of critical plant molecules, and, most importantly, the host plant cannot readily alter the target to counteract the toxin's activity. In addition, toxins many times have evolved ways that allow them to be easily transported across barriers--often times they mimic essential metabolites or illicitly utilize common transport mechanisms. There are other reasons why phytotoxins should have great interest to those concerned with herbicides: they are active at extremely low concentrations, and generally appear to be unaffected by potential degrading enzymes within the host cell; they often are novel in structure; furthermore, the amount of information we have on structure-activity relationships is limited and mostly qualitative, making it likely that much more can to be learned for our benefit; and lastly, mechanism of action studies by defining the specific targets within the cell being perturbed, pinpoint critical reactions that can be taken advantage of. Truly, non host-selective toxins can be very important sources of knowledge as well as progenitors of biocides. Since they may also inhibit organisms other than plants, they may prove to be useful in other arenas as well.

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Chapter 4

Satellite Metabolites and Synthetic Derivatives of Abscisic Acid as Potential Microbial Product Herbicides

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The recent isolation of abscisic acid (ABA) from the fungi *Cercospora cruenta* and blue light-irradiated *Botrytis cinerea* has led to renewed interest in the development of ABA and its derivatives as practical plant growth regulators. The discovery of relatively large quantities of ABA has led to two developments: first, the synthesis of ABA derivatives that have plant regulatory activity and second, the isolation and identification of satellite metabolites. A review of the work and possible synthetic suggestions are presented and include the synthetic cyclization of ABA to produce bicyclics with distinct biological activities and the isolation of metabolites, including (2Z,4E)-(+)-4'-hydroxy-gamma-ionylideneacetic acid, from *C. cruenta* and *Stemphylium sp*. The potential exists, with the bicyclic derivatives, to produce herbicides.

In the late summer of 1957, I.D.J. Phillips joined our laboratory at the Boyce Thompson Institute, Yonkers, New York, as a pre-doctoral student. He was an exchange student from the laboratory of Sir P.F. Wareing, who had recently moved from the University of Manchester to Aberystwyth, Wales, and Phillips was seconded to Professor A.J. Vlitos, for two years. During that period I had the exceptional honor, and luck, of working with Phillips during which time he constantly referred to the β-inhibitor that was present in the dormant buds of *Acer pseudoplatanus*. He explained, in detail, that the secondary metabolite was present in the buds when they were laid down in the summer and that the amount of inhibiting substance, on a gram weight basis of collected buds, remained high through the winter months, then proceeded to drop in December and January. By March when Spring started in the British Isles (Phillips assured me that there is a spring in England), the titre of β-inhibitor had dropped to exceedingly low levels. At a point when the inhibitor was no longer present, buds broke dormancy and

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stem and leaf growth appeared. The experiments designed by Phillips were elegant and included the separation, from crude extracts, of the inhibitor in zones on paper chromatograms which, at the time, had recently been introduced into the laboratory. In a typical separation, the crude material, usually obtained from a methanol extraction of buds (and, later, leaves), was spotted onto paper chromatograms, developed in a suitable solvent (iso-propanol:ammonia:water), the chromatograms were then dried, cut into ten equal parts (Rf 0.0-1.0) and the cut pieces were introduced into test tubes with approximately 2 ml of water. To these were added ten etiolated wheat coleoptiles, under subdued green safelight and, following treatment, the extension of the coleoptiles was recorded. The β-inhibitor present in sycamore buds was extremely active against etiolated wheat coleoptiles and these findings were published (1,2). Further, Phillips stated that the growth of leaf disks of _Acer pseudoplatanus_ could be inhibited by the dormancy factor that had been eluted, in water, from paper chromatograms. Discussions centered around the practical use of the inhibitor to control the emergence of weed seeds by pre-planting application to tilled fields and other "herbicidal" applications. There was no doubt that Wareing in the United Kingdom and Addicott in the United States, who had discovered an identical compound in cotton (_Gossypium hirsutum_ L.) bolls, were hot on the trail of an important secondary metabolite. The British work implied that dormancy in tree buds could be chemically explained, while that of the Americans clarified, in cotton, the acceleration of abscission in leaves and bolls. Importantly, the methods by which this chemically-unknown substance had been detected involved the exogenous application of the material to either etiolated wheat (_Triticum_) coleoptiles in the case of _Acer pseudoplatanus_ buds, or _Avena_ coleoptiles, in the case of the cotton metabolite. In both bioassay species indole-3-acetic acid (IAA) caused curvature when applied to the etiolated tips of the seedlings but, IAA in combination with the inhibitor induced far less bending. The inhibitor also accelerated abscission when applied to cotton fruit or pedicels from which the fruit had been removed. In all these cases, the effects were those observed on _higher plants_ following application, and not microorganisms or vertebrates.

The structure of the inhibitor was elucidated by Ohkuma et al., in 1965 (3) and given the trivial name abscisin II, later, this was changed to abscisic acid (Figure 1). In 1965, Cornforth synthesized (±)-abscisic acid (ABA) (4), and relatively large samples of this isomeric mixture became available for further work, as opposed to the very limited milligram quantities of (+)-ABA that had been obtained from plant sources. The biologically active species is the 2-cis(+) form; the 2-trans(-) isomer is relatively inactive. This means that any evaluations wherein the synthetic form (±) is used must be clearly stated as such.

Over the intervening 25 years, there have been no major developments with respect to the use of abscisic acid, either in the native or derivatized form, in agriculture. The early promise that the compound showed for controlling the breaking of dormancy in stored products such as Irish and sweet potatoes, onions, carrots, and other commodities, has not materialized. Neither has a use been

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found for abscisic acid in prolonging dormancy in fruit trees, especially in the transition zones of the world where bud break brought on by unseasonably warm weather followed by a freeze leads to complete yield or crop destruction. Even the effects of (±)-ABA on seed germination appears to have gone unexploited as a possible selective pre-emergence herbicide. This despite the fact that (±)-ABA seems relatively non-toxic (LD_{50} figures are not given in the Merck Index). Paradoxically, 109 ABA analogs and metabolites have been isolated, synthesized, and their biological activities in various assay systems reported without much advancement in practical application (5). As if to complicate the conundrum, abscisic acid has been implicated in controlling abscission, bud dormancy, seed dormancy, tuberization, flowering, stomatal closure, drought resistance, root growth, fruit ripening, senescence, and membrane transport (6). Practical use of ABA seems to stick at one point: abscisic acid is not readily transported across leaf cuticles. Earlier work demonstrated that ABA is so poorly transported across leaf barriers that insuffcient material penetrates the cell to induce a response. Experiments with astomatous cuticles isolated from tomato fruit, and the upper epidermis of apricot leaves, pear, and orange, showed that penetration was linear with time, was greater through dewaxed than non-dewaxed cuticle and was more facile as the undissociated ion. Importantly, naphthalene acetic acid and 2,4-dichlorophenoxyacetic acid were 3 - 6 times more effective that (±)-ABA in cuticular penetration (7). More significant was the finding that ABA was not metabolized to another chemical species during transport across the cuticle.

If the cause of response to ABA in reaching its target site(s) when applied to higher plants is due to leaf penetration, we are faced with an apparent paradox. ABA has recently been discovered in the brains of pigs, rats (8), rodents and ruminants (9), and there are only two possible origins for this material. First, it may be obtained from dietary sources, in which case the ABA must survive passage through the digestive system, cross into the blood stream, be carried to the proximity of the brain and pass through the blood brain barrier into the brain where it is, presumably, slowly metabolized. It is difficult to transfer chemical compounds across the blood brain barrier, a constant nemesis for the medicinal chemist who must synthesize compounds with precise lipophilic and hydrophilic properties to reach the receptor sites in the brain. If this is the case for ABA reaching the brain in the mammalian system, then it is something of a contradiction that ABA can be transferred across a number of membranes and barriers, all possessing differing properties, yet not penetrate leaf and stem tissues in plants in sufficient quantity to induce a significant response. However, it is also possible that ABA in brain tissue is synthesized de novo, implying that the genetic code for manufacturing ABA exists in microorganisms, plants and animals and has not been deleted during the evolutionary process.

Two points have governed the possible development of ABA as an agricultural chemical. First, the relatively low yields of ABA from higher plants , as opposed to microorganisms, has precluded the isolation of biosynthetically dependent satellite metabolites which occur in less quantity which may also have potent biological activ-

ity. Second, there has been a preoccupation, especially in Western science, with the mode of action of ABA; and some of the earlier questions concerning the practical use of ABA have been held in abeyance. This may now be changing.

ABA and Associated Metabolites in Fungi.

The recent discovery of (+)-cis-ABA (Figure 1) in relatively large abundance in fungi, especially *Cercospora rosicola* (10), *C. cruenta* (11), and a blue light (360 nm) sensitive strain of *Botrytis cinerea*, which produced 9.3 mg/100 mL of liquid culture (12), may mark the turning point for the discovery of homologs and analogs of ABA for agricultural use. Because yields of ABA can be increased by subjecting producer microorganisms to stress, the associated satellite metabolites may be concomitantly produced in greater quantities, isolated in pure state and then utilized or derivatized for specific biological use. Concurrently, the associated metabolites may be used to deduce, by suitable labelling, the biosynthetic pathways for ABA. Isolation of the satellite metabolites, followed by introduction of those metabolites to other fungi which, presumably, more efficiently convert them to other ABA precursors, has furnished valuable information. For example, in addition to the discovery of (+)-ABA in *C. rosicola* a further compound, (+)-(2Z,4E)-4'-oxo-α-ionylideneacetic acid (13) (Figure 2), which occurred in small quantities, was discovered. Later, it was found that another fungal species, *C. cruenta* could produce large quantities of (+)-ABA and (+)-(2Z,4E)-4'-oxo-α-ionylideneacetic acid (11). There followed the discovery that by feeding (+)-(2Z,4E)-α-ionylideneacetic acid (Figure 3) to *C. cruenta* the microorganism could convert the compound to (+)-4'-hydroxy-α-ionylideneacetic acid; (+)-(2Z,4E)-4'-oxo-α-ionylideneacetic acid; and (+)-ABA (14) (Figure 3), thereby establishing some of the biosynthetic sequence for (+)-ABA. Two points of interest are that the 4' position of (+)-α-ionylideneacetic acid has been partially oxidized to produce the OH derivative, or completely oxidized to biosynthetically make the (+)-4'-oxo product. The metabolite (+)-α-ionylidene acid has been shown to elicit the same plant growth inhibitory activity as (+)-ABA(14); and the (+)-4'-oxo derivative does not appear to have been tested. A further development with respect to (+)-ABA metabolites was the isolation of (+)-(2Z,4E)-trans-1,4'-dihydroxy-γ-ionylideneacetic acid (Figure 4) from *C. cruenta*. The major difference between this compound and those previously mentioned is the 1' position which has been oxidized to produce an hydroxyl function. This metabolite more closely resembles (+)-cis-ABA with the exception that the 4'-oxo is now an hydroxyl, the 5'-6' position is saturated (this corresponds to the 2'-3' unsaturation in ABA, which is numbered anticlockwise in the cyclohexene ring), and there is a methylene function at 6' (the corresponding position is 2' in ABA from which is subtended a CH_3 group). These changes are substantial enough to alter the biological activity so that (+)-trans-1',4'-dihydroxy-γ- ionylideneacetic acid is only, approximately, one-tenth as active as (+)-ABA in rice seedling assays (15). It is somewhat surprising that the minor addition of two protons at the 4' position and transition of the methyl to methylene reduces biological activity of the molecule by 90% compared with (+)-ABA.

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(Cercospora rosicola)

(C. cruenta)

Figure 1. (+)-S-abscisic acid

(Cercospora rosicola)

(C. cruenta)

Figure 2. (+)-(2Z,4E)-4'-oxo-α-ionylideneacetic acid

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H_3C CH_3 CH_3 H H CH_3 COOH

(+)-(2Z, 4E)-α-ionylideneacetic acid

(Cercospora cruenta)

ABA

(+)-(2Z, 4E)-4′-oxo-α-ionylideneacetic acid

H_3C CH_3 CH_3 H HO CH_3 COOH

(+)-4′-hydroxy-α-ionylideneacetic acid

Figure 3. (+)-(2Z,4E)-α-ionylideneacetic acid
(+)-(2Z,4E)-4′-oxo-α-ionylideneacetic acid
(+)-4′-hydroxy-α-ionylideneacetic acid

H_3C CH_3 CH_3 2′ 3′ 1′ OH 4′ 6′ 5′ HO CH_2 COOH

(Cercospora cruenta)

Figure 4. (+)-(2Z,4E)-*trans*-1′,4′-dihydroxy-γ-ionylideneacetic acid

There exists a monohydroxy congener, (2Z,4E)-ethyl- 1'-hydroxy--γ-ionylideneacetic which also inhibits rice seedlings and this differs structurally by the absence of a 4'hydroxyl function and, the COOH free acid moiety function has been modified to ethyl ester (16). It should also be pointed out that the methyl ester ($COOCH_3$) of (+)-trans-1',4'-dihydroxy-γ-ionylideneacetic acid exhibited selective inhibitory behavior, fully inhibiting the germination of lettuce seed (cv. Great Lakes) when applied at concentrations of 10^{-4} M, for a period of 5 days at 28°C (16).

Another dihydroxy natural metabolite of (+)-ABA is 1',4'-trans diol (Figure 5) which has been isolated from _Botrytis cinerea_ and proposed as a metabolite in _Cercospora cruenta_ and _C. rosicola_ (17): it also occurs in _Pisum sativum_ and _Persea americana_ (18). There is no record of this compound having been assayed for plant growth regulatory activity in either study.

During the search for a lettuce seed germination inhibiting substance from a species of _Stemphylium_ (unidentified) a novel compound was isolated, (2Z,4E)-(+)-4'-hydroxy-γ-ionylideneacetic acid (Figure 6) (19), which differed from the previously described compound (+)-(2Z,4E)-trans-1',4'-dihydroxy-γ-ionylideneacetic acid, by the absences of the 1' hydroxyl (Figure 4) and, from (+)-4'-hydroxy--γ-ionylideneacetic acid by a methylene group at position 6' (Figure 3). The metabolite, (+)-4'-hydroxy-γ- ionylideneacetic acid, had been discovered earlier in _Cercospora cruenta_ (20,21), and it had been determined that it inhibited the germination of lettuce seed (cv. Great Lakes) 50% when applied at rates greater than 50 ppm, whereas more than 5 ppm of (+)-ABA achieved the same results in identical bioassays (19).

Another natural ABA homolog that has been isolated from _Cercospora cruenta_ is (2Z,4E)-γ-ionylideneethanol (Figure 7), and this compound has also been synthesized, as the (±)-isomeric mixture, starting from (±)-γ-ionone (20). In bioassays with rice seedlings, the natural metabolite inhibited growth 60% at 5 x 10^{-4} M relative to controls, but the data for the (±)-isomeric mixture were not given (20). Additionally, another metabolite from the identical fungal source was (2Z,4E)-γ-ionylideneacetic acid (Figure 8) which proved to be less active than the homologous alcohol, inhibiting rice elongation only 42% compared to controls at 5 x 10^{-4}M (20). Comparison with (±)-ABA and (2Z,4E)--γ-ionylideneacetic acid in the same set of experiments demonstrated that both substances inhibited rice 100% at 10^{-4} M concentration. Neither ester derivatives of (2Z,4E)-γ-ionylideneacetic acid, nor ether derivatives of the corresponding alcohol appear to have been synthesized or tested for biological activity and because of the selective biological responses obtained with the methyl ester of (+)-trans-1',4'-dihydroxy-γ-ionylideneacetic acid (_vide supra_), this synthetic exercise may prove worthwhile.

(+)-ABA Synthetic Products.

Examination of the ionylideneacetic acid model leads to the question of other possible substitutions on the cyclohexane structure and the concomitant effect on biological activity. Again, _Cercospora_

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch004

<u>cruenta</u> has been shown to produce a stereoisomeric mixture of 3'-hydroxy-γ-ionylideneacetic acid (Figure 9) (unpublished, cf [22]), though earlier this compound was shown to be produced by <u>C</u>. <u>cruenta</u> when it was fed [2-^{14}C]-(2Z,4E)-1',4'-dihydroxy-γ-ionylideneacetic acid (23). While the biological data were not reported for this unusual metabolite, synthesis of four chiral stereoisomers was successfully accomplished using (+)-(S)-3-hydroxy -2,2-dimethyl-1-cyclohexanone as the chiral starting material (22). Of these, the (+)-(1'S,3'S)-(2Z,4E)--3'-hydroxy- -ionylideneacetic acid (Figure 10) was only mildly inhibitory to the germination of lettuce and radish seed to the extent that it was approximately 1% as active as (+)-ABA. In rice seedlings, no growth inhibition was observed. The remaining three chiral isomers were either not tested, or were inactive. As noted earlier, the 4'-hydroxy-γ-ionylideneacetic acid has 10% of the germination inhibition activity of (+)-ABA in lettuce.

Synthesis of (±)-deoxyabscisic acid isomers has also been accomplished as an exercise to prove the structure of a C_{15} ABA metabolite, found in wilted pea seedlings (<u>Pisum</u> <u>sativum</u>) (24). The synthesis proved that the C_{15} metabolite was trans-1',4,-dihydroxy--γ-ionylideneacetic acid and not, as had previously been reported, the deoxy-abscisic metabolite of (+)-ABA (25). An added result of the synthetic program is that both the 2Z and 2E-deoxy-abscisic acids (Figure 11) were tested for biological activity on rice seedlings and germinating lettuce seed. The 2Z acid was moderately active against second leaf sheaths of Sasaminori cultivar rice plants at 10^{-3} M (~80% inhibition). Lettuce seed germination was inhibited 100% at 10^{-4} M and ~30% at 10^{-5} M. The 2E acid was mildly inhibitory at 10^{-3} M against second leaf sheath of rice (~20%) and fully active against lettuce seed germination at 5 x 10^{-4} M (25).

Some of the most recent syntheses have been those of ABA analogs that were made to probe the receptor sites of (+)-ABA in plants. These included the three compounds shown in Figure 12. While the substitutions remain in the same position on the phenyl ring for each analog, the substitutions on the cyclohexene ring are altered at the 1' and 4' position so that in the first molecule the 1'=H and 4'=H_2; in the second, 1'=H and 4'=O; and in the third, 1'=OH and 4'=O. All the synthetics elicited only weak responses in suppressing germination in cress at 10^{-3}, and 10^{-4} M. In mung bean cutting bioassays, analog 2 (Figure 12) inhibited epicotyl extension to a greater extent than ABA, and analog 3 (Figure 12) increased transpiration by 60% at 10^{-4} M (26). Thus, a synthetic analog of ABA now exists that is more potent in a specific assay than ABA, when applied exogenously. Because of the success of these experiments it is probable that other analogs will be forthcoming. The group that synthesized these analogs has postulated that the design was based on the thesis that the sp^3 bonds in the ABA side chain might rotate under certain cellular environmental conditions in proximity of the receptor sites. Structurally, this means that the side chain almost forms a ring structure when folded "upward". Be that as it may, recent correspondence (Dr. S. Yoshida, Tokyo University) now reports that phenolic analogs of ABA have been made. Using Figure 12 as a reference, these analogs have the fol-

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(Botrytis cinerea)
(Pisum sativum)
(Persea americana)

Figure 5. 1',4'-*trans*-diol of ABA.

(Cercospora cruenta)
(Stemphylium sp.)

Figure 6. (2Z,4E)-(+)-4'-hydroxy-γ-ionylideneacetic acid.

(Cercospora cruenta)

Figure 7. (2Z,4E)-γ-ionylideneethanol.

Figure 8. (2Z,4E)-γ-ionylideneacetic acid.

(Cercospora cruenta)

Figure 9. 3'-hydroxy-γ-ionylideneacetic acid.

[synthetic]

Figure 10. (+)-(1',3'S)-(2Z,4E)-3'-hydroxy-γ-ionylideneacetic acid.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch004

lowing substitutions on the phenyl ring: the CH_3 function is replace by an OH, and the OH is replaced by either a proton, methyl, or ethyl function. The corresponding biological acitvity is changed accordingly. Also, carboxylic analogs have been prepared exchanging the CH_3 function, just mentioned, with a COOH group. It becomes evident that further substitutions, including carboxylic acid esters and hydroxyl derivatives to produce ethers may change biological activity. Of course, from the practical herbcidal standpoint the presence of a phenyl group leads to the obvious consideration of a 2,4-dichloro substituted product and, for other purposes, the synthesis of a dinitro analog.

The total synthesis of four stereoisomers of (±)-methyl phaseate from (±)-4-(6'-acetoxymethyl-2',6'-dimethyl-1-cyclohexen -1'-yl)-but-3-en-2-one was accomplished in 1986, but the biological activity was not reported (27). Later, (+)-methyl phaseate was successfully synthesized from (-)-β-pinene (28); but, again, no biological data were given. The specific isomer (-)-phaseic acid (Figure 13) was originally isolated from _Phaseolus_ _multiflorus_ (29). Earlier experiments with phaseic acid, obtained from bean embroys, demonstrated that the metabolite was as effective in inhibiting α-amylase production in barley (_Hordeum_ _vulgare_) aleurone layers as (±)-ABA (30), but other work with phaseic acid indicated that it was only 10% as effective as (±)-ABA in accelerating abscission in the excised nodes of cotton seedlings (31).

Analogs of ABA and Practical Use

With the exception of the research of Yoshida et al. (26) which represents the coalition of two ideas, that is the elucidation of the mode of action of (+)-ABA relative to receptor sites and production of practical analogs of (+)-ABA for possible agricultural use, virtually all endeavors concerning (+)-ABA have been concerned only with the possible mode of action of (+)-ABA. Thus, all of the structures that we have just examined have primarily been evaluated to determine their relative role in (+)-ABA formation and metabolism. This in no way detracts from the difficult and elegant chemistry that has been carried out.

Analogs of (+)-ABA and Practical Use

Our interest in (±)-ABA has been twofold. First, because there are obvious possible synthetic derivatives yet to be made which may have practical agronomic application and second, because we find that certain existing analogs have practical application. It will be noted that in the discussion by Walton concerning the structure-activity relationships of ABA analogs and metabolites (5), reference is made to α-ionol, and α-ionone, and their effects on stomatal closure in tests conducted on epidermal strips. In 1985, results of the effects of β-ionone, another analog of ABA, on _Aspergillus_ _flavus_ were published and this proved to have patentable application (32). Specifically, β-ionone (Figure 14) inhibited sporulation of _A_. _flavus_ at concentrations ranging from 10^{-4} to 10^{-5} M. This was a significant discovery because _A_. _flavus_ is the producer of the aflatoxins, a series of related compounds that are

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Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch004

Figure 11. 2Z-deoxyabscisic acid
2E-deoxyabscisic acid

Figure 12. ABA analogs

(Phaseolus multiflorus)

Figure 13. (−)-phaseic acid

Figure 14. β-ionone

potent hepatic carcinogens which are present in some food products. An aflatoxin concentration of parts per billion may produce cancers in mammals and fish. More importantly, the aflatoxins concentrate in the spores of A. flavus and are not found in the mycelium. Beta-ionone, while allowing the mycelium to grow at an arrested rate, inhibits spore formation (33), thereby eliminating aflatoxins production. As an interesting aside, β-ionone is the predominant fragrance in English violets, the scent of which permeates the atmosphere of a room almost to the point of becoming cloying when bouquets of the flowers are present. A patent for the use of β-ionone to control A. flavus as a fungistatic agent was granted in 1982 (34). The alcohol, β-ionol, was approximately half as active at β-ionone against A. flavus (32).

Because (+)-ABA was first discovered using the wheat coleoptile assay, the latter is always a suitable reference point for assaying ABA analogs and homologs to determine their relative activity. We have recently reevaluated (±)-ABA in our etiolated wheat coleoptile bioassay (Triticum aestivum L. cv. Wakeland) and found that it inhibits 100, 100, 81 and 62% at 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M, respectively, relative to controls (Figure 15). The elements of the etiolated wheat coleoptile consisted of sowing wheat (Triticum aestivum L. cv. Wakeland) on moist sand in trays, covering the trays with aluminum foil to maintain high humidity, and placing them in a dark room at 22±°C for four days. Following germination and emergence, the shoots were removed from the caryopses and roots (which were discarded) and the shoots were put into a Van der Weij guillotine. The first two apical millimeters were discarded and only the next 4 mm were saved from each etiolated shoot. Ten of these coleoptiles were placed in a test-tube containing 2 mL dipotassium phosphate-citrate buffer, pH 5.6, supplemented with 2% sucrose, plus the compound to be tested, and incubated for 18 hours at 22°C. All manipulations were carried out under a safelight at 540 nm. Following incubation, all sections were blotted on paper towels, placed on a glass plate and put into a photographic enlarger to produce images X 3, which were measured and recorded. All data were statistically analyzed. In contrast, β-ionone inhibits etiolated coleoptiles 100 and 23% at 10^{-3} and 10^{-4} M, respectively (Figure 16). It is not clear from the literature as to whether other homologs or analogs of ABA have been tested for potential use as specific fungicides in stored products.

Conclusion

The homologs and analogs of ABA are candidates for the production of practical agricultural chemicals. The primary problem with respect to the use of ABA as an agrochemical appears to be mainly that of translocation across leaf surfaces into subepidermal cells and, from there, movement to the receptor sites. While the predominance of the research involved with ABA, and its congeners, has been directed toward the mode of action of the parent molecule (ABA), it now appears that a few laboratories are once again re-examining these non-toxic secondary metabolites for practical purposes. The most important endeavour with respect to the production of a pre-, or post-emergence herbicide are the bicyclic ABA analogs synthesized by

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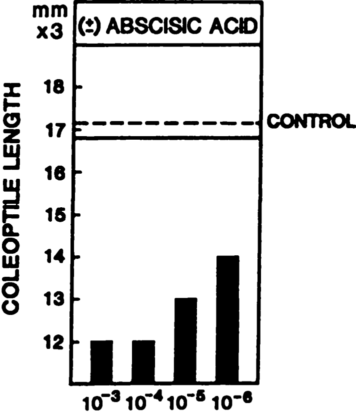

Figure 15. Effects of (±)-abscisic acid on growth of etiolated wheat (Triticum aestivum L. cv Wakeland) coleoptiles. Dotted line: control. Values below solid line are significantly inhibited ($P < 0.01$)

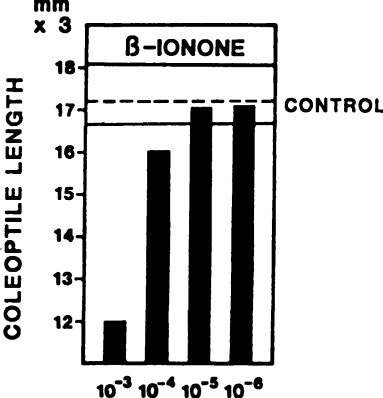

Figure 16. Effects of β-ionone on growth of etiolated wheat (Triticum aestivum L. cv Wakeland) coleoptiles. Dotted line: control. Values below solid line are significantly inhibited $P < 0.01$)

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch004

Yoshida et al. which, in due course, should generate practical agrochemicals which, it is hoped, will retain the non-toxic, biodegradable properties of the parent molecule.

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Chapter 5

Biochemical Responses of Plants to Pathogens

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Various enzymes have been implicated in host plant resistance to disease. Among the more important of these are chitinase, glucanase, glycosidase, NADPH oxidase, and several enzymes of secondary plant metabolism including phenylalanine ammonia-lyase, peroxidase, and polyphenol oxidase. Studies on enzymatic resistance to pathogen attack of crop species indicate the lack of a universal resistance mechanism(s). That is, specific enzyme activities in various plant species have often been both positively and negatively correlated with infection by various pathogens. There is a great need to examine resistance mechanisms in pathogen-weed situations if the potential manipulation of such interactions for biological control is to be realized. Responses of these enzymes to pathogen attack and the biochemical processes regulated by these enzyme reactions are surveyed. Particular emphasis is on enzymes and constituents of secondary plant metabolism since their involvement in defense has been studied most. Regulation of secondary metabolism by agrochemicals and enzyme inhibitors as related to pathogen infection and recent research on biochemical interactions of some weeds and pathogens are also discussed.

Plants defend themselves from attack by nearly all microorganisms, but some microorganisms (plant pathogens, i.e., fungi, bacteria, and viruses) have infection processes that can evade plant defense mechanisms and cause infection that may lead to plant death. These pathogens may confound defense mechanisms by suppression of the mechanism(s) or by not being recognized by the host plant. Once the plant does react to invasion by a pathogen, several enzyme activities are usually increased (mostly enzymes of secondary plant metabolism) which results in the synthesis of compounds such as phytoalexins, phenolics, lignin and others which are known defense chemicals.

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Data from whence knowledge of these defense mechanisms originated have nearly all been from studies on crop plants and their respective disease organisms. There are virtually no studies of such mechanisms in weed-pathogen interactions. This represents a serious void in the full utilization of biological weed control using bioherbicides (pathogens or their phytotoxic products).

Because these defense mechanisms differ widely depending on plant species and disease organisms, and because most plant pathogens have specific or a very limited host range, there is a need to examine weed pathogen-host interactions individually. Knowledge of specific weed-pathogen interactions could possibly lead to chemical manipulation or controlled regulation of the defense mechanisms to increase bioherbicide efficacy and provide better weed control. Knowledge of agrochemical effects on biocontrol microorganisms is also lacking. The scope of this chapter will be to examine some of the plant defense mechanisms that have been reported in the literature. Particular emphasis is placed on enzymes and products of secondary plant metabolism [i.e., phenylalanine ammonia-lyase (PAL), polyphenyl oxidase (PPO), peroxidase (PO) and general and specific phenolic compounds] and on noted instances of chemical interactions of these enzymes and secondary plant products with enzyme inhibitors, herbicides, and plant growth regulators.

Some Important Enzymes Implicated in Plant Defense

The enzymes most commonly studied in plant defense reactions can be divided into two major groups (lytic enzymes and enzymes associated with plant phenolic metabolism) and a lesser third group of generally unrelated enzymes (Table I). Discussion of some of the salient information regarding the roles and action of these enzymes when plants are infected by pathogens follows. Instances where these enzymes are thought not to play roles in plant defense are also presented.

Chitinase and 1,3-β-Glucanase. Chitinase and 1,3-β-glucanase are lytic enzymes that degrade chitin and glucans, two major components of fungi and fungi imperfecti cell walls. Both enzymes have been implicated for some time as antifungal defense proteins in plants (1-4), although pathogenic fungi also produce similar enzymes (5). Generally, this implication relys on several pieces of indirect evidence: (1) even though chitinase has no substrate in plants and callose (1,3-β-glucanase substrate) is extremely low in plants, high activity levels of both enzymes commonly occur (3, 4); (2) isolated fungal cell walls are degraded by purified fractions of these enzymes (6-8); (3) pathogen infection, elicitors from pathogens, and ethylene induce these enzymes (9, 10); and (4) at least one fungal pathogen produces proteinaceous inhibitors of 1,3-β-glucanase (11). Furthermore, physiological concentrations of these two enzymes are inhibitory to the growth of pathogenic fungi (12). Recently chitinase and 1,3-β-glucanase have been shown to act synergistically to inhibit fungal growth (13).

When the host and pathogen come in contact, each produces lytic enzymes as a defense mechanism (Figure 1). Pectinase and cellulase are major emzymes of pathogens that are related to infection

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Table I. Some Enzymes of Plant Defense Mechanisms

Enzyme Name	Reaction
Lytic enzymes	
Chitinase (EC 3.2.1.14)	Random hydrolysis of β-1,4-acetamido-2-deoxy-D-glucoside linkages in chitin and chitodetrin
β-1,3 Glucanase (EC 3.2.1.39)	Hydrolysis of 1,3-β-D-glucosides in 1,3-β-D-glucans
Enzymes that metabolize phenolics	
Peroxidase (PO) (EC 1.11.1.7)	Donor + H_2O_2 ⇒ oxidized donor + H_2O; ex. donor: pyrogallol, guaiacol, gallate, other phenolics and some amines
Phenylalanine ammonia-lyase (PAL) (EC 1.11.1.7)	Phenylalanine ⇒ t-cinnamate + NH_4^+
Polyphenol oxidase (PPO) (EC 1.14.18.1)	L-Dihydroxyphenylalanine ⇒ "DOPA chrome" (one of many possible reactions)
β-glycosidase (EC 3.2.1.21)	phenolic glycosides ⇒ fungitoxic aglycones (ex. arbutin ⇒ hydroquinone)
Other less-studied defense enzymes	
NADPH oxidase "system"	$2O_2$ + NADPH + H^+ ⇒ $2O_2^-$ + $NADP^+$
Protease	Hydrolysis of amide linkages in proteins and peptides

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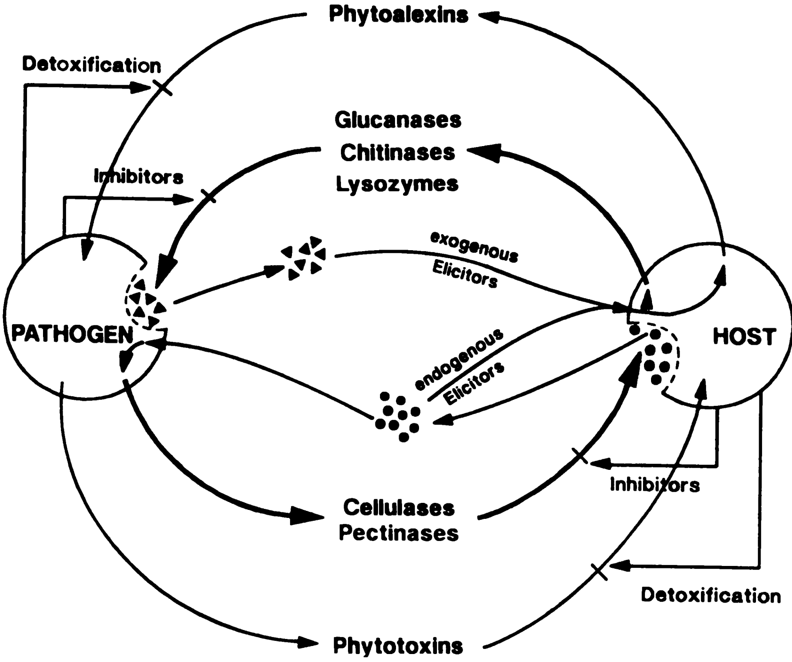

Figure 1. Schematic representation of lytic enzyme action in plant-pathogen interactions. (Redrawn with permission from Ref. 13. Copyright 1988 American Society Plant Physiologists).

processes. Both the host and pathogen may also have defensive constituents against each other's enzymes, as in plant-produced pectinase inhibitors (14) and fungal-produced 1,3-β-glucanase inhibitors (11). Another analogy in this mechanistic scheme is the production of toxic chemical constituents by the host (phytoalexins) and pathogen (phytotoxins). Chitinase (15) and 1,3-β-glucanase (16) can cause (phytoalexin) elicitor release from cell walls of fungi.

Peroxidase (PO). Peroxidase (PO) is found in both plants and microorganisms. Although the physioloigcal role of the enzyme is not completely understood, it catalyzes several reactions, including polymerization of hydroxycinnamyl alcohols which leads to lignin formation; suberization; oxidation of many mono- and diphenols to toxic quinones; and oxidation of indoleacetic acid (IAA) (17). PO has been studied with regard to disease resistance in many plants (Table II) and the enzyme itself is toxic to some microorganisms (26). High inherent PO activity in apical leaves and root tips of potato plants was strongly correlated with resistance to *Phytophthora infestans* (18), whereas other leaves, tuber peels, and pulp had low PO activity and were susceptible (18). This correlation of resistance and high PO activity was extended to explain differences in susceptibility of potato varieties.

Table II. Selected Instances of Peroxidase Activity Relative to Disease Resistance

Disease Organism	Host	Reference
Peroxidase Involved in Disease Resistance		
Phytophtora infestans	potato	17, 18
P. phaseolicola	bean	19
Ceratocystis fimbriata	sweet potato	20
Pseudomonas tabaci	tobacco	21, 22
Puccinia graminis f. sp. *tritici*	wheat	23
TMV	tobacco	24
Diplodia gossypina	cotton	25
Xanthomonas phaseoli var. *sojensis*	soybean	26
Rigidoporus lignosus	rubber tree	27
Peroxidase Not Involved in Disease Resistance		
Helminthosporium carbonum	maize	28
Puccinia graminis tritic	wheat	29, 30
CMV	cucumber	31
Fusarium	tomato	32
Pseudomonas solanacearum	tobacco	33
Pseudomonas syringae pv. *tomato*	tomato	34

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Cotton bolls of intermediate age were more resistant to Diplodia gossypina than young or old bolls (25). PO activity was higher in these intermediate bolls and PO increased several fold when resistant bolls were inoculated with this pathogen. More recently, PO was shown to be involved in root-rot (Rigidoporus lignosus and Phellinus noxium) infection in rubber trees (Hevea brasiliensis) (27). PO activity increased more rapidily in resistant cucumber hypocotyls than in susceptible tissue when inoculated with the pathogen Cladosporium cucumerinum Ellis & Arthur (35). PO isozyme patterns in infected resistant tissue were similiar to wound-induced isozyme patterns. Similar types of studies have suggested a direct role of PO in disease resistance. However, some host-pathogen studies indicate that PO is not involved in host defense (Table II). PO activity was increased in both resistant and susceptible maize plants inoculated with Helminthosporium carbonum, but the increase was greater in susceptible plants (28). Recent studies with Pseudomonas solanacearum infection of tobacco have concluded that PO increases are not involved in induced resistance (33).

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Phenylalanine ammonia-lyase (PAL). The conversion of L-phenylalanine to t-cinnamate and ammonia by PAL represents a key branch point from primary to secondary plant metabolism (Figure 2). PAL, therefore, is pivotal for regulation of the synthesis of various phenolic compounds, including flavonoids, hydroxycinnamates, etc., some of which are phytoalexins. The enzyme has wide distribution in plants (and some microorganisms) and its activity in plants is increased by exposure to various factors, including light, wounding, temperature changes, and infection by pathogens. Studies on its properties and behavior have been reviewed (36, 37). In a variety of plants, there is often an increase in PAL activity associated with increased production of specific phenolic compounds early after inoculation and infection as summarized in reviews (38, 39). PAL activity levels were increased several fold in resistant vs. susceptible potato tuber tissue after inoculation with Phytophthora infestans, indicating a positive defense mechanism (40). Fusarium solani infection caused similar PAL increases (41). PAL in resistant lucerne increased rapidly in response to infection by Verticillium albo-atram, but not in a susceptible variety (42).

PAL induction and phytoalexin accumulation is common to a wide range of host-parasite interactions when plants are treated with elicitors (43). Studies with several plant cell cultures also indicated that PAL activities are readily increased by elicitors (44).

Other studies do not show a strong correlation of increases in PAL activity and disease resistance. For example, PAL levels increased in both susceptible and resistant hosts during disease interactions (45-47). In other instances, PAL activity was stable after infection, even though phenolics have been shown to be involved in host response (48, 49). PAL activity increases are also not correlated with resistance in other studies (50-52). Additionally, PAL activity was shown to increase in susceptible maize tissue treated with Helminthorsporium maydis, but not in resistant maize treated with H. maydis or H. carbonum (53).

Few studies examining enzyme responses in host defense mechanisms have been applied to biological control of weeds with plant

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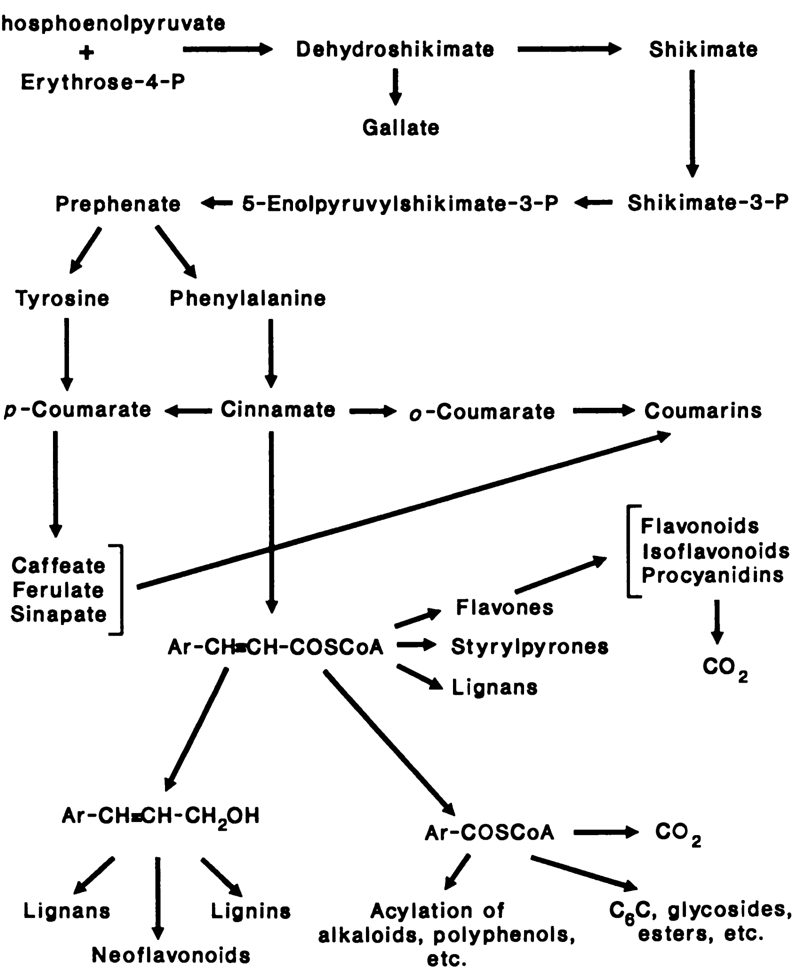

Figure 2. Biosynthetic pathway of shikimate- and cinnamate-produced phenolic compounds in plants.

pathogens. Recently, however, phenylpropanoid metabolism has been implicated in a weed-pathogen interaction [i.e.; sicklepod (Cassia obtusifolia) and the fungal pathogen Alternaria cassiae] (54). PAL activity was increased about 3-fold in weed seedlings challenged by spores of this pathogen (Figure 3). Enzyme activity increases generally preceeded increased soluble phenolic compound production and the appearance of necrotic lesions. However, inundative spore levels necessary to kill plants overwhelmed any defense response mediated by PAL or other enzymes.

Polyphenol oxidase (PPO). It has been suggested that PPO plays a role in disease resistance due to its oxidation of various phenolic compounds into toxic quinones (55). Although PPO activity levels did not differ in leaves of healthy apple varieties with a range of resistance to apple scab, there was a significant increase in activity of resistant vs. susceptible varieties when challenged by the disease organism (56). PPO was also inhibitory to viral infection of cowpea (57). PPO activity was higher in resistant vs. susceptible tomato cultivars and in mature vs. young tomato leaves (34). Mechanical injury induction of PPO increased pathogen resistance, and resistance and PPO levels were directly correlated in 20 tomato cultivars with a wide range of resistance and susceptibility. PPO has been shown to be important for synthesis of dihydroxyphenols in plastids (58). However, recent studies indicate that PPO is not involved in the o-hydroxylation of phenolic compounds in mungbean (Vigna radiata) (59). Some studies of PPO (catechal oxidase) generally suggest the enzyme increases after infection by virus, bacteria, or fungi (60). Although PPO may in some instances be involved in plant defense, in many cases there is inadequate evidence to prove a significant role for PPO in such mechanisms (60).

β-Glycosidases. One way plants are protected from autotoxic phenolic levels is by formation of phenolic glycosides. The glycosides are usually inactive and are compartmentalized, but membrane damage and infection cause release and hydrolysis. Host glycosidases, if induced by elicitors, can release fungitoxic phenolics at the onset of infection. β-Glycosidase in apple trees has been shown to be responsible for resistance to apple scab disease (61). The enzyme converts phloridzin (non-toxic glycoside) to phloretin (toxic aglycone). In pear trees, arbutin is a major glycoside and hydrolysis by β-glycosidase yields the aglycone hydroquinone, which is very toxic to Erwinia amylovora, the pathogen causing fire blight (62). Tree parts most susceptible to this organism had low enzyme levels, whereas sites of high enzyme activity were resistant.

Oat leaves contain avenacosides A and B, which are inactive saponins that are hydrolyzed after wounding by a β-glucosidase specific for glucose at carbon 26 (63-65). Activation requires only minutes and, thus, this is an effective defense mechanism. Other studies indicate that microbial glycosidases can also catalyze release of antifungal products from cyanogenic glycosides (66). Sterols in fungal cell membranes are receptors for the toxic aglycones (67), and glycosidase inhibitors such as 1,5-aldonolactones reduce fungal membrane damage (68).

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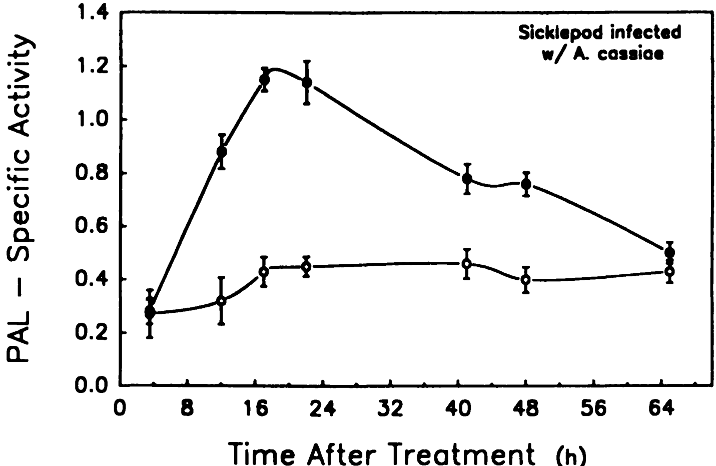

Figure 3. Extractable PAL activity (specific activity, units/mg protein) from sicklepod seedlings with (●) and without (○) *Alternaria cassiae* spores. (Reprinted with permission from Ref. 54. Copyright 1990 Paul Parey Scientific Publications).

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Some pathogenic fungi are resistant to saponins because fungal glycosidases are secreted or are cell wall-bound rather than membrane-bound. This causes glycoside hydrolysis to occur at a distance from sensitive membranes and, thus, aglycones do not reach membranes due to lack of solubility and transport. Alternaria solani possesses this system and its glycosidase hydrolyzes tomatine to tomatidine without harm to the fungus (69).

NADPH oxidase. This defense mechanism has not received much attention, but has been implicated in some host-pathogen interactions. NADPH oxidase in potato tuber tissue was shown to be activated immediately after invasion by an incompatible race of Phytophthora infestans, causing simultaneous superoxide production, hypersensitive cell death, and phytoalexin production (70-72). This enzyme system was not activated by a compatible P. infestans race (71). This enzyme was activated in potato leaves by both compatible and incompatible races before penetration, but superoxide was only increased when penetration occurred by incompatible races (73). A water-soluble glucan, found to be a hypersensitivity-inhibiting factor from compatible races, prevented activation of the superoxide-generating reaction in the protoplast (71). Digitonin could activate the superoxide- generating enzyme system which might enhance resistance to P. infestans (74). Other research will be necessary to evaluate this enzyme system as a host defense mechanism in other host and pathogen species.

Proteases. Proteolytic enzyme activity (hydrolysis of amide bonds of proteins and peptides) has received very little study related to disease resistance. Proteolytic activity is widespread in both hosts and pathogens. A high correlation was found with tomato cultivars infected by Pseudomonas syringe pv. tomato and disease severity (75). Other studies also suggest proteolytic activity correlations with disease (76, 77).

Ethylene. Ethylene is a ubiquitous plant hormone that regulates many plant growth responses and the activity of many enzymes (78). Some responses to ethylene of enzymes implicated in defense against pathogens include, increased PAL activity (79) and increased PO (80) and 1,3-β-glucanase (81). Some plants enhance ethylene levels in response to pathogen attack (82-84). Exogenous ethylene can trigger elevated biochemical defense reactions against various pathogens (85). This evidence lead to the hypothesis that endogenous stress-produced ethylene may be a signal for the plant to activate defense systems (82, 83). Recently, ethylene increases in tomato plants infected by Phytophthora infestans during the hypersensitive response have been attributed primarily to elevated ACC (1-aminoclyclopropane-1-carboxcylic acid) synthase activity (86). Other studies using aminoethoxyvinylglycine (AVG) to suppress ethylene production in elicitor-treated soybean (Glycine max L.) tissue and in infected cantaloupe (Cucumis melo) seedlings (84) did not reduce phytoalexin production and only marginally reduced levels of a defense glycoprotein, respectively. These latter results tend to suggest that endogenous stress ethylene plays only a minor role in plant biochemical defense; however, exogenous ethylene had little or no effect on these two defense reactions. Exogenous ethylene markedly increases both chitinase and 1,3-β-glucanase in some

plants (1). Overall, ethylene levels are elevated in infected plants, causing elevated defense enzyme activities. This leads to increased production of phenolics, phytoalexins, and glycoproteins which can provide resistance in some cases (87).

Problems in Assessing Defense Factors. Some caution should be exercised in assessing some of these overall results pertaining to the involvement of enzymes in resistance mechanisms. Resistance expression of plants after pathogen attack is a very complex process involving many closely integrated and regulated factors. Many host enzyme studies involving pathogen effects either were not attempting to prove the role of a particular enzyme in defense against a pathogen, or were not properly designed to accomplish that end. Furthermore, the data may be further confounded by compartmentalization (subcellular or plant organ) of the enzyme or its product(s), enzyme isolation and extraction techniques, and the fact that many pathogens possess the same enzyme systems implicated in resistance. Compounds (especially phenolics) released by pathogen action and by homogenation and extraction procedures can alter enzymes activities (88-90). Inoculum size and environmental factors also influence the dynamics of infection which can, in turn, influence defense response. Isozymes of these enzymes may also be numerous [as for PO (91)] and may be differentially induced, not affected by pathogen action (92), or identical in resistant and susceptible cultivars. Since viruses do not have PO and PPO activity, these pathogens may be useful to evaluate the roles of those enzymes in primary infection processes (93).

Enzyme Inhibitors to Manipulate Infection

Control of enzymes related to plant defense against pathogens could be effective in regulating plant disease. The use of specific or non-specific enzyme inhibitors (or activators) could result in regulated disease initiation and development that could promote or synergize the effects of microbial pathogens for biological control of weeds. Although some naturally occurring and synthetic enzyme regulators are known, relatively few studies have examined their potential for disease regulation. Of these studies, most have focused on crop-pathogen interactions.

Inhibition of PPO, PO, chitinase, glucanase, and β-glucosidase. Few synthetic or natural inhibitors of these enzymes have been elucidated. Some natural products such as curcurbitacin I and D can repress induction of laccase by the pathogenic fungus of cucumber, *Botrytis cinerea* (84). It is not known how this compound is involved in resistance or if these compounds are active inhibitors that could reduce defense in plant species by causing PPO or PO inhibition. Oxalates are natural product inhibitors of chloroplastic PPO (95) and 4-chlororesorcinol has also been used as a PPO inhibitor (96) in adventitious root promotion studies (97). The synthetic compound, diethyldithiocarbamate is a well known PPO inhibitor (98) that might be effective in blocking PPO activity in cases where PPO is involved in defense. Glyphosate [*N*-(phosphonomethyl)glycine] caused minor elevation of PPO activity in plant tissues (99, 100), but it is not known if this has potential to increase the defense of plants to

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disease. Direct effects of glyphosate on pathogens with potential for weed control would be important to determine.

Perhaps one of the most interesting PPO inhibitors is tentoxin, a fungal phytotoxin produced by the pathogen _Alternaria alternata_, which can totally eliminate PPO activity in some plant species (_101_) while exhibiting no effect on _o_-hydroxylated phenolic levels (_102_). This phytotoxin does not alter _in vitro_ PPO activity (_103_). Phytotoxicity caused by tentoxin is low even though PPO is inhibited and chlorosis is induced with little or no effect on plant morphogenesis (_102_). PPO oxidation of phenolics can play a role in plant resistance to pathogens (_104_). The use of PPO inhibitors to study weed-pathogen interactions could provide useful information for biological weed control and for determining the role of PPO in weed-pathogen interactions.

Inhibition of plant chitinases and/or glucanases without inhibition of similar enzymes in fungal weed pathogens could dramatically reduce plant resistance to pathogens. Recently a streptomycete antibiotic, allosamidin, was found to be a potent competitive inhibitor of chitinase from _Candida albicans_, a human fungal pathogen (_105_). The insecticidal properties of this antibiotic may also arise from its potent inhibition of insect chitinase. Plumbagin, an analog of juglone, is reported to be a chitinase inhibitor (_106_, _107_). These compounds have apparently not been tested in plants.

Various phenolic compounds have been observed to inhibit peroxidase-catalyzed IAA oxidation (_108_) and β-glucosidase (_109_). However, no synthetic or specific inhibitors for these enzymes are available that could be used to reduce weed resistance to plant pathogens.

Inhibition of PAL. PAL has been widely studied, and many of these investigations have centered on the regulation of PAL activity by natural or synthetic inhibitors. Naturally-occurring phenolic compounds such as _t_-cinnamate, _p_-coumarate, and other PAL regulators act via feedback inhibition (_110_). D-Phenylalanine and _p_-fluorophenylalanine were also discovered in early research to inhibit PAL _in vitro_ (_111_). Some other inhibitors useful in studying secondary metabolism via PAL inhibition have been found (Figure 4). Some of the compounds depicted here, as well as many others with varying inhibitory activity, have been used or discovered in Amrhein's laboratory (_112_). Aminooxyacetate (AOA) was used as a transaminase inhibitor in the development of a PAL assay in intact cells and was found to be a weak inhibitor of PAL (_113_). AOA inhibition of PAL and various transaminases results in phytotoxicity to many plants; hence, it is not a very useful _in vivo_ inhibitor in some cases due to lack of specificity resulting in pronounced phytotoxic effects (_114_). In fact, AOA has been registered for use as a herbicide (_115_). An analog of AOA, L-α-aminooxy-β-phenylpropionic acid (AOPP), was found to be a very potent and more specific inhibitor than AOA and could be used _in vivo_ without deleterious effects on plant growth (_116_, _117_). O-Benzylhydroxylamine was also found to be an effective PAL inhibitor (_118_). Another inhibitor of PAL, 3-(1,4-cyclohexadienyl)-L-alanine, has also been rigorously studied (_119_). One of the newest and perhaps most useful PAL inhibitors is (1-amino-2-phenylethyl) phosphonic acid (APEP) (_120_) which has potency about

equal to AOPP, but a K_i which is 10^3 times higher than the K_i of AOPP.

Some PAL inhibitors (especially AOA and AOPP) have been used to test the role of PAL in disease resistance. These PAL inhibitors did not block resistance expression or glyceollin accumulation in soybean tissue treated with an incompatible race of Phytophthora megasperma f. sp. glycinea (121, 122). AOA and AOPP reduced phenylpropanoid flux by inhibiting PAL in vivo, and decreased localization of the hypersensitive reaction of TMV (tobacco mosaic virus) in tobacco; i.e., lesion size was increased and virus progressed with the lesion edge (123, 124). This suggests that enhanced PAL activity and production of phenolic metabolites is directly involved in hypersensitive resistance to viral infection. AOPP reduced glyceollin levels in soybean, causing increased susceptibility to Phytophthora megasperma (125, 126).

The PAL inhibitor APEP also prevents glyceollin induction and transforms an incompatible pathogen interaction to a compatible one, suggesting a role for PAL and toxic phenolic compounds in resistance (127). AOA inhibited PAL activity in vivo, but not its synthesis, resulting in increased extractable PAL activity from maize mesocotyls which prevented resistance expression. This suggested that PAL is involved in resistance (128).

Phosphite was found to increase PAL activity and to inhibit growth and infectivity of Phytophthora cryptogea on cowpea leaves. Pretreatment of leaves with AOA, prior to phosphite and P. cryptogea, lowered PAL, reduced kievetone and phaseollidin accumulation, and caused significant increases in disease symptomology (129).

It is evident from these studies that the use of PAL inhibitors can weaken plant defense in some cases, thereby increasing disease severity. In biological weed control, such compounds might be useful as pathogen synergists. This could provide better weed control with less pathogen inoculum. Apparently none of these PAL inhibitors have been tested for this utility in weed control. It is also possible that more efficient inhibitors may be synthesized in the future as knowledge of PAL and secondary metabolism increases.

Xenobiotic Compounds That Alter Secondary Plant Products

Xenobiotics can alter various metabolic pathways in plants. Chemical alteration of phenolic compounds for agricultural purposes has been suggested to have potential (130, 131). Since several major plant enzymes of secondary metabolism are involved in resistance to pathogens, alteration of secondary metabolism could influence disease development. Herbicides, plant growth regulators (synthetic and natural), and enzyme inhibitors such as those presented for PAL (Fig. 4) have been reported to alter secondary plant products (Table III). Results here demonstrate that the levels of many phenolic derivatives are influenced by such compounds when applied to a variety of plant species. If such xenobiotics are to be used to regulate levels of secondary products, application concentration, uptake, translocation to active sites, and concentration at active sites are critical factors that need to be determined. Lack of rapid metabolism (or the nature of metabolism) or degradation within plants would also be important.

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$H_2N—O—CH_2—COOH$

Aminooxy acetic acid

$H_2N—O—CH(C_6H_5)—COOH$

α-Aminooxy-β-phenylpropionic acid

$H_2N—O—CH_2—C_6H_5$

o-Benzylhydroxylamine

$H_2N—CH(COOH)—CH_2—C_6H_7$

3-(1,4-Cyclohexadienyl)alanine

$H_2N—CH(PO_3H_2)—CH_2—C_6H_5$

(1-Amino-2-phenylethyl)phosphonic acid

Figure 4. Chemical structures of several PAL inhibitors.

Table III. Effects of Synthetic and Naturally Occurring Chemicals on Secondary Plant Product Levels in Intact, Excised, or Cell Culture Tissues

Chemical	Plant	Secondary Plant Product	Qualitative Effect	Ref.
Herbicides				
acifluorfen	spinach	ferulate amine	+	132
	soybean	glyceollins	+	133
		glyceofuran	+	133
	bean	phaseollin	+	133
	pea	pisatin	+	133
	broadbean	medicarpin	+	133
	celery	xanthotoxin	+	133
	cotton	hemigossypol	+	134
alachlor	sorghum	anthocyanin	-	134
		lignin	-	134

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Table III. (con't)

chlorsulfuron	soybean	anthocyanin	+	135
	sunflower	p-coumarate	+	136
		caffeate	+	136
		ferulate	+	136
		sinapate	+	136
2,4-D	tobacco	scopolin	+	137
	sunflower	scopolin		138
DSMA	soybean	anthocyanin	+	139
glyphosate	soybean	anthocyanin	-	140
	buckwheat	shikimate	+	141
	tobacco	shikimate	+	142
	ryegrass	shikimate	+	143
	various	gallate	+	143
		4-hydroybenzoate	+	143
metribuzin	soybean	anthocyanin	-	139
propanil	soybean	anthocyanin	-	139
sethoxydim	corn	anthocyanin	+	144
Fungicides				
WL 28325	rice	momilactone A	+	145
		momilactone B	+	145
Plant growth regulators				
ethylene	sorghum	anthocyanin	-	146
	turnip	anthocyanin	-	146
	cranberry	anthocyanin	+	146
	poinesttia	anthocyanin	+	146
	lettuce	flavonoids	+	147
kinetin	*Amaranthus* sp.	amaranthin	+	148
IAA	soybean	anthocyanin	-	149
Dimethipin	soybean	anthocyanin	-	150
PAL inhibitors				
AOPP	mint	caffeate	-	151
	soybean	glyceollin	-	125,126
	coleus	rosmarinate	-	152
	carrot	anthocyanin	-	153
	buckwheat	anthocyanin	-	111
		vanillin (fr. lignin)	-	154
	cabbage	anthocyanin	-	117
	radish	feruloyl deriv.	-	155
		kaempferol	-	155
		pelargonidin	-	155
	sphagnum moss	sphagnorubin	-	156,157
	cucumber	hydroxycinnamates	-	158
	tobacco	secondary phenolics	-	102
AOA	soybean	anthocyanin	-	114
OBHA	mungbean	anthocyanin	-	118

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Xenobiotics That Alter Extractable PAL Activity. PAL inhibition has been chosen as a site of action for herbicide design; however, several herbicides and other chemicals were found to have no specific effect on PAL (159). Analogous results were obtained when herbicides encompassing the major herbicide classes were examined (139, 160). The high increase of PAL by glyphosate makes this herbicide unique in these correlations. Generally PAL activity correlates well with the production of secondary products, and this has also been demonstrated in PAL-phenolic (Fig. 5) and PAL-anthocyanin (Fig. 6) correlations in soybean seedlings as affected by seventeen herbicides (139). Other herbicides, fungicides, insecticides, and adjuvants can alter secondary plant compound levels via indirect effects (150). Some herbicides have more dramatic effects on extractable PAL activity levels from various plants and have been examined for some interactions with plants and disease organisms (Figure 7). Because glyphosate reduced aromatic amino acid (phenylalanine, tyrosine, and tryptophan) pools in Rhizobium japonicum and duckweed (Lemna gibba L.), and supplementation of these amino acids reversed glyphosate action, inhibition of this pathway was proposed as this herbicide's mode of action (161). Induction of PAL activity can also reduce aromatic amino acid levels sufficiently to reduce growth, and reversal is possible by feeding phenylalanine (162). Thus, increased PAL activity was examined as a possible mode of action of this herbicide (163). Extractable PAL activity in plants was found to increase dramatically after treatment with glyphosate, but no in vitro effect was noted (99, 163). Although glyphosate's primary mode of action has now been shown to be inhibition of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (164), increased PAL, and reduced EPSP synthase, resulting in phenolic product alteration by glyphosate could affect disease development in weeds or crop plants or in genetically altered glyphosate-resistant crop plants. Sublethal glyphosate concentrations were found to block glycelloin accumulation and to lower resistance of soybean to a bacterial pathogen (121, 122). Glyphosate has also been examined in other instances for interactions of plants and disease organisms (165-167).

The herbicide 2,4-D (2-4-dichlorophenoxyacetic acid) has been known for some time to inhibit PAL in vivo but not in vitro (168). 2,4-D also interacts with IAA to reverse glyphosate growth inhibition in tissue cultures (169, 170), but not in intact plants (171). Extractable PAL activity in soybean seedlings was inhibited by 2,4-D (171) and 2,4-D lowered PAL activity increases caused by glyphosate (171). The herbicide has also been examined for interactions of induction and secretion of pathogenesis-related proteins in tobacco suspension cultures (172).

Acifluorfen {5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid} is a diphenyl ether herbicide which requires light and oxygen for its toxic action (173). This compound affects secondary plant metabolism by increasing extractable PAL activity (132, 174, 175). For instance, it increases N-feruloyl-3-methoxytyramine (FMT) levels (132, 133, 176). Modification of enzymes and products of secondary plant metabolism by acifluorfen (133, 174) is similar in some host-pathogen interactions (177). Acifluorfen has been applied with the weed pathogen Colletotrichum gloeosporides (Penz.) Sacc. f. jussiae as a mixture or sequentially and shown not to inhibit disease development on northern jointvetch [Aeschynomene virginica (L.) B.S.P.] (178).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch005

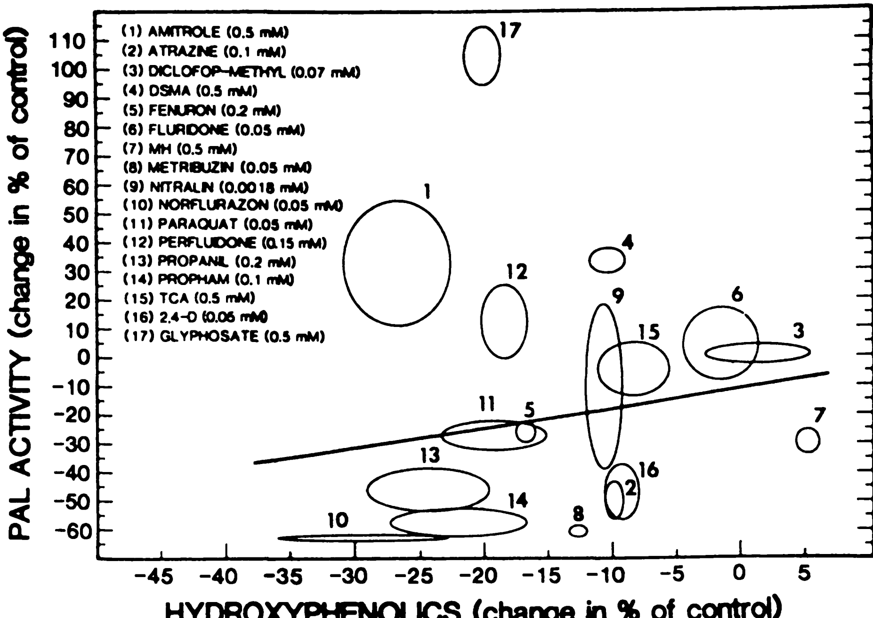

Figure 5. Correlation of extractable PAL activity with soluble hydroxyphenolic levels in axes of light-grown soybean seedlings 48 h after herbicide treatment. (Redrawn with permission from Ref. 139. Copyright 1983 Weed Science Society of America).

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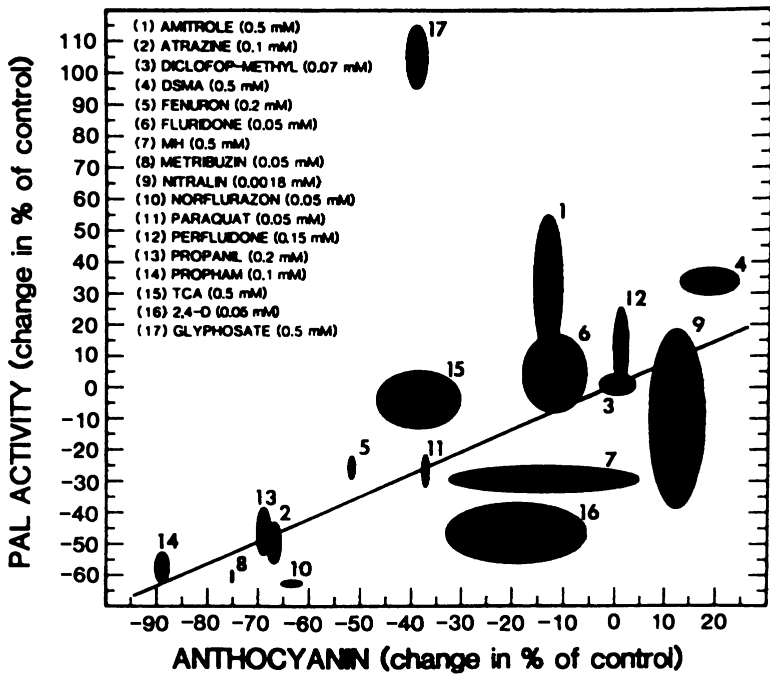

Figure 6. Correlation of extractable PAL activity with anthocyanin content in hypocotyls of light-grown soybean seedlings 48 h after herbicide treatment. (Redrawn with permission from Ref. 139. Copyright 1983 Weed Science Society of America).

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$HO-P(=O)(OH)-CH_2-NH-CH_2-COOH$

Glyphosate

Cl—C_6H_3(Cl)—O—CH_2—COOH

(2,4-Dichlorophenoxy)acetic acid

F_3C—C_6H_3(Cl)—O—C_6H_3(COOH)—NO_2

Acifluorfen

C_6H_4(Cl)—SO_2—NH—CO—NH—triazine(CH_3, OCH_3)

Chlorsulfuron

Figure 7. Chemical structures of some herbicides that affect in vivo PAL activity and phenolic levels in plants.

Chlorsulfuron {2-chloro-N-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide} causes large increases in total phenolic compound levels in hypocotyls of sunflower seedlings (136) and increases in anthocyanin levels of soybean (135). This compound has been suggested as an exceptional agent for altering PAL (135, 136) and secondary plant products for studying ecological interactions (136).

Plant Growth Regulator Interactions. Plant pathogenesis-related (PR)-proteins (some of which are enzymes) are a group of host-encoded inducible proteins which are synthesized in certain cases of resistance to pathogens or stress (179). The expression of PR-genes in plant tissues can be affected by plant hormones. Ethephon (2-chloroethylphosphonate) degrades to produce ethylene which induces PR-proteins in tobacco (180). AVG, which blocks ethylene production by plants, prevents this induction of PR-proteins (181). Other growth regulators such as IAA, benzyladenine, and the herbicide 2,4-D, induce PR-proteins in tobacco callus or in intact tobacco leaves (182). Kinetin (181) and gibberellic acid (183) are also PR-protein inducers.

Ethylene induces PAL (79), and toxic levels of the ethylene precursor ACC and ethephon cause increases of FMT in spinach (133) Anti-ethylene treatment; i.e., $AgNO_3$ (184) and heat shock (185) can counteract increases in FMT caused by the herbicides acifluorfen and oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl) benzene] (133). FMT levels caused by these herbicides are also counteracted by AOA and AOPP, which inhibit ethylene production and PAL activity (186), but not by AVG (133).

Prospects

The importance and complexity of various aspects of host defense dynamics, including phenolic substances, phytoalexins, and lignification, have been studied for some time, but their precise roles in disease resistance are still not fully understood, as summarized in the present discussion and many reviews (e.g., 187-189). Detailed discussion of these areas is beyond the scope of this chapter. Phytoalexins and their elicitors, as related to defense and the development of secondary metabolic products as herbicides are discussed elsewhere in this book (chapter by N. Keen).

The present review clearly shows that many compounds can alter enzyme activities and products of secondary plant metabolism which can, in turn, significantly modify plant responses to stress, such as attack by pathogens. Although many plant pathogens have been identified that have potential as biological weed control organisms (190-192), some of these may lack sufficient virulence factors relative to aggressiveness. Since both agrochemicals (herbicides, plant growth regulators, etc.) and pathogen infection can modify secondary metabolism in plants, chemical regulation could modify pathogen virulence. This research area holds great promise for understanding how to improve weed control via integrated management. Data on the biochemistry and physiological aspects of herbicide action as well as on specific molecular sites of action of herbicides is accumulating. This information, coupled with an understanding of pathogen-weed interactions should expand our ability to provide more

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efficacious weed control by manipulative integration of chemicals and biological agents such as plant pathogens.

Because certain herbicides can predispose plants to disease (193) and because efficient integrated weed control (biological and chemical) demands that there be little or no adverse interactions when these two systems are used simultaneously, further research in this area is necessary. Indeed it would be useful, for example, if a herbicide and pathogen interaction was synergistic against weeds.

The importance of secondary plant metabolism in plant disease resistance suggests that it as an area that will provide fundamental answers to questions allowing us to manipulate weed host-pathogen interactions for improved biological weed control.

Conclusions

The use of chemicals (synthetic or natural) for the manipulation or regulation of weed resistance and/or weed pathogen infectivity could have an important impact on weed control strategies. There is potential for host range expansion of host-specific weed pathogens via chemical or agrochemical manipulation. Mechanisms of chemical synergy or antagonism between herbicides and pathogens for weed control could lead to a more complete understanding of herbicide mode of action, plant defense against pathogens, and pathogen infection processes. Knowledge of such mechanisms could be used for crop protection (e.g., control of crop pathogens). Synergistic interactions could also reduce amounts of herbicide and bioherbicide (plant pathogen or microbial phytotoxin) needed for efficient weed control.

Acknowledgments and Disclaimers

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Chapter 6

Phytoalexins and Their Elicitors

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Phytoalexins are an important component of the plant disease defense reaction called the hypersensitive response. Successful pathogens have evolved methods for dealing with plant phytoalexins, including suppressors of their production, detoxification of the phytoalexins and in some cases avoiding elaboration of substances, called elicitors, that would otherwise initiate the defense reaction. Elicitors obtained from pathogens are of considerable utility for study of various aspects of plant biology because of their interaction with the products of plant disease resistance genes. Substantial information has been obtained on how elicitors are perceived by plant cells and how they function, but much remains to be done. Finally, elicitors may prove of value for the economic production of exotic plant secondary metabolites and as specific herbicides.

Phytoalexins and elicitors that stimulate their production by plant tissues have received increasing attention as a consequence of several recent developments. First, after years of controversy, phytoalexins are now generally accepted as a *bona fide* mechanism of disease defense in higher plants. Second, race specific elicitors have been isolated that confer the plant specificity of the pathogen race which produces them. This specificity implies that the elicitors are determinative of the interactions and that they should be useful for the elucidation of recognition and signal transduction mechanisms in plants. Third, recent evidence has illuminated the mechanisms whereby elicitors lead to derepression of genes encoding phytoalexin biosynthetic enzymes and other defense response genes. Fourth, progress has occurred in the use of elicitors to produce exotic natural products by plant cell cultures.

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Disease Resistance in Plants

Disease defense in plants is a complex of mechanisms, including anatomical and morphological features as well as preformed chemical substances. Phytoalexin production is usually associated with a plant defense system called the hypersensitive response (HR), which is only invoked following pathogen infection. The induction of hypersensitive reactions in plants is under several control circuits and thus is involved at several levels of pathogen recognition and exclusion. Two such levels are classed as general resistance (in which an entire plant species reacts against all members of an entire pathogen taxon) or as specific resistance (in which certain cultivars of a plant species are resistant to only certain biotypes or races of a pathogen species). Incompatible plant-pathogen combinations result from the occurrence of the host defense reaction and plant resistance, which are in turn presumed to result from recognition of the infecting pathogen. Compatible host-pathogen combinations, on the other hand, are those in which recognition and host defense do not occur, the pathogen develops to a considerable extent in the plant tissue and disease occurs.

Phytoalexin production is only one component of the hypersensitive response. Others include the production of enzymatically active proteins such as chitinases, antiviral proteins and β-1,3 glucanases as well as plant cell wall structural elements, including hydroxyproline rich glycoproteins, callose and lignin. The deposition of these substances into plant cell walls surrounding the pathogen infection site presumably constitutes a barrier to further pathogen ingress through the plant. While none of these mechanisms have been convincingly implicated as physiologically important in disease defense, it is likely that they contribute toward resistance to at least certain types of pathogen.

Phytoalexins are low molecular weight, antibiotic secondary metabolites that, as noted above, are produced inducibly by many plant species and are involved with defense against pathogens [for more detailed recent reviews, see (1-10)]. Phytoalexins are not present in vegetative plant parts in significant quantity but accumulate rapidly at infection sites of incompatible pathogens and in response to treatment with various substances or stimuli called elicitors. Phytoalexins are general toxicants which affect the plasma membrane integrity of microorganisms, animal cells and, indeed, cells of the plants that make them.

Over 200 species of plants have been shown to produce phytoalexins and this substantial number *a priori* indicates that they are widely distributed, physiologically important components of plant disease defense. Phytoalexins have recently been isolated from several plant taxa in which they had not previously been found [e.g. citrus (11); *Coleostephus* (12); sorghum (13); and crucifers (14,15)]. It was also recently observed that all of 61 naturally collected accessions representing 10 species of *Glycine* produced one or more members of the family of closely related phytoalexins trivially called glyceollins (16). While the production of glyceollins is rare outside *Glycine*, these phytoalexins are highly conserved among members within the genus. This high degree of conservation implies a crucial role for the phytoalexins in *Glycine* spp. Furthermore, while phytoalexins generally exhibit antibiotic properties, many of

them have relatively unusual chemical structures. In addition to the large number of novel structures already described, recent reports have noted new and chemically unique phytoalexins (Figure 1). These include brassilexin, a sulfur containing indole derivative (14), the oryzalexins from rice (17), a biphenyl phytoalexin, magnolol, from *Cercidiphyllum* (15), the nitrogen containing compound dianthalexin (18) and the novel spiroketalenol ether phytoalexin, mycosinol, from *Coleostephus* (12). In addition, certain secondary metabolites previously considered as preformed metabolites have been shown to be produced as phytoalexins [e.g. protoanemonin by *Caltha palustris* (19) and anthocyanidins in sorghum, shown to behave as phytoalexins (13)].

Our understanding of phytoalexins and their biological role has been aided by the recent finding that some of them are photo-activatable to molecules of greater biological activity (20). For instance, it was previously thought that phytoalexins did not directly affect viruses in hypersensitively responding plant tissue. However, 2,7-dihydroxycadalene, a photoactivatable cotton phyto-alexin was recently shown to inactivate cauliflower mosaic virus *in vitro* (21). This raises the possibility that certain phytoalexins may cause virus inactivation during hypersensitive resistant plant reactions.

Evidence for the Involvement of Phytoalexins in Resistance

After years of frequently contrived controversy on the role of phytoalexins in disease resistance, they are now generally regarded as *bona fide*, physiologically important agents. This emerging consensus has resulted from the continued accumulation of data suggesting importance in resistance and from genetic experiments showing that the degradation of host phytoalexins by certain patho-gens is required for high virulence (22,23). An important correlary of these genetic experiments states that if a pathogen elicits phytoalexins but cannot otherwise tolerate or detoxify them, then the phytoalexins constitute an effective disease defense mechanism. It is not possible in this paper to cover the extensive literature linking phytoalexins with disease resistance, but the review articles cited earlier discuss much of it. The general lines of evidence linking phytoalexins to resistance expression (with references that are illustrative but far from comprehensive) are:

(i) Genetically resistant plants invariably produce higher levels of phytoalexins than do susceptible genotypes (24,25).

(ii) pathogen produced suppressors of phytoalexin production decrease resistance (26).

(iii) chemical inhibitors of protein synthesis or inhibitors of enzymes in phytoalexin biosynthetic pathways decrease phytoalexin production and negate resistance (27,28).

(iv) race specific elicitors have been isolated that reproduce the specificity for phytoalexin induction of the pathogen race from which they were isolated (29,30).

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(v) removal of phytoalexins from a pathogen infection site decreases resistance while exogenous addition of phytoalexins increases resistance (31).

(vi) localization studies have shown that phytoalexins are made at the right place at the right time and at sufficient concentration to account for inhibition of the pathogen (32,33).

(vii) electron microscope studies have shown that pathogens in resistant-reacting plant tissues exhibit ultrastructural abnormalities that are extremely similar to those shown by the same organisms grown in culture and exposed to the pure phytoalexins (34,35).

Elicitors of Phytoalexins and the HR

Plant pathogens elaborate metabolites into the culture medium or contain substances that elicit plant tissues to produce phytoalexins in the absence of the pathogen itself. Generally, this involves several components (e.g. 36) that may appear in culture fluids, most of which are not race-specific--that is they are produced by various isolates of the pathogen and cause similar effects on a range of host cultivars. The physiologic importance of such elicitors has generally not been established, and DeWit and Spikman (37) showed that non-specific glycoprotein elicitors present in culture fluids of *Cladosporium fulvum* were not detectable at all in infected host tissues, but a race specific elicitor was isolated from infected leaves, as will be discussed later. Despite the resulting suspicion that non-specific elicitors found in culture fluids may not necessarily be of physiological importance in plant-pathogen interactions, roles have nevertheless been claimed for some of them.

The major types of non-specific elicitors characterized have been glucans and chitin derivatives. The most studied of these are branched β-1,3-glucans extracted from the cells walls of various fungi, particularly *Phytophthora* spp. Albersheim's group extracted glucans from cell walls of *P. megasperma* f.sp. *glycinea* by autoclaving them in water or with mild acid hydrolysis (38). The extracts were carefully fractionated into the smallest elicitor-active component, identified as a seven membered glucan composed of a β-1,6-linked pentaglucan with β-1,3-linked glucose residues on the second and fourth residues (39) (n=1, Figure 2).

Yoshikawa et al. (40) recently isolated homologues of the heptaglucan elicitor from *P. megasperma* f.sp. *glycinea* cell walls treated with a purified β-1,3-endoglucanase from soybean plants. These glucans shared the basic heptaglucan structure of a β-1,6-linked glucose backbone with the penultimate residues substituted with β-1,3-linked glucose residues. However, the enzyme-released elicitors included those with longer β-1,6 backbones than the heptaglucan (Figure 2). Significantly, the larger glucans with n=2 or greater were much more potent elicitors than the heptaglucan (n=1). These more active elicitors were not detected in the earlier work by Albersheim's group (39), probably because they exposed cell walls to the relatively harsh treatment of acid extraction. Unlike acid, which attacks both β-1,3 and β-1,6 glycosidic linkages, the glucanase used by Yoshikawa and co-workers (40) only attacks internal β-1,3 linkages, thus preserving the longer β-1,6-linked elicitor back-

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BRASSILEXIN

ORYZALEXIN A

MAGNOLOL

MYCOSINOL

DIANTHALEXINE

Figure 1. Recently described phytoalexins.

Figure 2. Homologous polyglucan elicitors isolated from cell walls of *Phytophthora megasperma* f.sp. *glycinea*; n = 1 to 5 or greater and all glycosidic linkages are β.

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bones. While these glucan elicitors have considerable activity in phytoalexin elicitor assays and are suspected to play a role in disease defense, no evidence has yet definitively linked them to the occurrence of defense reactions in soybean plants infected with an incompatible race of *P. megasperma* f.sp. *glycinea*. Indeed, all races of the pathogen contain the elicitors and all soybean cultivars respond similarly to glucan preparations. Finally, while glucans efficiently elicit phytoalexins in some plants such as soybean, they are relatively poor elicitors in other plants (e.g. 41).

Chitin and chitosan are also fungal wall components that elicit phytoalexins and/or lignin accumulation (42,43). As with the glucan elicitors, the physiologic role of chitosan has not yet been established and no race specific properties have been noted. Another elicitor which has not yet been shown to possess race specific features is a glycoprotein from *Puccinia graminis* f.sp. *tritici* cell walls that elicits the HR in wheat leaves (44). A final class of non-specific elicitors worthy of note are oligogalacturonide elicitors isolated from plant cell walls. Bailey (45) obtained evidence suggesting that wounded plant tissues produced 'endogenous elicitors' which subsequently elicited phytoalexin responses in surrounding healthy cells. Bruce and West (46) and later several others showed that certain microbial pectic enzymes released elicitor-active oligogalacturonide fragments from plant cell walls. Again, the physiologic relevance of such elicitors has not been established, but it is appealing to think that they may be of importance in the case of plant attack by pathogens that produce relatively low activities of pectin degrading enzymes.

Since they cannot account for the specificities observed in gene-for-gene plant-pathogen systems, the physiologic role of non-specific elicitors, if any, must be in general resistance. For instance, if plant tissues respond rapidly enough to glucans, chitin or other non-specific elicitors from an invading pathogen, the resulting defense reaction may be sufficient to impede further pathogen development. Similarly, pectic enzymes produced by the pathogen may liberate plant oligogalacturonide elicitors that lead to resistance. The situation might also be complex since plant enzyme inhibitors, unfavorable pH conditions or other factors that reduce the efficiency of pathogen produced pectic enzymes might increase the probability that effective levels of oligogalacturonide elicitors could be formed at the pathogen infection site (47,48).

A wide range of 'abiotic' elicitors that are not obtained from pathogens function at varying efficiencies as phytoalexin elicitors. These include heavy metal ions, polyanions, various antibiotics and detergents and red-ox agents (see reviews noted earlier for details). The latter group includes glutathione, reported to be an effective elicitor in *Phaseolus vulgaris* by Wingate et al. (49). It is probable that most of the abiotic elicitors function by causing non-specific damage to plant tissue which releases endogenous plant elicitors, by blocking phytoalexin turnover (50) or by mimicing the plant cell signal transduction machinery (see below).

Suppressors and Enhancers

Metabolites have been isolated from pathogens that suppress phytoalexin production by a host plant in the presence of otherwise functional elicitors (e.g. 26). The elaboration of such suppressors

is a logical pathogenic strategy for overcoming general resistance and they may accordingly be more widespread among pathogens than is currently appreciated.

Doke, Kuc and associates have presented evidence indicating that suppressors may also function in a gene-for-gene system involving single disease resistance genes. The potato-*Phytophthora infestans* system involves the interplay of several single disease resistance genes and many pathogen races. The suppressor model as applied to this system holds that all fungus races elaborate non-specific elicitors when infecting potato [possibly arachidonic and related fatty acids (51)]. Races that are not restricted in a certain plant resistance genotype have been proposed to produce specific suppressor substances that repress the phytoalexin response. Doke et al. (52) presented evidence suggesting that the race specific suppressors might be mycolaminarans, small oligoglucans present in the cytoplasm of various *Phytophthora* spp. The suppressor work has been criticized because it is at odds with genetic data on gene-for-gene systems and the suppressors have neither been isolated and characterized nor proven to account for specificity.

To complicate the potato-*P. infestans* system further, Maniara et al. (53) and Preisig and Kuc (54) reported the presence in fungal homogenates of substances, again identified as oligoglucans, that behaved as 'enhancers' of the defense reaction elicited by arachidonic acid in potato. Additional work will be required to determine whether the various suppressors and enhancers are physiologically important in resistance expression.

Race Specific Elicitors

As noted earlier, specific resistance occurs in only certain plant cultivars against certain pathogen biotypes. In classic work, Flor (55) and others showed that invocation of the HR is determined by the activity of single genes in the host (called disease resistance genes) and in the pathogen (called avirulence genes). Because resistance genes are widely used in agriculture for the control of plant diseases, there is considerable interest in understanding the biochemical basis of this specificity. The elicitor-receptor model has frequently been proposed to explain specificity in such gene-for-gene systems and is supported by considerable genetic and biochemical evidence (56-58). The model predicts that a pathogen race carrying a specific avirulence gene produces a unique metabolite, called a race specific elicitor, while races lacking the functional avirulence gene do not make this elicitor. Plants carrying the complementary disease resistance gene produce a unique receptor for the elicitor molecule. Following elicitor-receptor binding, unknown intermediate events result in specific derepression of certain plant genes, called disease response genes. Their expression leads to the various consequences of the HR, including phytoalexin accumulation (58).

Our own research group reported the first evidence suggesting occurrence of race specific elicitors in *Phytophthora megasperma* f.sp. *glycinea* (59) and *Pseudomonas syringae* pv. *glycinea* (60). Preparations from various races of these pathogens exhibited greater elicitor activity on incompatible soybean cultivars than on compatible cultivars. Despite the promising results, however, it was not possible to isolate and characterize the elicitors, a problem that has been noted with other plant-pathogen systems as well. Such

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difficulties may be caused by lability of the relevant elicitors or their production in quantity only when the pathogens have been inoculated into host tissue. More recent research in several laboratories has illuminated these possibilities.

Crucefix et al. (61) searched intensively for race specific elicitors in the *Bremia*-lettuce host-pathogen system. Infection structures of the obligately pathogenic fungus were assayed for effects in lettuce cotyledons and protoplasts of known resistance genotypes. While the presence of race specific elicitors of the HR was indicated from certain isolates of the fungus, bioassay inconsistency and the difficulty in isolating significant quantities of the putative elicitors posed serious problems. The authors also speculated that the specific elicitors in this system might be labile metabolites that were only secreted at certain stages of the infection process in the plant host. The production of race specific HR elicitors only during host infection was also suggested by the earlier work of Jones and Deverall (62) with the wheat-*Puccinia recondita* system and has been dramatically shown to occur in the interaction of *Cladosporium fulvum* and tomato by De Wit and co-workers.

Several investigators had searched unsuccessfully for race specific elicitors in *C. fulvum* by fractionating fungal cultures grown on common laboratory media. De Wit and co-workers (37,63) inoculated the fungus into a compatible tomato cultivar and subsequently extracted intercellular fluids from the inoculated leaves. These leaf extracts contained metabolites of presumed fungal origin that elicited the hypersensitive reaction only in subsequent bioassays on tomato cultivars carrying complementary resistance genes to avirulence genes in the originally inoculated fungal race. Thus, a specific elicitor was produced only by fungal isolates carrying a unique avirulence gene and only when the fungus grew in the host. One of the putative race specific elicitors, associated with pathogen avirulence gene *avr*Cf9, has recently been isolated and identified as a 27 amino acid, cysteine-rich peptide (29) (Figure 3). The peptide may be a processed product of the pathogen A9 avirulence gene, but this has not yet been tested by critical genetic experiments.

Another characterized race specific elicitor is victorin, produced by *Helminthosporium victoriae*. This metabolite specifically elicits the HR at picomolar concentrations only on oat cultivars carrying the gene *Pc*-2, that confers resistance to the rust pathogen, *Puccinia coronata* (64). The structure of victorin has been determined by Wolpert et al. (65) as a basic peptide containing unusual amino acid residues. Mayama et al. (64) showed that victorin elicited the hypersensitive response and phytoalexin production only in *Pc*-2 oat lines. It therefore appears that victorin is a race specific elicitor of the oat HR and that *P. coronata* races carrying

Tyr- Cys-Asn-Ser-Ser-Cys-Thr-Arg-Ala-Phe-Asp-

Cys-Leu-Gly-Gln-Cys-Gly-Arg-Cys-

Asp-Phe-His-Lys-Leu-Gln-Cys-Val

Figure 3. Race specific elicitor from *Cladosporium fulvum*. All relevant amino acids are assumed to be (L) and the first amino acid is the N-terminus.

the avirulence gene complementing *Pc*-2 must contain an analogous elicitor. Wolpert and Macko (66) recently identified a protein that may function as a specific receptor for victorin in the *Pc*-2 oat genotype. However, it remains to be established whether this protein is the primary product of the *Pc*-2 resistance gene.

Recent results with tobacco mosaic virus (TMV) have illuminated the basis of host range specificity with this virus. Knorr and Dawson (67) identified mutations in the coat protein gene of TMV that resulted in hypersensitive resistant reactions on tobacco plants carrying the N' resistance gene rather than the normal susceptible response. More recently, the same group has provided evidence indicating that the coat protein itself and not viral RNA or other viral gene products is a specific elicitor of the HR. The most elegant of these experiments involved introduction of the mutant coat protein gene into N' tobacco plants. The regenerated, transgenic plants exhibited numerous necrotic foliar lesions, similar to plants inoculated with the virus (Culver, J. and Dawson, W., Univ. of California, Riverside, personal communication, 1989). While it cannot be entirely ruled out that the TMV coat protein may have an unknown enzymatic function which leads to an active elicitor, the results indicate that the coat protein itself functions as an elicitor of the HR.

Avirulence Genes and Specific Elicitors

Our understanding of gene-for-gene complementarity and the role of race specific elicitors has been considerably furthered by the cloning of avirulence genes from several bacterial pathogens (for review see 57). Avirulence (*avr*) genes have been cloned from several members of the *Pseudomonas syringae* and *Xanthomonas campestris* groups and some of them have been sequenced and their gene products characterized. Thus far, all sequenced *avr* genes encode single protein products. Surprisingly, all of the proteins lack signal peptide sequences, indicating that they occur in the bacterial cytoplasm and are not secreted to the medium or to the cell surface. Coupled with the absence of detectable elicitor activity from certain *avr* proteins (e.g. 68,69), this indicates that the gene products themselves may not directly elicit the plant HR. Research with the *avr*D gene cloned from *P. syringae* pv. *tomato* has offered further insight into this aspect of *avr* gene function.

Four different avirulence genes have been cloned from *P. syringae* pv. *tomato* (70, Shen and Keen, unpublished) that, when introduced into the related bacterium *P. syringae* pv. *glycinea*, caused them to elicit hypersensitive reactions on some but not all soybean cultivars. These bacterial genes therefore behaved as race specific *avr* genes in *P.s. glycinea*. Surprisingly, one of them also turned out to be identical to *avr*A, previously cloned from race 6 of *P.s. glycinea* (69,61). Thus, the *avr* genes from *P.s. tomato* not only function in *P.s. glycinea*, but one of them is identical to an *avr* gene present in a *P.s. glycinea* race. On the other hand, a functional copy of one of the other *P.s. tomato* avirulence genes (*avr*D) has not been identified in any isolate of *P.s. glycinea*.

Kobayashi and Keen (manuscript in preparation) sequenced the *avr*D gene from *P.s. tomato* and found that it encoded a single protein product of 34 kD. It was also observed that *Escherichia coli* cells

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expressing *avr*D elicited HR in precisely the same cultivars as did *P.s. glycinea* race 4 cells carrying the gene. Neither the partially purified *avr*D protein nor lysed *E. coli* cells expressing *avr*D elicited the HR in the appropriate soybean cultivars. However, supernatant fluids of these *E. coli* cultures contained a low molecular weight factor that specifically elicited the HR only in incompatible soybean cultivars (Keen, N. T., Tamaki, S., Thordal-Christensen, H., Gerholt, D., and Kobayashi, D., unpublished data). The *avr*D gene product may therefore possess an enzymatic function that converts a normal *E. coli* metabolite to an elicitor-active compound which is secreted from the cells. Further work has also shown that wildtype *P.s. tomato* but not *P.s. glycinea* produces the elicitor. However, introduction of the cloned *avr*D gene into *P.s. glycinea* leads to production of the elicitor and these cells elicit the HR in appropriate soybean cultivars.

The *avr*D elicitor has been purified by the use of C18 and phenyl HPLC columns (Tamaki, S., Stayton, M., Gerholt, D., and Keen, N. T., unpublished data), but its structure is not yet known. Low levels of the elicitor are produced by *P.s. tomato* cells, but an *avr*D mutant strain is deficient. These and other genetic experiments prove that the elicitor specified by *avr*D mediates avirulence gene function and is the agent which initiates plant defense in soybean cultivars carrying the complementary resistance gene. It is therefore the first race specific elicitor that has been isolated and proven to account for gene-for-gene specificity. The results with *avr*D are also consistent with the elicitor-receptor model discussed earlier. It will be of further interest to see if, as predicted, a specific receptor for the *avr*D elicitor occurs in soybean cultivars carrying the complementary resistance gene.

Since *avr*D leads to HR on some but not all soybean cultivars, the gene-for-gene relationship predicts the presence of a classical single resistance gene in those cultivars that are resistant to bacteria carrying *avr*D. This possibility has been tested in more recent work involving crosses of compatible and incompatible soybean cultivars. F2 progeny of crosses involving soybean cultivars Acme x Norchief and Flambeau x Merit segregated in a 3:1 manner for both resistance to *P.s. glycinea* carrying *avr*D and for sensitivity to the *avr*D elicitor (Keen, N. T., and Buzzell, R. I., unpublished data). Sensitivity to the elicitor and resistance to Psg carrying the gene were dominant and co-inherited in all of ca. 600 segregating F_2 and F_3 progeny tested. The tight linkage of elicitor sensitivity and resistance implies that the elicitor is the bacterial agent which physiologically initiates the HR in resistant plants. The presence in soybean of a resistance gene complementary to *avr*D is surprising in view of the fact that a functional *avr*D gene has not been identified in any race of *P.s. glycinea*. What therefore is the function of this soybean resistance gene? Does it confer resistance to other bacteria carrying *avr*D, such as *P.s. tomato*?

How Elicitors Initiate Phytoalexin Production

Recent progress has occurred in understanding the biochemistry that occurs in plant tissues treated with phytoalexin elicitors. This work has been facilitated by the use of cloned plant genes encoding enzymes in phytoalexin biosynthetic pathways. Thus, in legumes, it

has been possible to use cloned genes of phenylalanine ammonia lyase and chalcone synthase as probes to monitor expression of these defense response genes following pathogen infection or elicitor treatment (see 58). These experiments have demonstrated increased expression of the defense response genes rapidly after elicitor treatment (72), therefore suggesting the occurrence of few intermediate steps between elicitor recognition and plant gene activation.

While plant disease resistance genes involved with gene-for-gene resistance as well as resistance genes conferring general resistance have been postulated to encode cell surface receptors, very little direct evidence is at hand. Yoshikawa et al. (73) produced the first evidence directly indicating that soybean cells possess receptors for the fungal glucan elicitor, mycolaminaran. Since mycolaminaran is not race specific, the putative receptor must be associated with general and not specific resistance. Schmidt and Ebel (74) and Cosio et al. (75) confirmed occurrence of the glucan receptor on the soybean plasma membrane, setting the stage for its isolation. It should also be noted that receptors for the *Cladosporium* peptide, for the oat *Pc-2* resistance gene affected by victorin, and the putative soybean receptor for the elicitor specified by *avr*D could also be isolated by using affinity techniques with the various elicitors as probes.

It is not yet clear whether receptor/elicitor complexes directly interact with DNA to derepress phytoalexin biosynthetic genes or whether other second messengers exist. For instance, work by Hadwiger (76) suggests that elicitors or elicitor-receptor complexes may directly interact with plant DNA, thus changing its transcription. Recent experiments in Chris Lamb's laboratory have also illuminated the basis for gene derepression. *Phaseolus* phenylalanine ammonia lyase and chalcone synthase genes were found to contain DNA elements upstream of the coding regions that functioned as silencer and activator regions (see 58). These regions are assumed to interact with as yet unidentified proteins to activate gene expression in response to elicitors or inoculation with incompatible pathogen races.

Several groups have obtained data indicating that changes in calcium flux from elicitor-treated plant cells may serve as a second messenger in phytoalexin initiation (77-80). The altered calcium flux may in turn be caused by rapid depolarization/polarization and redox perturbation of the plant cell membrane (56; 81-85). For instance, production of superoxide anion, hydrogen peroxide and hydroxyl radicals have been observed in plant tissues undergoing hypersensitive reactions (86-89).

Uses of Elicitors

Elicitors have previously been useful in studies on the mechanisms of plant defense and promise greater utility in the future. This should be especially true as additional race specific elicitors are isolated and their effects tested on incompatible plant genotypes. In addition, elicitors may prove useful in other aspects of plant biology. Some of the established and potential uses of elicitors are shown in Table I.

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Table I. Uses of elicitors

1. As agents for the specific induction of plant enzymatic pathways leading to phytoalexins (3,4).
2. For the production of exotic secondary metabolites by cultured plant cells (19,90).
3. Screening plant cultivars and breeding lines for pathogen resistance (91,92).
4. For studies on receptor biology in plant cells (66,73).
5. In studies on plant-pathogen specificity and cell-cell signalling (57,58).
6. For use as specific herbicides (93).

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Elicitors have been widely used in studies of defense gene activation (for reviews see 3,58). Since it is simpler to treat plant cells with elicitors, as opposed to intact pathogens, results are not compromised by other components of the pathogen. There has been the tendency, however, to assume that if a substance causes induction of early defense genes such as phenylalanine ammonia lyase, then it is necessarily a phytoalexin elicitor. Ebel et al. (94) showed, however, that some substances which activate early defense genes do not lead to phytoalexin accumulation. Therefore, elicitors should be carefully checked to ensure that they in fact activate phytoalexin pathways.

There has recently been considerable interest in the use of elicitors to activate biosynthetic pathways leading to relatively exotic plant secondary metabolites (19,95-98). Indeed, it may be possible to efficiently isolate rare metabolites from cultured plant cells following their exposure to appropriate elicitors. For example, Tyler et al. (90) used fungal elicitors to produce sanguinarine from *Papaver* cell suspension cultures and reused the same cells with a new round of medium and elicitor. Such semicontinuous production schemes may permit the economic isolation of rare secondary metabolites that behave as phytoalexins.

While non-specific elicitors may have utility in selecting disease and insect resistant breeding lines (91,92), race specific elicitors would seem to have the greatest potential use in this area. Thus, breeding lines segregating for a certain disease resistance gene could be screened relatively easily if a specific elicitor complementing the resistance gene was available.

As noted earlier, limited experimental results indicate that plant receptors may perceive elicitor stimuli. Race specific elicitors are also being used as affinity probes for isolation of the corresponding plant receptors. These in turn are postulated to be the primary products of plant disease resistance genes, and their isolation would present a rationale for cloning the resistance genes.

Plant-pathogen specificities involving either specific resistance or general resistance clearly involve differential recognition of pathogens. In some cases this may be more complicated than pathogen display of a constitutive metabolite that is recognized by the resistant plant. For instance, certain bacterial avirulence genes

are inducibly expressed following infection of the plant. This implies that either the specific plant environment or certain plant signal molecules may be required to activate the expression of avirulence genes.

The specific elicitor, victorin, was considered for many years to be a host selective toxin, until it was shown to interact with the *Pc-2* disease resistance gene by eliciting the hypersensitive response in oat plants. Similarly, the peptide elicitor associated with the *ACf9* avirulence gene of *Cladosporium fulvum* and the *avr*D elicitor could be considered as host specific toxins if information was not available linking them to resistance expression. Since purified preparations of all these elicitors cause extensive necrosis on sensitive plant cultivars, it may be possible to use the elicitors themselves or suitable derivatives as specific herbicides (93). For example, injection of the *avr*D elicitor into a single primary soybean leaf of a sensitive cultivar result in necrosis not only of the infiltrated leaf but also the other primary leaf on the plant (Tamaki, S., and Stayton, M., Univ. of Wyoming, unpublished data, 1989). This indicates a degree of systemicity for the elicitor which would be useful in a potential herbicide.

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Chapter 7

Pathogens with Potential for Weed Control

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Biological control with plant pathogens is an effective, safe, selective, and practical means of weed management that has gained considerable importance. The recent success in using certain rust and smut fungi as classical biocontrol agents, the registration and commercial use of two facultatively parasitic fungi as mycoherbicides in the United States, and the potential registration of two more mycoherbicides in the USA and Canada have further stimulated worldwide interest in biological weed control with plant pathogens. Approximately 45 species of fungi are reported to be under evaluation or development in 19 countries and 44 locations against about 65 weeds. Included are 14 of the top 18 world's worst weeds, and several others of regional and specific importance. Among pathogens with potential, several species have been found that attack more than one weed species. Efforts should be made to develop these pathogens as broad-spectrum mycoherbicides through strain selection or genetic engineering. Certain weed-pathogen systems have been shown to be suitable for low-input weed control technology that could be developed with public support and dispensed through user's efforts. Many others may be suitable for specialty or niche markets in which the target weed lacks suitable chemical or other means of control. It is anticipated that public institutions and private industries will continue to support research and development, respectively, of classical and mycoherbicidal candidates and thus promote this field of alternative weed control methods.

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This chapter focuses on pathogens that hold most potential for practical use in the near future, and the research and industrial trends that appear to influence this potentiality. Since 1970, considerable progress has been made towards practical use of plant pathogens as safe and selective agents of weed management (1-3). The interest in using plant pathogens is increasing (4,5) partly because this approach offers an exploitable biotechnology and is an effective supplement to conventional weed control based on chemical and mechanical methods. Often the lack of suitable chemical herbicides serves as an impetus for biological controls. This could result from the unavailability of chemical herbicides, unsuitable performance of existing chemicals in terms of efficacy or environmental safety, their high cost to return ratio, or the buildup of resistant weed biotypes. Under such situations, pathogens could offer specificity in control, environmental safety, and less drastic changes in the ecosystem compared to chemical herbicides. They may also be less costly to develop as herbicides than chemicals (6). Microbial pathogens offer several additional biological and technical advantages which are reviewed in Chapter 18 in this book. Examples of weed control obtainable with pathogens are listed in Table I. Included in this table are agents that are presently used or are expected to be used as commercial herbicidal products as well as different types of pathogens, weeds, and habitats.

Self-disseminating pathogens (e.g., rusts, smuts, and certain dry-spored foliar fungi) as well as poorly disseminating pathogens such as soil-borne and mucoid-spored fungi are being developed for use. The former are generally intended to be used as classical agents capable of causing self-sustaining epidemics following inoculative releases. Typically, they are spread by spores or other infective propagules that are disseminated by wind, water, or insect vectors. The latter are commonly pathogens that have co-evolved with their weed hosts in particular regions resulting in homeostatic relationships characteristic of endemic diseases. Normally, they do not produce self-sustaining epidemics of destructive potential sufficient to afford weed control. But by applying inundative doses of inoculum, as bioherbicides, they can be used to create a temporary but highly destructive level of disease (3). It is also possible to use native rust fungi in an augmentative strategy to increase the level of epidemic and weed control (25) or to increase biotic stress on the weed thereby augmenting crop competition towards the weed (26).

Inoculative (Classical Biocontrol) Agents

Generally, the following considerations determine the decision to seek and deploy classical (inoculative) agents: (1) The weed is an exotic, lacking native pathogens; (2) no alternative controls are available; (3) sufficient economic incentive exists to justify biocontrol efforts; (4) epidemiologically, the eco-climatic factors in the region of introduction indicate reasonable chances of success of the candidate pathogens; (5) no conflict of interest exists, i.e., everyone is in favor of controlling the weed; and (6) the introduced pathogens are extremely host specific. Historically, inoculative agents have been used against exotic weeds growing in areas that are

Table I. Examples of Weed

Pathogen	Disease/Damage and Pathogen Type
Alternaria cassiae[b]	Foliar blight, seedling kill, imperfect fungus
Alternaria sp.	Foliar blight, seedling kill, imperfect fungus
Araujia mosaic virus	Systemic mosaic, reduced plant productivity, virus
Cercospora rodmanii	Leaf-spot, reduced plant productivity, imperfect fungus
Chondrostereum purpureum	Wilt, wood-rot, plant kill, basidiomycete
Colletotrichum orbiculare (Berk. et Mont.) v. Arx.	Stem and leaf lesions, anthracnose, seedling death
C. gloeosporioides f.sp. aeschynomene[b]	Anthracnose, shoot and plant kill imperfect fungus
C. gloeosporioides f.sp. malvae[b]	Anthracnose, shoot and plant kill, imperfect fungus
Entyloma compositarum	Leaf blight, defoliation smut fungus
Fusarium culmorum	Chlorosis, plant kill and lysis, imperfect fungus
F. solani f.sp. cucurbitae	Vascular wilt, plant kill, imperfect fungus
Phytophthora palmivora[b]	Root rot, plant death death, phycomycete
Puccinia chondrillina	Foliar pustules, reduced plant productivity, rust fungus

[a]Recalculated or compiled from the data in the references cited. Control = reduction in weed density, weed problem, or cost savings; averages or the range of reported values are presented here. [b]Registered or expected soon to be registered as commercial mycoherbicides. [c]Eight years after release of the fungus. [d]Laboratory data only. [e]Four years after release of the fungus.

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Control Obtained with Plant Pathogens

Weed	Plant Type	% Control[a]	Ref.
Cassia obtusifolia L. (sicklepod)	Annual herb	80	7
Carduus pycnocephalus L. (Italian thistle)	Annual herb	0–100	8
Morrenia odorata (stranglervine)	Perennial vine	26[c]	9
Eichhornia crassipes (waterhyacinth)	Floating aquatic macrophyte	11–99	10
Prunus serotina (black cherry)	Tree	90	11
Xanthium spinosum L. (spiny cocklebur)	Annual herb	70	12
Aeschynomene virginica (northern jointvetch)	Annual herb, semiaquatic	76–100	13, 14
Malva pusilla (round-leaved mallow)	Annual herb	73	15
Ageratina riparia (Hamakua-pamakani)	Annual herb	50–95[c]	16, 17
Hydrilla verticillata (hydrilla)	Submerged aquatic macrophyte	100[d]	18
Cucurbita texana (Texas gourd)	Annual vine	20–97	19, 20
Morrenia odorata (stranglervine)	Perennial vine	90–100	21, 22
Chondrilla juncea (skeletonweed)	Perennial herb	88[e]	23, 24

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relatively unmanaged, inaccessible, or of low economic return. The following are examples of successful classical introductions which are still effective. Since success with a classical agent in one region of the world tends to promote the use of the same agent in other regions against the same or closely related target weed, these pathogens may find additional use in other areas in the future.

Puccinia chondrillina Bubak. & Syd., a rust fungus from Europe, determined to be a virulent, host-specific, and safe biocontrol agent, was introduced into Australia in 1971 to control Chondrilla juncea L., rush skeletonweed (27, 28). In 1976 it was introduced into California and later into other western states in the U.S. (29-31). The biology of this pathogen, weed control success, and assessment of the economic impact of controlling rush skeletonweed in Australia have been reviewed (23, 24, 27-31). In summary, the rust has been remarkably successful (Table I) in controlling one of three biotypes of the weed, the narrow-leaf type, with consequent beneficial returns to the local economy and ecology (24). Recent exploration in the Mediterranean region has yielded two races of the rust that are virulent on the intermediate-leaf type. These have also been introduced into Australia (32). Additional surveys are underway to find races virulent on the broad-leaf type as well (32).

As in Australia, the rust is the most effective of three introduced biocontrol agents in the United States (29-31); the other two are a gall midge, Cystiphora schmidti Rubsaamen, and a gall mite, Aceria chondrillae Canestrini. Only minor problems have been encountered following the introduction and establishment of the rust in new areas of the world. These being the reduced economic returns due to the prevalence of the resistant weed biotypes in Australia and the relatively modest efficacy of the rust in the United States.

Phragmidium violaceum (Schultz) Wint., a European rust pathogen of blackberries, Rubus spp., was introduced into Chile in 1973 to control Rubus constrictus Lef. et M. and R. ulmifolius Schott. The two hosts differed in their susceptibility to the rust; R. ulmifolius was more susceptible. The rust caused extensive defoliation, a significant decrease in seed production, and increased the invasion of canes by secondary pathogens. About five years after its introduction, the pathogen had spread to over nearly 800 km in a north-south direction along the Chilean coast providing high levels of weed control (33). In 1984 this fungus was reported to have been introduced unlawfully into Australia, presumably for controlling weedy European blackberries of the R. fruticosus L. aggregate. The rust is now spreading rapidly and providing considerable biocontrol (34). Meanwhile, Australian scientists have determined it to be host specific to Rubus spp. within the Rosaceae (35). Although it is too early to assess the biocontrol efficacy of this fungus, and there is the danger of infecting cultivated blackberries and other economically important Rubus spp., the prospects for legitimate use of this rust in Australia are said to be good based on the overall risk-benefit considerations (35).

Puccinia carduorum Jacky, a rust fungus from Turkey, was released in 1987 on a limited basis in Virginia to control musk thistle, Carduus thoermeri Weinm. (=C. nutans L. ssp. leiophyllus [Petrovic] Stoj. & Stef.), a problem in about 15% of all counties in the United States and in Canada. Detailed studies in quarantine

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

greenhouse revealed that the rust race considered for introduction into the United States was capable of infecting globe artichoke (Cynara scolymus L.) and some native, North American Cirsium spp. However, the rust does not attack artichoke under its native conditions in Eurasia where it coexists with artichoke and several other members of the Compositae. It is possible that the limited susceptibility of artichoke and the reaction of Cirsium spp., is a greenhouse anomaly (36). Hence, the rust is considered to be sufficiently host specific and safe for use in the United States (36,37). Initial observations confirm that the rust has overwintered and established itself in Virginia (Baudoin, A. B. and Bruckart, W. L. personal communications), but it is too early to assess its effect on thistle control.

Entyloma compositarum Farlow, a smut fungus, was introduced from Jamaica into Hawaii in 1974 to control Hamakua pamakani, Ageratina riparia (Regel) King & Robinson (=Eupatorium riparium Regel), a weed in pastures, forests, and rangelands (16, 17). It is capable of causing chlorotic to reddish-brown angular lesions on the upper leaf surface and massive white sporulation on the lower leaf surface. Inoculum becomes airborne during cool, moist (>98% relative humidity) nights. Two to three months after the pathogen's release, devastating epidemics were recorded in dense stands of the weed in high rainfall sites in Oahu, Hawaii, and Maui, reducing weed populations up to 80%. Eight years after release, the smut is reported to have provided up to 50% control even in areas with low rainfall but cool temperatures (Table I) (16). No environmental or nontarget damage has resulted from this introduction (16).

Several other obligate and facultative parasites have been reported as potential inoculative biocontrol agents; these are summarized in Table II and reviewed previously (50, 51).

Inundative Agents (Microbial Herbicides)

The key consideration in the decision to develop mycoherbicides is the weed control efficacy of the pathogen (52). Efficacy should be assessed by the speed, amount, and ease of weed control (52). Factors such as the ease of disease initiation and epidemic build-up independent of very specialized epidemiological requirements also impinge upon the mycoherbicidal potential of pathogens. Following efficacy, host specificity of the agent ranks next in importance. Although pathogens with ill-defined or unrestricted host range are not recommended for biocontrol use, host specificity to an extreme degree is not needed in the case of native pathogens that are to be used in site-directed inundative applications (53). In fact, as will be discussed later, a level of host nonspecificity is desirable to develop microbial herbicides capable of controlling more than one economically important weed.

Several other considerations influence the potential success of a mycoherbicide. For example, the organism should grow well in culture and yield infective mycelia or spores for inoculum. The inoculum must have good viability and shelf life, and yield infections and disease cycles over a range of environmental conditions, whether formulated or not. The pathogen also must be genetically stable to preclude loss of virulence or a change in host range. Survival of inundative agents is not a requirement. In fact, lack

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

Table II. Pathogens with Potential as Inoculative Biocontrol Agents

Pathogen	Weed	Ref.
Cercospora sp.	*Heliotropium europaeum* L. (common heliotrope)	38
Melampsora euphorbiae (Schub.) Cast.	*Euphorbia esula* L. (leafy spurge)	39
Puccinia abrupta Diet. & Holw. var. *partheniicola* (Jackson) Parmelee	*Parthenium hysterophorus* L. (false ragweed)	40
P. cardui-pycnocephali Syd.	*Carduus pycnocephalus* (Italian thistle)	41
P. expansa Link	*Senecio jacobaea* L. (ragwort)	42
P. jaceae Otth	*Centaurea solstitialis* L. (yellow starthistle)	43
	C. diffusa Lam. (diffuse knapweed)	44
P. obtegens (Link) Tul.	*Cirsium arvense* (L.) Scop. (Canada thistle)	45
P. punctiformis (Str.) Roehl	*C. arvense* (Canada thistle)	46
P. xanthii Schw. f.sp. *ambrosia-trifidae*	*Ambrosia trifida* (giant ragweed)	47
Uromyces heliotropii Sredinski	*H. europaeum* (common heliotrope)	38
U. rumicis (Schum.) Wint.	*Rumex* spp. (curly docks)	48
Uromycladium tepperianum (Sacc.) McAlp.	*Acacia saligna* (Labill.) Wendl. (acacia tree)	49

See also ref. 50 and 51.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

of prolonged survival is desirable for safety. Short-term survival would minimize inoculum buildup in treated sites and the potential for long-term genetic interactions in the field. Finally, economic considerations, such as the lack of other suitable controls for the target weed; a profitable market size, and patent protection; technical feasibility of large-scale production; and marketability will determine the ultimate decision to register and commercialize potential inundative agents. More discussion on these points can be found in Chapter 17 of this volume and in Bowers (54), Churchill (55), and Bannon (56, 57).

Presently two mycoherbicides are registered for commercial use in North America. These are: DeVine (Abbott Laboratories, North Chicago, IL 60064) and Collego (Ecogen Inc., Langhorne, PA 19047) used respectively against stranglervine, Morrenia odorata (H. & A.) Lindl., and northern jointvetch, Aeschynomene virginica (L.) B.S.P. Two others, Casst (Mycogen Corp., San Diego, CA 92121) and BioMal (Philom Bios, Saskatoon, Saskatchewan, Canada S7N 2X8), are undergoing evaluations for possible registration in the near future for controlling sicklepod, Cassia obtusifolia L., and round-leaved mallow, Malva pusilla Sm., respectively. In addition, several other pathogens have been evaluated to various extents, including many that have undergone field testing and evaluations under the Experimental Use Permit (EUP evaluations) issued by the U.S. Environmental Protection Agency (58, 59).

DeVine, discovered and developed by Ridings, Burnett, and others, was the first registered mycoherbicide (60-62). It was registered in 1981 for use as a post-emergent directed spray and is marketed as a liquid concentrate of chlamydospores of a pathotype of Phytophthora palmivora (Butler) Butler native to Florida. The pathogen is capable of killing seedlings and adult stranglervine, a weed of South American origin found in Florida's citrus groves. Extensive host range and efficacy trials done in the laboratory and field supported the safety and biocontrol potential of this fungus (60). Safety to nontarget susceptible plants was addressed by label restrictions which prescribe selective, site-specified applications of the mycoherbicide. DeVine is not to be used where susceptible nontarget plants are grown or occur (22), and to date no adverse nontarget effects have been reported. The level of weed control (Table I) has been good to excellent (21, 22, 61, 62). However, the market for DeVine is small, specialized, and concentrated in the citrus-growing areas of Florida.

Collego, based on Colletotrichum gloeosporioides (Penz.) Sacc. f.sp. aeschynomene, an anthracnose-inciting pathogen of northern jointvetch discovered in Arkansas, was developed by Daniel, Templeton, Smith, TeBeest, and co-workers in collaboration with the Upjohn Company and the U.S. Department of Agriculture (2, 6, 63, 64). The fungus is capable of killing both seedling and mature northern jointvetch, a hard-seeded non-nodulating leguminous weed in rice and soybean crops. The commercial product, registered in 1982, is a wettable powder of dried spores produced by liquid fermentation (54, 55, 64). It is applied post-emergence, aerially or with land-based sprayers (14). The history, development, registration, integrated use, and post-registration status of Collego have been reviewed (2, 54, 55, 65). Collego has provided consistently high levels of weed

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

control (Table I) and is well accepted by users (64). Although the fungus has a wider host range than originally thought (65), and was found to be capable of infecting but not killing several economically important legumes (66), it has not posed any danger to nontarget plants under field use. It spreads poorly due to the sticky nature of its spores, and the chance of uncontrollable epidemic is nonexistent. The inoculum declines to very low levels in soil and irrigation water following inundative applications, but the pathogen may persist on infected host tissue and overwinter (67, 68). This low survival rate, which may be typical for inundatively applied inoculum, is an added safety feature of mycoherbicides, but it also necessitates annual applications for effective weed control (2, 3). No environmental or user hazards have been found with Collego during nearly two decades of use (2).

Alternaria cassiae Jurair & Khan, a dematiaceous fungal pathogen inducing foliar blight, was discovered in Mississippi. Walker and co-workers (7, 69, 70) showed it to be safe and efficacious against sicklepod. Subsequently, in a two-year region-wide collaborative field trial, it was demonstrated to be highly effective in controlling the weed in soybean (Table III) (71). It has a fairly narrow host range, and is capable of controlling three economically important leguminous weeds (sicklepod, coffee senna [Cassia occidentalis L., and showy crotalaria [Crotalaria spectabilis Roth,]) which are important in the southeastern United States (70, 72). The fungus is currently under commercial development as Casst, a wettable powder formulation, by Mycogen Corporation. Details of this development are discussed in Chapter 17 of this book and elsewhere by Bannon (56).

Colletotrichum gloeosporioides (Penz.) Sacc. f.sp. malvae, an anthracnose-causing pathogen of round-leaved mallow, is being developed as a mycoherbicide by Philom Bios, a Canadian biotechnology company (15, Gantotti, B. and Mortensen, K., personal communications). The fungus, native to mid-western Canada, is highly virulent, very efficacious, and easy to culture and apply with conventional equipment (Mortensen, K., personal communication). Registered chemical herbicides do not give satisfactory control of this weed, so the market outlook for this mycoherbicide is good. The fungus is also pathogenic to velvetleaf, Abutilon theophrasti Medic., although its potential to control this weed is yet to be confirmed (15). Safflower (Carthamus tinctorius L.), an economically important plant, is susceptible to this pathogen under optimum growth chamber conditions. The risk to safflower is being addressed through extensive host range and pathogenicity trials (Mortensen, K., personal communication) to decide the question of risks vs. benefit.

As previously stated, numerous pathogens are being investigated as potential biocontrol agents for many annual and perennial weeds and it would be impossible to review all of the research here. Fungi representing nearly all of the major fungal classes are being studied (Charudattan, unpublished data). A small proportion of these candidates may reach commercial or practical use in the near term. A few in this category, Cercospora rodmanii Conway (for controlling waterhyacinth, Eichhornia crassipes [Mart.] Solms), Fusarium culmorum (W. G. Smith) Sacc. and an unidentified sclerotial

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

Table III. Average Percentage of Sicklepod (Cassia obtusifolia) Control Obtained with Alternaria cassiae in a Region-wide Field Trial

Year	No. of Applications[a]	Arkansas	Florida	Mississippi	N.Carolina	S.Carolina
1982	1	25	81	78	100	0[b]
	2	85	94	74	100	0[b]
1983	1	86	95	97	98	71
	2	78	98	80	100	82

Data from the 21st day after the first application of inoculum. [a]One or two applications of the fungus at 1 x 10^6 conidia per ml (9.4 kg conidia per hectare) were made, the latter at 7-day intervals. [b]Weather-related experimental failure was reported. From Charudattan et al. (71).

fungus (for hydrilla, Hydrilla verticillata [L.f.] Royle), and Fusarium solani (Mart.) Appel & Wollenw. f.sp. cucurbitae (for Texas gourd, Cucurbita texana [Scheele] Gray), are discussed in more detail in Chapters 8 and 9 in this volume.

In my view, the pathogens most likely to reach commercial or practical use, hence the most noteworthy, are those that are (1) candidates against weeds of economic importance on a very large scale (economically attractive weeds), (2) pathogens that may be amenable for development as broad-spectrum mycoherbicides, (3) mycoherbicides for low-input technology, and (4) mycoherbicides for specialty or niche markets.

Potential Pathogens of Economically Attractive Weeds. Table IV includes weeds that are considered to be of major worldwide importance. These weeds belong to Group 1 of the world's worst weeds according to Holm et al. (73). Due to this ranking they may be attractive targets for mycoherbicide industries seeking economically viable products. It is noteworthy that several candidate pathogens are available for this group of weeds. Among these, Colletotrichum coccodes (Wallr.) Hughes (74, 75) and Cercospora rodmanii (76-78) have undergone extensive field testing and/or EUP evaluations. Others in this group, such as Fusarium lateritium Nees (79), Phomopsis convolvulus Ormeno (80), and Bipolaris sorghicola (Nisik. & Miyake) Shoem. (81) have also undergone (or are undergoing) some measure of commercial development and evaluation.

Potentially Broad-spectrum Mycoherbicides. Among pathogens reported to hold promise as mycoherbicides are strains or pathotypes of the same species or subspecies that are pathogenic to more than one weed target. Examples are listed in Table V. In some cases, individual strains or pathotypes host specific to particular weeds have been developed as mycoherbicides (e.g., the stranglervine pathotype of Phytophthora palmivora used as DeVine [61]). In other instances, individual strains putatively host specific to different weeds have been reported, but no attempt has been made to screen these strains against a collection of economically important weeds. Thus, it might be worthwhile to screen the strains of Fusarium lateritium reported from velvetleaf, giant ragweed (Ambrosia trifida L.), spurred anoda (Anoda cristata [L.] Schlecht.), prickly sida (Sida spinosa L.), and pondweeds (Potamogeton spp.) (Table V) to determine whether a strain with broad-range capability for biocontrol could be found. In other instances, subspecies or formae speciales of the same species (e.g., Colletotrichum gloeosporioides [Penz.] Sacc.) have been found to be effective against different weeds. There has been no exploration of the feasibility of recombining or hybridizing these subspecies into broad host-range pathotypes which could be then used against more than one weed target. Both somatic and genetic recombination techniques could be used for this purpose (13, 58, 94-96), and this possibility should be explored. Bioengineering can open not only greater market potential for mycoherbicides based on recombinant organisms, but also provide us a better understanding and ability to monitor the safety, specificity, and survival of the recombinants.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

Table IV. Potential Mycoherbicide Candidates of Economically Attractive Weeds

Pathogen	Weed[a]	Ref.
Colletotrichum coccodes	Abutilon theophrasti (velvetleaf)	74, 75
Fusarium lateritium		79
Fusarium lateritium	Ambrosia trifida (giant ragweed)	82
Septoria tritici Desm. f.sp. avenae	Avena fatua L. (wild oat)	83
Ascochyta caulina Sacc.	Chenopodium album L. (common lambsquarters)	84
Cercospora chenopodii Fres.		84
C. dubia (Riess) Wint.		84
Phomopsis convolvulus	Convolvulus arvensis L. (field bindweed)	82
Cercospora caricis Oud.	Cyperus esculentus (yellow nutsedge)	82
Phyllachora cyperi Rehm	C. rotundus L. (purple nutsedge) (in certain cases)	82
Pyricularia grisea (Cke.) Sacc.	Digitaria sanguinalis (L.) Scop. (large crabgrass)	82
Cochliobolus lunatus Nelson & Haasis	Echinochloa crus-galli (L.) Beauv., (barnyardgrass)	85

Continued on next page

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

Table IV. Continued

Alternaria eichhorniae Nag Raj & Ponnappa	Eichhornia crassipes (waterhyacinth)	86
Cercospora piaropi Tharp		87
C. rodmanii		76-78
Bipolaris setariae (Saw.) Shoemaker	Eleusine indica (L.) Gaertn. (goosegrass)	82
Pyricularia grisea		88
Dichotomophthora indica Rao	Portulaca oleracea L. (common purslane)	89
D. portulacae Mehrlich & Fitzpatrick ex M. B. Ellis		90, 91
Curvularia sp.	Roettbollia cochinchinensis L. f. (itchgrass)	92
Phaeoseptoria sp.		92
Sphacelotheca holci Jack. (S. cruenta (Kuehn) Potter	Sorghum halepense (L.) Pers. (johnsongrass)	26
Bipolaris halepense Chiang, Leonard & Van Dyke		93
B. sorghicola (Lefebvre & Sherwin) Alcorn		81
Colletotrichum graminicola (Ces.) G. W. Wils.		82
Gloeocercospora sorghi D. Bain & Edg.		82

[a] These, arranged alphabetically, are in Group I of the World's Worst Weeds listed by Holm et al. (73).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

Table V. Potentially Broad-spectrum Mycoherbicides

Pathogen	Weed	Ref.
Alternaria cassiae	*Cassia obtusifolia* (sicklepod) *C. occidentalis* (coffee senna) *Crotalaria spectabilis* (showy crotalaria)	69-72
Amphobotrys ricini (Buchw.) Hennebert	Weeds in Euphorbiaceae	97, 98
Colletotrichum gloeosporioides various f.spp.	Weeds in Leguminosae, Malvaceae, Dodders, etc.	13, 15, 63 99, 100
C. dematium (Fr.) Grove f.spp. (incl. f. *truncata* sensu von Arx; ref. 103)	Weeds in Leguminosae	101, 102
C. graminicola	Weeds in Gramineae	82
Fusarium lateritium	*Abutilon theophrasti* (velvetleaf)	79
	Ambrosia trifida (giant ragweed)	82
	Anoda cristata (spurred anoda)	79
	Sida spinosa (prickly sida)	79
	Potamogeton spp.	104
Pyricularia grisea	*Digitaria sanguinalis* (large crabgrass), *Eleusine indica* (goosegrass)	82, 88
Sclerotinia sclerotiorum (Lib.) de Bary	Several weeds across taxonomic lines	105, Chapter 10, this book

Mycoherbicides for Low-input Technology. Some mycoherbicide candidates, even if their efficacy and other biological attributes are acceptable, may not be attractive commercially due to a very localized prevalence of the weed problem, small market size, competition from chemical herbicides, or a projected low return on investment. Yet they could be developed by public-supported agencies and dispersed through individual users. Since the agents may not be used widely, they could be developed with low-input technology through small-scale or cottage industries. Examples are given in Table VI.

Table VI. Mycoherbicides for Low-input Technology

Pathogen	Weed	Ref.
Cephalosporium diospyri	*Diospyros virginiana* (persimmon)	106
Chondrostereum purpureum	*Prunus serotina* (black cherry)	11, 107
Colletotrichum gloeosporioides f.sp. *clidemiae*	*Clidemia hirta* (Koster's curse)	108, 109
Puccinia canaliculata	*Cyperus esculentus* (yellow nutsedge)	25, 110,

This approach has been taken in dispensing *Cephalosporium diospyri* Crandell, a wilt-inducing pathogen of persimmon (*Diospyros virginiana* L.), considered weedy in rangelands of Oklahoma (106). The fungus is provided to ranchers who inoculate clumps of persimmon trees with the aid of shot-gun pellets filled with inoculum. The pathogen causes wilting and death of inoculated trees and spreads to nearby trees presumably through root contacts.

Chondrostereum purpureum (Pers.:Fr.) Pouzar, a wound-infecting basidiomycete, is proposed to be used as a biocontrol for black cherry, *Prunus serotina* Ehrh., a weedy tree in the forests of the Netherlands (Table I) (11, 107). A few milligrams of mycelial inoculum per tree, applied to stumps, is sufficient to insure plant kill and prevent resprouting (11). In view of the need to inoculate individual trees to obtain control, this fungus may also be best suited for dispensing through individual users.

Colletotrichum gloeosporioides (Penz.) Sacc. f.sp. *clidemiae*, which incites a leaf blight and defoliation of young Koster's curse plants, *Clidemia hirta* (L.) D.Don, (108) is said to be distributed through hikers who are provided with liquid inoculum and instructions to apply the fungus (109). The weed is a problem over a widespread area in Hawaiian forests but the pathogen does not disseminate readily due to the sticky spores. In the absence of industrial mass-production and application, spreading this biocontrol agent through individual users appears to be a novel and rapid means of achieving practical success.

Phatak et al. (25) have demonstrated that a native rust fungus, *Puccinia canaliculata* (Schw.) Lagh., can be used in an augmentation

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

strategy to control yellow nutsedge, Cyperus esculentus L., in crops such as corn. As little as 5 g of uredospores of the fungus per hectare, applied through a center-pivot irrigation system, can lead to high levels of disease and weed control (110). Presently, the only means of producing inoculum of this rust is from infected plants in nurseries or greenhouses. The lack of in vitro methods of inoculum production and the need for relatively small amounts of inoculum suggest that this fungus is a good candidate for low-input weed control technology (111).

Mycoherbicides for Niche Markets. Certain weeds are likely to remain attractive targets for mycoherbicides due to peculiarities involved in control and user demands. For example, weeds of regional importance may be suitable for mycoherbicidal control if no other method provides economical control. In some cases, chemical herbicides may be precluded from use due to environmental concerns. The growing trend in organic farming may also favor biological over chemical herbicides. Indeed, the mycoherbicides mentioned earlier, DeVine, Collego, Casst, and BioMal, are all developed for special needs of niche markets in which chemical herbicides were lacking or unsatisfactory. Hence, despite the small market size and the specialized nature of these markets (e.g., citrus, rice, and irrigated peanut) industries were willing to develop these mycoherbicides. Table VII includes other pathogen-weed combinations that may fit this specialty designation. In these examples, specific demands of the weed control system, i.e., a need to protect vulnerable aquatic habitats from chemical pollution, or the sensitivity of crop plants to chemicals used for dodder (Cuscuta sp., a parasite of the crop plants) control, are expected to serve as an impetus for the development of mycoherbicides.

Problems in Practical Use of Mycoherbicides. Despite the promise held by mycoherbicides and the experimental successes achieved to date, several biological, technological, and economic problems exist which may limit the practical utilization of this technology. The following are some important examples.

Fungal propagules must be hydrated in order to germinate and infect their hosts. Dew or other forms of free moisture on the plant surface enable this hydration. A pathogen that needs prolonged contact with free moisture (roughly for more than 6 hr), or several cycles of wetting and drying, is more subject to the vagaries of nature and therefore is likely to be less competent in infecting the host than one that needs shorter exposure to moisture. Empirical knowledge with many foliar diseases suggests that fungi with dry (not sticky) and large spores are more affected by the moisture factor than those with sticky and small spores. The latter appear to be more capable than the former at adhering, remaining, and hydrating on the plant surface. Thus, the type of propagule used as inoculum and its epidemiological requirements for germination and infection can pose a serious limitation.

Pathogens that are difficult to mass-produce offer a technological challenge. Many fungi such as Alternaria spp. and some Colletotrichum spp. do not sporulate under liquid fermentation, the preferred method of commercial inoculum production. Labor-intensive

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Table VII. Mycoherbicides for Specialty Markets[a]

Pathogen	Weed	Ref.
	Aquatic weeds:	
Fusarium culmorum,	Hydrilla verticillata (hydrilla)	18
Rhizoctonia sp.?		112, Chapter 8 in this book
Colletotrichum gloeosporioides,	Myriophyllum spicatum (eurasian watermilfoil)	99, 113
Mycoleptodiscus terrestris (Gerdemann) Ostazeski		114
Pythium spp.	Potamogeton spp. (pondweeds)	115
	Lemna spp. (duckweeds)	116, 117
	Parasitic weeds:	
Colletotrichum gloeosporioides (Penz.) Sacc. f.sp. cuscutae,	Cuscuta spp. (dodders)	13
Alternaria sp.		118, 119

[a] Many other candidates under research and development are also likely to fall in this category; the above are merely examples.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch007

and costly methods of inoculum production may be impractical from a commercial perspective. Therefore, technological difficulties in inoculum production may preclude development of a pathogen that is otherwise desirable from safety and efficacy standpoints.

Integration of mycoherbicides with chemical-based pest management practices can be complicated due to incompatibility between the biological and chemical pesticides. This problem and practical solutions to it have been discussed by Smith (14) from his experience with the Collego-northern jointvetch system in rice and soybean. Similar empirical solutions will be required for every mycoherbicide that is to be used in integration with chemical pesticides.

The ability of a weed to compensate for disease-induced growth-loss and thereby overcome pathogenic attack has been demonstrated in the case of *Cercospora rodmanii* leaf spot of waterhyacinth, a plant with a dominant clonal growth habit (77). Similar tendency has been reported with velvetleaf, a vigorous terrestrial annual infected by *Colletotrichum coccodes* (74, 75). This problem, not too uncommon among weeds, stems from the inability of the pathogen to kill the host completely or severely curtail its regrowth. In such situations, one of the ways to improve the pathogen's weed control ability may be to include a plant growth regulator or a sublethal dose of a chemical herbicide to the mycoherbicide formulation (10, 75, 78). For example, Hodgson et al. (75) reported that thidiazuron (*N*-phenyl-*N*'-1,2,3-thiadiazol-5-ylurea), which promotes defoliation and retards plant regrowth, might be used to retard velvetleaf growth, thereby improving the effectiveness of *C. coccodes*. Similar adjuvant effect of thidiazuron has been found in the waterhyacinth-*C. rodmanii* system as well (Charudattan and DeValerio, unpublished data). More on this point may be found in Chapter 16 of this volume.

Several economic factors affect commercialization of mycoherbicides (54, 64), the ultimate consideration being the projected economic returns from the mycoherbicide. A pathogen that is prone to be unprofitable or unpredictable in this respect, whether due to small market size, cost of production and marketing, competition from other alternative controls, or efficacy, is unlikely to be developed or offered for sale by commercial enterprises.

Conclusions

Biological control with plant pathogens offers a feasible alternative and supplement to chemical weed control. Research, regulatory, and industrial trends suggest that some of the pathogens reviewed here may become usable through the efforts of private industries or public agencies. Prospective pathogens that do not meet commercial criteria for development could still be made available to the users through low-input technology outlets. Economic and public demands for affordable, effective, and safe herbicides are expected to stimulate and sustain the interest in this field. Future research efforts must focus on the problematic aspects discussed here.

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Chapter 8

Biological Control of Aquatic Weeds with Plant Pathogens

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The use of plant pathogens as biocontrol agents of aquatic plants has only just begun to be considered as a major area of research in the United States. Prior to the 1970's virtually no work was being conducted in this specialized area. Since that time the US Army Engineers (USAE) has been given the added responsibility of maintaining weed-free waterways by non-chemical alternatives. The Biomanagement Team at the USAE Waterways Experiment Station and the University of Florida have established programs to study biological control of aquatic weeds using plant pathogens. Hundreds of microorganisms have been evaluated for their potential as biological control agents of waterhyacinth, alligatorweed, eurasian watermilfoil, hydrilla, algae, and numerous other aquatic weeds. As a result, several fungal and viral diseases of these troublesome aquatic plants have been identified. Information will be presented on current efforts in the development of these plant pathogens for aquatic weed management.

The study of plant pathogens as biocontrol agents for aquatic plants has reached the end of nearly 20 years of research. During this time the subject has evolved from a "commencement period" (1) to a period in which a substantial backlog of data has been accumulated. However, the use of plant pathogens for aquatic weed control has not reached past the experimental stage. Most problem aquatic weeds have been to some degree associated with at least one plant pathogen. However, the problems associated with infestations of the three most noxious water weeds, waterhyacinth (Eichhornia crassipes (Mart.) Solmes.), eurasian watermilfoil (Myriophyllum spicatum L.), and hydrilla (Hydrilla verticillata (L.f.) Royle) and

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the growing problems resulting from infestations of numerous others have not been sufficiently reduced.

Problems Caused by Aquatic Weeds

The aquatic weed problem is one of worldwide proportion which is increasing despite enormous expenditures of money and human resources in the application of chemical and mechanical controls. The U.S. Army of Engineers (CE) spends nearly $6 million annually to maintain weed free waterways with an additional $2.5 million going into research (Decell,J.L. U. S. Army Engineer Waterways Experiment Station, personal communication, 1989).

The major aquatic weeds in the United States were introduced from various parts of the world and have spread so rapidly that eradication has become impossible. Aquatic plants are a valuable part of many aquatic systems, but when in excess, they become problems for man and wildlife. Excessive populations block our navigation canals and clog hydroelectric plant inlets and irrigation canals. They make recreational water activity difficult and potentially unsafe. Excessive aquatic weed populations may harbor arthropods which transmit infectious human diseases such as malaria and encephalomyelitis. Wildlife may be affected through the elimination of habitat and game fish may be competitively disadvantaged or eliminated altogether because of depleted oxygen levels caused by respiration and decomposition of vegetation (2).

Causes of Aquatic Weed Problems

Man is responsible for nearly all of the problems caused by aquatic weeds (3). Man has intentionally or unintentionally contributed to the aquatic weed problems. For example, alligatorweed was unintentionally introduced by transport ships to the United States from South America (4). Waterhyacinth was deliberately introduced as an ornamental plant; excessive plants from aquatic gardens were simply discarded in waterways where they reproduced and spread unchecked (5). African salvinia became a nusance aquatic plant after the construction of damns. The lack of natural predators allowed their populations to reach nusiance proportions.

Many aquatic weeds reproduce vegetatively and the capacity to produce seed may be insignificant or eliminated. Some of these introduced species have different nutrient, light and temperature requirements which give them a competitive edge over native species. Hydrilla, for example, can thrive under very low light conditions (6) in oligotrophic to highly eutrophic waters with low to high salinity, and at depths of a few centimeters to more than 15 meters.

Another man-made cause of the aquatic weed problem has been the pollution of waterways. Over-nutrification is caused by the dumping of human, industrial, municipal, and agricultural wastes. The build-up of nutrient-rich sediments in many lakes has made them havens for unchecked vegetative growth of noxious aquatic weed species. The excessive aquatic weed problems are also encouraged through reduction in water flow by the construction of reservoirs and irrigation canals. By reducing water flow, nutrient loads in

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waterways may be concentrated, thus providing excessive nutrients for growing vegetation (7).

Advantages of Plant Pathogens for Aquatic Weed Biocontrol

Various species of bacteria, fungi, and viruses have been or are being investigated for their biocontrol potential on problem aquatic plants. As pointed out by Zettler and Freeman (7), plant pathogens have many characteristics compatible with biocontrol technology. Plant pathogens are numerous and diverse, frequently host specific, may be easily disseminated and self-perpetuating, will not eradicate the host, and do not infect man or other animals. Biological control of weeds using plant pathogens would also eliminate undesirable consequences of chemical herbicide use including residue or toxicity problems and accumulation of the chemicals in the soil and ground water (1).

Approach to Research

Two approaches have been used for biological control of aquatic plants using plant pathogens. The first approach has been to use native plant pathogens as biological herbicides. This approach seeks to produce rapid but temporary control through massive inoculation with pathogens that may cause endemic diseases. Biological herbicide development has also been favorably received by commercial producers of biological control products. The other is the "classical" approach, which requires that control organisms introduced from the target weed's natural range, establish permanent populations large enough to provide long-term control. Classical biocontrol using exotic insects has been successful in controlling several problem weeds. The most publicized example is the biological control of the prickly pear cactus in Australia by the insect *Cactoblasitis cactorum*. More recently introduction of the exotic biocontrol organisms *Puccinia chondrillina*, *Cystiphora schmiolti*, and *Aceria chondrillae* on skeleton weed (*Chondrilla juncea*) in California has resulted in more than 50 percent reduction of this weed in rangelands (8). Once these biocontrol agents were established, no further introductions were necessary. Although less expensive, this approach takes longer to achieve control and it is often difficult in the long term to demonstrate a link between the presence of the control organism and reductions in the host population. Classical biological control is also less appealing to commercial producers of biocontrol products, since the control agent is only applied once and in relatively small amounts.

Obstacles in Using Plant Pathogens in Aquatic Weed Biocontrol

Several problems have been discovered which must be overcome before the use of plant pathogens as biocontrol agents of an aquatic weed can succeed, especially for submersed species such as hydrilla or eurasian watermilfoil. First, the level of inoculum which must be applied in an aquatic system is generally extremely high as compared to terrestrial environments since sufficient inoculum must be added to bring the entire water column to the desired inoculum

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density (Joye, unpublished data). High levels of inoculum may affect other life in the system, not necessarily due to direct toxicity, but from the extreme turbidity and resultant depletion of oxygen created by such treatment. In treatment of weeds with chemical control agents a similar problem results from increased turbidity and oxygen depletion from decomposing vegetation.

Development of delivery systems which enable the inoculum to infect the target weed is needed. Propagules dispersed in water are at the mercy of prevailing currents, which may remove propagules before they can adhere to the plant surface. A pelletized formulation or a viscous paste of the biocontrol agent can theoretically minimize loss of inoculum. Such formulations should help pathogens attach to the target weed so that they can infect the host. We are currently evaluating alginate pellet formulations similar to those developed by Walker and Connick (9). Results are pending experimental conclusions. We must utilize herbicide application techniques similar to those developed by the chemical herbicide industry to optimize biocontrol formulations for placement of the inoculum on the plant.

Another potential problem is the fragmentation of the plant after attack by a biocontrol agent. Submersed plants fragment readily, and fragments can survive for considerable periods since they do not desiccate after being separated from the root system and can acquire nutrients from the water through adsorption (3). Fragments created by the breaking up of the plant could increase the rate of vegetative propagation by the target weed. The immediate control may have been satisfactory but the overall weed infestation may be increased.

Optimal timing of application of the biocontrol agent must be addressed. Infection processes dictate that special attention be given to the timing of application of biocontrol agents. Most microorganisms associate with plants are recovered during senescence as temperature and light intensity drop during the fall. This is also the time when plant pathogens or opportunistic parasites are most abundant on the plant surface as well as many saprophytic microorgnaisms. In addition, if plant pathogens are present they should occur during the active growing period of the plants. As with so many crop diseases where some of the most damaging disease occur on young actively growing plants, virulent pathogens of aquatic plants should be manifested on growing plants causing diseases if they are present. However, application of the plant pathogens for biocontrol during the fall would only be useful if the pathogen prevented reestablishment of the weed the next growing season. In the case of hydrilla this would be unlikely unless the pathogen attacks the vegetative propagules (tubers and turions) that will regenerate the plant population the following spring. More typically biocontrol agents must be effective during the early part of the growing season. This is comparable to the control of terrestrial weeds in field crops; unless the weeds are controlled within the first few weeks after planting, crop production may be so low that harvesting would not be economically feasible.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

Control of Specific Weeds

Waterhyacinth.

Waterhyacinth (Eichhornia crassipes [Mart.] Solmes) is one of the most troublesome aquatic weeds in the world (Figure 1). Its exponential increase is attributable to rapid spreading by seeds and vegetative propagation, a high degree of morphological plasticity, adaptability to a wide range of environmental conditions (e.g., water depth, pH, nutrients, light, and temperature), and competitive ability. Because of these quallilties, waterhyacinth continues to demand constant vigil. Management of waterhyacinth continues to be almost exclusively through the use of chemical herbicides. No less than twenty chemical herbicides have been used commercially for controlling waterhyacinth, with 2,4-D and diquat being the most commonly used (2).

In the United States, problems of waterhyacinth seem to have been somewhat alleviated during the past 15 years. This reduction has been attributed to the introduction of the two weevil species (Neochetinia eichhorniae and N. bruchi). The elimination of some populations of waterhyacinth in Florida, Louisiana and Texas has been attributed to the weevils (10,11). In general, the effects of these weevils have resulted in a slow reduction of waterhyacinth infestation (12). As expected with biocontrols, the insects reproduce slowly in relation to waterhyacinth, and optimal environmental conditions are required for several years before the insects exert a significant impact (12).

Since the early 1970's, the list of fungi with potential use in biocontrol of waterhyacinth has grown to more than 100 isolates (2). Of these, Cercospora rodmanii Conway (Figure 2) initially isolated in Florida (13) has shown the greatest promise as a biocontrol agent (14,15). C. rodmanii is capable of inducing leaf spots, leaf necrosis, and secondary root rot to waterhyacinth (Figure 2) (13). The disease spreads to uninoculated plants by wind-disseminated conidia. The host specificity of C. rodmanii was examined under greenhouse and field conditions in which 81 speices and cultivars in 58 families were tested. Under greenhouse conditions, only after repeated application of the inoculum did nontarget species become infected. However, none the the nontarget species were affected under field conditons (16). In addition, inoculation with mycelia and conidia of C. apii and C. capsici produced only hypersensitive responses on older leaves of waterhyacinth. Isolates of C. hydrocotyle and C. nymphaea has no effect on waterhyacinth. Based on these studies C. rodmannii was not considered to be a threat to economically or ecologically important plants in Florida (16).

The availability of nutrients was found to have a significant effect on the disease epidemic development of C. rodmannii on waterhyacinth. Under nutrient poor conditions, the disease epidemic was able to severely restrict growth of waterhyacinth. However, under nutrient rich conditons, in addition to the reduced rate of epidemic development, the waterhyacinth actually grew at a rate faster than the epidemic rate.The plants were apparently able to compensate for disease and dying leaves with a rapid leaf turnover.

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Figure 1. A waterhyacinth population in bloom somewhere in Florida.

Figure 2. Leaf spots on waterhyacinth caused by *Cercospora rodmanii*.

This rapid leaf generation appeared to render additional infection initials unavailable. From these results, the authors concluded that multiple inoculum application would generally be necessary when waterhyacinth is in an early growth phase and the incorporation of other biotic and abiotic controlling agent would be useful (14).

Commercialization of this pathogen as a mycoherbicide has been explored by Abbott Laboratories. Preliminary results indicated that a wettable powder formulation was acceptable pending standarization of infectivity and virulence of the propagules (17,18,19). Largescale studies have demonstrated that control of waterhyacinth using C. rodmanii in combination with the weevils significantly increased through a synergistic response (18,19).

Integration of biological and chemical control agents may provide a promising approach to waterhyacinth control. The combination of the herbicides 2,4-D and diquat with C. rodmanii indicate significantly more damaged to waterhyacinth can be obtained. Implementation of an integrated approach to control waterhyacinth is pending further test results (Charudattan, R. University of Florida, personal communication, 1989).

Hydrilla.

Hydrilla (Hydrilla verticillata [L.f.] Royle) (Figure 3) is a submersed aquatic plant native to Asia that has become a problem in the tropical and subtropical regions of the world. In addition, hydrilla does not seem to have reached it potential distribution in the U. S. (Figure 4).

The expanding range of hydrilla and its subsequent management may be made even more exasperating by the fact that two morphotypes exist in the United States. A dioecious form occurs mainly in the south and the recently introduced monoecious form is reported along the eastern seaboard in the Washington D. C., Maryland, and North Carolina regions (17). The proliferation of the two types of hydrilla is attributable to vegetative reproduction from stem fragments, tubers, and turions. Tubers and turions enable the species to survive adverse conditions such as cold temperatures and drought (20). Although hydrilla is capable of sexual reproduction by seed, male flowers are rarely seen in either morphotype, suggesting that sexual reproduction is of limited occurence in nature.

Hydrilla tolerates a wide range of ecological conditions. It occurs in water that is clear to very turbid, oligotrophic to highly eutrophic and at depths of more than 15 meters. It can tolerate alkaline to acidic conditions and moderate salinity. Its low light requirement enables it to successfully compete with other submersed aquatic plants (6).

As with most aquatic weeds, hydrilla control is usually through the application of chemical herbicides. However, hydrilla has not been deterred significantly by this type of management. Therefore, biological control measures have been investigated more intensively during the past decade.

Charudattan (University of Florida, personal communication, 1989) has identified more than 100 microorganisms or microbial

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

Figure 3. Hydrilla growing in a cove of Lake Nacogdoches, Texas.

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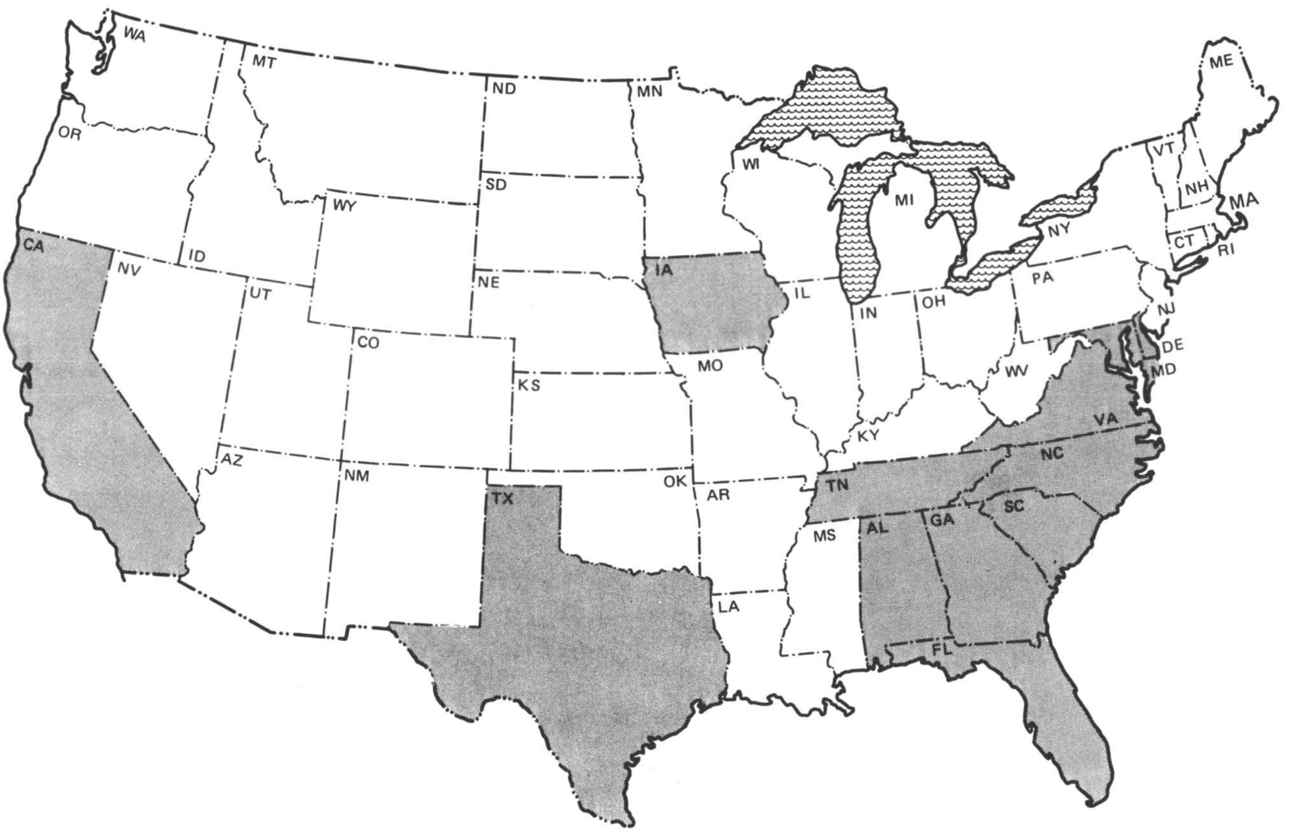

Figure 4. The distribution of *Hydrilla verticillata* in the United States.

products which could potentially damage hydrilla. Joye (unpublished data) has collected more than 200 fungal and bacterial isolates from hydrilla populations within the United States which are being screened for potential biocontrol use.

An isolate of *Fusarium roseum* 'Culmorum', originally collected from *Stratioides aloides* L. (Hydrocharitaceae) in the Netherlands has shown potential as a mycoherbicide (21,22). Since this pathogen is not a native of the United States, its use for biocontrol in the United States remains unresolved (22). Further extensive research is still needed for this pathogen. Even though this fungus may not be host specific (22), it may have some application in specific cases where other nontarget species would not be affected.

In 1987, a fungus identified as *Macrophomina phaseolina* was isolated from an apparently healthy hydrilla plant growing in a lake in southern Texas. Laboratory, greenhouse, and field tests have indicated that this fungus is capable of infecting and destroying inoculated hydrilla populations over a relatively short period of time. In greenhouse experiments, test plants growing in column aquaria exhibited symptoms of disease within 7 days after inoculation. Within three weeks 99 percent of the hydrilla tissue had been destroyed (Figure 5). In a field test, biomass of inoculated plants growing under natural conditions was reduced 61 percent four weeks after inoculation (23).

This fungal isolate may be described more as an opportunist rather than as a true pathogen since it is present in low densities in the natural mycoflora of hydrilla. Only through augmentation of this organism into the hydrilla population does significant damage result (23).

The fungus was found to be host specific only to hydrilla and duck lettuce (*Ottelia alismoides* L., Hydrocharitaceae) in a field of 46 species from 22 families. Since only members of Hydrocharitaceae, have been affected, this fungal isolate appears to be a good candidate for use in biocontrol (23). However, diseases caused by *M. phaseolina* are generally associated with plant stress as other diseases of economically important species are susceptible to isolates of this species. Thus, host range studies will have to be more intensively and extensively conducted to determine the true potential that this pathogen could be used for biocontrol of hydrilla.

In acute toxicity tests, the white amur (grass carp) was not affected at three times the recommended rate of 1×10^6 colony forming units per milliliter. Rates of ten times the recommended rate caused significant mortality (90 %) to fish (Joye, unpublished data). However, mortality may be due to excessive turbidity and reduced levels of dissolved oxygen rather than to any toxic effects. Further work is planned to determine the cause of the observed mortality.

Future work will concentrate on expanding host range tests, evaluating vertebrate toxicity, and developing an appropriate formulation. Ongoing surveys will continue to search for new organisms within the United States and abroad.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

Eurasian watermilfoil.

Myriophyllum spicatum L. (eurasian watermilfoil) was first positively identified in North America from collections made in widely separated locations during the 1940's (24) (Figure 6). Since then it has become a severe problem in many areas of the United States (Figure 7). Eurasian watermilfoil has many characteristics similar to hydrilla which also make it a formidable competitor. It survives much colder temperatures than hydrilla and is the most important submersed weed in Canada and northern United States. The search for pathogens of eurasian watermilfoil began about the time the search began for pathogens of hydrilla. Initially naturally occurring declines of milfoil populations were observed (25). However, attempts to discover pathogens responsible for these declines have not been successful (26). However, several pathological conditions have been described (27) Early investigations suggested the possibility of a virus being responsible for the "Northeast disease" of eurasian watermilfoil in the Chesapeake Bay during the 1960's, but this was never confirmed (28). Another decline of eurasian watermilfoil in the Chesapeake Bay, "Lake Venice disease", was again not satisfactorily explained. It has been suggested that pollution, siltation, autotoxins and/or a combination of factors were involved (28). Eurasian watermilfoil is no longer a significant problem in the Chesapeake bay and the cause of its demise will likely never be determined.

In 1970 the University of Florida began a search for plant pathogens of eurasian watermilfoil. Fungal isolates taken from terrestrial plants were tested, including several species of Fusarium, Pythium, and Phytophthora. However, none of these fungi were pathogenic to eurasian watermilfoil. Other workers have since reported fungal-host associations with isolates of fungi from the genus Acremonium (29) and several weak pathogens have been identified (30,31) such as Fusarium sporotrichioides and Colletotrichum gloesporioides but no promising control agents have been reported (31).

An alternative approach stresses the use of microorganisms capable of degrading eurasian watermilfoil by cellulolytic or degenerating properties of enzymatic means. There are organsims naturally occurring on the surface of eurasian watermilfoil (32). Instead of introducing pathogens from other sources, microorganisms native to the phyllosphere of the target plant may be manipulated to produce enzymes lytic to tissues of the plant host. The induced pathogens would be applied to the plant host where host damage would commence. The damage would theoretically be triggered by compounds excreted from the plant host. Induction of host damage would cease with the destruction of the plant host. Thus, no new pathogenic population would be introduced and no residues would accompany the control process (32). Results of these experiments remains to be seen.

Studies of lytic enzyme-producing microorganisms from milfoil led to the discovery of an isolate of Mycoleptodiscus terrestris that accelerates necrosis of the plant when inoculum is derived

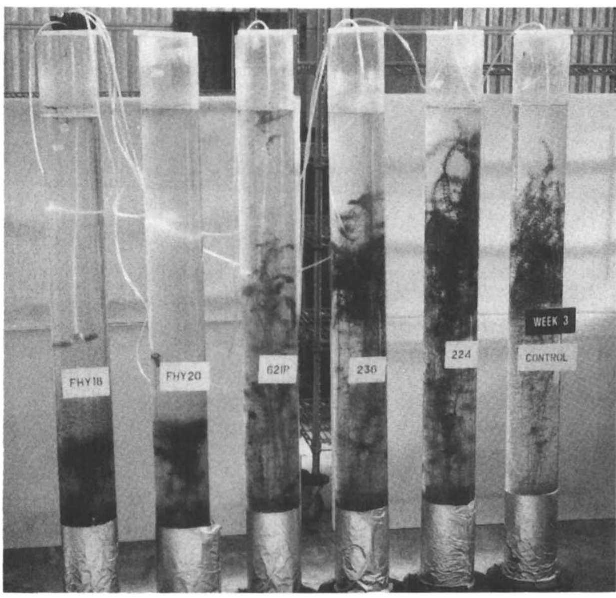

Figure 5. The effects of various fungal isolates on hydrilla 3 weeks after inoculation under greenhouse conditions. Isolates FHy18 and FHY20 are Macrophomina phaseolina. Isolates 621P, 236, and 224 are Fusarium roseum var. culmorum, F. moniliforme var. subglutinans, and Cladosporium cladosporioides, respectively.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

Figure 6. Eurasion watermilfoil, Myriophyllum spicatum, with flower spikes.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

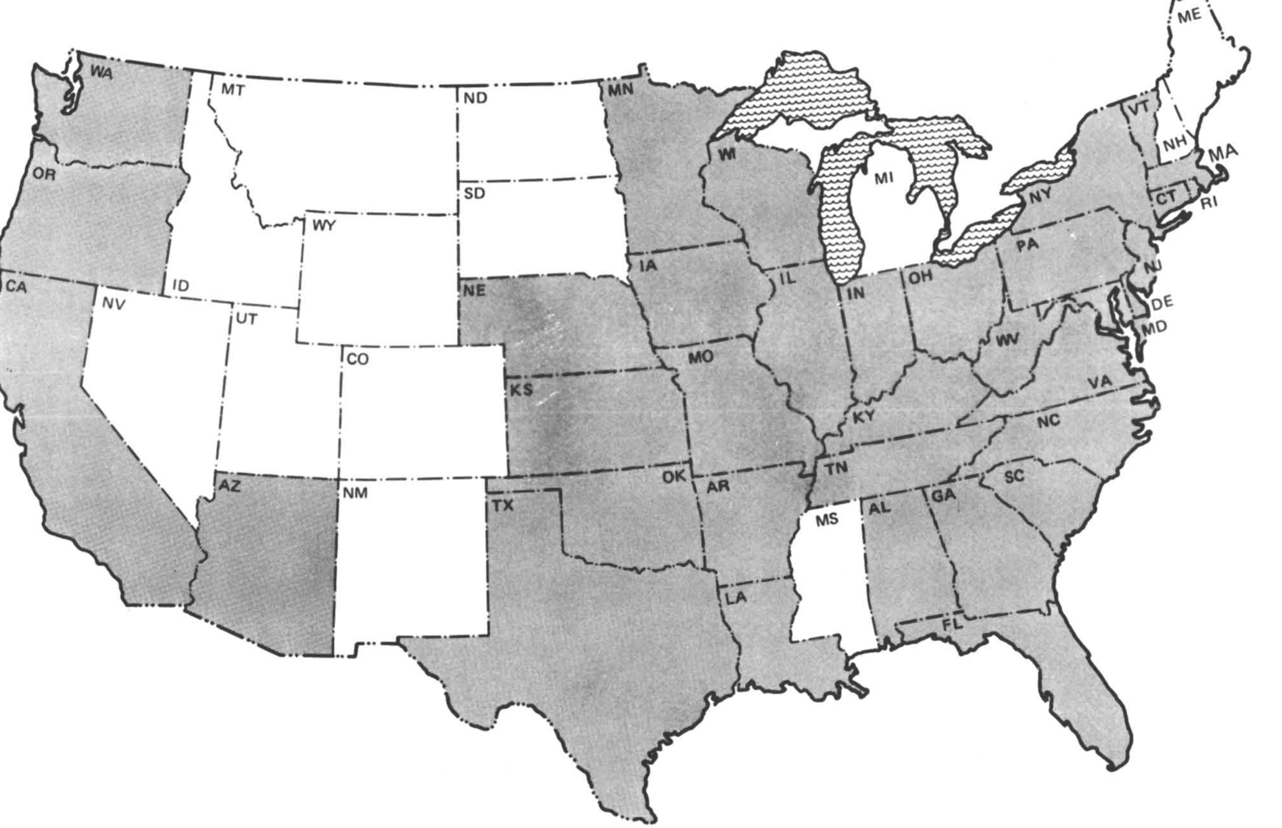

Figure 7. The distribution of eurasian watermilfoil, *Myriophyllum spicatum*, in the United States.

from cultures grown on medium containing cellulose (32). Preliminary laboratory and field tests of the fungus revealed that it has a substantial impact on eurasian watermilfoil. A biotechnology company and the U.S. Army of Engineers have initiated a cooperative agreement to conduct field tests of this fungus. Results of these experiments and definitive tests for determining that no environmental constraints exist will determine whether this organism will become commercially available for biocontrol of eurasian watermilfoil.

Waterlettuce.

Pistia stratiotes L. (waterlettuce) (Figure 8) is a free-floating aquatic plant introduced to the United States from West Africa. With the demise of waterhyacinth in many waterways, waterlettuce has become a problem weed in many lakes and streams of Florida, southern Louisiana and Texas. From 1967 to 1969 waterlettuce in the Kainji Lake in Nigeria was observed to slowly dieback. The dieback was associated with the presence of an aphid (*Rhopalosiphum nymphaeae* L.), and a virus was proposed as the causal agent (33). Transmission of the disease by this insect was demonstrated in greenhouse studies and theoretically the disease could be spread in natural population of water lettuce if infected plants were placed in noninfested populations. The causal agent has not been positively identified, presumably because of lack of funding. However, there still remains the potential for development of biocontrol agents of water lettuce.

Duckweeds.

Lemna sp.(Figure 9), *Spirodela* sp., and *Wolffia* sp. are free-floating aquatic plants common to almost every region of the temperate world. Duckweeds are found growing in stagnant or slow-moving water (Figure 10). Severe infestations restrict boat traffic and growth of more desirable aquatic species. Duckweeds are, however, considered a valuable food source to waterfowl (34).

Several plant pathogens have been reported to be associated with duckweeds, but little is known about the degree of pathogenicity or the relationship between the duckweed and the fungus. There are several reports of duckweed kills in natural populations (34). While the causal agents have been assumed to be fungi, descriptions of infection are scarce. In Louisiana, duckweed kills have been observed and the fungus *Pythium myriotylum* has been implicated as the causal agent. An investigation of this fungus on *Lemna* spp. and *Spirodela* spp. determined that at temperatures over 22° C, (optimum 32° C) in dense populations of duckweed this fungus grew exponentially and could destroy entire populations of duckweed within several days. In pathogenicity tests using six different duckweeds, *Lemna gibba* and *L. minor* were very susceptible, while *Spirodela polyrrhiza* and *L. valdivini* were resistant and *L. aequinoctialis* and *S. punctata* were immune to infection (35).

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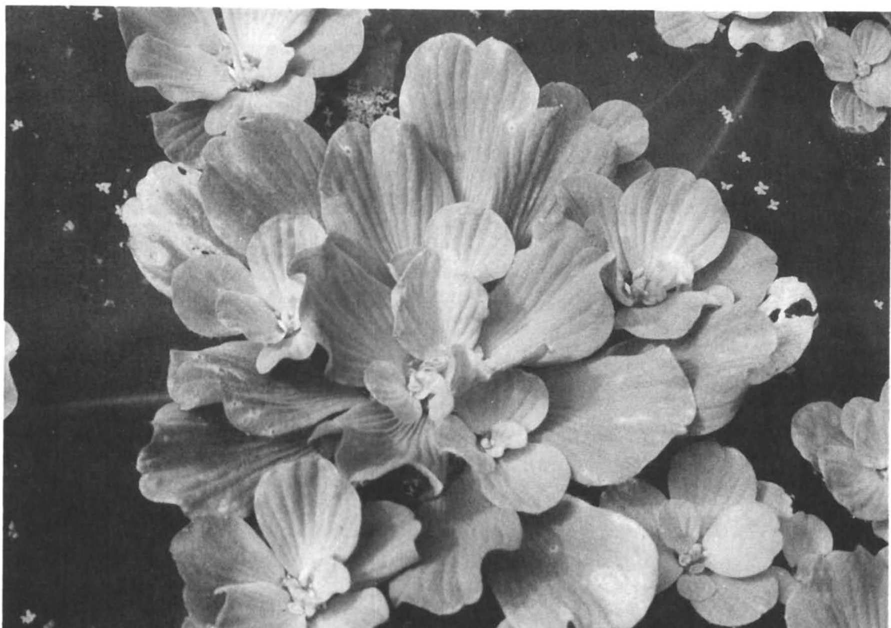

Figure 8. Waterlettuce, Pistia stratioides, growing in greenhouse culture.

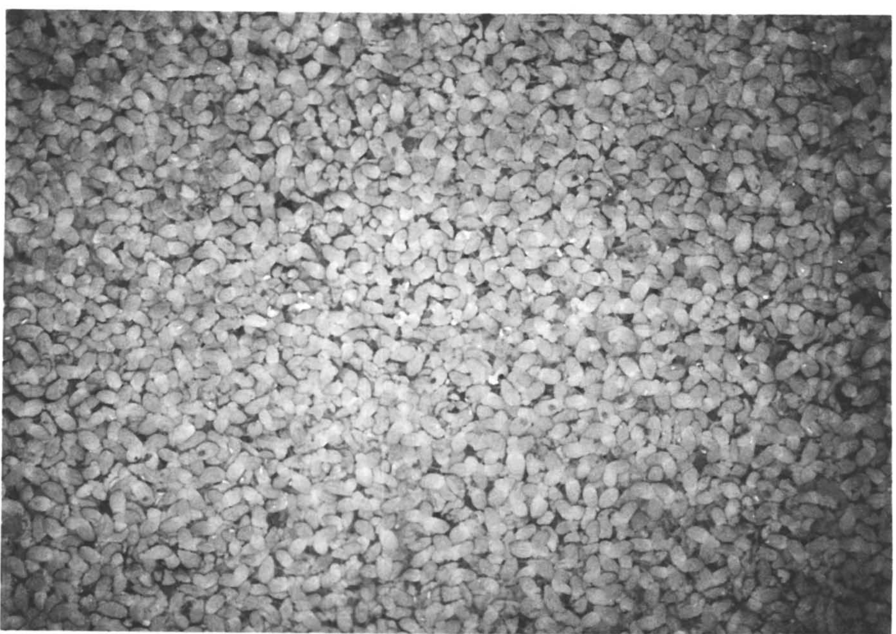

Figure 9. Lemna minor L., a duckweed growing in greenhouse culture.

There are no other reports on the potential use of pathogens for biocontrol of duckweeds, and it is not known if P. myriotylum could be developed into a biocontrol agent.

Alligatorweed.

Alternanthera phyloxeroides (Mart.) Grisb. (Alligatorweed) is a rooted emergent aquatic plant which also has the capability to thrive as a terrestrial plant. This weed grows prolifically in many irrigation and drainage canals, on riverbanks and streambanks, and on the shores of lakes and ponds (Figure 11).

In 1976 a new species of Alternaria was found on alligatorweed and was designated as A. alternantherae Holcomb & Antonopoulos (36). This fungus produced purple leaf-spots with tan, necrotic centers which enlarged over time (Figure 12). It was evaluated for potential biocontrol use but was found to be unsatisfactory because it would not attack stem tissue and conidial production was reduced through reculturing. Recent studies of this organism suggest that it may have some potential for use in integrated weed control when used with insects and chemical herbicides (Joye, G. F. USAE Waterways Experiment Station,unpublished data).

Blue-green algae.

Algal blooms caused by such alga as Anabaena sp., Lyngbya sp., Microcystis sp., Plectonema sp., and Phormidium sp. occur as the result of eutrophication and are related to such factors as water temperature, solar radiation, inorganic nutrients and dissolved organics (Figure 13). Most investigations of algal management have been concentrated on controlling inorganic nutrients. Success in preventing the introduction of nitrates and phosphates seems unlikely, since major sources of these nutrients are difficult to control.

In 1963, the virus designated LPP-1 (Lynbya, Plectonema, Phormidium) was discovered to lyse cells of several blue-green algae (37). The virus was able to lyse 13 species of filamentous blue-green alga. Algal filaments were randomly lysed by the virus, resulting in the algal filaments fragmenting into progressively smaller units until only scattered cells remained.

In 1987 Phlips reported that LPP viruses available through culture collections were not active against strains of Lyngbya birgei but that naturally occurring viruses in Florida were active against this alga (38). This suggests that waste stabilization ponds could be a source of highly effective LPP virus (37).

Only a few algal virus studies are presently being conducted, however, such studies should be encouaged and expanded. The use algal viruses as a new mechanism for the protection and cleaning of the waterways is a relatively unexplored area. Further research is necessary to determine if these algal viruses will be useful for man's purposes (37).

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Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

Figure 10. A duckweed population in a small lake near Delta, LA.

Figure 11. An alligatorweed population on the bank of a lake in Vicksburg, MS.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch008

Figure 12. Leaf spots on the foliage of alligatorweed caused by Alternaria alternanthera.

Figure 13. An algal bloom in a bayou near Delta, LA.

Conclusion

Research in aquatic weed biocontrol will continue to be limited for some time in the future, making progress in this area slow. Successful application of biocontrol techniques to aquatic systems will encourage new participants in this field of research and will help enlighten funding agencies and the general public about this important area of pest management.

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Chapter 9

Genetic Variability of Fungal Pathogens and Their Weed Hosts

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Successful use of a fungal pathogen as a bioherbicide is dependent on the genetic make-up of both the fungus and its target weed. The genetic stability and variability of each must be carefully considered prior to commercial use. The weed host may consist of a genetically heterogeneous population and vary in susceptibility to the biocontrol agent within its geographic range. Fungal pathogens can vary in several important characteristics such as sporulation, virulence, host range, or tolerance to environmental extremes. Specific examples will be used to illustrate the advantages and disadvantages of genetic variability in biological weed control.

The successful use of a fungal plant pathogen for biological weed control is dependent, in part, on the genetic composition of both the fungal pathogen and its target weed. Prior to commercial use, consideration must be given to the extent of genetic diversity in populations of the fungal pathogen and its host. For those working on biological control of weeds, genetic variability can pose potential problems that must be resolved before use, but can also provide for the genetic improvement of biocontrol fungi.

Genetic Variability of Weeds

The expected genetic variability in the target weed population can be an important consideration in the development of a biocontrol agent. The widespread appearance of herbicide resistant weeds (1) and use of genetic resistance in plant breeding programs (2) suggests that host resistance will occur in target weeds. The amount of expected genetic variability in a target weed population can be related to its reproductive system (3,4), selective pressure exerted by the pathogen population (5), and ecological factors (3).

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In general, less genetic variability would be expected with asexually reproducing species than with those reproducing sexually (3,4), and among sexually reproducing species, outbreeding populations are typically more variable than inbreeding populations(3). Burdon and Marshall (4) examined 81 examples of biological control of weeds and found that substantial weed control was attained more frequently with weeds having asexual reproductive systems than with those reproducing sexually. However, weeds reproducing asexually also exhibit genetic variability for characters important in biological control. For instance, rush skeletonweed (Chondrilla juncea L.) is an apomict, producing viable seed without fertilization, yet consists of at least three ecotypes in Australia that differ in leaf morphology and resistance to the rust, Puccinia chondrillina Baker & Syd.(6,7). An extensive search throughout the Mediterranean was required to find a rust isolate virulent to the major ecotype of this weed in Australia (6).

Disease is a strong selective force in plant populations that involves a complex genetic relationship between both the pathogen and its host resulting in genetic diversity in both populations (8-10). In the absence of disease pressure, disease resistance may decline (9). For example, Dinoor and Eshed (11) found less resistance to powdery mildew (Erysiphe graminis DC.) in wild barley (Hordeum spontaneum K. Koch) populations from arid regions of Israel where disease pressure was limited compared to regions favorable to disease development.

In some instances, plants can avoid disease in time and space (8) reducing the need for genetic resistance. The success of Colletotrichum gloeosporioides (Penzig.) Sacc. f. sp. aeschynomene as the bioherbicide Collego for control of northern jointvetch (Aeschynomene virginica (L.) B.S.P.) can be attributed to the uniform susceptibility of the weed host (12). In nature, the pathogen is poorly disseminated such that disease incidence is low (12). Therefore, little selective pressure has been exerted on the weed and the population appears uniformly susceptible throughout the rice (Oryza Sativa L.) production regions of Arkansas (13).

Ecological factors can influence the genetic diversity of weed populations (3). Many weeds are immigrant species that may have less genetic diversity than plants native to the geographical region due to the limited number of introduced individuals (3). The amount of diversity that subsequently develops depends on factors such as the introduced population size, reproductive system, ability to outcross to related species, selection pressure on the weed population and amount of time since the introduction (3). For example, the three rush skeltonweed ecotypes in Australia may each represent separate weed introductions (7). Although importation and release of the rust , P. chondrillina, has resulted in control of the most widely distributed ecotype of rush skeletonweed, the distribution and importance of the other two resistant ecotypes has increased (14). New fungal isolates, virulent to the other two ecotypes of rush skeletonweed, are being sought (15, 16). Such a program would be nearly impossible if numerous ecotypes of the weed existed in Australia.

Resistance to disease is not restricted to pathogens introduced for classical biocontrol, but also applies to endemic pathogens.

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Colletotrichum malvarum (A. Braun & Casp.) Southworth is an endemic pathogen that has been studied as a potential bioherbicide for control of prickly sida (Sida spinosa L.) (17). Plants from seed collected from several locations throughout the U.S. tested for susceptibility to a single isolate of the fungus, resulted in control ranging from a low of 51% to a high of 99% (Table I) (TeBeest D. O., unpublished data). Comparable studies with a range of fungal genotypes would be needed to determine whether sufficient virulence exists in the fungal population to overcome resistant genotypes of the weed.

Table I. Control of Sida spinosa with Colletotrichum malvarum

Seed Source	Percent Control
Rohwer, AR	51
Stillwater, OK	63
Griffin, GA	75
Stuttgart, AR	76
Ben Hur, LA	81
Marianna, AR	89
Stoneville, MS	90
Baton Rouge, LA	91
Burbon, MS	93
Urbana, IL	99

The genetic variability observed in weed populations must be considered during the development of a bioherbicide. Pathogen isolates must be selected that will provide control of the full range of weed genotypes. There is no reason to expect that disease resistant genotypes will not arise over time. The advantage of biological control agents is that the pathogen may also be genetically variable, and new virulent genotypes can be selected for use.

Genetic Variability of Fungal Pathogens

In addition to the target weed, the genetic variability of the fungal biocontrol agent also must be considered.

Both asexual and sexual reproductive systems exist in fungi (18). In contrast to many higher eukaryotes, the potential for extensive genetic variability in asexually reproducing fungal species may be high. This is due to the high reproductive potential of many fungi, short generation time, expression of mutations in the haploid vegetative phase and potential for asexual genetic exchange. Mechanisms for asexual genetic exchange in fungi include the exchange of nuclei following anastomosis, potential mitotic recombination in heterokaryons, and potential exchange of extrachromosomal genes following vegetative fusion (18-20). Genetic exchange within asexually reproducing species may be limited by vegetative incompatibility such that fungal populations may consist of genetically isolated groups (21-23). Although the extent of asexual genetic exchange in nature remains speculative, it does occur readily in laboratory studies and may be operative in natural ecosystems (20).

Problems of Genetic Variability

The potential of a biocontrol agent to adapt to a new, economic host related to the target weed has been a source of concern for regulatory authorities. This is particularly true for imported pathogens introduced into the U.S. from overseas for classical biological control (24). Therefore, stringent regulatory requirements must be met to ensure the safety of imported pathogens to economic plants (25). Pathogens considered a particular risk would include those that are readily disseminated, those attacking economic hosts closely related to the weed host, those having closely related pathovars attacking economic hosts, and those having a demonstrated ability to hybridize with related pathovars (24). Studies with *Cochliobolus* species on grasses (26, 27) and with the grass rusts (28, 29) have demonstrated that common hosts of related pathogens can serve as a source of genetic diversity by inducing hybrids with altered host ranges. The risk is considered much less for endemic pathogens because they are already adapted to host populations in the area of use (24).

The most complete study to date of genetic stability and potential host adaption of an endemic weed pathogen was conducted with *Phytophthora palmivora* (Butl.) Butl. for control of stranglervine (*Morrenia odorata* (H. & A.) Lindl.) in Florida citrus groves (30, 31). Because several related *Phytophthora* species and pathovars were pathogenic to citrus or other economic hosts, concern was expressed for the possible adaptation of the stranglervine pathovar to citrus or possible hybridization with related crop pathogens. Repeated passage of *P. palmivora* through citrus fruit did not increase adaptation of the fungus to citrus(30, 31). In culture, the stranglervine pathovar remained unique morphologically and pathologically(30,31). Mutagenized zoospores failed to give rise to more aggressive strains(31). Experimental matings with compatible isolates of *P. nicotianae* B. Haan var. *parasitica* gave rise to oospores that were non-germinable(31). In mating experiments on citrus roots, oospores were not recovered and only *P. nicotianae* could be reisolated from root tissue(31). Based on these studies, *P. palmivora* was considered sufficiently safe for use in stranglervine control and subsequently commercialized as the bioherbicide DeVine.

Care must be taken to ensure the commercial formulation of the biocontrol agent remains genetically stable. The commercial strain must be carefully preserved and continually monitored throughout the production process for viability, virulence and genetic uniformity (32). Production procedures may require modification to maintain pathogen uniformity and genetic stability.

As molecular genetic techniques continue to be adapted for use in fungal systems, the general public and regulatory authorities have become increasingly concerned with the risk of release of genetically-engineered fungi and potential genetic exchange with related wild-type strains in the environment. Currently, our understanding of gene flow and genetic exchange in natural ecosystems is limited and in need of further study. However,

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several studies of fungal pathogens tend to support the concept that host-parasite interactions are genetically complex and highly co-evolved, such that single gene changes are unlikely to result in pronounced changes in host preference (10, 24).

The potential for genetic exchange in biocontrol fungi through somatic cell fusion and heterokaryosis has been studied with *C. gloeosporioides* f. sp. *aeschynomene* and related pathotypes (Chacko R., Weidemann, G.J., and TeBeest, D.O., unpublished data). Some isolates of this fungus can produce heterokaryons following anastomosis of nutritionally deficient auxotrophs. However, only parental genotypes could be recovered from asexual progeny obtained from heterokaryotic colonies (Table II).

Table II. Growth of conidia from heterokaryotic colonies of *Colletotrichum gloeosporioides* f. sp. *aeschynomene*

	Percent Recovery		
Heterokaryons[a]	MM[b]	MM+ PUR, MET	MM+ LEU, INS
R111	0	16	84
R116	0	18	82
R114	0	92	8
R113	0	24	76
R115	0	40	60
R117	0	100	0
R120	0	64	36

[a] Heterokaryons obtained from crosses of auxotrophic isolates 881 (Leu-, Ins-) and 885 (Pur-, Met-).
[b] MM- minimal medium, PUR-purine, MET-methionine, LEU-leucine, INS-inositol.

Heterokaryotic hybrids also can be produced by fusing protoplasts to overcome genetic barriers to anastomosis (33, 34). Plasmid-mediated transformants have been successfully produced (TeBeest D.O., unpublished data) in this fungus and with several other *Colletotrichum* species (35-37). To date, genetic exchange appears limited in *Colletotrichum* and hybrid isolates often exhibit reduced fitness characterized by reduced growth and sporulation and decreased virulence.

Benefits of Genetic Variability

The genetic variability in fungal biocontrol agents provides a heterogeneous gene pool that can be utilized to select for desirable genetic characters to improve the efficacy of a biocontrol agent. Fungal genotypes can be selected or developed to increase virulence to the target weed, increase or decrease host range, improve cultural characteristics or improve tolerance to environmental extremes (38). Genetic improvement techniques include selections from natural populations, or utilizing laboratory methods such as mutation, hybridization, or gene cloning (39).

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A great deal of natural variability often is present in a fungal population. Webb and Lindow (40) found isolates of Ascochyta pteridis (Bres.) Sacc. from bracken fern (Pteridium aquilinum (L.) Kuhn) to vary in virulence, spore production, and tolerance to desiccation. Isolates of biocontrol fungi are routinely screened for desired characteristics such as increased virulence, abundant spore production, and environmental tolerance (38, 41).

Host-range limitations may not inhibit development if sufficient genetic variability exists in the pathogen population. For example, Nikandrow and coworkers (unpublished data) demonstrated that isolates of Colletotrichum orbiculare (Berk. & Mont.) Arx pathogenic to spiny cocklebur (Xanthium spinosum L.) varied in host specificity. Some isolates were pathogenic to economic hosts such as safflower (Carthamus tinctorius L.) while others were not such that isolates could be selected with a host range that excluded important economic hosts (Table III).

Table III. Pathogenicity of Colletotrichum orbiculare isolates obtained from Xanthium spinosum

		Disease Severity (0-3)[a]		
		Isolate		
FAMILY	HOST	1	4/15b	5/11
Asteraceae	Xanthium spinosum	3	3	3
	X. cavanillesii	1	1	1
	X. orientale	0	0	0
	X. italicum	1	1	1
	X. occidentale	1	0	1
	Carthamus tinctorius	2	0	1
	Bellis perennis	0	2	2
	Silybum marianum	1	1	3
Cuburbitaceae	Cucumis melo	0	0	0
	C. sativus	0	0	0
Mimosaceae	Acacia dealbata	1	1	1
Myrtacea	Eucalyptus cinerea	1	1	1

[a] Disease rating: 0—no symptoms, 1—leaf lesions only, 2—leaf and stem lesions, 3—plant death.

Even broad host range pathogens may not present an absolute barrier to use if methods can be developed to restrict host range. Sands (Chap. 10 this volume) has proposed limiting the host range of Sclerotinia sclerotiorum, a broad host range pathogen, by producing genetically modified isolates unable to parasitize economic hosts or persist in the environment.

Because screening natural populations of a biocontrol fungus can be tedious and time-consuming, techniques such as mutation or hybridization offer attractive alternatives for inducing genetic variability for selection. For example, a potential limitation to the use of Collego as a bioherbicide is the application of fungicides, such as benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate), for disease control. A benomyl tolerant

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strain was obtained by chemical mutagenesis that could be used by growers applying benomyl (42). Protoplast fusion (33,43) and molecular genetic techniques (44,45) offer additional promise for strain improvement of biocontrol agents but are constrained by our limited understanding of the molecular genetics of plant disease.

Summary

The genetic variability of a fungal weed pathogen and its target host constitutes an important challenge to those developing bioherbicides. Genetically heterogeneous weed populations may be difficult to control throughout their entire geographic range or disease resistant genotypes may develop over time. Pathogens must be genetically stable so that new pathotypes do not arise that could threaten desirable plant species. On the other hand, the ability to manipulate fungal pathogens genetically provides great promise for improving the control of serious weed problems by biological means.

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Chapter 10

Biotechnological Approaches to Control of Weeds with Pathogens

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Plant pathogens have rarely been successfully used as biocontrol agents of weeds. One reason for this is that they are usually not lethal enough at low concentrations. In addition, they are usually not host specific. Our approach has been to mutate lethal broad host-range pathogens to obtain isolates that are still lethal to target hosts, but reduced in host range, survival capacity, or otherwise biologically contained. Two such types of biological containment are presented in a fungus, *Sclerotinia sclerotiorum*, a lethal pathogen of 40 different weeds.

Modern genetic technology has the potential to advance the practices of medicine, agriculture, and environmental protection. Potential dangers of this new technology do exist and must be avoided. Opponents to releases of genetically altered organisms cite examples of the destruction incurred by unintentional releases of plant pathogens such as the Dutch elm fungus. During the course of our studies, mutants of *S. sclerotiorum* induced with ultraviolet irradiation were obtained that exhibited increased host-ranges (Table I). The fact that many induced mutants (chemical or irradiation) exhibit enhanced virulence (1,2) supports the use of reasonable caution before releasing certain types of genetically modified organisms. Our charge is to determine if it is possible to avoid the kinds of disasters that often accompany new technologies via containment systems, genetically engineered or otherwise.

Natural Containment Systems

Containment of microorganisms within a specific niche or within a certain area is not a novel phenomenon. Most plant pathogens, for instance, are genetically delimited to a specific niche, such as plant host species or climate. The mechanisms of host range

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delimitation are complex and not understood in their entirety. Nutritive fastidiousness, absence of trigger metabolites, specific binding, and enzyme production are all involved. Whatever the mechanisms, the general practice of crop rotation (forcing host-specific pathogens to survive in absence of their host), is often an effective method of control of many plant pathogens. Perhaps these natural cases of self-containment can be used as models in the development of containment systems for safely releasing genetically altered microbes.

A Non-Engineered Approach to Containment

Our own research involves development of genetic constraints in broad host-range plant pathogens. The pathogen we work with, *Sclerotinia sclerotiorum*, attacks over 40 noxious broad-leafed weeds, and as many crops in most countries. By deletion mutagenesis, we have obtained an auxotrophic mutant, Al-pyr, that has pyrimidines as an absolute growth requirement (3). This nutritional requirement is complemented fully by cytosine supplements and to a lesser extent by uracil (Fig. 1) added to a modified Czapek solution agar (4). Thymidine supplements were ineffective in overcoming the auxotrophy of this mutant. The effect of cytosine, concomitantly with the mutant fungus on seven hosts under greenhouse conditions, is shown in Fig. 2. Preliminary evidence from limited field trials indicates that when Al-pyr is applied with an external source of cytosine, it kills plants in the target area; but the fungus failed to infect plants when external cytosine is absent.

Table I. Expanded Host Ranges of *S. sclerotiorum* Mutants

	Percent Killed Plants		
	Wildtype	Mutant	
Host	Parent	B-326	B-850
Alfalfa	0	0	100
Bean*	0	70	70
Canada Thistle	50	100	100
Clover	·0	0	60
Dandelion	75	75	50
Leafy Spurge*	0	30	65
Lentil	85	70	100
Lettuce	100	85	100
Lupine	0	0	65
Poppy	100	65	100
Rape	85	85	100
Safflower	100	100	100
Spotted Knapweed	40	65	85
Sunflower	85	100	100

*Lesions developed with wildtype parent but did not succumb to the disease. (Reproduced with permission from Ref.9. 1986 Environmental Protection Agency.)

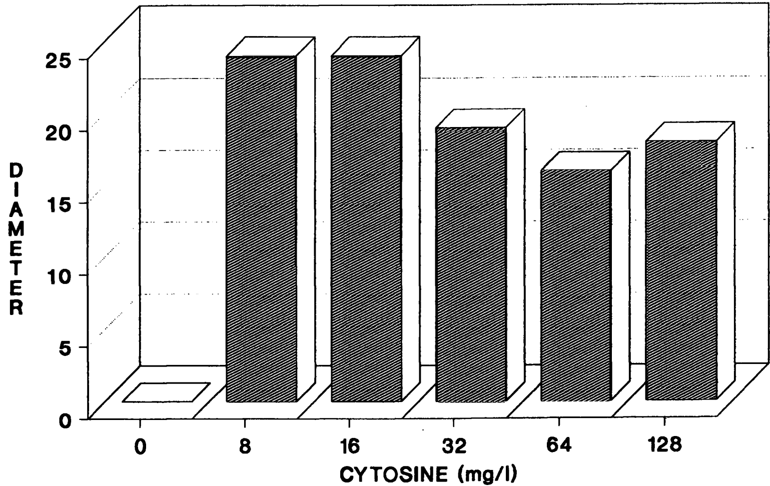

Figure 1. Radial growth response of *S. sclerotiorum* to pyrimidine supplements added to modified Czapek solution agar. Colony diameter (mm), 48 hr after inoculation onto modified Czapek solution agar. Mean of three replications.

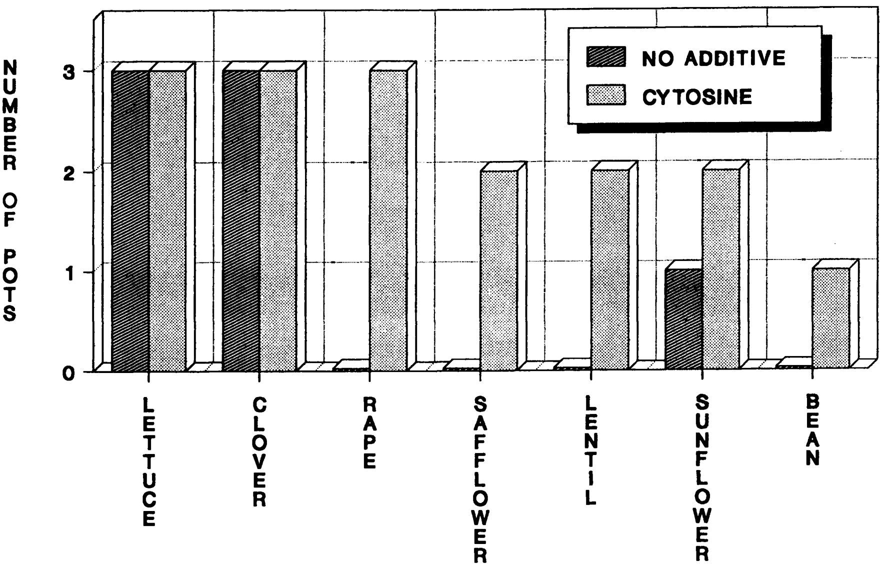

Figure 2. Effect of external cytosine (50 mg/l) on virulence of *S. sclerotiorum* A1-pyr. Applied on cotton plugs to site of inoculation (PDA cultures) of *S. sclerotiorum* A1-pyr. Number of pots showing disease on seedlings. Two to ten seedlings of each species per pot.

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Another *S. sclerotiorum* mutant obtained in this study, SL-1, cannot form sclerotia. Sclerotia are morphological structures that serves both as precursors for fruiting bodies (ascocarps) and for dormant survival during adverse conditions (5). This mutant is unable to make spores, or survive the winter, or undergo sexual recombination, resulting in its demise during the winter months. SL-1 may be slightly less virulent evidenced by its tendency to cause disease in lower percentages of host plants (Fig. 3). However the apparent host range was unchanged in respect to the plants tested (Fig. 3). As the wildtype fungus is widely endemic, the unlikely occurrence of back mutations to prototrophy or sclerotial formation would not significantly increase risk to crops. Neither of these mutants are genetically engineered and both have met requirements necessary for safe experimental releases. They are presently being tested in EPA reviewed small scale field tests in Montana. The value of such organisms lies in their broad host-range, lethality, ease of culture, and restricted dissemination. In the future, they may be engineered with the cytosine requirement or sclerotial formation attached behind a host specific promoter.

Genetically-Engineered Containment Systems

Containment systems, useful, but often not critical in non-engineered situations, may well be critical prior to release of many genetically-engineered microorganisms. To date, three strategies have been proposed for containing these organisms.

The first strategy, (6,7), involves incorporation of recombinant genes to a suicide vector. By methylation, the suicide portion of the vector is turned off while the organism is in the presence of a specific substrate (e.g. crude oil). The carrier microorganism then self destructs once the substrate become limiting in the environment. Similarly, Bej et al. (8) constructed a suicide vector that is turned off in the presence of carbenicillin. Neither of these systems exclude the possibilities that recombinant DNA could be transferred to other microorganisms while in the presence of the necessary substrate, and that alternate organisms might allow persistence of the cloned genes without self-destructing. These suicide systems do not preclude mutations around the suicide module which could result in non-destructive retention of the cloned genes.

The second strategy, proposed by Peter Siderius (9) involves co-induction of a suicidal gene with the gene of interest (Fig. 4). This results in concomitant death of the organism with gene expression. Thus, the desired gene product is produced but the gene is totally contained. The major limitation to this proposed system is that it is not applicable to areas such as biological weed control where a viable organism is required for activity.

The third strategy proposed by our laboratory (9) attempts to overcome some of the problems incurred by the other two systems. This strategy involves splitting gene expression between two or three different loci, usually different plasmids or genomes (Fig. 5). As all loci must be present for gene expression, the risk of concurrent transfer is very low and the gene is effectively contained.

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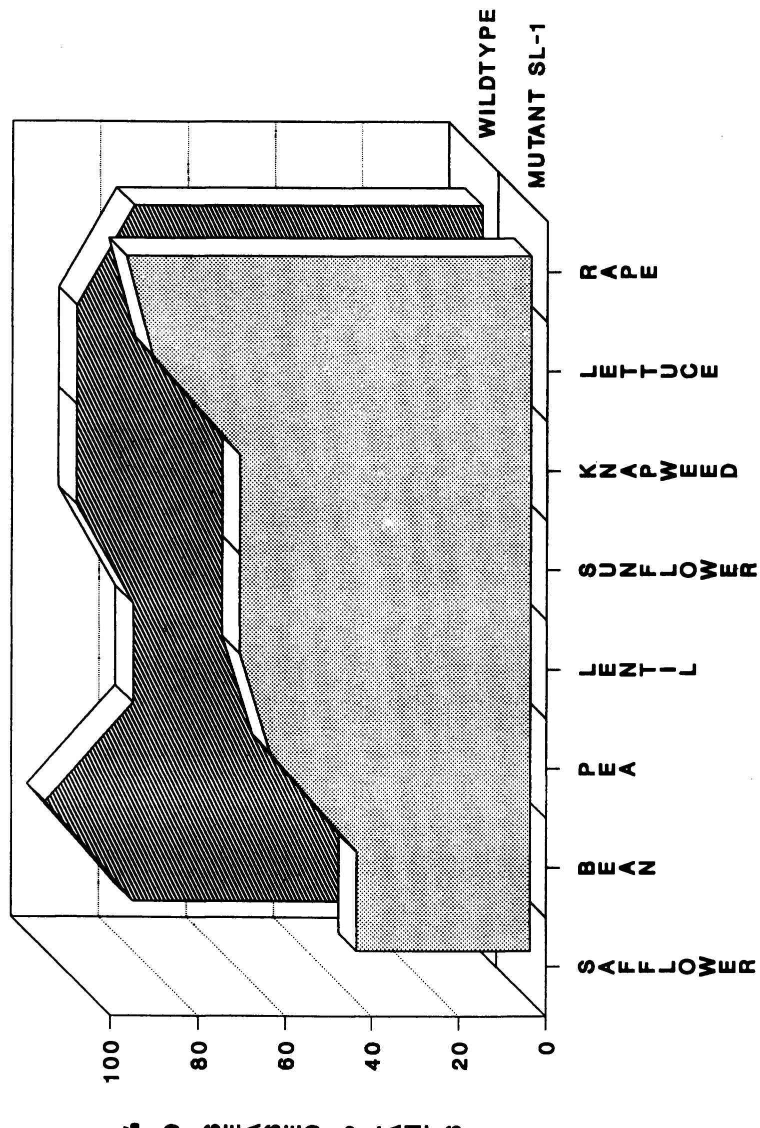

Figure 3. Virulence of the non-sclerotia forming *S. sclerotiorum* mutant SL-1 on eight plant hosts. Five plants per replication. Mean of three replications.

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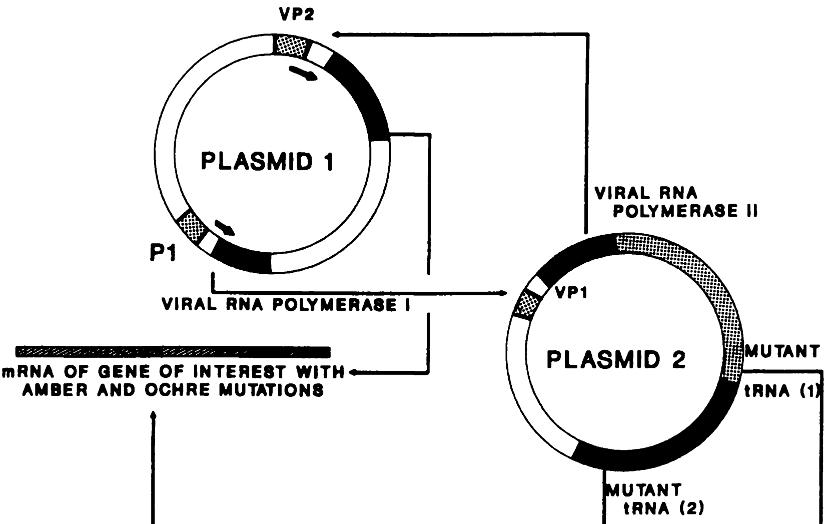

Figure 4. A suicide gene expression containment model system. P1 - Promoter of inducible operon. VP2 - Viral promoter 1. VP2 - Viral promoter 2.

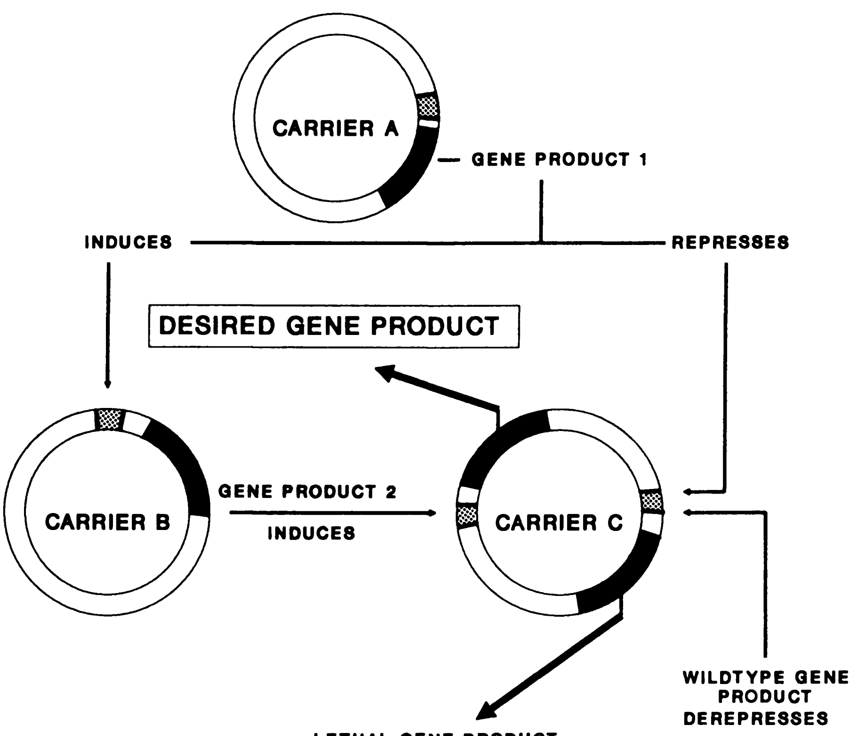

Figure 5. A model triplicate safeguard cassette for fungi. Note: Carriers include genomic or autonomous elements.

Viral promoters, restriction nucleases, and host specific promoters may all be borrowed from plant pathogens for use in development of cellular self-destruct systems (6,10).

Reasons for Containment

From our own analysis of potential risks, we find it difficult to see how any public institution or private corporation would consider release of genetically modified microbes without incorporating some sort of containment system. First is the problem, perceived or actual, of liability. Secondly, they may find that their product persists too long, interfering with future sales and with introductions of improved strains. Finally, government approval may take an inordinate amount of time reviewing the risk factors of a non-contained system, as compared to one with a proven type of containment.

Reasons Against Containment of Engineered Microbes

Few containment systems currently exist, and none have been extensively tested. A requirement for containment might hinder research and investment in biocontrol agents considered safe by virtue of host specificity. Single deletions, done by recombinant methodology, rather than by chemical mutagenesis, also seem to present low risk and should not require containment systems.

Conclusions

Containment systems may be valuable addenda to released microbe genetic systems to reduce liability exposure, enhance investment, and expedite evaluation of environmental safety. Even non-engineered microbes can be effectively constrained by auxotrophy or developmental blocks.

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Chapter 11

Interactions of Pathogens on Plant Leaf Surfaces

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Leaves of terrestrial plants support a diverse assemblage of microbes, predominantly filamentous fungi, yeasts, and bacteria. The composition of this microbial community changes continuously as species enter by immigration and exit by death or emigration. Population densities fluctuate over several orders of magnitude and are influenced by many variables, including climatic and seasonal factors, host species, air spora, and leaf position within the canopy. Nonpathogens in the community buffer against infection, mainly by competing directly with pathogens for nutrients (exploitation competition) or by indirectly inhibiting pathogen growth (interference competition). These interactions are strongly influenced by the environment. As such they differ not only between greenhouse and field, but can be expected to vary in different microbial habitats. The development of successful bioherbicides will require a thorough understanding of the ecology of potential agents and their interactions with other phylloplane associated microbes within the phylloplane environment. This understanding will provide the base for defining the task expected of the bioherbicide and the combination of attributes necessary to accomplish the task.

Interactions among nonpathogenic and pathogenic microorganisms on the leaf surface (phylloplane or phyllosphere) are dynamic and influence the outcome of infection by plant pathogens, whether the pathogen population is unmanipulated or is deliberately increased, as is the case with microbial herbicides. The physical and chemical characteristics of the plant surface set the stage for this microbial community by presenting morphological features and exuding compounds that enhance or inhibit leaf colonization by pathogens and the phylloplane microbes. Finally, the environment acts on all the microorganisms, as well as on the plant. Successful biocontrol of pathogens or weeds necessitates a broad understanding of the phylloplane microbial community and how it interacts with the leaf and microclimate. The issue of biocontrol of weeds by microbial herbicides is really the same as biocontrol of foliar pathogens, only viewed from the perspective of increasing rather than decreasing disease development.

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Physical and Chemical Characteristics of Plant Surfaces

Anatomy. The anatomical and morphological features of a leaf that surface-dwelling or epiphytic microbes encounter include the roughness or pattern of the surface as determined by the topography of the epidermal cells, superficial wax and cuticular layers, various cavities (stomata), and projections (trichomes). These features are continually changing as a result of leaf growth, aging, microbial activity, weathering, plant nutrition, and cultivation practices.

In combination with the cuticle, the epicuticular waxes reduce transpiration and control gas exchange (Figure 1). Waxes, which consist of complex mixtures of various long-chain aliphatic compounds and triterpenoids (1-5), also reduce surface wettability and possibly offer a chemical barrier to infection by plant pathogens (3,6,7) (Figure 2). The morphological appearance of surface waxes varies among plants and may be amorphous-, tube-, ribbon-, or plate-shaped. These patterns are caused primarily by the differences in the chemical nature, composition and distribution patterns (2,4,8). In the case of johnsongrass (*Sorghum halepense* L.), the leaves at emergence are covered with a smooth amorphous wax, but plates of crystalline wax form on the amorphous wax within 1 or 2 days (9). After 3 or 4 weeks from emergence, a smooth layer of coalescence wax is deposited over the wax plates (9). The thickness also varies with plant species, age of the plant, surface of the leaf assayed, and environmental conditions, especially light. Wax thickness and ornamentation have recently been correlated with contact angles of droplets of spray formulations applied to the adaxial (upper) surface (1,2,4,5).

An inhibitory effect (generally a measure of spore germination and germ tube growth) of leaf wax extracted from several plants (e.g. chrysanthemum, *Chrysanthemum morifolium* L.; sugar beet, *Beta vulgaris* L.; apple, *Malus pumila* Mill.) has been demonstrated against such pathogens as *Botrytis cinerea* and *Podosphaera leucotricha* (7,10,11). Conidia of *B. cinerea* were completely inhibited from germinating when applied with nutrients in aqueous droplets to cavities containing dried deposits of wax material at concentrations of 0.05 to 0.25 mg/cm^2 of cavity surface (7). However, too few studies have been conducted to assess the significance of these compounds in relation to their influence on microbial colonization of leaves. Wax chemistry, as it determines wax structure, affects the wettability of the leaf surface. Surface wetting is important in foliar uptake and spread of chemicals and nutrients and is undoubtedly involved in distribution of microbes on leaf surfaces. Chemicals are generally deposited either uniformly over the entire droplet area or as an annulus at the periphery of the droplet. Annular patterns of deposition will decrease the area of interface between the leaf and the droplet and create chemical concentration gradients which do not result if the compound is uniformly distributed. This effect on pesticide uptake and distribution has been examined with some compounds and found to vary with species and the chemical (4). The influence of wax on nutrient concentration gradients and deposition of microbes in aersols or rainfall, and their subsequent distribution on leaf surfaces, has not been investigated.

Beneath the epicuticular wax layer is the cuticle which bounds the epidermal cells and lines the sub-stomatal cavities. Structurally, it is noncellular and often multi-layered, comprising an inner region which merges with cellulose fibrils of the epidermal cell wall (cuticular layer, fibrillar in organization) (2). The chemical component of the cuticle proper is an

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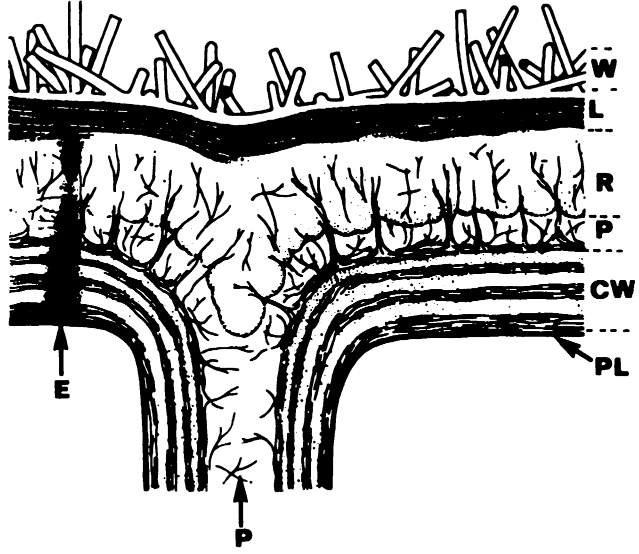

Fig 1. The outer covering of land plants. W, wax rodlets; L, lamellae of cutin and wax; R, reticulate region of cutin and wax with cellulose fibrils; P, pectin; CW, cell wall of alternating layers of cellulose fibers and layers of hemicellulose plus pectin; PL, plasmalemma; E, ectodesma. (Reproduced with permission from Ref. 2. Copyright 1983 Edward Arnold.)

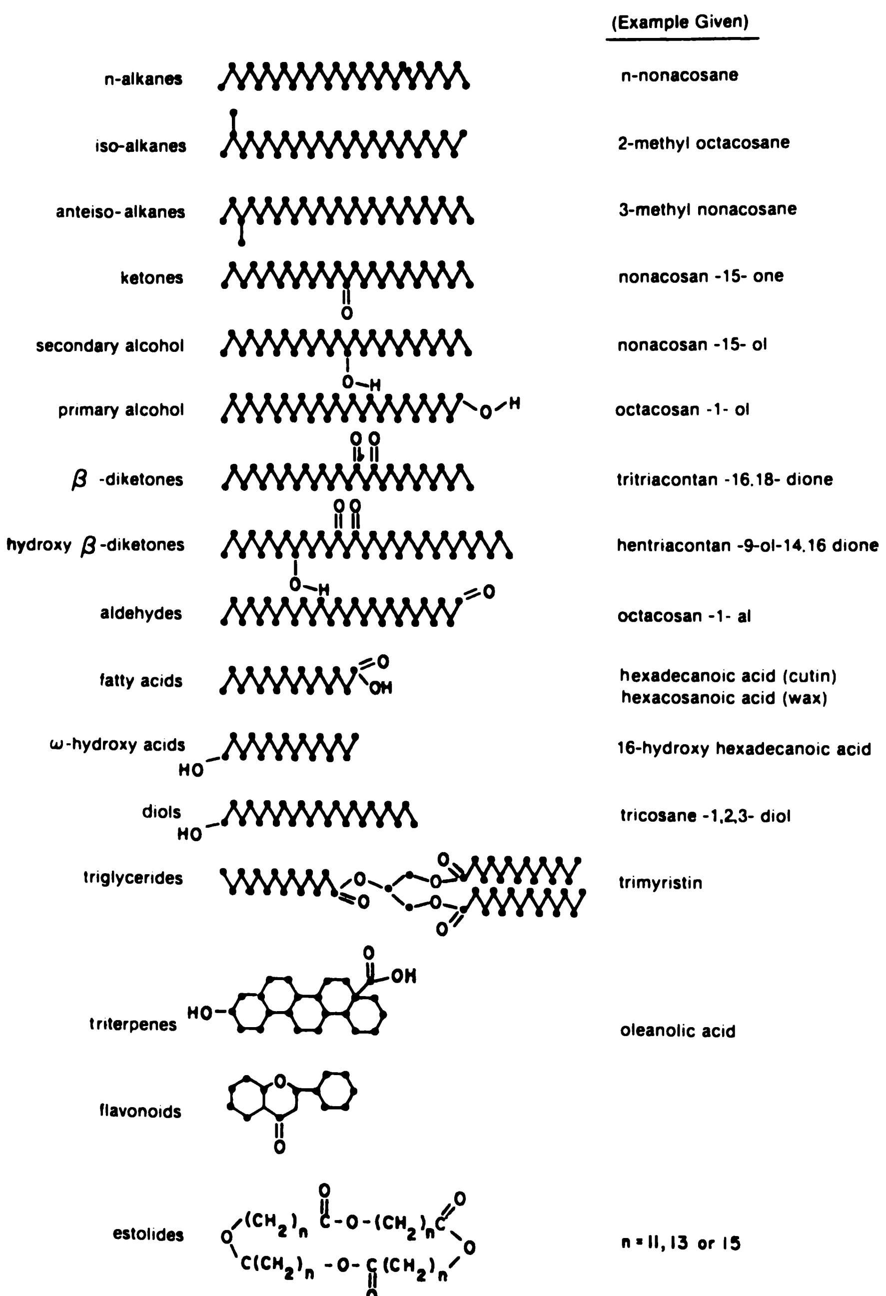

Fig 2. The major classes of compounds in plant waxes. (Reproduced with permission from Ref. 2. Copyright 1983 Edward Arnold.)

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insoluble polymer (cutin) derived from interesterified hydroxy and hydroxy epoxy fatty acids (12,13). The cuticle may be homogenous and amorphous in some species or lamellate or reticulate in others (2,4,9). Platelets (intracuticular wax) are often embedded within the cuticle. In some species, the cuticle is separated from the cellulose of the epidermal cell wall by a pectin layer (12).

The cuticle offers some deflection of solar radiation (2) and has been described as a physical barrier to pathogen invasion (11-13). However, some pathogens produce cutinase which may be involved in overcoming this physical barrier (13). Utilizing an immunological approach (ferritin-conjugated antibodies prepared against the enzyme) combined with electron microscopy, Kolattukudy et al. (13) showed that germinating spores of *Fusarium solani* f. sp. *pisi* and *Colletotrichum gloeosporioides* secrete cutinase at the infection site. Cutinase inhibitors have also been used to stop the penetration process by these fungi and the addition of cutinase to germinating spores of cutinase-less mutants of *F. solani* f. sp. *pisi* and *C. gloeosporioides* resulted in infection. Normally, these mutants were not able to infect hosts with an intact cuticle (13). Many pathogenic fungi and at least on bacterial pathogen (*Streptomyces scabies*) produce cutinase (12). There is little evidence to suggest that leaf saprophytes have the same ability (14,15). Saprophytic yeasts (*Cryptococcus* spp.) have shown cutinolytic activity in vitro (14), but no in situ experiments have been reported that would indicate epiphytic microbes in general use the cuticle as a nutrient source.

The epidermis contains holes (stomatal pores) whose size is regulated by the swelling of adjacent guard cells. Stomata regulate transpiration and exchange of CO^2, O^2, and other volatiles. They are generally more abundant on the abaxial (lower) surface. These pores provide a portal of entry for active penetration by fungal germ tubes as well as passive entrance by fungal spores and bacterial cells in surface water. The number, spatial arrangement, structure, and time of opening and closing of the stomata have been related to resistance and susceptibility of plants to pathogens (11). Other openings on the aerial surfaces that have been implicated in infection by pathogenic bacteria are nectaries and hydathodes (9).

Trichomes are cells that originate solely from and project out of plane of the epidermal cells. These projections can secrete various compounds such as salts, nectar, and terpenes. They apparently provide some protection against excessive sunlight and insect damage (2). However, their role in the life history of epiphytic microbes is unknown.

Certain morphological features (eg. stomata, cuticular wax patterns, cell junctions between epidermal cells) are recognized by various fungal pathogens and should be considered when adopting fungi as microbial herbicides. Two types of thigmodifferentiation have been recognized among the rust fungi: (i) directional changes, the reorientation of the direction of growth by hyphae from rust urediniospores (16-19); and, (ii) differentiation, the formation of infection structures such as appressoria and vesicles, which facilitate the positioning of fungal hyphae over the infection court (eg. stomata) (18,19).

The growth of urediniospore germlings of most rust fungi (*Uromyces*, *Puccinia*, *Melampsora*) on leaves or artificial substrates (prepared with certain topographical features) is perpendicular to the ridges and furrows on these surfaces. The perpendicular orientation is well-documented and an indication of a fungal response to the host (16,18). Gradients of pH may also be involved in spatial orientation. An inverse correlation between stomatal

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aperture and the ability of germ tubes of the rust *Uromyces viciae-fabae* to locate stomata of common spiderwort (*Tradescantia virginiana* L.) and dayflower (*Commelina communis* L.) has been reported (20). More germ tubes ended over pores on leaf surface replicas where a pH gradient was created (replicas of nail varnish placed onto water agar of varying pH), suggesting a role of pH in stomatal location.

When the germling of a rust fungus grows over a specific topographic feature (eg. stomatal guard cell lip, depression on an artificial substrate), swollen, adhesive, pre-infection structures called appressoria develop. Certain species of rust fungi appear only to form appressoria and vesicles (fungal structures formed within the substomatal cavity from continuous hyphal growth of the appressorium) in response to contact stimuli (*Uromyes appendiculatus*), whereas other fungi (*Puccinia graminis*) apparently recognize chemical stimuli (18). For *U. appendiculatus* the surface signal that most enhanced growth orientation was ridges or furrows that were spaced 0.5 m to 15.0 m apart. Appressorial formation was initiated when germlings encountered a ridge that raised the elevation by 0.5 m (17).

Some fungi can directly penetrate leaf tissue. Within this group there are some species that appear to respond to topographic features. Hyphae of *Rhizoctonia solani* and *Botrytis squamosa* have been shown to orientate in the furrows of cell wall junctions on the host surface. This response has been referred to hyphal attachment-directional growth and induced by contact with the plant surface. Several lines of evidence have been presented for such fungi as *Colletotrichum*, *Helminthosporium*, *Botrytis*, *Erysiphe*, and *Sclerotinia*, where appressorium formation over or near epidermal cell wall junctions is the result of a contact stimulus with the leaf surface (19). Conidia of *Alternaria cassiae*, a pathogen of sicklepod (*Cassia obtusifolia* L.) (21), produce germ-tubes of viable length (50-300 m 18 hr after field inoculation) and form appressoria directly over epidermal cells or stomata (22). Host-surface chemistry has been suggested as playing a role in determining the growth response of this pathogen (22).

Thus, pathogenic fungi have the ability to respond to leaf topography in initiating infection. The influence other microbes have on these responses and how fungi are capable of sensing the topographical signals are but two areas of research that can provide information necessary for the understanding of the microbial interactions on leaf surfaces. Additionally, an understanding of the physiological and morphological processes of spore germination, hyphal growth and infection of bioherbicides on aerial plant tissues will assist in the development of formulations for these microbes (23,24).

Chemicals on the Leaf Surface. Numerous compounds have been isolated from leaf washings. Many of these compounds are listed in Table I. Morris and Rouse (26) suggest that concentrations of carbohydrates and amino acids leached from leaves range from about 3-74 μg/ml and 3-52 μg/ml free moisture, respectively. Most of these substances have a stimulatory effect on microbes (10,25,27,28). They may difuse from plant leaves (leachates); be exogenously leaked from microbial propagules; be secreted form aphids as honeydew; or occur as fall-out (primarily pollen) from the atmosphere (28,31). The types and amounts of nutrients available influence microbial growth (possibly through competition if nutrients are limited) and alter microbial patterns in space and time (26). The application of nutrients to leaves can, but does not necessarily, affect microbial populations quantitatively (32) and qualitatively (26). Spore germination and sporulation of several fungi have been demonstrated to be enhanced by the nutritional level available (33,34).

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Table I. Metabolites leached from plant foliage

Inorganic	Organic		
	Carbohydrates	Amino Acids	Organic Acids
boron	fructose	alanine	aconitic
calcium	galactans	arginine	adipic
chlorine	glucose	asparagine	ascorbic
copper	lactose	aspartic acid	citric
iron	pectic substances	β-alanine	fumaric
magnesium	raffinose	eysteine	glutaric
nitrogen	sucrose	γ-aminobutyric	glycolic
phosphorus	sugar alcohols	glutamic acid	lactic
potassium		glutamine	maleic
silica compounds		glycine	malic
sodium		histidine	malonic
strontium		hydroxproline	pyruvic
sulfur		isoleucine	succinic
zinc		leucine	tartaric
		lysine	acidic glycosides
		methionine	
		phenylalanine	
		proline	
		serine	
		threonine	
		tryptophan	
		tyrosine	
		valine	

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Leachates that accumulate in water on the phylloplane include various organic and inorganic substances (eg. amino acids, carbohydrates, vitamins, growth regulators, minerals) (25,28). The amounts and forms of these compounds are influenced by the plant species, plant age, environmental conditions (eg. occurrence and amount of dew, relative humidity, precipitation, light intensity and duration, temperature), and damage or tissue disruption by insects, pathogens and saprophytes (10,25,35,36). Populations of bacteria, yeasts and *Cladosporium* spp. were all shown to increase after fungal structures had ruptured the lower epidermis of leaves (37).

The spores of several genera of fungi (eg. *Botrytis*, *Uromyces*, *Colletotrichum*) have been shown to leak exogenous nutrients resulting in increases in populations of surrounding bacteria and yeasts (33). Using C^{14} tracing methods, Blakeman (33) showed that conidia of *Botrytis cinerea* suspended in water leaked substantial amounts of nutrients (2.5 to 20% of total C^{14} label) into the ambient solution (33). Clark and Lorbeer (38) demonstrated that sterile distilled water in which conidia of *B. cinerea* and *B. squamosa* were incubated for 4 hr, supported moderate growth (10^3 to 10^6 cells/ml increase) of bacterial cells added to the suspension. In contrast, addition of bacterial cells to sterile distilled water resulted in a decrease in the number of recoverable cells by plate dilution (38). Urediniospores of *Uromyces viciae-fabae* varied in the amount of amino acids and carbohydrates that were leached from them following a 24 hr hydration. Urediniospores

taken from the oldest pustules (70-85 days) leaked the greatest amounts of substances. There appeared to be an increase in loss of amino acids and carbohydrates with an increase in age of the pustules (31). An analysis of leachate over 24 hr, revealed a large initial nutrient loss within the first hour of hydration. Interestingly, there was a greater loss from young than mature urediniospores during this first hour (31).

Another potentially significant source of nutrients is insect honeydew (excretions of certain insects consisting of about 90% simple sugars) (29,36,39). A substantial increase in nutrient level on the adaxial surface of sycamore leaves (*Acer pseudoplatanus* L.) was correlated with arrival of aphids in June which fed on the abaxial surfaces and deposited honeydew on the leaves below them (40) (Figure 3). This source of nutrition greatly increased populations of saprophytic fungi such as *Aureobasidium pullulans*, *Cladosporium* spp., and white and pink yeasts (40). Populations of *Sporobolomyces roseus* and *Cryptococcus* spp., common leaf inhabitants, can be stimulated by honeydew deposition (29,32,36). The addition of honeydew to the inoculum of *Cochliobolus sativus* or *Septoria nodorum* increased the degree of infection of wheat leaves by 2.5 to 4 times water controls (29). Spore germination, formation of the number of germ tubes per conidium and growth of the germ tubes of *C. sativus*, were enhanced. Similarly, pathogens such as *C. sativus*, *S. nodorum*, *Fusarium graminearum*, *Alternaria longipes*, *Botrytis cinerea* and saprophytes (eg. *Sporobolomyces* spp., *Cryptococcus* spp., *Cladosporium* spp.) have all been stimulated by the presence of pollen (10,36). This nutritional source promotes germination, growth of germ tubes, and colonization (10).

There is some evidence for inhibitory compounds in plant leachates that reduce germination of plant pathogens (27,28,41). For instance, water-soluble leachates from two cultivars of pecan (*Carya illinoensis* (Wangenh.) C. Koch) inhibit germination of conidia of *Cladosporium caryigenum*. These compounds can be recovered from immature and mature leaves (27). Juglone (5-hydroxy-1, 4-naphtoquinone) was identified as one inhibitory compound, but its effect can be overcome by other phylloplane-associated substances. What role compounds such as juglone have on saprophytic populations remains to be determined. This example indicates the complexity of interactions on the plylloplane which can influence the infection process (27).

To summarize, there are physical and chemical barriers (eg. cuticle, antimicrobial leachates) of the leaf surface that may prevent infection by phylloplane microbes. In contrast, there are topographical features (eg. ridges and furrows, stomata) that are recognized by fungal pathogens and appear important for successful infection. An understanding of how these surface features, the prepenetration development of pathogens, and possibly the nonpathogenic epiflora should assist in selecting or genetically improving microbes as biological herbicides. Essentially nothing is known about how saprophytic or resident microbes may alter these barriers as they colonize the phylloplane. Manipulation of microbial populations by altering the quantity or quality of nutrients, ideally selectively, could promote the effectiveness of mycoherbicides. However, success of nutrient applications for enhanced bioherbicide activity will depend upon being able to predict the response of other phylloplane microorganisms to the introduced substrate.

Microclimate of Leaf Surfaces

The microclimate of the leaf surface has been described as a "hostile" environment where only a limited group of organisms regularly occurs. This

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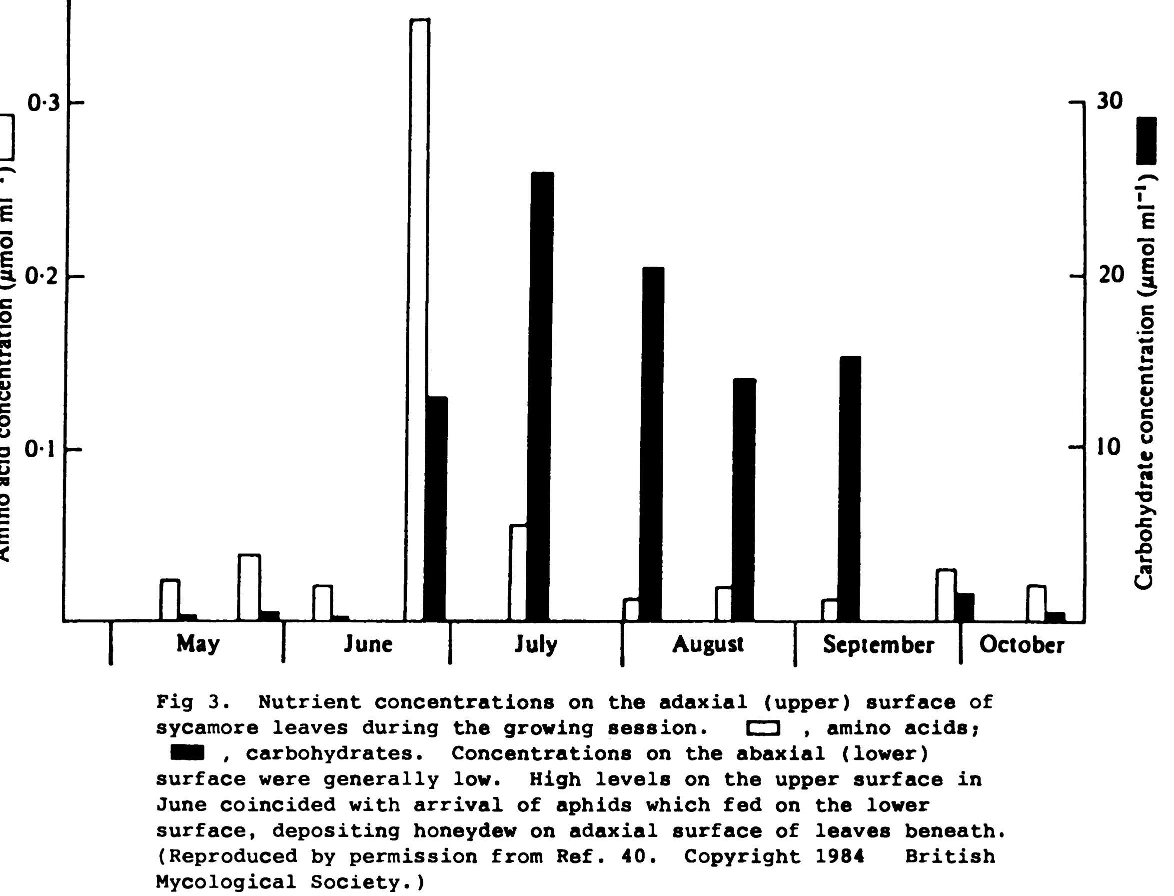

Fig 3. Nutrient concentrations on the adaxial (upper) surface of sycamore leaves during the growing session. ▭ , amino acids; ■ , carbohydrates. Concentrations on the abaxial (lower) surface were generally low. High levels on the upper surface in June coincided with arrival of aphids which fed on the lower surface, depositing honeydew on adaxial surface of leaves beneath. (Reproduced by permission from Ref. 40. Copyright 1984 British Mycological Society.)

is a rather anthropomorphic view in the same sense that we would consider the Arctic to be "hostile", yet polar bears are quite happy there. Just as polar bears are adapted for their environment, so too are the microbes that actually grow on the phylloplane (as opposed to merely being isolated from it as transients).

Fluctuations in the leaf environment are normal; the exposed plant can not offer the degree of stability or moderation of extremes as can soil. Temperature, humidity (relative air humidity and free water), precipitation, radiation and wind speed have the greatest impact on the leaf microclimate (42-44). These factors can be modified by plant density and development, and by the growth of individual plant leaves and shoots (45-47). The influence of plant density in turn depends upon such factors as direction of the crop row relative to the prevailing wind, irrigation or precipitation, and the plant height (45). Increasing plant density tends to reduce the fluctuations in temperature and relative humidity, and to alter the distribution of dew.

The effect of crop microclimate on pathogen development (eg. dispersal, germination, colonization, sporulation, survival) is well known (48-54). Models have been developed for forecasting the occurrence and severity of plant diseases. The ability to monitor various environmental parameters has improved greatly with innovations in electronic sensors (44,55,56). However, given the dynamic interaction between microclimate and plant growth, any ecological approach designed to understand microbial interaction on plant surfaces should begin to incorporate both processes (55,57). Unfortunately, there are few data relating microclimate to microbial interactions and even less information exists on interactions among plant growth, microclimate and microbial interactions. Thus, we discuss here some of the major meteorological factors and how they affect microorganisms on the leaf surface.

Leaf temperature is influenced by air temperature which is in turn modified by wind speed and radiation. Leaf temperature follows a diurnal cycle, being cooler at night and warmer during the day than adjacent air temperatures. This illustrates the need to measure the meteorological events of interest at the site of interest (within the plant canopy or on individual leaf surfaces) instead of relying on weather stations some distance from the source of the microbial interaction. Also, numerous fluctuations in leaf temperature occur during a 24 hr period, illustrating the rapid response of leaves to changes in radiation and wind speed and the variation in temperature regimes to which microbes are constantly exposed (42). No single temperature can be recorded for a leaf as there is a periphery effect of leaves resulting in warmer temperatures in the center than on the edges of a leaf (42). Hence, it becomes exceedingly difficult to design experiments that simulate even a portion of the variability in temperature that microbes encounter on a leaf surface.

For fungal and bacterial pathogens, temperature has been shown to influence germination, infection, latent period, lesion development, sporulation, dispersal and survival. For instance, air temperature influences the ability of the mycoparasite *Dicyma pulvinata* to colonize lesions induced by *Cercosporidium personata*, causal agent of late leaf spot of peanuts (*Arachis hypogaea* L.) (58). On detached peanut leaflets colonization was greatest at 23-28 C, and progressed over time in a logistic pattern (58). At temperatures >28 C, the percentage of lesions colonized declined markedly, accompanied by a slower rate of colonization (58). At temperatures >31.5, no colonization was

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observed. Sporulation of *D. pulvinata* on leaf spot lesions followed a similar course with respect to temperature (58).

Air temperature can also affect the rate and extent of lesion development on weeds by microbial biocontrol agents. Disease development on velvetleaf (*Abutilon theophrasti* Medik.) by *Colletotrichum coccodes* was most severe at air temperatures of 24 or 30 C which corresponded to the optimal ranges for spore germination and mycelial growth of this mycoherbicide (59). Air temperatures cooler than 24 C resulted in less disease and longer times before diseased leaves abscised from the plant (59). TeBeest et al. (60) demonstrated that the rate of anthracnose development (caused by *Colletotrichum gloeosporioides* f. sp. *aeschynomene*) on northern jetch (*Aeschynomene virginica* L.) was reduced if inoculated seedlings were incubated in alternating day/night temperature regimes of 32/24 or 28/20 C compared to a constant incubation temperature of 28 C. Between the two alternating temperature regimes, the disease-index values were significantly lower with the 32/24 than 28/20 C regime 6 days after inoculation (60). Certain day/night temperature regimes were also found to be more conducive for the development of *Alternaria crassa* on jimsonweed (*Datura stramonium* L.) (61). Weed control was significantly reduced by day/night air temperatures of 35/24 C at all inoculum concentrations tested. With inoculum concentrations of 10^5 or 10^6 conidia/ml and day/night temperatures of 29/18, 100% mortality was recorded (61). Severity of disease on Italian thistle (*Carduus pycnocephalus* L.) inoculated with *Alternaria* sp. was reduced at all inoculum levels as incubation temperature was decreased (20,13 or 5 C) (62). This effect was overcome with higher levels of inoculum (in this case conidia of the *Alternaria* sp.) (62), illustrating the importance of understanding key environmental parameters in formulating a control strategy. Unfortunately, there is no information on how temperature fluctuations on the leaf surface favor one microbe over another or what variables of temperature (eg. daily mean temperature, hourly recordings, temperature sums, or temperature response functions) are most appropriate for considering microbial interactions.

The duration of relative humidity or free moisture (fog, dew, precipitation) is of great importance to certain microbial life stages. Humidity of the air is a function of temperature and is generally higher within the canopy than outside. This results in gradients throughout the canopy. The wetting and drying processes (eg. interception, evaporation), as well as the duration of the wetness, vary according to leaf morphology (eg. wax layer, number and type of trichomes) and the structure and density of the canopy. These are among the key components that need to be considered in developing models that explain the interactions among plant, microbe, and the environment on the leaf surface.

As the presence and duration of moisture on leaves has been shown to be critical in the germination and infection of several mycoherbicides (59,60,61,63), formulations are being developed that provide moisture and retard evaporation of water from the leaf surface (64). Quimby et al. (64) showed that without a dew period conidia of *A. cassiae* applied in an aqueous carrier alone and in an aqueous carrier followed by an overspray of an invert emulsion resulted in 0 and 88 percent mortality, respectively, in sicklepod seedlings. The development of this technology may provide a system to overcome some of the environmental constraints for mycoherbicide application that currently exist.

Not all microbial propagules respond similarly to humidity or free water. This is contrasted by the hydrophobic conidia of the powdery

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mildews versus the hydrophylic nature of many conidia borne in an acervulus or pycnidium such as the spores of *Colletotrichum* spp. Humidity can have a direct effect on germination, infection, sporulation, dispersal, and survival of microbial propagules. It can affect the rate of germination and the ability of arriving propagules to colonize. Peanut plants with lesions caused by *C. personatum* were inoculated with conidial suspensions of the mycoparasite, *D. pulvinata*, and placed in a dew deposition chamber either at the beginning of the day cycle (11 hr light, 55% RH, constant 26 C, initial period of dry leaves) or night cycle (13 hr dark, 10.5 hr dewpoint, constant 26 C, initial period of wetted leaves) (65). Leaves placed into the initial dry leaf period had more lesions colonized by the mycoparasite and were colonized faster (65). Apparently the presence of free moisture on the leaves inhibited the germination of the conidia of *D. pulvinata* as light exhibited no effect on the rate of spore germination of *D. pulvinata* (65). On the other hand, an increase in free moisture periods for up to 24 hr increased the severity of infection of Italian thistle by conidia of *Alternaria* sp. At least 4 hr of free moisture on the leaves were required to obtain infection (62). A similar trend of the effect of dew period on disease development was observed with seedlings of northern jointvetch inoculated with *C. gloeosporioides* f. sp. *aeschynomene* (60). At least 12 hr of free moisture following inoculation by a conidial suspension was required to obtain 100% infection at 28 C. Disease developed more slowly and lesions remained relatively small (1 cm or less) when inoculated plants were given less than an 8 hr dew period (60). Walker and Boyette (66) reported that two successive dew periods of 4 and 8 hours enhanced mortality of sicklepod inoculated with *A. cassiae*. Greatest increase in mortality was observed when the second dew period occurred within 2 days of the first dew. Also, the initial dew period had a greater impact on disease development than the second dew period (eg. successive dew periods of 6 and 4 hours resulted in greater mortality than 4 and 6 hour successive periods) (66).

The survival and epiphytic distribution of pathogenic bacteria on buds and leaves of cucumber plants (*Cucumis sativus* L.) are influenced by the ambient relative humidity. The terminal buds of cucumber seedlings were inoculated with an isolate of *Pseudomonas syringae* pv. *lachrymans*, placed into different levels of relative humidity (alternating, 50-60% during the day and >90% at night; high, >80%; and, low, 30-50%) and not wetted (67). At the low RH the pathogen was not detected on healthy leaves or terminal or axillary buds. However, at the high RH the bacterium could be found on most of the buds as well as on the leaves (67). Similarly, the ice nucleation frequency of ice nucleation-active bacteria appears to be controlled by interacting factors such as environment, strain, and host (68). Epiphytically isolated strains had higher ice nucleation activities if the plants were maintained under wet conditions (moistened by spray inoculation and maintained in plastic bags for 48 hr); pathogenic strains were favored after dry incubation (unbagged plants) (68). Obviously, environmental components are interactive with microbial genotype. This confounds the task of understanding the role humidity may play in propagule activity and survival.

Radiation is the ultimate energy source for all plant processes. The daily light:dark cycle and seasonal variation in day length can also directly influence microbial activities, e.g. sporulation in numerous fungi (69). The intensity and wavelength of light may directly affect the survival of propagules on the leaf surface, and indirectly influence the probability of infection by pathogens, by moderating host metabolism (48,70). Only about 3% of detached and 47% of attached sporangia of the blue mold pathogen,

Peronospora tabacina, survived a 3 hour exposure to solar radiation of 8.6 MJm^{-2}. Approximately 75% of detached and 98% of attached sporangia protected by a UV filter germinated. Longer exposures were required to kill conidia of *Alternaria solani* and urediospores of *Uromyces phaseoli*: spores of *A. solani* were the most resistant (70). Spores of all three species survived much better if shaded by a leaf piece than when exposed to full sunlight. Based on these results, the magnitude and duration of solar radiation and the spore type applied should be considered to maximize the chances of survival of an applied biocontrol agent.

The influence of temperature together with light intensity on the colonization of uredinia of *Melampsora larici-populina* by four species of *Cladosporium* were tested in a leaf disc assay. With respect to the variance ratio, the species of *Cladosporium* was the greatest contributor, followed by light x temperature interaction, and light intensity and temperature, respectively, in determining the variation in colonization of uredinia (71). The combinations of temperature and light intensity favoring colonization varied with the species of *Cladosporium* and cultivar of poplar used in the test (71). These results emphasize the need to incorporate the interactive effects of environment and microbes to obtain an understanding of the microbial ecology of a leaf surface.

Wind speed has a strong influence on spore liberation and on the dissemination of fungal propagules. Spores that are deposited on leaf surfaces by impaction are greatly affected by wind speed (44). As sporangia of *Peronospora tabacina* need to remain wet for approximately 2 hr to germinate and infect tobacco (*Nicotiana tobacum* L.), growers have traditionally attempted to decrease the leaf drying time in shade tents by raising the walls of the tents (72). However, this practice was found to increase wind speed in the first 20 m inside the tent. With walls up the wind just inside the tent was 60% of wind outside compared to 30% if walls were down, but by 20 m inside the tent the wind was 15% of the outside regardless of wall position. Thus, the spores spread, but drying of leaves was not significantly affected. Elevated wind speed also increased the inoculum deposited on the plants (72). Dispersal of bacteria in aerosols driven by prevailing winds is well documented (73). The interaction between plants (as major sources of bacteria) and canopy wind speed on the introduction of viable bacterial cells into the atmosphere have demonstrated the need to reconsider the concept of bacterial dispersal (74), which heretofore has emphasized the role of splash and droplet mechanisms and the soil as a repository.

From the above mentioned examples, it is apparent that the environment influences all stages in the life history of a microbe. If the goal is to develop reliable and efficacious bioherbicides, then an understanding of which components of the life history (eg. germination, colonization, infection) are most affected by the environment would seem appropriate. Additionally, some knowledge of how these same environmental parameters affect other phylloplane micobes would improve success of the applied bioherbicide. Environmental windows may exist that favor the biological agent over competing microbes. Even though very little of this information exists, recent advances in electronic technology make it possible to accurately analyze environmental data. There is also the possibility that the phylloplane microclimate could be modified by applying encapsulated microbes, by altering plant leachates quantitatively and qualitatively, or by altering plant growth conditions (e.g. greenhouse and orchard environmental manipulations).

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Microbial Populations of the Leaf Surface

Qualitative and Quantitative Data. The predominant phyllosphere microorganisms in temperate zones are filamentous fungi, yeasts, and bacteria, which commonly occur at densities of from 10^4 to 10^7 propagules per gram fresh weight or per cm^2 of leaf material (75). Most estimates of surface coverage of temperate deciduous leaves are well below 1% (76). Although this may appear to be quite low, it could simply mean that most or all of the favored colonizable sites are occupied. In the tropics, densities are much higher. Ruinen (77) reported continuous microbial layers up to 22 µm thick on the phylloplane.

There have been very few detailed taxonomic studies describing saprophytic bacteria on the leaf surface (35,78). Genera that have been described reflect the ability of particular bacteria to grow on culture media, as no in situ methods are yet used in phylloplane taxonomic studies. The traditional research emphasis has been placed on identifying and enumerating pathogenic bacteria and the environmental conditions most conducive for disease development (79,80). More recently, the population dynamics and relationships of pathogens with saprophytic or other pathogenic strains have begun to be examined (35,81). Genera that have been found associated with leaf surfaces as pathogens and saprophytes include *Erwinia*, *Pseudomonas*, *Xanthomonas*, *Flavobacterium*, *Bacillus*, *Corynebacterium*, and *Lactobacillus* (78,82). An approach that could offer a greater potential for understanding the ecology and interactions of leaf surface bacteria with other microbes would be to study them as functional groups in terms of physiological properties such as substrate utilization and enzyme production (26).

In contrast to the few studies detailing the identification of phyllosphere bacteria, there have been numerous studies on the taxonomic identification and seasonal occurrence of fungi (83-90). The number of species recovered is somewhat dependent on the methods used to collect the individuals (eg. spore fall, leaf washing, direct observation) as well as the media that may be employed. In spite of methodological differences among studies, certain genera of filamentous fungi and yeasts are almost invariably recorded (eg. *Aureobasidium*, *Sporobolomyces*, *Cladosporium*, *Alternaria*), whereas certain pathogens demonstrate host specificity (eg. rusts, powdery mildews, downy mildews, smuts). The species richness of phyllosphere microbes isolated from a plant host during a growing season can be relatively high. Stadelmann (91) found 107 "types" of bacteria, 18 species of yeasts and 190 species of filamentous fungi on apple leaves. Pal and Sharma (87) recovered 91 and 69 species of fungi from sunnhemp (*Crotalaria juncea* L.) and garden pea (*Pisum sativum* L.), respectively. However, most species may be uncommon. Only 7 of the more than 100 species of filamentous fungi and yeasts recovered by Andrews et al (92) from apple leaves were on virtually every leaf sampled.

Several classification schemes have been proposed to describe these fungi. Dickinson (83) separated them into three categories (pathogens, non-pathogenic epiphytes, and exochthonous fungi) and further distinguished within the categories based on growth and colonization ability. The non-pathogenic fungi recovered from ribbongum (*Eucalyptus viminalis* Benth.) were classified by Cabral (85) as ruderals, residents, or primary saprophytes by combining the ideas of Dickinson (83) and Pugh (93). This scheme is based on activity and to a certain extent the frequency of recovery. Davenport (94) divided the species of yeasts on developing apple and grapes (*Vitis* spp.) into resident and transient groups based on a biological calender,

which compared species on a functional basis at similar stages of plant development. By a mathematical analysis based on the level of association (association matrix) and analysis of recurrent groups, Thomas and Shattock (95) identified seven recurrent groups for species of filamentous fungi found on senescent or damaged leaves of perennial rye grass (*Lolium perenne* L.). The seven groups presented a mathematical expression of the association of pathogens and primary and secondary saprophytes. As the approach was used with fungi on sensescent or damaged leaves, an interesting extension of the work would be to examine how fungi on living leaves would be grouped, particularly with regards to their temporal position.

Spatial Distribution. Observational and statistical analyses have been made of spatial distribution of epiphytic microbes. The difficulty has been in determining what constitutes a sample, the scale of the distribution, the mechanics of selecting the sample (position on the plant, age of leaf, etc.), and the assay used for quantitating microbial propagules or microbial cell mass. Carroll (96) studied the distribution patterns and microbial cell biomass of microepiphytes on Douglas fir needles (*Pseudotsuga menziesii* (Mirb.) Franco). Thin, free-hand sections were made from needles removed from various positions within the tree canopy. Estimates of cumulative microbial volumes were made by photographing the sections, overlaying the photographs and tracing microbial patterns onto Mylar film as they appeared from microscopic observation, cutting out the patterns, and weighing the tracings from each zone of the needle. The microbes were found to be concentrated over stomata and in midrib grooves (96). The highest concentrations occurred in the lower portions of the canopy and decreased with height (96). Thus, there was a nonrandom occurrence of microbes with regard to surface of individual needles, age class of the needles, and canopy stratum. With the exception of 1-and 2-year old needles, microbial biomass estimates were greater on the bottom surface of needles than on the top (for age classes 3 through 8 yr) (96). Other studies have shown that position on the leaf or within a canopy influences the amount of microbial growth. Numbers of fungal propagules and hyphal development were greater on the adaxial than the abaxial surface of mature leaves of aspen popular (*Populus tremuloides* Michx.) (97). A similar trend was found on leaves of sycamore, where the number of fungal spores and mycelial biomass was generally greater on the adaxial than abaxial surface (40). A significant effect of height and lateral position of apple leaf samples was found for the densities of epiphytic filamentous fungi and yeasts (98). Wildman and Parkinson (97) also found that the height within the canopy influenced the isolation of some fungi from aspen popular leaves.

Subsequent colonization patterns, after application of microbes to leaves, appear to be nonrandom. Leben (67) observed that 19-28 days after inoculation of leaf buds of cucumber seedlings with suspensions of *Pseudomonas syringae* pv. *lachrymans*, the bacterium appeared to be distributed uniformly on the two expanding leaves below the bud (by leaf imprints on a selective medium). However, on older leaves the bacterium was usually distributed nonrandomly, often found associated with epidermal cells on leaf veins and with basal cells of trichomes on veins. Bacterial aggregates were generally less prevalent on older leaves. The distribution on older leaves was thought to be related to leaf moisture (67). Blakeman (79) reported that bacterial or yeast cells immediately after inoculation onto bean leaves (Vicia faba L.) were evenly distributed on anticlinal walls of epidermal cells. Later, differential colonization was detected (79). When

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch011

yeasts were inoculated alone, the cells were mainly associated with leaf veins. Bacteria inoculated alone were found associated with veins and stomatal pores. However, when the two microbes were combined, yeasts were found to colonize the anticlinal walls over the leaf lamina, whereas the bacteria were primarily on anticlinal walls of veins and in stomatal pores (79). Whether this situation is typical of the distribution of these two types of microbes on other leaf surfaces remains to be determined.

The influence of leaf pathogens on the saprophytic microflora has been insufficiently examined, but Stadelman and Schwinn (99) showed that lesion areas on scabby apple leaves contained between the orders of 10-100 fold the microbes than did non-scabby portions. Presumably the microflora were altered qualitatively. Changed patterns of microbial colonization have implications for bioherbicide applications, eg. in the role that opportunistic microbes may play in invasion and death of leaves.

There may be as great as a 1,000-fold variation in bacterial counts from leaf to leaf assayed from apparently similar leaves in similar canopy positions at the same sampling period (35,100). Most data sets obtained from plate counts of bean and corn leaves are described by a log normal distribution, indicating that on individual leaves or leaflets the spatial distribution of bacterial cells are not uniform within a canopy (35,100). A log normal distribution tends to reflect the fact that chance effects of random variables are acting on a large and diverse collection of objects (in this case bacteria). Interestingly, a similar study on wheat leaves indicated that bacterial counts were best described by a log normal distribution, but counts of yeasts (mainly *Cryptococcus* spp. and *Sporobolomyces* spp.) and *Cladosporium* spp. colonies appeared normally distributed (101). Much more work needs to be done, particularly with regard to host, host age, environmental factors, and position of the sample within a canopy, before the spatial distribution of these microbes can adequately be described.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch011

Seasonal and Diurnal Patterns. Seasonal distribution of microbes on leaves is well established (35,81,88,94,101,102); microbial populations typically increase as the season progresses and plant organs mature. These studies are useful in monitoring impact of environmental disruptions on microbes, assisting in selecting the most appropriate time-frame for the application of introduced microorganisms (particularly in terms of nutrients and potential competition), and suggesting sampling strategies to monitor microbes. Successional phases for bacteria, yeasts, and filamentous fungi have been proposed (79), but these proposed patterns ignore the fluctuations that occur in the periods between typical sampling intervals. Rather than total colony counts from dilution plating, biomass measurements or doubling times may be more appropriate for understanding the dynamics of microbial colonization and determining growth rates. The degree to which this might be important is evident from changes in population size and ice nucleation activity of *Pseudomonas syringae* on bean leaflets harvested at 2-hour intervals during three, 26-hour periods (103). Overall population fluctuations differed during each of the periods and in one case, during several hour intervals, population growth rates in situ were on the same order of magnitude as optimal rates in vitro. In addition, doubling times of several bacterial species under favorable conditions on leaf surfaces had previously been found to be similar to that in culture (104,105).

With regard to fungal systems, Breeze and Dix (89) examined numbers of germinated spores and total mycelial length of fungi and found several colonization events before a stable community was established on Norway

maple leaves (*Acer platanoides* L.). These results suggest that seasonal trends are the sum of numerous iterations of dispersal, growth and death which may be species-specific. Research that can explain these fluctuations in terms of environmental factors, plant growth, and microbial interactions should illustrate the most favorable biological windows for application of introduced microbes for biocontrol.

In overview, evidence to date on phylloplane microbial populations suggests the following: (i) that quantitative seasonal trends for the microflora in general, and for certain species in particular are relatively well known; (ii) that qualitative trends, particularly for the bacteria, are poorly documented; (iii) that spatial and short-term temporal variation is not well understood, but probably substantial and; (iv) that the community is highly dynamic.

Microbial Interactions

Evidence for naturally occurring microbial interactions comes mainly from plant pathological studies involving either fungicide applications, or the isolation or observation of mycoparasites in situ. There are several cases where fungicide application caused an increase in disease. This appears to be the result of reducing the non-target antagonistic leaf microorganisms (106). Fokkema et al. (107) demonstrated in a two-year study that inoculation of water-sprayed leaves of rye (*Secale cereale* L.) with *Cochliobolus sativus* at the time of flowering resulted in 60% less necrosis compared with inoculations on benomyl-sprayed leaves. Microbial populations of filamentous fungi and yeasts on water-sprayed leaves at flowering were 10,000 and 3,000 propagules per cm^2 leaf surface in the first and second year of the study, respectively. This contrasts with those of the benomyl-sprayed leaves, which were only 1,200 and 400 propagules per cm^2, respectively. The authors suggested that increase in disease incidence was a loss of the buffering capacity of saprophytic fungi towards the pathogen (107). Further evidence suggesting that loss of buffering effect can lead to increased disease is provided by the surface sterilization experiments of Spurr (108).

Dieback or "gummosis" disease of apricot (*Prunus armeniaca* L.) caused by *Eutypa armeniacae* was an insignificant disease in South Australia until routine applications of copper fungicides were introduced for the control of "shothole" disease (*Clasterosporium carpophilum*) of apricot leaves (106). Experiments by Carter (109) supported the hypothesis that the copper fungicides were detrimental to naturally occurring antagonists of *E. armeniacae*. The incidence of dieback was reduced with the application of a saprophyte to pruning wounds (infection courts of *E. armeniacae*). Elsewhere, there is evidence that as resistant strains of *Botrytis cinerea* to benomyl emerge, continued applications of benomyl to cyclamen (*Cyclamen persicum* Mill.) reduce the naturally occurring antagonists, resulting in more severe infections (106,110). All of these instances are correlative, but do illustrate the likely involvement of antagonistic interactions on leaf surfaces.

Numerous fungal hyperparasites of rusts and powdery mildews apparently exist in nature and may act to decrease populations of the pathogens (111,114). Some of the more studied species include *Ampelomyces quisqualis*, *Darluca filum*, *Tuberculina vinosa*, and *Verticillium lecanii* (111). There appears to be no host specificity among those most frequently examined, although differences in aggressiveness have been found among isolates of *D. filum* (111). The spatial distribution of *D. filum* is erratic and clustered on individual leaves with a vertical gradient within the canopy (greatest number of sori infected on lower leaves) (111). Successional

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch011

patterns have been suggested to exist with the hyperparasites of powdery mildews (112).

Hyperparasites can adversely affect sporulation, the infectious period, and dispersal of pathogens (111,114,115). Freeman (114) reported that constant parasitism by *D. filum* of *Puccinia stenotaphri* on St. Augustinegrass (*Stenotaphrum secundatum* (Wilt.) Kuntze) was responsible for maintaining this rust at a low level of destructiveness. Kuhlmann et al. (115) found that in pure stands of dwarf live oak (*Quercus minima* (Sarg.) Small) in Florida, 93% of the sori of *Cronartium strobilinum* were naturally infected by *D. filum*. A greater understanding of the population biology of these hyperparasites will be necessary if biotrophic fungi are to be successfully used for the biocontrol of weeds.

Most evidence demonstrating the interactions among phylloplane microorganisms comes from experiments on the biological control of plant pathogens. Typically, a stage of the life cycle, such as spore germination, germ tube growth, appressorium formation, spore formation or lesion development is inhibited or reduced. Three forms of antagonism have been described (116): exploitation competition, interference competition, and mycoparasitism.

Exploitation competition involves the depletion of a resource (most commonly space or nutrients) without imparied access to it. The extent of exploitative competition will increase proportionally with the degree of ecological niche overlap (117). Because certain sites on a leaf surface (eg. leaf veins, junctions of epidermal cells) are more likely to harbor microbes than others, competition for space may be of some importance during colonization (67,79). The initial occupation of selected microsites by massive applications of introduced microorganisms may well alter the community structure. Lindemann and Suslow (118) demonstrated that ice nucleation-active (INA^+) strains of *Pseudomonas fluorescens* or *P. syringae* inoculated onto strawberry blossoms (*Fragaria* x *ananassa* (Duchesne) 'Douglas') at concentrations of 10^2 colony-forming-units (cfu) per blossom could be inhibited by elevating the bacterial population to carrying-capacity by applying large numbers (10^7 cfu per blossom) of ice nucleation-deficient (INA^-) deletion mutants. However, growth cessation of INA^+ was not accompanied by a significant decrease in its population (118). INA^- derivatives were inhibited by INA^+ parental strains when the inoculum doses were reversed. The authors suggested the inhibition of growth resulted from colonizable sites being saturated and/or nutrient depletion (106).

Certain strains of bacteria can competitively exclude other strains. Lindow (117) found that Ice^+ (ice nucleation-active) populations of *Pseudomonas syringae* on corn plants (*Zea mays* L.) were reduced by prior colonization of the leaves by Ice^- strains (ice nuleation-deficient). Interestingly, the total bacterial population was not affected by the prior inoculation of Ice^- strains (117). For these reasons, Lindow (117) concluded that the antagonism between these strains resulted from competitive exclusion of new arrivals to an occupied niche rather than competitive displacement. As the carrying capacity of leaf surfaces for bacteria is low relative to other plant habitats, particularly roots (101), preemptive colonization by one strain may be sufficient to reduce the population size of similar strains. The importance of competition for space among naturally colonizing microbes remains unknown.

Several experiments have examined the role of nutrient depletion in microbial interactions. Mixed C^{14}-labeled amino acids were added to droplets on sugar beet leaves (wetted 24 hr prior to application) and uptake

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of the labeled compounds by epiphytes was determined. Approximately 80% of the amino acids were absorbed by leaf-associated microbes. Inhibition of germination of conidia of the fungal pathogen, *Botrytis cinerea*, was positively correlated with the uptake of amino acids by the leaf-associated microbes (33,119). Similarly, isolates of *Phoma betae* and *Cladosporium herbarum* were inhibited from germinating on sugar beet leaves when incubated with 10^5 cells/ml of a *Pseudomonas* sp. Substantial uptake of amino acids by the bacterium was also noted (119). However, the percentage of germinating conidia of an isolate of *Colletotrichum dematium* f. sp. *spinaciae* was not reduced by this bacterium even though the uptake of amino acids was high. Germ tube length was reduced, but appressorial development was stimulated (119). The same *Pseudomonas* isolate also induced appressorial formation in *C. acutatum* on glass slides and on sugar beet leaves (120). The induction was eliminated in the presence of added nutrients, but occurred if conidia were leached by creating a steep diffusion gradient away from them (120). In vitro studies with *Sporobolomyces roseus* and *C. sativus* suggested that yeasts can also compete for endogenous conidial reserves, thereby inhibiting germination of the pathogen conidia (121). Germination was restored by transferring the conidia to yeast-free solutions, indicating that yeast may impose a nutrient stress by acting as a nutrient sink. On wheat leaves, the reduction of germ tube length was more pronounced than inhibition of germination (121).

In determining which organisms may be most affected by competitors on the leaf surface and thereby the best candidate for biological control, it may be useful to consider the stage in the life cycle likely to be influenced, and the duration of that phase. For pathogens this entails considering their various infection strategies (36). For example, the only pre-infectional stage of biotrophic pathogens (those pathogens requiring a living host for their nutrition) that may be affected is spore germination. These pathogens usually infect rapidly without forming an extensive hyphal network for substrate utilization that could be inhibited by antagonistic microbes. In contrast, some necrotrophic pathogens tend to be vulnerable during the spore germination phase as well as during their period of epiphytic mycelial growth (36).

Late stages in the infection process by necrotrophic pathogens may be influenced by competition. Yeasts, such as *S. roseus* and *Cryptococcus laurentii* var. *flavescens*, when sprayed onto corn leaves 2-3 days prior to inoculation with *Colletotrichum graminicola*, reduced lesion density and necrosis by approximately 50% compared to untreated leaves (122). Neither conidial germination, superficial mycelial growth, nor appressorium formation were inhibited, but the number of infections on the leaves was reduced (122). The authors suggested that appressorium germination and/or development of the penetration peg was affected by the nutrient stress imposed by the yeasts (122). Numerous other studies have implicated nutrient deprivation as the cause for reduction in propagule germination or infection (123,127). One category of these involves the addition of honeydew to leaves. Populations of pathogens (*C. sativus* and *S. nodorum*) and saprophytes on wheat leaves (*Triticum asetivum* L.) were increased, as was infection (29). However, if honeydew was applied 4-5 days before inoculation with the pathogens, development of the saprophytic microbes counteracted the stimulatory effect on the pathogen (29). Thus, it may become important to understand the influence and to monitor the deposition of honeydew prior to the application of agents for biocontrol of weeds to ensure that successful

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch011

infection by these agents is not inhibited by the development of a saprophytic community.

Other chemicals, such as iron chelating compounds (siderophores), on the plant surface are produced by epiphytic bacteria and can stimulate fungal germination and appressorium formation (128). Presently, the evidence is correlative, as no siderophores have been detected in situ, but sufficiently intriguing to reexamine the role of the leaf microflora with respect to fungal infection. A *Pseudomonas* isolate (UV3) stimulated conidial germination and appressorium formation when added to conidial suspensions of *C. acutatum* on strawberry stolons (129). A siderophore was purified from the bacterium and found to produce a similar, but enhanced response (129). Conidia of *C. musae* and *B. cinerea* have also been stimulated to germinate and form appressoria in the presence of another siderophore produced by *P. fluorescens* (128,130). There is also some evidence that these compounds enhance aggressiveness of the pathogens, as lesion development can be more pronounced when inoculation is accompanied by siderophores (128). If this response to siderophores can be demonstrated among many fungi, the effectiveness of biocontrol fungi for weeds may be enhanced by applying fungi with synthetic iron-chelating compounds or bacteria that produce them. The studies on nutrient competition also illustrate the complex interactions between nutrients on the leaf surface and the qualitative and quantitative composition of the microbial community.

Interference competition implies that the access to a resource by a microbe is limited, generally by a chemical (antibiosis) (116). Few studies have demonstrated the direct role of antibiotic production in microbial interactions in the soil (131). Only indirect evidence has been presented for its importance on leaf surfaces. This is based either on in vitro assays and/or the effect of applying the antibiotic or culture filtrate to a microbial population. Cullen and Andrews (132) showed a positive correlation between levels of antibiotic production in vitro by strains of *Chaetomium globosum* and antagonism to *Venturia inaequalis* on seedlings in growth chamber studies. The antibiotic, identified as chetomin, was inhibitory in vitro to *V. inaequalis* and when applied in vivo (132). Two antibiotics have been isolated from a strain of *Penicillium frequentans* that was antagonistic towards *Monilinia laxa* (133). Spore germination and germ tube growth were inhibited when conidia were incubated in a partly purified preparation of the antibiotics (133). Similar results were obtained in vivo by inoculating wounded surfaces of sterilized peach twigs (*Prunus persica* L.) with the pathogen and the antibiotics (133). Activity of these compounds against other components of peach twig mycoflora was also demonstrated (133).

The above examples are typical of the many studies suggestive of the role for antibiosis in biocontrol (134-138). However, in most cases the data are circumstantial and there is frequently a lack of correlation between antibiotic production on laboratory media and biological control on a plant surface (139). Until the putative compounds are shown to occur in situ and to occur naturally, at inhibitory levels, from growth of antagonistic but not non-antagonistic strains on the leaf surface, their role in antagonism will not be confirmed.

Use of introduced mycoparasites for biological control is well documented and illustrates that this type of antagonistic interaction can potentially occur on a leaf surface (113,134,140-144). For example, *Aphanocladium album*, when introduced as a spore suspension onto uredinium of the rust fungus, *Puccinia graminis* f. sp. *tritici*, invades the urediniospores through opened germ pores or directly through the spore wall (143,144). The equatorial plane of the urediniospre collapses and the cytoplasm is disintegrated (144). Aecidiospores

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch011

are also invaded and totally collapse, but the number of teliospores penetrated is apparently very small (143). Depending upon the number of hosts and the spore stage involved with each host, the role of mycoparasites such as *A. album* in development of biocontrol of weeds may be minor. However, before introducing the pathogenic fungi for weed control it would be well to document any naturally occurring mycoparasites, and the environmental conditions wherein they may exhibit their greatest destruction.

In overview, the relationship between microbial interactions on leaves and bioherbicide development can be summarized as follows. Two issues are relevant to the development of a bioherbicide. First, the task that is expected of the bioherbicide, given the leaf surface environment, needs to be clearly understood. Second, the most appropriate combination of attributes of the microbes that can accomplish the defined task need to then be developed and tested in a screening assay which has good predictive power. Attributes that may be sought in a microbe if it is to be applied repeatedly or if the host has emerged would include rapid germination of spores, topographic or chemotactic response for early invasion of stomata, extensive colonization of plant cells with accompanying plant necrosis, and possibly toxin production. However, if the agent is expected to remain viable until the host emerges or to cycle within a time period, attributes of interest would include competitive ability and tolerance to fluctuating environmental conditions such as alternating water availability, nutrient flux and levels of UV radiation, an ability to respond as rapidly as does the seed or seedling and high spore production per lesion. This is not a complete list, but it does provide an idea of the range of combinations that could be sought, depending upon the objectives defined. As attributes are defined, the feasibility of applying pathogens with different attributes simultaneously or in a sequence needs to be considered. For example, Crawley et al. (145) obtained the highest level of mortality (100%) on spurred anoda (*Anoda cristata* L. Schlecht.) when *Fusarium lateritium* was applied 5 days after *A. macrospora*. This application sequence resulted in significantly greater mortality than when the agents were applied alone or if *F. lateritium* was applied 5 days before *A. macrospora*. When the agents were applied simultaneously, an average of 79% of the plants were killed, but there was a 5- to 10-day lag in disease development compared to the treatment of applying *F. lateritium* 5 days prior to *A. macrospora* (145).

As the desirable attributes are combined (either through a microbial selection and improvement, or by applying a microbial consortium), the impact of the environment and associated leaf microorganisms on the bioherbicide(s) need to be examined. Strategies that alter or avoid (in space and time) high populations of antagonistic or competitive resident microorganisms can be implemented. These would include the application of encapsulated microbes with or without nutrients (either that directly affect the resident population selectively or influence the production of toxins or antibiotics by the bioherbicides), chelating agents, or combinations of microbes that occupy overlapping niches. Chapter 16 in this volume reviews formulation and application technology related to biological weed control with microbes. There are ranges of environmental conditions that favor the development of any microbe. Defining the ranges for such variables as temperature and available water that promote the activity of the bioherbicide, but are restrictive to antagonistic microbes, would enable predictive models to be developed and weather monitoring to be of practical significance. Thus, a fundamental knowledge of the bioherbicide in terms of its ecology and interaction with other phylloplane associated microbes within

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch011

the phyllosphere environment should make its deployment more consistent and effective.

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Chapter 12

Functional Significance of Adhesion to the Preparation of the Infection Court by Plant Pathogenic Fungi

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Adhesion is a component of fungal survival. For plant pathogens it represents an early event in host recognition, and may be host-specific, as with the nematophagous fungi, or non-specific, with attachment to any surface. Regardless of specificity, adhesion initiates the infection process, and in its absence infection is prevented. Adhesives are components of the fungal extracellular matrix. Adhesion often involves the binding of lectins and carbohydrate polymers on the fungus and host surfaces and may involve the morphological differentiation of specific fungal structures. Stimulation of adhesion often results from developmental changes in the fungus that are stimulated by the host. Enzymes in the fungal matrix are also determinants of attachment and penetration. Cuticular erosion may expose aspects of surface topography necessary for recognition of the infection court. Other components of the matrix ensure survival by their antidesiccant properties and others by the selective binding of toxic polyphenols by specific proteins.

In a traditional sense, plant pathologists seek to prevent disease in economically important crops and to this end we often study why an organism is successful. Ideally we hope to learn information that will be useful in the application of disease control measures. The subject of this volume is the control of weeds through the use of plant pathogens. Thus, the goal is quite different from that of traditional pathology as we are attempting to ensure the success of a disease

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relationship to control an unwanted plant. A phenomenon of great importance to parasitism is the infection process, an area that encompasses the earliest events in the interaction of a pathogen with a host. One of these events is the adhesion of the pathogen to the plant. The significance of adhesion to parasitism is evident when one realizes that for most pathogens adhesion must occur if the organism is to penetrate and successfully cause disease. In the present review I will consider the complexity of the adhesion process. It seems appropriate that adhesion should be considered as a target of importance for the successful use of a pathogen as a bioherbicide.

For some time, it was considered that attachment of fungal pathogens was an event that occurred by chance, often through simple entrapment of propagules amongst leaf hairs or in anticlinal grooves on the leaf surface. This is in spite of the fact that it was recognized early that the hyphae of fungal germlings are typically surrounded or encased in an amorphous matrix or film (1). We now know that attachment is an active process that involves the secretion of adhesive materials by the fungal conidium or mycelium, materials often referred to as components of extracellular matrices. In some cases the secretion of adhesives occurs in response to specific stimuli from the host plant whereas in other instances adhesion occurs in a non-specific manner to any substratum. Where adhesion is triggered by a specific stimulus it is often accompanied by the formation of stages of morphological development that are necessary for the attainment of adhesive competence. Adhesion may also involve another critical phenomenon. It is likely to be associated with events necessary for host recognition and eventual penetration, a phenomenon best termed preparation of the infection court.

Adhesion is most easily recognized during the formation and attachment of appressoria, the structures from which penetration occurs, to the host surface. Indeed, appressoria are often referred to as adhesive structures (2). However, adhesion may begin prior to conidial germination and occur during the process of germ tube elongation that leads up to appressorium formation (3).

Functional significance of adhesion and mucilages.

The significance of adhesion to fungal survival and spread, as well as the chemistry of adhesives and extracellular mucilages have been discussed extensively in recent reviews (3, 4, 5). Perhaps the most important aspect of fungal adhesion, regardless of the mechanism by which it occurs, is the prevention

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch012

of displacement. Fungi encounter plant surfaces in both dry and aqueous environments. It seems reasonable that the movement of wind and water can easily displace inoculum from the potential infection court and must be dealt with by the organism. Thus, attachment of propagules to the host is an important prerequisite to penetration. Attachment or adhesion may also establish the first physiological contact between a host and a pathogen and ensure that the stimuli that account for directional growth or the necessary changes in morphogenesis (appressorium formation) are recognized (6, 7). The physical contact between a pathogen and a host also provides a site at which recognition of the compatibility or incompatibility of the host is established (8).

As most fungi require water as a medium for germination and growth on the plant surface it is also reasonable to assume that adhesives must, to some extent, be water insoluble. Mims and Richardson (9) have presented evidence that in *Gymnosporangium juniperi-virginianae* the adhesive that binds basidiospores changes from a diffuse network to a compacted, electron dense substance with time. Thus, adhesives may at first be relatively water soluble but their composition may change rapidly after secretion from the fungus.

In some fungi, such as *Colletotrichum musae*, the appressorium functions as a dormant structure that may be bound to the host surface for a considerable period prior to the event of penetration (10). The need for a firm anchoring of the pathogen during the period of latency is obvious. Other fungi, such as *Zygophiala jamaicensis* the causal agent of flyspeck of various fruits, grow entirely on the plant cuticle and their firm adhesion to this surface is necessary for their saprophytic mode of growth. *Z. jamaicensis* produces copious amounts of extracellular mucilage, apparently as an adhesive, and degrades the host cuticle (Figure 1) which it uses as its sole source of carbon (11, 12, 13).

Zoospore Encystment in the Oomycetes.

The best examples of adhesion resulting from the stimulus of contact are found among the Oomycetes where zoospore encystment occurs upon contact with the host. Zoospores are motile propagules that lack cell walls. These cells often require specific sites on a host root for attachment and eventual penetration. For example, zoospores of *Pythium aphanidermatum* and *Phytophthora cinnamomi* adhere primarily in the zone of root elongation (14, 15) where terminal fucosyl

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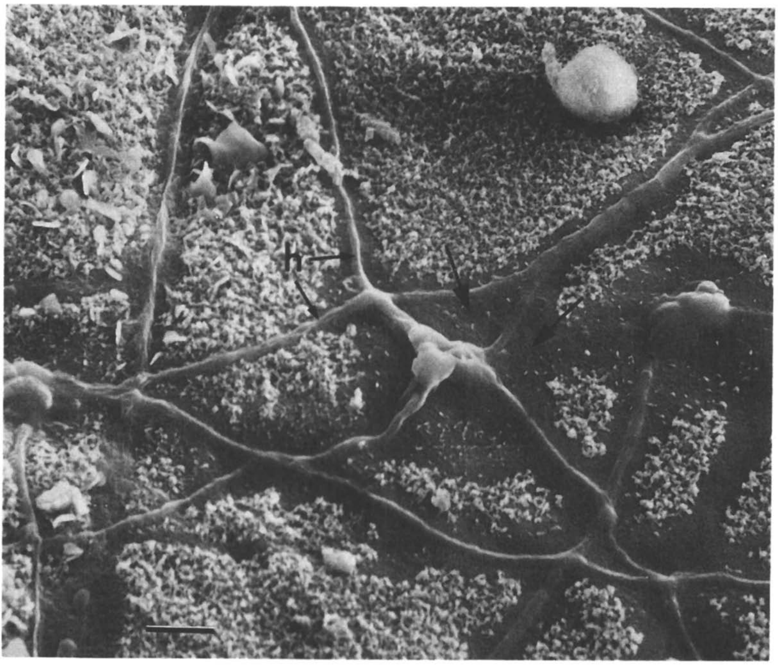

Figure 1. Scanning electron micrograph of *Zygophiala jamaicensis* growing on the surface of a plum fruit. Note the extensive areas free of wax crystals (arrows) surrounding the hyphae (h) of the fungus. Bar represents 10 µm. Reproduced with permission from Ref. 12. Copyright 1987 American Phytopathological Society.

residues in the root mucilage act to bind a lectin-like substance on the zoospore surface. Encystment can be artificially induced in P. cinnamomi by treatment of zoospores with the lectin Concanavalin A (16). Evidence from studies with Pythium spp. in association with host and non-host roots suggests that species pathogenic to graminicolous hosts are stimulated to encyst specifically by roots of grass species (17, 18) indicating that host recognition is related to the specificity for encystment and adhesion. Contact triggers encystment of the zoospore which eventually results in the formation of a cell wall. Encystment also results in the release of an adhesive film that covers the cyst and allows for the initial binding of the cyst to the host surface.

Generally, encystment occurs within minutes, as in Pythium aphanidermatum (19), and results in the formation of a cyst with an adhesive outer coat. The origin of the adhesive coat has been demonstrated to be peripheral vesicles in the encysting zoospores. Hardham (20) showed that binding was specific to the cyst, and not the zoospore, of Phytophthora cinnamomi and that the adhesive was preformed and present in small vesicles at the zoospore periphery (21) The adhesive was composed of high molecular weight glycoproteins that bound specifically with soybean agglutinin suggesting that the hapten was N-acetyl-D-galactosamine. In contrast to the apparent involvement of lectins in adhesion of Phytophthora is the adsorption of Fusarium moniliforme and Phialophora radiciola to maize root mucilage. In these fungi adhesion appears to occur through ionic interactions between mucilage polymers on the root and fungal conidia (22). As in the case of Phytophthora, however, binding appeared to depend on the stage of fungal development suggesting that only specific propagules are capable of developing adhesive competence, and it is precisely those propagules that are involved in attachment and penetration of the host.

In Plasmodiophora brassicae, an organism closely related to the Oomycetes, Aist and Williams (23) have demonstrated that adhesion and penetration occurs in a succession of organized steps that are highly specific. Zoospores release an adhesive coat that binds the cyst to the cabbage root hair. The bound cyst then initiates the process of penetration which also involves adhesion. A vesicular swelling called the adhesorium forms, and through a bulbous extension binds to the host surface. Penetration occurs when the cyst forces a rod-like structure, termed the Stachel, through the adhesorium and into the host

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch012

cell. These events occur in a period of but 2.5 to 3.5 hours.

Preformed adhesives and adhesives formed in response to host stimuli.

The infectious propagules of some fungi are produced with a preformed adhesive which ensures that the propagule, generally a conidium, will bind upon contact. This binding often occurs non-specifically and to any substratum. For example, in *Magnaporthe grisea*, the causal agent of rice blast, the apex of the conidium contains a preformed adhesive material located outside the conidial plasma membrane but within the conidial wall (24). The wall at the apex of the conidium bursts upon hydration and releases the adhesive which allows for binding to any surface. The nematophagous fungus *Meria coniospora* has a mechanism for attachment similar to that of *M. grisea*. The apices of conidia possess a knob-like structure that is covered with a layer of adhesive mucilage that allows attachment to the nematode host (25). It was later shown that adhesion occurs only with specific nematode species and only to the tail of males or to the cephalic region of both males and females (26). Adhesion of *M. coniospora* to the nematode *Panagrellus redivivus* occurred specifically at the sensory organs in the region of the nematode mouth and appeared to be mediated by the presence of sialic acid in these regions (27), again suggesting that a lectin-hapten relationship mediates the process of adhesion.

The plant pathogenic fungus *Dilophospora alopecuri* produces conidia with extensively convoluted terminal appendages. Adhesion to the nematode *Anguina agrostis* (the vector of annual ryegrass toxicity) effects entry of the fungus into the plant and occurs by attachment of conidial appendages to the nematode. Binding is mediated through a mucilagenous fibrillar material so that conidia collect in the transverse annulations of the nematode (28). Although there is no apparent damage to the nematode cuticle by this adhesion, attachment does alter the nematode life cycle and results eventually in nematode death. This apparent co-parasitism has been suggested as a control for annual ryegrass toxicity as the nematode serves as the vector for the causal bacterium, *Corynebacterium rathayi*. The fungus itself is not directly involved in ryegrass toxicity.

A carbohydrate binding lectin has been demonstrated to occur on the surface of the nematophagous fungus *Arthrobotrys oligospora* (29).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch012

Synthesis of the protein occurs in the fungus when it produces trap structures for nematode capture. The lectin, which has specificity for binding to N-acetyl-D-galactosamine, occurs only on the surface of traps. Adhesion only occurs at these sites and penetration of the nematode by the fungus only occurs at those sites (30). That lectins may be involved as a general mechanism for adhesion of nematophagous fungi is consistent with findings that the surface of nematodes is interspersed with various saccharide residues often specific to a stage of nematode development (31). As the nematode cuticle is also rich in lipids, free hydroxyl groups in these compounds may also be involved in adhesion.

Lectin-hapten relationships are also important to the establishment of mycorrhizal relationships. In the ericoid mycorrhizal fungus *Hymenoscyphus ericae* there are binding sites specific for Concanavalin A in an extracellular matrix that surrounds hyphal walls (32, 33). When cultured in the presence of host roots the matrix and associated hapten (mannose) increase in abundance but gradually disappear after penetration and establishment of symbiosis (34). Noninfective strains of the fungus lack the hapten even when grown in the presence of a potential host (35).

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Preparation of the infection court by fungal pathogens.

The establishment of fungal pathogens, especially those that occur on aerial plant parts, depends on the recognition of the plant as a potential host. In most cases such recognition is subtle and not well understood. An exception is the interaction of the rust *Uromyces appendiculatus* with its bean host *Phaseolus vulgaris*. Urediospores germinate and subsequent germ tube growth occurs in a directed manner so that when a stomate is encountered penetration can occur. Such directed growth (thigmotrophism) is probably stimulated by mechanical signals inherent to the leaf surface and perceived by the fungus (6, 7). The formation of appressoria, a change in morphogenesis, is also stimulated by a signal from the plant surface. This response is termed thigmodifferentiation and in *U. appendiculatus* it results when the germ tube encounters a guard cell lip of a specific height (36). Epstein et al. (37) presented evidence to show that an extracellular protein from the fungus is required for *U. appendiculatus* germling adhesion to a substrate and that adhesion itself is a prerequisite to directed growth and thigmodifferentiation. Of course, adhesion

is required for the successful penetration of the leaf.

Growth of germ tubes and attachment of germlings to the surface of aerial plant parts such as leaves is also conditioned by the hydrophobic nature of the leaf (38). Although below ground plant parts can easily be envisioned to effect adhesion through lectin-hapten binding, above ground parts do not appear to be amenable to such mechanisms. This is in spite of the fact that conidia of fungi can be shown to possess surface carbohydrates that could serve as haptens (39). Probably the lack of lectin mediated binding on foliage results from the hydrophobic nature and chemistry of waxes and cutin that cover the surfaces of leaves.

The establishment of contact on the leaf surface and growth of the germling over the surface often involves the erosion of the leaf cuticle. Two fundamental questions relative to such erosion are whether fungi produce extracellular matrices that contain cutinases or other enzymes capable of degrading cuticle components, and whether recognition of the host surface results from erosion. First is the question of whether fungi consistently produce an extracellular matrix that surrounds germ tubes. Evidence that this does occur is substantial (3) and is best visualized in *Bipolaris* spp. where multiple layered hyphal sheaths have been demonstrated (40, 41). Sheath deposition even occurs prior to the time that the germ tube emerges from the conidium (40, 41).

Cuticular erosion results in the formation of imprints on the leaf surface that can easily be detected by scanning electron microscopy (42). Comparison of *Erysiphe graminis* with *E. cichoracearum* demonstrated that *E. graminis* erodes both barley and cucumber cuticles, but that *E. cichoracearum* only eroded or left imprints on cucumber cuticles (43). These results suggest a level of host specificity, possibly for the enzymatic erosion of the cuticle, that may also be involved in the successful penetration of the leaf.

Erosion and wax degradation also occurs in the cuticle of rice in contact with *Helminthosporium oryzae* germlings (44) and the fungus produces excessive amounts of an extracellular matrix around germ tubes and appressoria. The extracellular sheath or matrix also was shown to adhere tenaciously to the cuticle and associated wax components.

Extracellular sheaths have also been demonstrated to be important in the interorganismal contact that occurs during fungal hyperparasitism. *Trichoderma* spp. parasitizing *Rhizoctonia solani* and *Sclerotium*

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rolfsii deposit a mucilage-like material on their hosts (45). Erosion of the surface of the host mycelium occurs in a manner apparently similar to that of fungal-plant interactions. The levels of extracellular chitinase and glucanase from the pathogen were also shown to increase when the pathogen was grown in the presence of either fungal host or their wall materials. Thus, erosion in this instance may be associated with the simultaneous production of these enzymes with the apparently adhesive sheath material.

The time at which release of extracellular matrices by conidia or germlings occurs is also important to understanding their involvement in disease development. In some cases extracellular mucilages or matrices are released upon hydration (24), at the time of germination (41, 46), as the germ tube grows along the leaf surface (9, 47), and in association with the morphogenesis of appressoria (47).

For Erysiphe graminis it is now known that the stimulus of contact, either with the leaf or with cellophane, results in the release of a liquid from the ungerminated conidium (48). The surface of the native, unstimulated conidium is covered by a reticulate network of ridges interspersed with spinelike wall projections. Within 10 min of contact the conidium releases a liquid film that accumulates to the extent that the reticulate network is covered. In less than 30 min after contact, the film is considerably thickened, and prominent globose bodies are found intermittently over the surface. Components of the film are deposited onto the contact surface and flow outward (15-20 µm) forming a zone around the conidium. The interface of the conidium and the leaf surface is covered with material released by the conidium suggestive of the adhesive mucilages produced by other fungi. Whether the material serves as an adhesive is unknown.

In the area of the leaf surface in contact with the E. graminis film, the cuticle loses its original integrity as evidenced by the apparent dissolution of surface wax crystals. Within 60 min the film disappears and the surface of the conidium again assumes the morphology of the unstimulated state. These events all occur prior to the time of conidial germination. Concurrent with the release of the liquid film (48) it was shown that there is a release of esterase with activity against p-nitrophenyl butyrate (49). Activity was released in two stages, the first occurring within 2 min of contact and the second between 10 and 15 min of contact (Figure 2).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch012

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch012

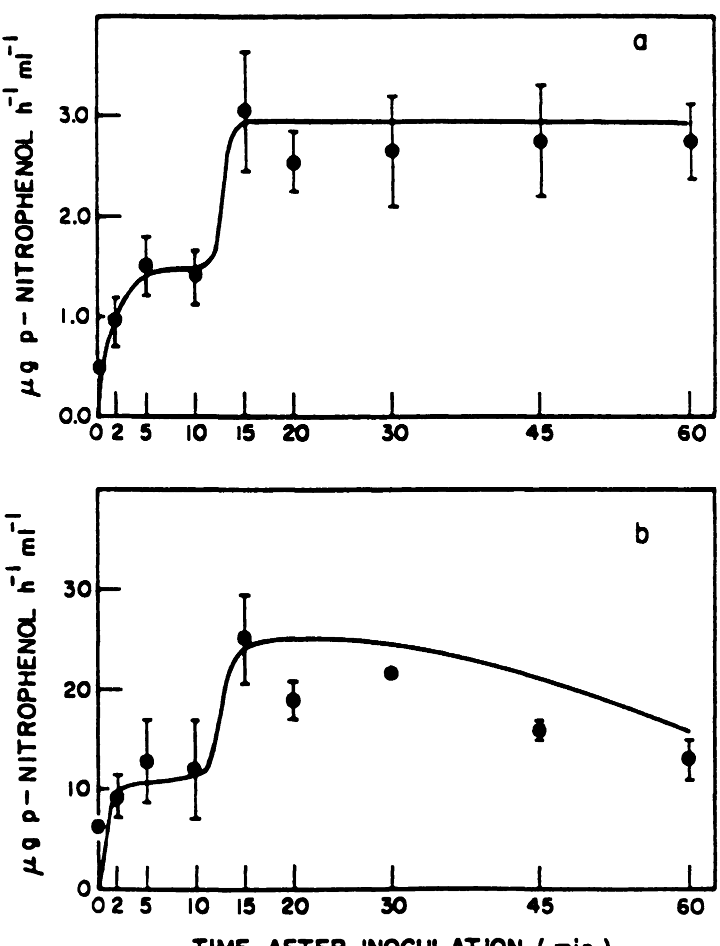

Figure 2. Contact stimulation of the release of esterase from conidia of *Erysiphe graminis*. a) conidia on barley leaves; b) conidia on a cellophane surface. Reproduced with permission from Ref. 49. Copyright 1988 Academic Press, Inc.

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The first phase of esterase release coincides with the appearance of the film on the conidial surface and the second phase of release coincides with the time of appearance of the globose bodies on the conidia. Analysis of the liquid for protein composition by native PAGE and SDS-PAGE gels demonstrated the presence of several peptides ranging in molecular weight up to 94 kDa and the presence of three esterase active components. It was suggested that the release of liquid was an aid to the establishment of the infection court by the fungus and that one result of the release was the degradation of components of the cuticle. Degradation of the cuticle was subsequently shown to occur after incubation of the partially purified esterase preparation on the barley leaf surface suggesting that the esterase was a cutinase type enzyme. The exact role that cuticular degradation plays to ensure the success of the pathogen is unknown; however, results indicate that the appressorial germ tube only elongates to the margin of deposition of the liquid film that surrounds the conidium (Kunoh and Nicholson, unpublished). Thus, either the liquid film itself or the enzymatic degradation of the cuticle may be involved in the recognition of a zone suitable for penetration.

Composition of Adhesives.

There is little work that definitively describes the composition of fungal adhesives. However, it is apparent that fungi do not share either common adhesives or mechanisms for adhesion. One factor that does appear with regularity is that the surface of fungal germ tubes are typically ensheathed by a glycoprotein/carbohydrate matrix (41, 50, 51, 52), and it appears that polysaccharides or glycoproteins are often involved in the process of adhesion. One study suggests that the appressorial adhesive of *Colletotrichum graminicola* is a hemicellulose (53); however, this may be questioned on the basis of the purity of enzymes involved in structure elucidation. In *Bipolaris sorokiniana* (54) and *Neurospora crassa* (55) a galactosaminoglycan appears to serve as a surface-binding compound and a protein or glycoprotein function in the binding of bean rust germlings (56), *Phytophthora cinnamomi* and *P. palmivora* zoospores (15, 57), *Buergenerula spartinae* hyphopodia (50), and *Candida albicans* cells (58).

Lectin binding to specific haptens has been reported for fungi that adhere to roots, nematodes, or other fungi. The chemistry of adhesion is better understood for lectin-hapten relationships than for

adhesion that occurs in the apparent absence of lectins. The firm attachment of nematophagous fungi to their hosts is required for parasitism (59) and the surface of nematodes has been shown to be interspersed with specific saccharides, often specific to stages of nematode development (31). As discussed above, a carbohydrate-binding protein (approximately 20 kDa) with specificity for N-acetyl-D-galactosamine, present on the nematode surface has been partially characterized from trap structures of the fungus *Arthrobotrys oligospora* (29). Synthesis of the lectin was shown to be regulated by fungal development as it occurred only on the surface of trap-bearing hyphae that form in response to the presence of the nematode.

In *Meria coniospora*, another nematophagous species, adhesion to the nematode is also mediated through a lectin. Conidia possess a knoblike structure at their apices which is covered by a layer of mucilage that attaches the conidium to the nematode (25). Adhesion of the fungus to specific nematode species and regions of the nematode body is mediated by a fungal lectin specific for N-acetylneuraminic acid (sialic acid) (27). It has since been suggested that steric configuration of sialic acid residues on the nematode surface serve as stimuli for the processes of adhesion and infection (26).

It has been demonstrated that in the mycoparasite *Trichoderma*, lectin binding probably accounts for the specificity of host identification (45, 60). Two host fungi (*Rhizoctonia solani* and *Sclerotium rolfsii*) possess surface lectins with binding specificity for galactose residues that are present on the mycoparasite surface.

Glucose- and mannose-rich sites for lectin binding have been detected on the surface of the fungal wall and in an extracellular material surrounding the wall of ericoid mycorrhizal fungi (33, 51). Concanavalin A binding sites were localized in an extracellular material that radiated from the fungal wall of the mycorrhizal fungus *Hymenoscyphus ericae*. The frequency of binding sites was significantly increased by growth of the fungus in the presence of host roots (35). In contrast, a noninfective strain of the fungus lacked the hapten regardless of the presence of the host. The presence of mannose binding sites in the extracellular fungal matrix was necessary for mycorrhizal establishment. Interestingly, once adhesion and host penetration occurred, the binding sites and extracellular matrix disappeared from the fungus (34). This induction of hapten binding sites prior to adhesion resembles the induction of adhesion in *Candida albicans* to human

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch012

epithelium, a process that is induced by contact and mediated by lectins binding to glucose and mannose haptens on the fungal surface (61, 62).

The extracellular mucilage of *Colletotrichum graminicola*.

Neither the composition nor the functions of fungal extracellular matrices has received significant attention. Numerous fungi, especially those within the genus *Colletotrichum*, produce their conidia embedded within a mucilagenous material. The best studied of these organisms is *Colletotrichum graminicola*, the causal agent of anthracnose of a variety of grasses. The mucilage possesses remarkable properties that allow it to function in the survival of the organism and to ensure its successful secondary spread.

The mucilage of *C. graminicola* is produced during sporulation on infected host tissue in acervuli (Figure 3) and in culture, and consists of a complex mixture of high molecular weight glycoproteins, enzymes, and small peptides (63, 64). Sporulation and mucilage production appear to be light-dependent as neither event occurs in dark grown cultures. The carbohydrate composition of the glycoproteins includes mannose, rhamnose, galactose, and glucose in levels of 66, 22, 10, and 2 mole %, respectively. The peptide portions of the glycoproteins are approximately 50% hydrophobic amino acids and contain very low levels of aromatic amino acids. A very high level of proline (11 mole %) is also evident.

This amino acid and carbohydrate composition closely resembles that of animal mucins (65, 66). One such glycoprotein of particular importance is a proline-rich protein (GP-66sm) that is produced by the mouse submandibular gland in response to high levels of dietary tannin (67). The protein selectively binds to tannins and precipitates them from solution. This protects the mouse from the harmful effects of dietary tannins which otherwise would reduce the effective uptake of necessary amino acids from dietary protein. The resemblance of certain of the *C. graminicola* glycoproteins to animal mucins suggested that the fungal mucilage would also contain proline-rich proteins capable of binding to potentially inhibitory toxic phenols. Indeed, we have demonstrated the presence of 3 proline-rich proteins in the spore mucilage that bind specifically to the phenylpropanoids *p*-coumaric and ferulic acid (68). Both phenols are produced by the corn plant in response to infection during the period of lesion

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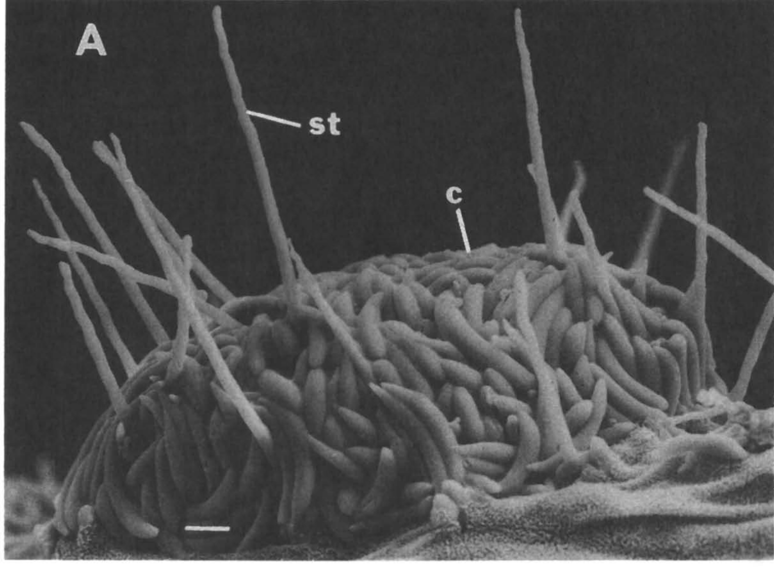

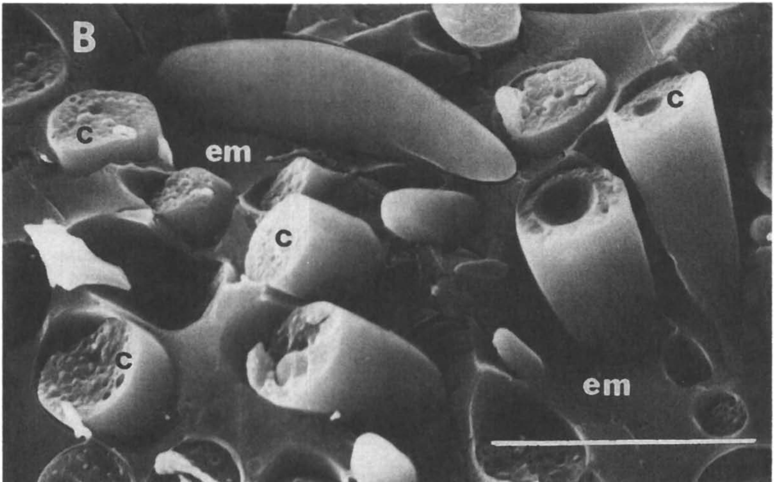

Figure 3. Scanning electron micrographs of Colletotrichum graminicola. A) Fully developed acervulus of the fungus on a corn leaf showing conidia (c) interspersed with sterile setae (st). Bar represents 10 µm. B) The cut surface of an acervular mass of conidia. The cut surfaces of conidia (c) are visible and demonstrate that conidia are embedded within an extracellular mucilage (em). Bar represents 10 µm. Fig. 3A reproduced with permission from Ref. 69. Copyright 1989 Academic Press, Inc.

restriction and aid in the containment of the fungus. In the absence of the mucilage and associated proline-rich proteins the fungus is inhibited by these phenols completely whereas in its presence the fungus is not inhibited (68). Comparison of the fungal proteins with the mouse protein showed that the fungal complex of proteins was five-fold more effective in sequestering phenols than the purified mouse protein (68).

The proline-rich proteins are thought to function as protectants of spores in the secondary spread of the fungus (69). Secondary spread occurs from leaves when spores are carried across necrotic tissue of lesion margins onto healthy, uninfected areas of the leaf. It has now been shown that both *p*-coumaric and ferulic acids as well as glycosides and esters of these compounds leach readily from the necrotic tissue (Figure 4) and inhibit fungal development *in vivo* if the mucilage is absent (69).

Enzymes in the mucilage include an invertase (70), non-specific esterase (63), ß-glucosidase (64), and DNase (71). Evidence suggests that the esterase aids in the erosion of the plant cuticle; roles for the other enzymes have not been ascertained.

A feature of the *C. graminicola* mucilage that may be common amongst numerous fungi is its antidesiccant property (63). Spore masses dry within minutes at relative humidities less than 90 %. Spores in the absence of mucilage become desiccated and die within hours, whereas in its presence spores survive for several months, even at relative humidities as low as 45 %. Upon drying the mucilage forms a thin film (< 0.01 µm) that surrounds spores and protects them (Figure 5). The mechanism through which antidesiccant activity is expressed is unknown. Dispersal of the fungal spores is also facilitated by the drying of the mucilage as spores are bound together in clusters that may be spread by wind as dry, particulate matter (63).

As the theme of this volume is the control of weed species it is appropriate to report the presence of a low molecular weight (< 5 kDa) peptide in the mucilage of *C. graminicola* that functions as an elicitor of phytoalexin synthesis in sorghum. Grain sorghum produces 2 phytoalexins of the deoxyanthocyanidin class, apigeninidin and luteolinidin (Figure 6) (72, 73). Importantly, synthesis of these compounds not only inhibits fungal growth and development but also kills the sorghum tissue itself (Snyder and Nicholson, unpublished). Both shattercane and johnsongrass are important weed species of the genus *Sorghum* and both have the capacity to produce the toxic deoxyanthocyanidins. We

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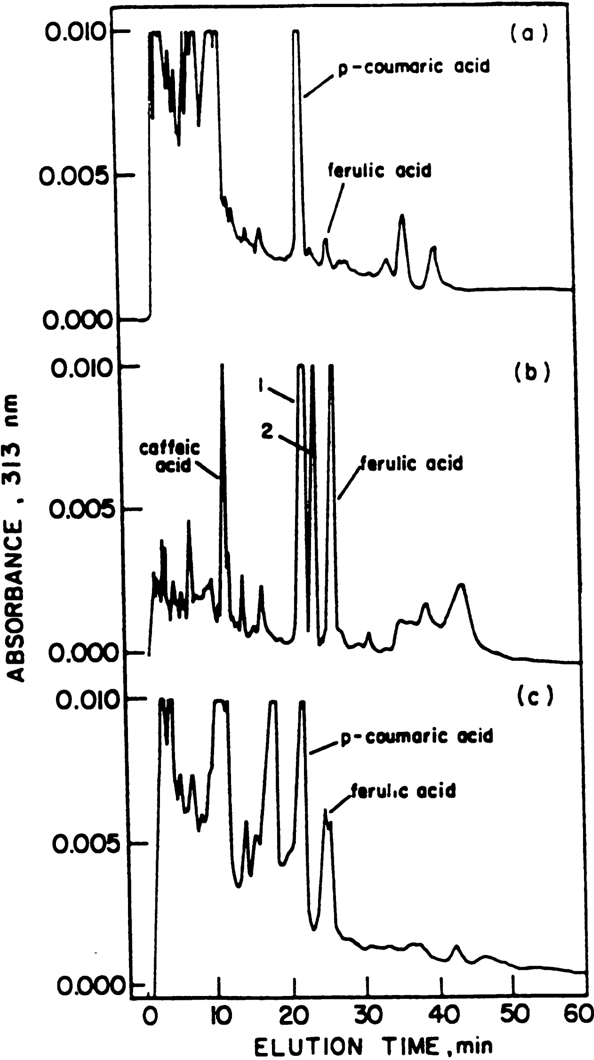

Figure 4. HPLC separation of phenolic components of an aqueous leachate from the surface of corn leaves infected with Colletotrichum graminicola. a) untreated leachate; b) base hydrolyzed leachate; c) acid hydrolyzed leachate. Compounds 1 and 2 are isomers of *p*-coumaric acid. Compounds separated isocratically on a reversed phase C-18 column with a 70 % to 30 % mixture of absolute methanol and 1 % acetic acid. Reproduced with permission from Ref. 69. Copyright 1989 Academic Press, Inc.

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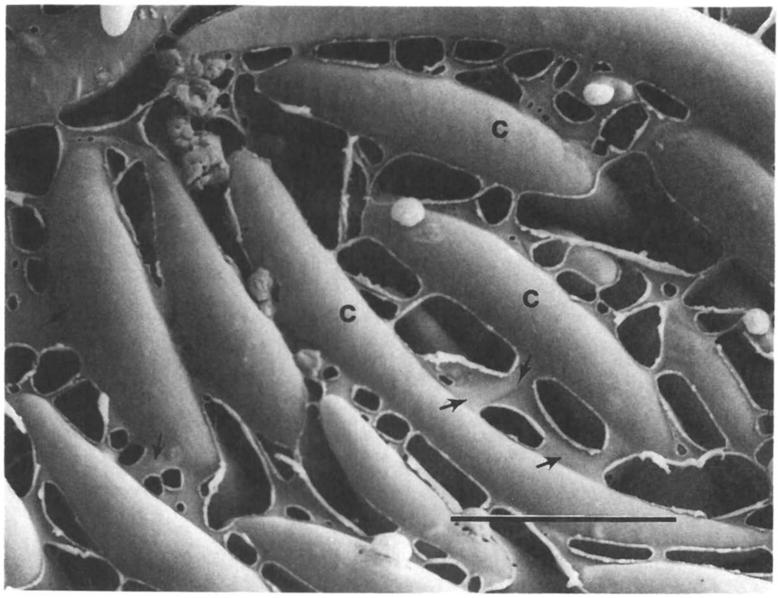

Figure 5. Scanning electron micrograph of the surface of a dried mass of Colletotrichum graminicola conidia showing that the conidial mucilage dries to a thin film (arrows) that surrounds conidia (c). Bar represents 10 µm. Reproduced with permission from Ref. 69. Copyright 1989 Academic Press, Inc.

HO OH HO (I)

HO OH OH HO (II)

Figure 6. Structures of the 3-deoxyanthocyanidin phytoalexins from sorghum. (I) apigeninidin; (II) luteolinidin.

have now demonstrated that the C. graminicola elicitor also stimulates deoxyanthocyanidin synthesis in these weeds with the result that the tissue is killed (Yamaoka and Nicholson, unpublished). To date no other plant species, including corn, cotton, soybeans and a variety of vegetables, are affected by the elicitor. Present investigations emphasize the potential of the elicitor to serve as a biocontrol agent for either shattercane or johnsongrass.

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Chapter 13

Herbicide–Pathogen Interactions and Mycoherbicides as Alternative Strategies for Weed Control

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Herbicides are used on crops and soils to control weeds in various crop production systems. They may also affect soil properties, plant pathogens and saprophytic microorganisms, as well as the host or non-target host plants. These effects normally have little influence on crop growth and generally prove beneficial. However, it has been shown that several herbicides can predispose crop plants to increased disease and as the use of herbicides on various crops increase it is important to evaluate the nature of herbicide-disease predisposing interaction. Procedures may then be taken to avoid or correct problems of detrimental interactions between herbicides and plant diseases. This paper explores the possibility of exploiting these interactions to improve weed control by mycoherbicide or mycoherbicide-herbicide combinations.

Weeds continue to cause annual losses of about 12% in American agricultural production resulting in a loss of $18.2 billion annually (1). The most successful weed control programs are those that combine the use of crop competition and rotation and chemical, mechanical, and other control techniques in an integrated weed management system. Many new control techniques are available, but many of these, including the biological control, have been explored to only a limited extent.

Chemical Weed Control

Chemical weed control technology has progressed greatly during the past 30 years. The discovery of new classes of highly selective, effective, safe and relatively inexpensive herbicides helped revolutionize modern weed control. In the United States herbicides are used on more than 80 million hectares of crop and noncrop lands for control of numerous species of weeds, and more than 50% of the total crop land was treated with herbicides in 1988. Chemical names and structures for most of the herbicides discussed in this paper are given in Table 1. Most weeds are

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currently controlled by chemical, mechanical or other methods because currently the use of biological controls is limited only for a few species of weeds (2).

It is estimated that in 1987, 356 million lbs of herbicides valued at $1.84 billion were used for weed control in various cropping systems in the United States (1). This high volume use of chemicals can sometimes result in problems such as (a) injury to non-target crops from spray drifts, herbicide residues in soil, herbicide residues on foliage; (b) injury to treated crops through adverse interactions with other chemical pesticides; and (c) retreatment of crops. Templeton and Smith (3) also include several other problems such as a narrow spectrum of weed control and alteration of environmental quality. A partial solution for this dilemma can be achieved by the use of biological control agents such as mycoherbicides that can replace chemicals in weed control programs.

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Biological Control of Weeds

Biological control of weeds with plant pathogens has been reviewed by Charudattan and Walker in 1982 (4), Charudattan in 1985 (5), Holcomb in 1982 (6), Quimby in 1982 (7), and more recently by Templeton *et al.* in 1986 (8).

Sands and Rovira in 1972 (9) were among the first to use a mutant form of *Pseudomonas solanacearum* as a biocontrol agent to control weeds. Since then, biocontrol of weeds has expanded using predominantly fungal pathogens.

Fungi have a number of advantages as weed control agents. They are the most commonly encountered pathogens of plants, many are destructive, most can be mass cultured and formulated, and according to Smith, they can be integrated into ongoing IPM and crop production systems (10). Unlike the bacteria and viruses which usually enter the host through openings or with the help of vectors, fungi are capable of actively penetrating the host. Biological weed control with plant pathogens makes use of three approaches termed classical, augmentative, and microbial herbicide strategies (11, 12, 13). The classical strategy involves deployment of pathogens that are capable of building up to epidemic (epiphytotic) levels following a limited number of inoculum releases. Target weeds are usually exotic and lack a complement of natural enemies in their adventive ranges. Rusts and other fungi obtained from the native range of the weed usually have efficient inoculum dissemination capacity through airborne spores. When conditions are favourable, the introduction of the pathogen into the new range of the weed results in a severe disease outbreak, stress on the weed, and weed control.

Examples of weeds controlled successfully with this strategy include rush skeletonweed, *Chondrilla juncea*, with the rust *Puccina chondrillina* in Australia (14), blackberries, *Rubus* spp., with the rust *Phragmidium violaceum* in Chile (15) and Australia (16), and river eupatorium, *Ageratina riparia*, reported in Hawaii by Trujillo in 1976 with a deuteromycete, *Cercosporella ageratinae* (17).

The classical strategy is best suited for controlling weeds in undisturbed areas, rangelands and waterways. It is less suitable for agricultural lands where man-made disturbances to the weed's habitat are likely to disrupt epidemics, thereby reducing efficacy of the pathogen. However, it may be possible to integrate classical biological control pathogens with sublethal rates of chemical herbicides in agricultural intergrated pest management (IPM) systems. This needs to be tested.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch013

Table 1. Information about the herbicides referred to in the text as associated with changes in plant response to disease

CHEMICAL GROUP	CHEMICAL STRUCTURE	CHEMICAL FORMULA	COMMON NAME
THIOCARBAMATES	$C_2H_5-S-C(=O)-N(C_2H_5)(C_6H_{11})$		CYCLOATE
	$(C_2H_5)(C_4H_9)NCOSC_3H_7$	*Butylethylthiocarbamic acid S-propylester*	PEBULATE
	$((CH_3)_2CH)_2NCOSCH_2CCl{=}CHCl$	*Bis(1-methylethyl) carbamothioic acid S-(2,3-dichloro-2-propenyl) ester;*	DIALLATE
	$CH_3CH_2S-C(=O)-N(CH_2CH_2CH_3)_2$	*Dipropylcarbamonthioic acid S-ethyl ester;*	EPTC
	$NaSC(=S)NH-CH_2-CH_2-NHC(=S)SNa$	*1,2-Ethanediylbiscarbamodithioic acid disodium salt;*	NABAM
DIQUAT	$H_3C-{}^+N(C_5H_4)-(C_5H_4)N^+-CH_3$	*1,1'-Dimethyl-4,4'-bipyridinium*	PARAQUAT

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch013

GLYPHOSATE	$HOOCCH_2NHCH_2-P(=O)(OH)-OH$	*N-(Phosphonomethyl)glycine; MON-*	GLYPHOSATE
SULPHONATED UREAS	$SO_2NHCONH$; Cl; N; OCH_3; CH_3	*2-Chloro-N-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl] benzenesulfonamide;*	GLEAN
PHENOXYALKANOIC ACID DERIVATIVES	Cl; OCH_2COOH; CH_3	*(4-Chloro-2-methylphenoxy)acetic acid;*	MPCA
	OCH_2COOH; Cl; Cl	*(2,4-Dichlorophenoxy)acetic acid;*	2,4-D
SUBSTITUTED UREAS	Cl; Cl; $NHCON(CH_3)_2$	*3-(3,4-Dichlorophenyl)-1, 1-dimethylurea;*	DIURON
TRIAZINE	$(CH_3)_3C$; O; N; NH_2; N; N; SCH_3	*4-Amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one;*	METRIBUZIN
DINITROANILINE	$N(CH_2CH_2CH_3)_2$; O_2N; NO_2; CF_3	*2,6-Dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine;*	TRIFLURALIN

The microbial herbicide strategy according to Templeton (12, 13) is the best option for weed control in agricultural IPM systems. The applicability of this strategy to commercial agriculture has been successfully demonstrated in citrus, rice, and soybean by the pioneering work of Ridings, Templeton, Smith, TeBeest, and co-workers (3, 18-23). In this strategy, the inoculum of a pathogen is mass cultured, standardized, formulated, and applied to weeds in perennial crops, the pathogen may be applied at any time when conditions are suitable for disease onset and weed growth. Because this method so far has relied on native, endemic diseases that are adapted to local conditions, environmental constraints on disease development are generally not present or can be surmounted with proper timing of inoculum application. Shrum states that deficiencies in the amount of natural inoculum available in the field can be overcome by the application of mass-produced inoculum. Both intensity and the speed of the epidemic can be increased to achieve the desired level of weed control by applying inundative doses of inoculum at the proper stage of weed growth (24).

Pathogens used as microbial herbicides include three anthracnose-inducing pathogens: *Colletotrichum gloeosporioides* f. sp. *aeschynomene* (CGA) for control of northern joint vetch in rice and soybean, *C. gloeosporioides* f. sp. *jussiaeae* for control of winged waterprimrose in rice, and *C. malvarum* for control of prickly sida in soybean and cotton. A soil-borne fungus, *Phytophthora palmivora* is used for control of strangler vine in citrus and a leaf spot-inducing fungus, *Cercospora rodmanii* has shown suppression of waterhyacinth. *Alternaria macrospora*, a blight-inducing fungus, has also been used for control of spurred anoda.

Although substituting a mycoherbicide for a chemical herbicide seems to be a relatively recent innovation, "many modern pest controls began in Socrates' garden". Smith and Secoy, researchers with Ag Canada, reported on several writings from the ancient Mediterranean period (25).

<u>Commercial Mycoherbicides: DeVine and Collego</u>

Presently only two pathogens are registered as microbial herbicides for commercial use in the United States. They are DeVine and Collego, used respectively for controlling the milkweed vine, *Morrenia odorata*, in citrus in Florida and northern jointvetch, *Aeschynomene virginica*, in rice and soybean in Arkansas and other southeastern states (26, 27, 28).

DeVine, produced by Abbot Chemical Company, is a liquid formulation of *Phytophthora palmivora* containing 6.7×10^5 live chlamydospores per ml. Ridings in 1985 (18) has reported that highly effective weed mortality could be achieved in the field with 8 chlamydospores per cm^2 of soil. In practice, DeVine is applied at the rate of 1.17 l of formulation in 450 l of water/ha. It is applied postemergence between May and September, after the vine develops from seeds or from rhizomes following winter-kill of the shoots. Application is made to the soil around citrus trees using a boom sprayer. The soil must be wet when DeVine is applied; either 5 cm of rainfall or irrigation before and after application should be provided. The pathogen in DeVine infects and wilts seedlings or established vines in 2-10 weeks following application. The vine populations gradually decline in treated areas, and complete control may be achieved in 18 months after one treatment (27).

However DeVine suffers from two commercial problems, the first is the rapid loss of viability which required Abbot to adopt a marketing technique akin to the handling of fresh milk. DeVine was "made-to-order"

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch013

and stored and distributed under refrigeration. It was necessary to monitor every lot to assure viability; inventories were not carried-over. The second problem relates to its long survival in soil which obviosly results in reduced sales.

Collego was the first postemergence microbial herbicide registered for use in an annual crop. Scientists of the USDA-ARS, University of Arkansas and the Upjohn Company were involved in the development of Collego. The history of research and development of Collego has been reviewed by Bowers (28) and Smith in (26). The pathogen *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, which causes an anthracnose, was originally found in 31 rice growing counties of Arkansas, where the disease was present at endemic levels (26). The pathogen causes lesions of northern jointvetch, mainly on stems, and also girdles the stem, resulting in wilting of the entire stem. Infection usually occurs seven to ten days after fungus application, and plant death may occur four to five weeks later. The pathogen appeared to be specific for only northern jointvetch, although infection occurred on a few other leguminous weeds in several species. Collego consists of dried spores of the pathogen obtained from submerged cultures. It is made up of two components. The component A is a spore rehydrating agent that is necessary to assure good viability. Component B contains the spores, at least 75.7 x 10^{11} per package, and inert ingredients (28).

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Improved Mycoherbicide Activity through Action of Herbicides

Control of Texas gourd, *Cucurbita texana*, with *Fusarium solani* f. sp. *cucurbitae* (*FSC*) has also been reported by Weidemann and Templeton in 1988 (29). The soil-born fungus, *FSC*, has shown potential as a mycoherbicide to control Texan gourd. Seedling emergence of Texas gourd was reduced significantly with trifluralin preplant incorporated either alone or tank-mixed with *FSC*, but *FSC* alone did not reduce seedling emergence effectively. Yu and Templeton reported that trifluralin is non-toxic to *FSC* at field rates and that tank-mix or sequential applications of *FSC* and trifluralin resulted in earlier disease incidence and higher seedling mortality than that achieved with *FSC* alone (30, 31). Thus, the integration of any mycoherbicide into existing weed management systems to overcome marketing constraints due to restricted host specificity or limited market size is an important industrial consideration. The ability of *FSC* to be formulated as either a solid or liquid and to control Texas gourd alone or as sequential applications at various times of plant growth suggest that *FSC* could be integrated into agricultural practices on cotton and soybeans to control Texas gourd all season.

The concept of integrating a chemical herbicide and a microbial herbicide to control a target weed can be illustrated by the example of waterhyacinth, *Eichornia crassipes*. The microbial herbicide candidate, *Cercospora rodmanii*, and sublethal rates of diquat or 3,4-D (0.3% and 6.4% respectively, of the current label rates) can be combined to achieve a better control of waterhyacinth than is possible with the microbial herbicide alone (Charudattan 1985, unpublished data). In this case, the integration is intended primarily to improve the efficacy of the microbial herbicide (5).

In 1985 in the Pacific Northwest, attempts have also been made to integrate *Puccinia chondrillina*, the classical biological control agent for skeletonweed, with chemical herbicides (32).

Increase in Disease on Non-target Hosts, due to Herbicides

Uneven herbicide distribution, coupled with the various effects of a herbicide on the metabolic activities and overall growth of a host and/ or a pathogen, could account in part, for an increased disease interaction resulting in greater pathogen damage be it *Fusarium, Rhizoctonia*, or even nematodes. Other factors, including temperature, moisture, soil type and light may also play a role in the remainder of the interaction.

Several types of interactions are possible when a herbicide is introduced into the plant environment. In addition to the desired effects of the herbicide on weeds, growth alteration of crop plants may occur. It follows then that any pesticide, applied to plants or soils to control a specific pest, may also affect nontarget soil microorganisms and plants. Therefore the phenomenon of disease increase due to herbicides is not restricted to a specific group of herbicides, pathogens, or crops.

Heitefuss (33, 34) discussed induce morphology and physiological changes in host plants which may ultimately alter resistance and susceptibility to plant disease. These changes included: (1) reduction of wax formation on leaves, (2) changes in carbohydrate, (3) nitrogen or glucoside metabolism, and (4) retardation to stimulation of plant growth stages of the host. If low doses of herbicides could physiologically and morphologically alter weeds in a similar manner then such weed hosts could be more susceptible to mycoherbicides resulting in more substantial weed control by such weed specific pathogens.

In 1960 monogerm (single germ) seed and sugarbeet herbicides were introduced. Sugarbeets grown in nematode infested soil treated with Tillam (a thio-carbamate herbicide) a 1967 had in 1973 a higher nematode population than beets growth in infested but nontreated soil. In 1976 and 1977, the use of cycloate (RoNeet) resulted in enhanced cyst development on *Beta vulgaris* and the wild beet species *B. patellaris* and *B. procumbens* in greenhouse tests. Although cysts did not mature readily on the wild beet species, roots of all species of sugarbeets grown in cycloate amended soil were more susceptible to larvel penetration (35-37).

Studies on another thiocarbamate fungicide (nabam), revealed that a 100 µg/ml solution of nabam increased hatching of sugarbeet nematode eggs compared to treatment with tap water alone (38).

Differences in the patterns of nematode multiplication in different *Beta* species suggest that the hatch-stimulating activity, if any, is not the only reason for the increased number of nematodes/ gram of root. Other factors that might be considered are (1) fewer roots available for penetration, (2) possible toxic effects of cycloate on roots, which predisposes them to increased penetration by the larvae and (3) an increased chemical and nutrient gradient which exuded from the roots which attracted more nematodes towards the roots. Increased nematode damage could be inferred from reports of Wheeler (39) on delay in maturation of sugarbeet seedlings under stress from cycloate as Johnson and Viglierchio found increased penetration of the nematode into the young seedlings (37). Altman also reported on the release of glucose to soil-plant-interface by seedlings growing in herbicide-amended soil (40-42). If similar responses occur in weed root systems, then soil-borne pathogens including nematodes could be induced to effect a better weed pathogenesis.

It seems to have been easy to accept the idea of some pesticides stimulating plant growth by eliminating weeds or by eliminating pathogens

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but more difficult to accept the concept that many pesticides are involved in predisposition of hosts, crop plants or weeds, to increasing disease. In 1977 in an article in Annual Review of Plant Pathology, Altman and Campbell reported that increasing disease occurred in 20 instances where herbicides had been used and at least 20 pathogens were involved (43). Van der Zweep (44) referred to the "all-system" approach for the interdisciplinary research mentality which would take into consideration all the consequences which a specific herbicide application might have on any other aspect of crop production. Altman and Campbell (43,45) suggested that in a world where protection of the environment is of major importance, methods of integrated control which would take into account the whole biological equilibrium should be sought for the efficient control of diseases, nematodes, insects, and weeds. If the above holds true then modification in weed physiology could occur when low rates of chemical herbicides that could make weeds more susceptible to a biocontrol agent are used (43). Table 2 lists the effects of some herbicides on plant roots.

Yarwood, in defining predisposition, wrote that if a chemical or physical treatment affects disease through its effect on the host, the condition is referred to as predisposition; if it acts directly on the pathogen the condition is called activation. Predisposition may be both beneficial when disease on the weed host increases or detrimental when the chemical stimulates the weed host to produce a phytoalexin which in turn prevents disease (46).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch013

Effects of Herbicides on the Pathogen/Plant interaction

Interactions between herbicides and plant pathogens have demonstrated that disease can be increased on host crops when recommended rates of herbicides are used.

Trifluralin is a selective, preemergent herbicide which is incorporated into the top 2-3" (5-10 cm) of soil to provide long-lasting control of annual grasses and broadleaf weeds. This herbicide normally affects weeds by inhibiting root development of germinating seeds. Continued inhibition may injure plants. In addition to direct herbicide injury from soil incorporation, there is also an effect on the soil microbiota, soil inhabitants, and plant pathogens. Since its introduction in the early 1970's, there have been many reports of root stunting accompanied by increased root rot and cereal cyst nematode damage where trifluralin has been applied.

Altman and Campbell, in a comprehensive review, described several interactions between herbicides and plant diseases. In 1983 Bareta and Altman working in Colorado (47) showed that trifluralin could alter the Fusarium disease syndrome in Pinto beans. In laboratory tests to evaluate the effect of trifluralin on the bean pathogen it was demonstrated that mycelial growth was not affected by the herbicide. However, sporulation was influenced by trifluralin resulting in a doubling of macroconidia at day 8 and day 12 of the tests. Although no comparable coults were made of the soil-borne Fusarium chlamydospores, germination of chlamydospores was evaluated and trifluralin at all concentrations was shown to increase germination. The germination effect was most pronounced in the absence of nutrients (47,48).

Trifluralin has also enhanced *Phythophthora* root rot severity in soybean, which Duncan and Paxton attributed to increased oospore production. Trifluralin (1-3 ppm) increased pre- and postemergence

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Table 2 Classes of herbicide shown to have direct effects on roots

Herbicide Class	Effect
Amides	Inhibition of protein synthesis and root growth. Inhibition geotropic responses. Root cell disorganisation and multinucleation.
Benzoics	Auxin-like responses, exudates, and initiation of adventitious roots affected. Cell elongation and division promoted or inhibited. Geotropic response lost.
Carbamates	Inhibition of cell division, lack of spindle formation, inhibition of protein synthesis, increased cell size, radial elongation of cells, polyploid nuclei chromosome contraction, cell wall thickening.
Dinitroanilines	Inhibition of primary and lateral root growth disruption of mitosis. Radial expansion near root tip. Root tip cells small, dense multi-nucleate, cells behind tip abnormally large, thin walled.
Nitriles	Reducing cell division, granulated nuclei, mottle chromosomes. Root swelling, discolouration, development of callus tissues.
Phenoxys	Incomplete cessation cell division, root tip swelling and adventitious root development. Root uptake reduced, roots softened and decayed.
Thiocarbamates	Enhanced root elongation.
Triazines	Indirect inhibition of root development by blocking photosynthesis.
Uracils	Root elongation inhibited. Roots swollen, necrotic, collapsed, cells multinucleate.
Unclassified	
Bensulide	Inhibition of root meristem mitosis, rapid elongation of epidermis – short, pitted trachea with lesions, root hairs at root tip only, considerable vacuolation and liqnification.
DCPA	Inhibited cell division.
Picloram	Roots swollen, split: deterioration of parenchyma, destruction of cambium and phloem and root primordia.

SOURCE: Adapted from Curry and Teem, ref. 86.

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damping-off due to *R. solani* apparently by increasing pathogen activity (49). Wrona *et al.* found an increase in number and size of *R. solani* lesions on *P. vulgaris* hypocotyls (50).

Mussa and Russell (52), however, studied the influence of trifluralin on *R. solani* f. sp. *phaseoli* and *P. vulgaris*, and found that growth was stimulated in culture and virulence increased on the host plant. Altman (1) also reported that lesion size and root rot symptoms increased on several Pinto bean varieties planted in soil infested with the pathogen and pretreated with field rates of trifluralin or EPTC.

In 1981 and 1982 potatoes, grown under contract in Idaho, Washington, and Wisconsin using Metribuzin for weed control, were found to exhibit an increase in potato early-dying. One of the causes of early-dying is *Verticillium dahliae*. Using microslerotial inocula of *V. dahliae* it was decided to evaluate the effect of a herbicide on the disease. Greenhouse tests using trifluralin (since Metribuzin was not available) were set up in 1983 and 1984. In all tests using the eggplant var. Black Beauty as an indicator host and microsclerotia from potatoes as inoculum, trifluralin did enhance *Vetricillium* wilt (53, 54).

In Australia an interaction between herbicides and soilborne diseases of cereals has been observed for several years. In 1986, Rovira and McDonald published a paper entitled "Effects of the Herbicide Chlorsulfuron on Rhizoctonia Bare patch and Take-all of Barley and Wheat" in *Plant Disease* (55). This paper described how residues of the herbicide chlorsulfuron (Glean) one year after application increased root disease in barley and reduced yield by up to 1 t/ha. Glean applied at 1/3 the recommended rate also increased root disease caused by *Rhizoctonia solani* in greenhouse tests. Although Rovira and McDonald could not detect any effect of Glean on the early infection of wheat seedling roots by the take-all fungus (*Gaeumannomyces graminis*), there were reports from Western Australian farmers of more "white heads" (plant symptoms seen at grain fill due to premature ripening and death of seed heads) occurring in those parts of fields sprayed with Glean than in unsprayed areas. There were also reports of increased losses from Rhizoctonia-root rot (Rovira, personal communication).

In 1984, extensive crop losses also occurred in wheat in Western Australia following the use of glyphosate (Round-up) before the direct drilling of cereals; these losses were largely due to a seedling root rot caused by the fungus, *Pythium* (56). Research in Washington State by the USDA/ARS has also demonstrated that *Pythium* can be a major problem in no-till wheat (personal communication). In addition to the above reports, there were many reports of high take-all in fields treated (postemergence) with herbicides containing Dicamba and MCPA during the 1986 wheat growing season.

The herbicides listed above represent both soil-applied herbicides (Glean and Treflan) and foliar-applied herbicides (Round-up, MCPA and Dicamba) and research with these herbicides has recently demonstrated an increase in root disease of several crops. Tottman and Thompson (57) showed that a mixture of dicamba 2,3,6, TBA, mecoprop and MCPA, and Mecoprop, Ioxynil and dicamba alone increased the severity of take-all infection of roots.

In a weed control program, combinations of low or sublethal rates of chemicals followed by biocontrol agents could result in a more comprehensive weed control since an affected plant could be prediposed to infection by a mycoherbicide. Furthermore, damage on leaves from sublethal herbicides may cause roots to be inefficient, thus making the

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weed more prone to infection by the mycoherbicide, resulting in more effective weed control.

In 1984 and 1986 Breay *et al.* (58) reported prolonged carryover of the herbicide chlorsulfuron (Glean) and trifluralin (Treflan). This does pose a warning for non-target host injury and persistence of herbicides thereby providing a potential for increased disease similar to incidents of disease enhancement reported by Altman and Rovira (59). This knowledge could be used in developing mycoherbicide weed control programs.

P.palmivora, the microbial herbicide used to control strangler vine in citrus in Florida, was inhibited by some herbicide treatments. In these tests, germinability of chlamydospores of the fungus was reduced when tank-mixed with the herbicides diuron, glyphosate, and paraquat. However, sequential application of spore suspensions and herbicides prevented the adverse effects of the herbicide on the fungus. In this research, spore germination was not reduced when water suspensions of chlamydospores were applied 3 weeks after spraying glyphosate. Thus, both glyphosate and the microbial herbicide were active and controlled the weed effectively (4,18).

The use of endemic fungi applied as mycoherbicides for weed control in agronomic crops offers some unusual opportunities for integration with weed and pest management systems. However, for successful use of endemic fungi as biological weed control agents in such systems, the pathogen, the weed, the environment, and the influence that mycoherbicides have on entire past management programs must be considered.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch013

Conservation Tillage and Herbicide Use

The CSIRO Division of Soils in Australia has an ongoing major research program on conservation tillage at two sites selected to represent major cereal-growing areas of southern Australia. Results of these trials have demonstrated that control of root disease through rotation with non-host crops and pastures increase yields of direct-drilled wheat to the levels obtained by conventional cultivation while improving soil structure and reducing erosion (Rovira, unpubl.). Table 3 lists wheat diseases that may increase under conservation tillage. However, conservation tillage is heavily dependent upon herbicide use as the use of tillage for weed control is minimized. It has been estimated that the principal cause of soil degradation (erosion and structure decline) in a cropping system is due to cultivation mainly carried out to control weeds (60). Therefore, as conservation tillage practices continue to expand, some means of reducing chemical herbicide use are warranted.

Table 3 Wheat diseases that may increase under conservation tillage

Common Name	Scientific Name
Tan spot	*Pyrenophora trichostoma*
Septoria leaf blotch	*Septoria tritici*
Take-all	*Gaeumannomyces graminis*
Bare patch	*Rhizoctonia solani*
Root rot	*Cochliobolus sativus*
Septoria glume blotch	*Septoria nodurum*
Powdery mildew	*Erysiphe graminis*
Bacterial blight	*Pseudomonas syringae*
Cephalosporium stripe	*Cephalosporium graminearum*

In 1984, worldwide there were 42 species of weeds reported as resistant to herbicides (61) and currently in Australia two species of resistant grass weeds have been identified, *Hordeum leporium* ssp. *glaucum* and *Lolium rigidum* (62, 63) with the resistant populations of *L. rigidum* showing cross resistance to herbicides from chemically similar and dissimilar groups (64). Use of mycoherbicides may become more important in management and prevention of herbicide resistant weeds.

Tillage has traditionally been used to stimulate germination of weeds which are killed by subsequent tillage. With conservation tillage, problems have arisen with poor control of in-crop weeds using postemergence herbicides. In southern Australia paradoxa grass, *Phalaris paradoxa*, brome grass, *Bromus diandrus*, silver grass, *Vulpia sp.* and barley grass *Hordeum sp.* are important winter cereal weeds poorly controlled by currently available herbicides. There is scope for mycoherbicides to aid in the control of these weeds.

Perhaps a more intensive research program to reduce or supplant the high levels of chemical use in conservation tillage programmes can be attained by substituting a mycoherbicide or combining low doses of chemical herbicides with selected mycoherbicides to achieve "synergistic" weed control.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch013

Exudates and Plant Disease

Sugars and minerals exuded from underground plant parts are a source of nutrients for soil microorganisms including soil saprobes and plant pathogens. Diseases may be affected through stimulation of fungi by exudates. Schroth and Cook, Schroth and Hildebrand (65,66) and Dodman and Flentje (67) have concluded that soilborne fungal diseases may be affected by root exudates. Dodman and Flentje (67) also reported that growth of *R. solani* was stimulated by root exudates and that this is important in the infection process by the fungus.

Hayman (68) showed apparent enhancement of fungal growth in the cotton seed spermosphere (the microhabitat around the seed in soil). Greater infection of young cotton seedlings by *r. solani* occurred when low temperatures caused a reduction in germination rate and, thus, an accumulation of exudates around the seed. Martinson (69) and Hunter and Guinn (70) also found seed exudates capable of stimulating growth of *R. solani*.

DeSilva and Wood (71) with several *in vitro* experiments also showed that exudates from young seedlings caused a greater stimulation of growth of *R. solani* than exudates from older plants.

If weeds can be shown to exude greater quantities or different sugars and minerals following normal herbicide or sublethal herbicide application then perhaps studies should be undertaken to develop methods of evaluating these exudates and their influence on the mycoherbicide.

Influence of Herbicides on the action of Mycoherbicides

Several herbicides have had a stimulatory effect on hyphal growth and spore production of pathogens. Trifluralin has been shown to increase growth of *Fusarium solani* f. sp. *phaseoli* and *Fusarium solani* f. sp. *cucurbitae* (*FSC*) and spore production of *F. solani* f. sp. *Phaseoli*. Glyphosate has also been shown by Watson *et al.* to increase enhanced pathogen growth (72).

Yu *et al.* (30, 31) have suggested that several hypotheses to explain this stimulatory effect on pathogens used as mycoherbicides: (1) the pathogen appears to utilise sugar better from enhanced media, (2) herbicides may neutralize or prevent the development of self-inhibitors by the pathogen and (3) the pathogen may use the herbicide as an energy source. The mechanism is unlikely to be (3), in studies using 50 mg trifluralin ml^{-1} it was concluded that the herbicide in the culture medium provided only a trace amount of additional C and N, which would be insufficient to account for the 100-130 mg increase in mycelial dry weight after 12 days. Therefore, utilization of trifluralin as an energy source was not considered to be a principal mechanism for the increased growth of *FSC* (30, 31).

Recovery of *FSC*, other soil fungi and bacteria from the rhizosphere and rhizoplane of trifluralin-treated inoculated roots indicated increased microbial activity in these zones. Although the growth of *FSC* was stimulated by trifluralin in liquid medium and soil culture, concentrations of trifluralin greater than those estimated to be in the soil at recommended rates of application were required to increase growth of *FSC*. Although some fungi can utilize herbicides as energy sources in the study by Yu *et al.* the added carbon from the herbicide was probably insignificant compared to the microbially-available organic carbon in the soil organic matter. They concluded that it was more likely that trifluralin stimulated root exudation from Texas gourd and subsequently increased the activity of *FSC*, soil fungi and bacteria in the rhizosphere and rhizoplane (31).

In determining the feasibility of using a herbicide in combination with a mycoherbicide (bioherbicide) the authors suggested that several prerequisites are required.

Selection of the best pathogen from those available and the choice of tactic to employ are based on knowledge of the pathogen and the weed. Considerable background information is available on diseases of economical crops, but much less is known of pathogens of weeds. A potential problem may be that some weeds are closely related to the crops in which they are found so that diseases of the weed may also affect the crop. However the problem is no less for the development of selective chemical herbicides, therefore detailed evaluation of potential pathogens must be made in laboratory and greenhouse tests. Intuitively, fungi would seem to have the greatest potential as bioherbicides because they offer a wide range of virulence, reproductive capacity, specificity, and stability. Also, a large base of technology on their production, handling, and storage exists; this can be readily adapted to mass produce most fungal pathogens (4-8). By working cooperatively the plant pathologist and the weed scientist can develop pathogens with high potential for control of weeds that are economically important. The thesis that pathogens can be employed to manage weeds appears to be sound both biologically and economically. Their use is based on basic principles of plant pathology. The challenge is to develop them for us in a weed management system, integrated with other control practices (26,28,31).

The integration of a mycoherbicide into existing weed management systems is an important industrial consideration. Studies with other mycoherbicides have shown that microbial pesticides can be integrated effectively into existing weed management programs. For example with Texas gourd the compatibility of *FSC* with several commonly used herbicides suggests that *FSC* could be integrated readily into existing

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weed management strategies to broaden the spectrum of weed control within the crop or to enhance the activity of herbicides recommended for control of Texas gourd all season.

The influence of trifluralin and its formulation blank of *Fusarium* dry rot of Pinto beans was investigated under greenhouse and laboratory conditions by Walker and Altman (47, 73). The formulation blank had no effect on either sporulation or vegetative growth of the pathogen. However, the herbicide stimulated germination of *Fusarium* chlamydospores in soil. The mechanism involved in the disease increases in the nontarget host were studied and shown to be due to the direct stimulation by the herbicide on the pathogen and the host, facilitating penetration and colonization by the pathogen (48).

If beans could be affected by trifluralin as described then it seems reasonable to assume that similar physiological and morphological injuries can occur in weeds making them more susceptible to mycoherbicides such as that described by Yu *et al.* (31). In fact, concentrations as low as 10% of recommended field rates could have affected hypocotyls and foliage of treated plants, including both host plants and weeds.

In 1979 Altman *et al.* (42) reported similar physiological changes in sugar beets using several carbamate herbicides: cycloate, eptam and diallate. Standard field rates of these compounds reduced growth and photosynthetic activity. Subsequent studies on wheat with eptam and diallate produced similar effects. In all instances when cycloate and diallate were used, in addition to the changes noted above, reducing sugars were increased three-fold and chlorophyll content of leaf tissue was increased from 34% to 58% over the controls. Foliar applied mycoherbicides, if used concomitantly or serially in a weed control program, could respond to such an additional energy source, resulting in more effective weed control.

Glyphosate has also recently been shown by Johal and Rahe to increase disease in beans, *Phaseolus vulgaris* (74,75). They suggested that glyphosate enhanced disease in beans caused by *Colletotrichum lindemuthianium* was due to a suppression of defence mechanisms in the host resulting in increased parasitization by fungal root-rot pathogens in soil. Such a response in weed hosts could make them more susceptible to specific mycoherbicides. Charudattan in 1986 suggested that treatment of water hyacinth with the mycoherbicide *Cercospora rodmanii* and sublethal rates of 2,4-D resulted in improved weed control compared to using *C. rodmanii* or 2,4-D alone (76).

Effect of Growth Regulators on the Action of Mycoherbicides

Currently thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-yl-urea), a plant growth regulator and cotton defoliant has enhanced control of velvetleaf with *Colletotrichum cocioides* in corn and soybeans. Wymore *et al.* (1987) observed that thidiazuron stimulated axillary bud development, caused curling and cupping of leaves, and reduced shoot height, weight, and leaf area of velvetleaf plants treated with as little as 0.07 kg ai/ha (77). This altered velvetleaf plant morphology may have been ideal for development of *C. coccodes* since short, bushy plants remained within the microclimate of the crop canopy and could have provided an optimum environment with *C. coccodes* to cause an epidemic and kill velvetleaf plants (77,78).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch013

Herbicide Safeners and Plant Diseases

Recently Craig *et al.* evaluated in two field trials the effect of two herbicide safeners (herbicide antidotes) (79) applied to sorghum seed, on diverse sorghum downy mildew, caused by *Peronosclerospora sorghii*. The pathogen can invade through roots and leaves. The researchers compared the safeners Concep II (CGA-92194, Ciba Geigy) and Screen (Flurazole, Monsanto) and the results of these trials indicated that seed treatment with Concep II increased the incidence of sorghum hybrids Dinero, and Tophand planted in soil infested with oospores had significantly higher levels of downy mildew than the untreated controls. Seed treatment of the hybrids Dinero and Tophand with the safener screen had no significant effect on the incidence of downy mildew when the seeds were planted in similarly infested soil (Table 4).

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Table 4 Effects of herbicide antidotes CGA 92194 and flurazole on the incidence of sorghum downy mildew induced by oospores of *Peronosclerospora sorghi*, pathotypes 1 and 3, in the sorghum hybrids Dinero, Tophand, and R920

Herbicide antidote	Disease incidence (%)[a]			
	Pathotype 1		Pathotype 3	
	Dinero	R920	Dinero	Tophand
Control	2	42	5	34
Flurazole	2	34	3	30
CGA 92194	5	90**[b]	50**	84**

[a] Frequency of diseased plants; each treatment value is the mean of three trials, 90 plants per trial.
[b] Asterisks indicate herbicide antidote values significantly different from the control treatment according to Student's *t* test ($P = 0.01$).

SOURCE: Adapted from Craig et al., ref. 79.

Table 5 Effects of herbicide antidotes CGA 92194 and flurazole on the incidence of sorghum downy mildew induced by conidial inoculation of sorghum hybrids Dinero, Tophand, and W839DR with *Peronosclerospora sorghi* pathotype 3

Herbicide antidote	Disease incidence (%)[a]		
	Dinero	Tophand	W839DR
Control	78	56	35
Flurazole	17*[b]	25	16*
CGA 92194	91	86*	93*

[a] Frequency of diseased plants; each treatment value is the mean of three trials, 45 plants per trial.
[b] Asterisk indicates herbicide antidote values significantly different from the control value according to Student's *t* test ($P = 0.05$).

SOURCE: Adapted from Craig et al., ref. 79.

Seed treatment with Concep II also significantly increased the incidence of downy mildew caused by conidial inoculation in the hybrids Tophand and W839DR. Screen seed treatments of Dinero and W839DR produced significant reductions in the percentage of plants with downy mildew (Table 5).

Although the capability of Concep II to increase the incidence of sorghum downy mildew did pose a problem for its use as a seed safener in regions where downy mildew is a threat, Ciba-Geigy, producer of Concep II, resolved this dilemma by coupling seed treatment with Concep II with Apron (Metalaxyl—Ciba-Geigy Corp.), a systemic fungicide which controls downy mildews. Apron alone did prove effective control of downy mildew.

The following mechanisms suggested by Craig *et al.* (79) by which Concep II increased down mildew might offer a means for more effective mycoherbicide weed control when using chemical safeners.

These mechanisms included the following:

1. Concep II could improve the germination of the oospore surrounding the seeds caused by the direct effect of the chemical on the spores or indirectly, by stimulation of the host to produce exudates which would stimulate spore germination.
2. Concep II could produce morphological or physiological changes in the host that would permit more rapid ingress and progress of the pathogen from sites of infection in the roots or the leaves to the apical meristem resulting in a systemically diseased plant.

Although Charudattan and DeLoach and others have suggested that low doses of chemical herbicides applied in conjunction with a specific mycoherbicide could improve the effectiveness of the mycoherbicide, the addition of the various herbicides safeners used by Craig *et al.* and the safeners listed by Hatzois and Hoagland in their text on crop safeners (1989) could expand the arsenal of chemicals that could be applied to weeds with or prior to the application of the herbicide-mycoherbicide combination to potentially improve the effectiveness of a particular mycoherbicide (80).

Naturally Produced Phytotoxins as Herbicides: Another Perspective for Weed Control

The need for more cost-effective, efficacious, selective, and environmentally safe herbicides is being increased by several current trends in agriculture and agricultural chemistry. The development of microbial toxins or allelochemicals (chemicals produced by plants, which inhibit germination, growth or development of other plants) as herbicides or as sources of new herbicide chemistries promise to help fill this growing need.

Although microbial biocontrol agents are actively being developed and commercialized [e.g., DeVine (*Phytophthora palmivora*) and Collego (*Colletotrichum gloesporiodes* f. sp. *aeschynomene*)], microbially reproduced toxins have several advantages over the living pathogens. The phytotoxic compound generally has a longer shelf life and usually requires less storage space and the process of formulation and application are usually simpler. Furthermore, the possibility of disease spreading to nontarget species does not exist.

Microbially produced phytotoxins also offer several advantages over allelochemicals as herbicides. Allelochemicals from higher plants generally have little selectivity and often are autotoxic to the reproducing species, and, most are not very effective when compared to

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phytotoxic microbial toxins (81). For a review of the topic see Thompson (82).

Conclusion

Two of the most important developments in weed control during the past two decades has been the discovery and development of safe and effective new herbicides that selectively control specific weeds in major crops and the use of mycoherbicides in weed control programs.

The feasibility of using mycoherbicides has been demonstrated for various crop-weed-pathogen combinations. Some of these feasibility studies were initiated as early as 1972 and included *Colletotrichum gloeosporioides* for control of northern jointvetch, *Aeschynomene virginica* in rice and soybean fields in Arkansas, *Phytophthora citrophthora* for control of strangler vine, in Florida citrus orchards; and Cercospora *rodmanii* for control of waterhyacinth, in Florida waterways. Other successful examples of pathogens used to control weeds include two rust fungi, *Puccinia chondrillina* for control of skeletonweed in Australia and in the western United States, *Uromyces rumicis* for suppression of curly dock in pastures in Europe and *C. gloeosporioides* f. sp. *Jussiaceae* for control of winged waterprimrose in rice. These studies resulted in the development and commercialization of two mycoherbicides "Collego" by the UpJohn Company and "DeVine" by the Abbot Corporation. All of the successful examples of mycoherbicides have not been sustained however, in the case of *P. chondrillina* rust of skeleton weed in Australia, there has been a gradual replacement of the susceptible narrow leaf forms by the resistant broadleaf and intermediate leaf forms.

Although only two fungi have become commercialized as weed control mycoherbicides, Charudattan and Walker (4) include several other potential mycoherbicide candidates in their appendix.

The process for evaluation of new mycoherbicides is described by Spurr *et al.* (84) as four decision making steps to arrive at a potential new mycoherbicide. These include (1) the selection of target weed, (2) selection and identification of a fungal pathogen (for the weed), (3) selecting an appropriate strategy for weed control that can be self-sustaining, and the artificial or bioherbicide strategy that requires annual application and is used on high value crops and (4) evaluation of the control tactic. Previously the choice of pathogen to control a weed has been largely due to chance observation, a more systematic approach is now needed for successful progress.

It is also obvious that further research is needed in the field of interactions between mycoherbicides and agricultural chemicals, as mycoherbicides applied in an intergrated pest management system need to be compatible with fungicides, herbicides and other chemicals, particularly as their narrow spectra will mean that they will often be combined with other weed control practices. Furthermore this area of research may result in gains in spectrum or effectiveness of mycoherbicides as many pathogens can act synergistically with herbicides (43,45). Understanding the mechanisms of synergistic reactions between mycoherbicides and chemical herbicides may also lead to the exciting prospect of genetically engineered organisms which are better able to take advantage of the effect of the chemical on the plant, again potentially increasing the spectrum or effectiveness of the mycoherbicide. As Greaves and Macqueen (85) pointed out however, there is a strong need for both interdisciplinary basic research in ecology, genetics, cell and

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molecular biology of weeds and pathogens as well as the broad fundamental aspects. However, shifts from the use of chemical herbicides to non-chemical herbicides e.g. mycoherbicides, is progressing slowly and the new technology involving non-chemical control will likely be most effective when it is integrated into conventional technology.

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Chapter 14

Synergistic Role of Soil Fungi in the Herbicidal Efficacy of Glyphosate

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The death of plants treated with the herbicide glyphosate involves certain soil fungi that function as glyphosate synergists. Glyphosate also kills plants without the participation of these fungi, but significantly higher doses of the herbicide are required. The contribution of glyphosate synergistic fungi to herbicidal efficacy is readily seen by comparing the doses of glyphosate required to kill plants in heat treated and untreated soils. Glyphosate renders the roots of treated plants permissive to fungal colonization well in advance of overt symptoms of phytotoxicity, presumably by interfering with natural defense mechanisms in the roots. Common airborne recontaminants of heat treated soils are ineffective as glyphosate synergists. The major glyphosate synergists of wheat and bean seedlings growing in diverse soil types were *Pythium* and *Fusarium* spp.

Plants have active and passive mechanisms for resistance to most microorganisms. Since chemicals used for weed control interfere with metabolic processes of plants, interactions between herbicides and plant pathogens are to be expected. Interactions commonly, although not always, result in increased disease associated with herbicide use. Several examples of disease enhancement by herbicides were described in the preceding chapter. We describe here a less common phenomenon where certain fungi that colonize plant roots enhance the efficacy of a herbicide.

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Glyphosate is the active ingredient of several broad spectrum commercial herbicides marketed by Monsanto, St. Louis, MO, that act by foliar absorption. Roundup is the most generally familiar of these products; others include Vision, a formulation used widely in silvicultural weed control in the Pacific Northwest, and Rodeo. These formulations are water soluble and under most conditions have negligible residual activity in soil (1).

The symptoms of glyphosate phytotoxicity are slow to develop. When applied in the field at rates equivalent to 0.8 to 2.5 kg/ha of glyphosate (a.i.), treated vegetation usually begins to show slight yellowing and flaccidity within 4 to 8 days. These first obvious symptoms become progressively more pronounced and the affected plants usually die within the next 3 to 7 days. Under laboratory conditions, and presumably in the field, growth of treated plants is arrested almost immediately following treatment even though other overt symptoms are delayed for several days.

It is now well established that the primary metabolic target of glyphosate is an enzyme of the shikimic acid metabolic pathway, enolpyruvyl shikimate-3-phosphate synthase (2,3). Via this action, glyphosate blocks the synthesis of the end products of this pathway, notably phenylalanine and tryptophan, but also various subsequent products (Figure 1) (4,5). It has seemed logical to conclude that the herbicidal effect of glyphosate is a direct result of its effect on the shikimic acid pathway.

What Causes Plant Death?

Some aspects of the behavior of glyphosate bring this conclusion into question, however. The efficacy of glyphosate seems to depend upon processes that occur in the roots. Why are roots more important than shoots if the ability of glyphosate to kill plants is nothing more than a direct result of its inhibition of the shikimic acid pathway? Glyphosate moves preferentially to metabolic sinks, and injury or stress to above ground portions of treated plants reduces the efficacy of glyphosate (6). The efficacy of glyphosate is sometimes inconsistent under essentially identical conditions of application, vegetation, and weather, where presumably identical amounts of glyphosate would be absorbed (7). It is not clear why these characteristics should be observed if the herbicidal action of glyphosate is a direct result of its effect on the shikimic acid pathway.

Sublethal doses of glyphosate can have some effects on plants in the long term that are difficult to attribute directly to inhibition of the shikimic acid pathway. Shortly after the introduction of glyphosate as a commercial herbicide, its use for control of suckers in commercial raspberry plantations was evaluated with initially promising results (8). The effects of these treatments in the long term is less well known. Similar

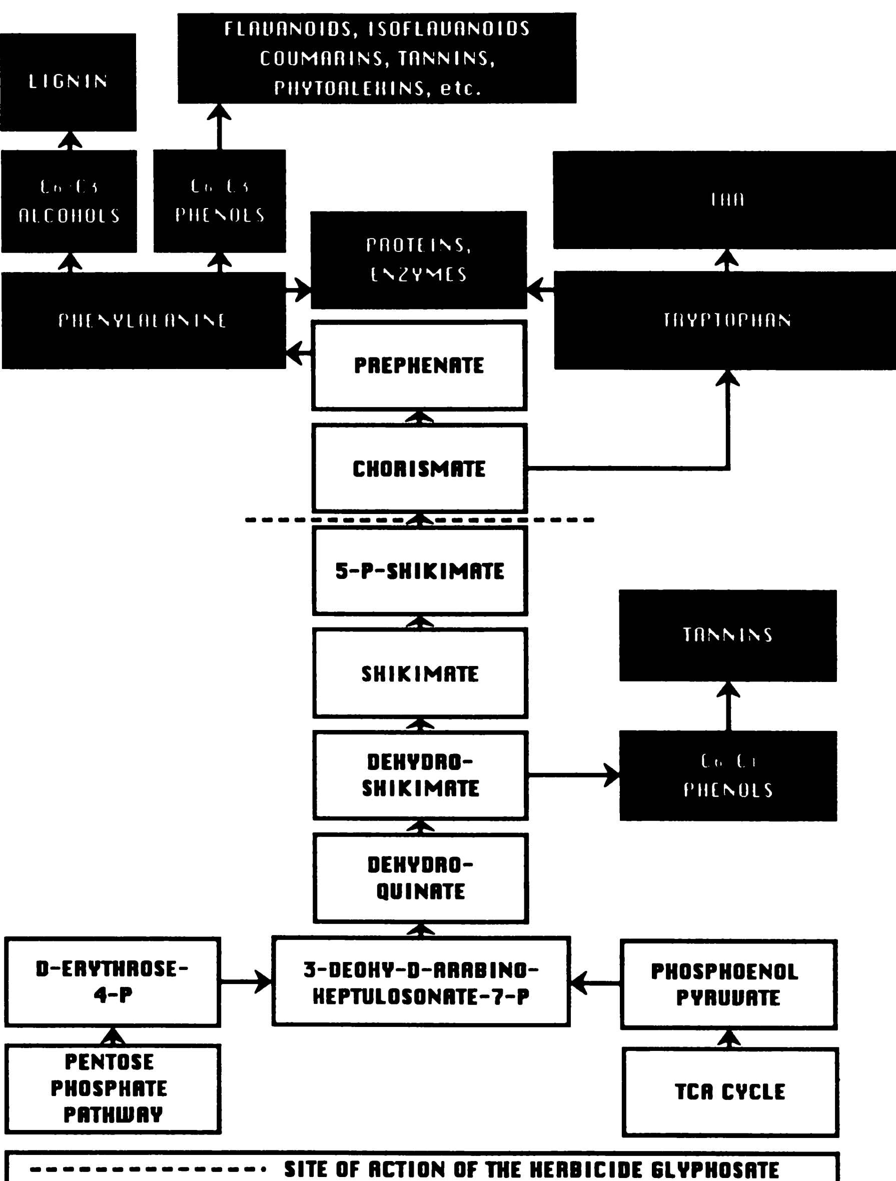

Figure 1. Major steps of the shikimic acid pathway represented in open boxes, and major products and some fates of these products shown in black boxes. The step of the pathway blocked by the herbicide glyphosate is represented by the dotted line.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch014

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promising results in British Columbia were followed in the next season with severely reduced growth of the mother plants that had appeared unaffected in the season of treatment (9). The hope that glyphosate might replace dinitro for control of suckers and the initial flush of primal canes quickly faded.

Such observations are not easily described as direct effects of inhibition of the shikimic acid pathway. Evidence that the herbicidal action of glyphosate can be enhanced by root colonizing microorganisms came while conducting dose response tests on bean seedlings (*Phaseolus vulgaris*) for studies of the effect of glyphosate on the expression of interaction phenotype in bean anthracnose disease (10). Seedlings growing in vermiculite survived doses of 10 µg per plant whereas similar seedlings growing in a freshly collected soil were killed (11). Subsequent tests revealed similar marked differences in the herbicidal efficacy of glyphosate on bean seedlings growing in raw soil and soil sterilized by autoclaving. The decreased efficacy of glyphosate observed on bean seedlings growing in autoclaved soil was restored by the addition of water extracts of raw soil. Filtration (0.2 µm pore size) or autoclaving of the raw soil extracts destroyed their ability to restore the efficacy of glyphosate (Table I). These results indicate that heat labile and filterable factor(s) present in raw soil contribute to the 'normal' level of efficacy of glyphosate observed on bean seedlings growing in raw soil.

The roots of glyphosate treated bean seedlings growing in raw soil were found to be colonized by *Pythium* and

TABLE I. Effect of soil extracts added to autoclaved mineral soil on % mortality of glyphosate treated bean seedlings (based on 20 plants)

GLYPHOSATE TREATMENT	SOIL EXTRACT[a] TREATMENTS		
	Untreated	Filtered[b]	Autoclaved
Control	0	0	0
Glyphosate[c]	100	0	0

[a] A 1:4 soil:water suspension passed through four layers of cheese cloth. Fifteen ml quantities of the indicated extracts were injected 5 days before and at the time of glyphosate treatment.

[b] Untreated extracts were prefiltered through Whatman filter papers and then passed through Millipore filter, 0.2µm pore size.

[c] 10µg/plant applied as four 1µl droplets on leaves.

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Fusarium spp. within 2 to 3 days, even though the treated plants showed no overt symptoms. Representative isolates of these *Pythium* and *Fusarium* spp. enhanced the herbicidal efficacy of glyphosate on seedlings growing in vermiculite or sterilized soil to levels comparable to those observed on seedlings growing in raw soil. The synergistic effect of the *Pythium* isolate on glyphosate efficacy was negated by the fungicide metalaxyl (11). Clearly, at the 10 µg per plant dose the herbicidal effect of glyphosate on bean seedlings is indirect. It is due to pathogenic activities of certain *Pythium* and *Fusarium* spp., resident in at least some soils, that colonize the roots of glyphosate treated but not untreated bean seedlings.

Glyphosate LD_{50} Values in Heat Treated and Raw Soils

Subsequent research in our laboratory has shown that differential sensitivity of plants to glyphosate in raw and heat treated soils is a general phenomenon. Significantly higher LD_{50} values for glyphosate have been observed in heat treated compared with untreated soil for every plant species tested (Figure 2). The plant species involved in these studies include both monocots and herbaceous and woody (one only) dicots. For each LD_{50} estimate, at least four different doses were used in order to obtain percentages of plant mortality ranging from 0 to 100 %. In total, five different soil types have been utilized, and comparative studies have been done using paired mineral (sandy loam) and organic (muck) soils. Autoclaving and microwave soil treatments have been compared. The magnitude of difference in sensitivity to glyphosate for plants growing in heat treated and raw soil varies in different soils, in different plant species and with different methods of heat treatment, but the direction of the difference is always the same. Plants growing in heat treated soil are less sensitive to glyphosate than are similar plants growing in raw soil of the same type.

Data for proportion of dead plants at different doses of glyphosate were analysed using GLIM and stepwise logistic regression (12). LD_{50} values were estimated by setting the regression expression for Y_i in the fitted regression model equal to zero and solving for X_{50} (when the proportion of dead plants is equal to 0.5, the logit Y_i equals 0.0) (Figure 3). The resulting expression for X_{50} was used to estimate LD_{50} by substituting the fitted regression coefficients. Standard errors were calculated using the propagation of errors formula for approximate variance of a transformed variable (13). Plant stem diameter measured at the times of treatment with glyphosate was included as a covariate in most analyses.

The increased tolerance of bean seedlings growing in heat treated soils is immense; they are typically 10- to 100-fold less sensitive to glyphosate in heat treated soil compared with raw soil. The tolerance of the other plant

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch014

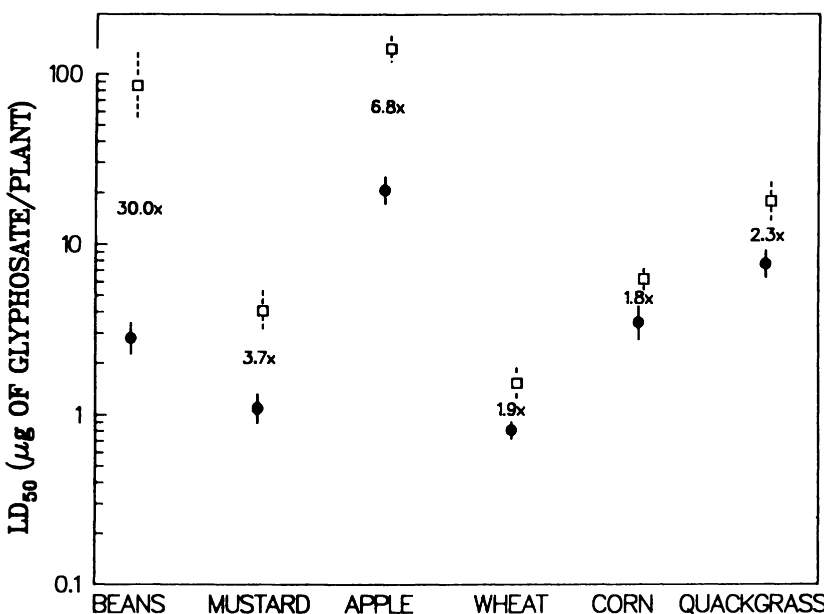

Figure 2. Dose of glyphosate needed to kill 50% of the plants (LD_{50}) grown in either control (● ———) or heat treated (□ — — —) soils. The ratio of LD_{50} values in heat treated over control soil is shown between the data points for each plant species. All heat treated soils were microwaved except for beans and apples where soils were autoclaved. All soils were mineral soils except for quackgrass seedlings that were grown in organic soil.

Table II. Residual effect of autoclave[a] and microwave[b] treatments of mineral and organic soils on populations of various components of soil microflora 2 weeks after seeding the treated and control soils with wheat. The heat treated soils were exposed to recontamination at seeding

SOIL		MICROFLORA COMPONENTS [c]			
TYPE	TREATMENT	*Pythium*[d]	*Fusarium*[e]	FUNGI[f]	BACTERIA[g]
Mineral	Control	1.9×10^3	2.6×10^4	1.9×10^5	1.7×10^7
Mineral	Autoclave	<13	<13	5.1×10^2	1.4×10^7
Mineral	Microwave	<13	<13	3.5×10^3	3.3×10^7
Organic	Control	1.1×10^3	3.9×10^4	1.2×10^5	1.1×10^7
Organic	Autoclaved	<16	<16	8.8×10^4	1.2×10^7
Organic	Microwaved	<16	2.6×10^2	2.9×10^3	7.7×10^7

[a] Three kg units of moist soil, 121°C, 15 p.s.i., 20 min, on 2 consecutive days.
[b] Two kg units of moist soil, 6 min, 540 watts.
[c] Colony forming units per g dry weight of soil estimated by dilution plating on:
[d] *Pythium* selective medium (14),
[e] *Fusarium* selective medium (15),
[f] Peptone dextrose rose bengal agar (16),
[g] Potato dextrose agar.

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species tested thus far is typically increased 2-fold to 10-fold by heat treatment of the soil being used for the tests (Figure 2). One of the rare instances where we failed to detect significantly increased tolerance in a heat treated soil involved apple seedlings and a 'virgin' soil - a benchland soil from the Okanagan Valley of British Columbia with no previous history of apples or cultivation of any kind. In contrast, apple seedlings growing in heat treated soil from a commercial orchard in the same general area had approximately 7-fold increased tolerance to glyphosate compared to those growing in the raw soil (Figure 4).

What causes the reduced sensitivity to glyphosate observed in plants growing in heat treated soils? Both autoclave and microwave treatments were effective in creating reduced sensitivity. Both types of heat treatment markedly reduced populations of resident *Pythium* and *Fusarium* spp. This effect persisted for at least 2 weeks following seeding of wheat (*Triticum aestivum*) and beans into the treated soils at the time when the soils were first exposed to recontamination (Table II).

While experiments of the type that established that *Pythium* and *Fusarium* spp. are the direct cause of death of

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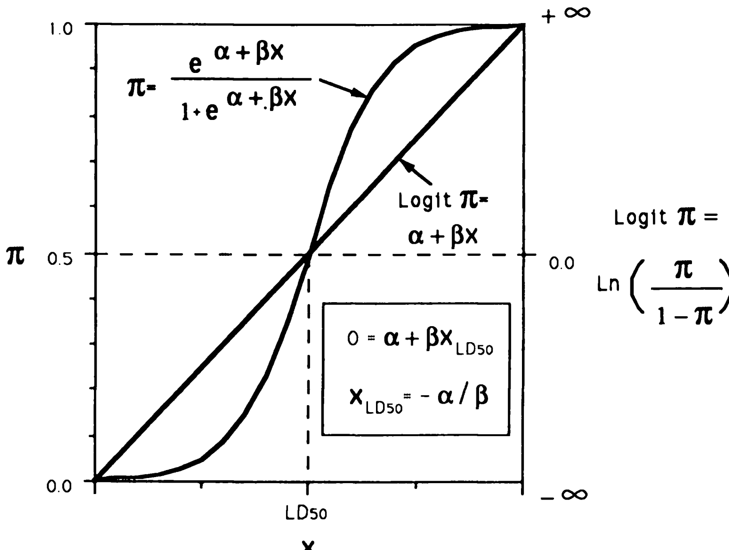

Figure 3. The logistic regression model used to estimate LD_{50} is represented on the left where π represents the proportion of dead plants. The logistic curve can be linearized by using the logit transformation shown on the right. LD_{50} values were estimated with the regression coefficients for logit π=0.0, as shown in the inset box.

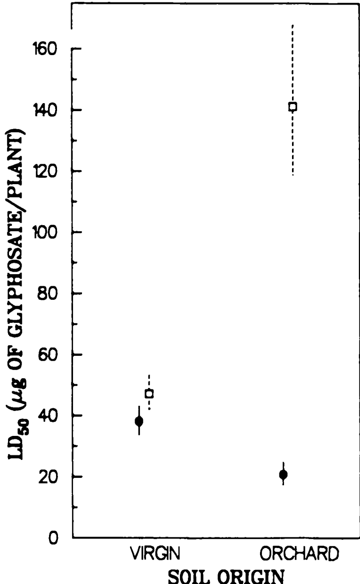

Figure 4. Effect of heat treatment of soil on LD_{50} values for glyphosate on 10-week old apple seedlings grown in either virgin soil or orchard soil. The soil was either left untreated (• ———) or autoclaved (□ — — —).

Table III. Effects of untreated, filtered and autoclaved extracts from mineral soils added to the same soil after autoclaving, on populations of various components of soil. microflora. The extracts were added 5 days prior to and at the time of glyphosate treatment, and assessment of microbial populations was done 1 day after the last amendment

SOIL EXTRACT TREATMENT	MICROFLORA COMPONENTS [a]			
	Pythium[b]	*Fusarium*[c]	FUNGI[d]	BACTERIA[e]
UNTREATED	6.0×10^1	7.9×10^2	2.7×10^3	8.5×10^6
FILTERED (0.2 µm)	<7	<7	1.2×10^3	8.1×10^6
AUTOCLAVED	<7	<7	3.0×10^2	9.8×10^6

[a] Colony forming units per g dry weight of soil estimated by dilution plating on:
[b] *Pythium* selective medium (14),
[c] *Fusarium* selective medium (15),
[d] Peptone dextrose rose bengal agar (16),
[e] Potato dextrose agar.

bean seedlings treated with certain doses of glyphosate have yet to be done for other plant species, we have obtained considerable circumstantial evidence suggesting that fungi within these genera function as glyphosate synergists in wheat seedlings as well as beans. Populations of *Pythium* and *Fusarium* were reintroduced into autoclaved mineral soil by amendment with water extracts of raw soil. At the time of glyphosate treatment the introduced *Pythium* and *Fusarium* spp. were at about 3% of their normal levels in raw soils (Table III and II), yet the fungi at these levels permitted full recovery of glyphosate efficacy on the treated seedlings (Table I). Filtration and autoclaving of the extracts precluded both the reintroduction of *Pythium* and *Fusarium* spp. (Table III) and the recovery of glyphosate efficacy (Table I).

Root Colonization

We developed a technique that permits plating of entire and intact surface sterilized root systems of seedlings (17). This has made it possible to estimate the number and location of fungal colonizations in the root systems of seedlings as a function of time after treatment with glyphosate. In both organic and mineral soils, *Pythium* and *Fusarium* spp. account for at least 50% and 25%, respectively, of total root colonizers of wheat and bean seedlings treated with glyphosate. Colonization of the root systems of glyphosate treated plants appears to begin

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch014

immediately following foliar application of the herbicide (Figure 5). *Pythium* colonizers sufficiently established within root tissues to survive surface sterilization with 1% sodium hypochlorite for 2 minutes were readily detected at 2 days after treatment with glyphosate. In wheat and beans not treated with glyphosate, fewer than one in 32 and one in six plants, respectively, yielded *Pythium* colonizers from their root systems. *Fusarium* spp. showed a similar pattern, but in this case superimposed on a significant background level (average of 0.5 colonies per root system) of natural colonization in plants not treated with glyphosate. Glyphosate treated plants of either wheat or beans typically yielded an average of one to three colonies of *Fusarium* per root system.

Pathogenicity of Root Colonizers. Are the *Pythium* spp. that rapidly colonize the roots of glyphosate treated plants pathogenic to these plants in the absence of glyphosate? Bean seedling emergence in autoclaved mineral soil was reduced by 83% when the soil was amended one day before seeding with a mixture of hyphal fragments from two *Pythium* isolates obtained from beans treated with glyphosate. A similar test involving two *Pythium* isolates from wheat reduced wheat seedling emergence by 45%. Two days after amendment, the numbers of colony forming units of *Pythium* spp. recovered from the amended autoclaved soils were less than one third of the numbers of colony forming units of *Pythium* spp. present in the soils before autoclaving. The levels of *Pythium* spp. in amended and autoclaved soils were similar 14 days after seeding. These results suggest that at least some of the *Pythium* spp. that function as glyphosate synergists can cause damping off in the absence of glyphosate.

Specificity of Root Colonizers. Two isolates of *Pythium* representative of the early colonists of glyphosate treated seedlings for both beans and wheat from organic and mineral soil were examined and identified by Dr. D.J.S. Barr of the Biosystematics Research Institute of Agriculture Canada, Ottawa. The total of eight different isolates yielded only the species *Pythium ultimum* (Campbell and Hendrix) and *Pythium sylvaticum* (Trow). The pattern of origin of these species did not suggest any particular relationship with plant species or soil type (Table IV). The isolates identified from the mineral soil were the isolates used in the pathogenicity tests described in the previous paragraphs and Tables I to III.

Glyphosate synergistic fungi (GSF) of 2- to 3-week-old seedlings of bean and wheat under the conditions of our tests thus appear to be certain *Pythium* and *Fusarium* spp. resident in diverse soil types. Organisms that readily recolonize heat treated soils from the air or from bean and wheat seeds sown into treated soils do not restore the efficacy of glyphosate to the levels observed in raw

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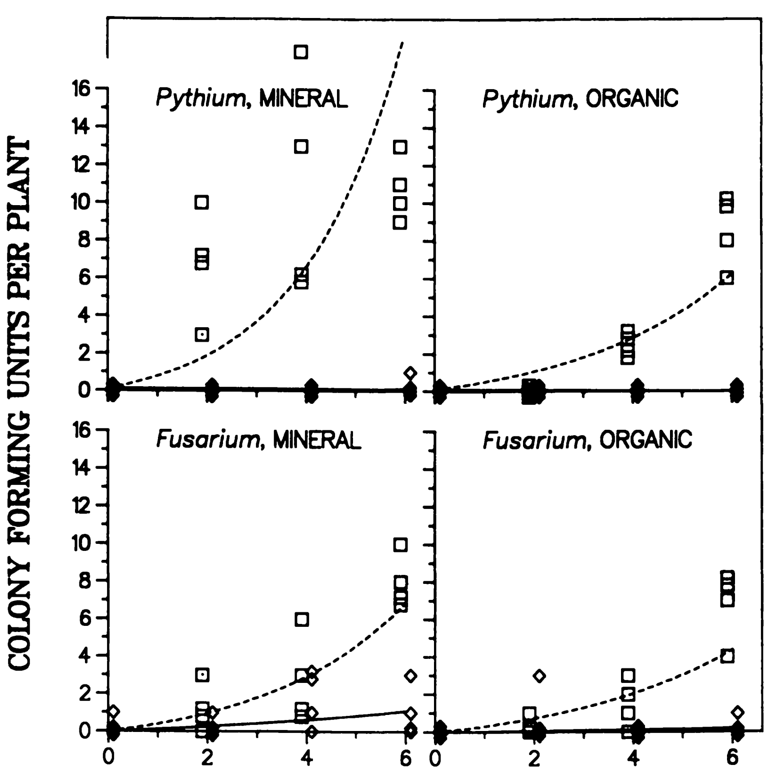

Figure 5. Rate of colonization of entire root systems of 2-week old wheat seedlings by *Pythium* or *Fusarium* spp. Plants treated with 2µg of glyphosate (□- - - -) were compared with control plants (◇———). The plants were grown at 25C, in either mineral or organic soils adjusted daily to field capacity (-0.06 Bar).

Table IV. Origin and identity[a] of representative *Pythium* isolates obtained from the roots of glyphosate treated seedlings[b]

PLANT SPECIES	SOIL TYPE	
	MINERAL	ORGANIC
Beans	*P. sylvaticum*	*P. sylvaticum*
	P. ultimum	*P. ultimum*
Wheat	*P. ultimum*	*P. sylvaticum*
	P. ultimum	*P. sylvaticum*

[a] Identifications made by Dr. D.J.S. Barr, Biosystematics Research Institute, Agriculture Canada, Ottawa.

[b] Two isolates submitted from each plant species-soil type combination for a total number of 8 isolates.

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soils. *Pythium* spp. colonizers of bean and wheat seedlings can cause damping off in the absence of glyphosate.

We have obtained evidence that treatment of established annual and perennial vegetation with glyphosate causes rapid colonization of the roots of diverse plant species by fungi in the field (18). Under the conditions of these tests and the methods used for assessment of colonizers, *Fusarium* spp. were the predominant colonizers. Populations of soil fusaria were increased significantly following treatment of the established vegetation on these plots with glyphosate.

One focus of our current research is the question of host specificity of GSF. The facts that only *P. sylvaticum* and *P. ultimum* were represented from among eight *Pythium* isolates from beans and wheat, and that both *Pythium* species were recovered from each plant species, suggest a lack of host specificity. The failure of heat treatment to reduce the sensitivity of apple seedlings on virgin soil while causing a 7-fold reduced sensitivity of seedlings growing in soil from an established apple orchard is indicative of possible specificity (the virgin soil may have lacked root colonizers specific to apples). We are currently using restriction fragment analysis to search for differences within *Pythium* species from different plant species. Information from this study will be used to select particular isolates for physiological tests for possible host specificity.

Significance of Glyphosate Synergistic Fungi

Do GSF figure significantly in the action of glyphosate in the field? There are at least three aspects of this question: the contribution of GSF to the herbicidal efficacy of glyphosate on target weed species, the

indirect effects of elevated activity of GSF on adjacent plants not treated with glyphosate, and the pathogenic effects due to increased activity of GSF in the rhizospheres of plants exposed to sublethal doses of glyphosate during 'selective' applications of the herbicide. While definitive answers to these questions are not yet available, there are some situations where involvement of GSF seems probable.

Under laboratory conditions, the contribution of GSF to the herbicidal efficacy of glyphosate is greatest at doses equal to the mean of the LD_{50} values observed for glyphosate on seedlings growing in heat treated and raw soils. These means ranged from 1.5 µg per plant for wheat seedlings to 80 µg per plant for apple seedlings (Figure 2). Do the rates recommended for application of glyphosate in the field fall within this range? Seedling shape and surface area, shielding by adjacent vegetation, spray droplet size and the extent of aerosol drift are among the many factors that contribute to the problem of estimating the amount of spray solution that would be intercepted by seedlings of a particular size in the field. The recommended rates for application of Roundup for most weed problems are equivalent to 8 - 25 µg of glyphosate per cm^2. Except for quackgrass (*Agropyron repens*), these rates would probably deposit amounts of glyphosate that would kill plants even without the participation of GSF. The combination of limited surface area and relative tolerance to glyphosate of quackgrass in our tests is such that the recommended rate for application of Roundup could easily require participation of GSF for field efficacy. Irrespective of whether the participation of GSF is needed to explain plant death in any particular case, colonization of glyphosate treated plants by these fungi occurs and probably contributes to herbicidal efficacy. More importantly, such colonization has potential consequences for untreated vegetation at the site.

The question of host specificity is of substantial importance in relation to optimizing efficacy and minimizing possible risks associated with the use of glyphosate. Understanding the nature of GSF will increase understanding of the effects of biotic and abiotic site factors on the efficacy of this herbicide. If GSF are not host specific, the increased inoculum potential of these synergists associated with the use of glyphosate could adversely affect untreated plants whose roots coexist in the same rhizosphere with roots of treated plants. This effect could occur in either space (selective treatment of vegetation) or time (subsequent crops). If glyphosate synergists are host specific, then adverse side effects would not be expected in plants of non host species whose roots coexist within the rhizosphere of a plant selectively treated with glyphosate.

There are several reports of reduced emergence and growth in crops seeded soon after treatment of established vegetation with glyphosate, and of impaired growth in

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untreated plants growing adjacent to plants treated with glyphosate. Various explanations for these effects have been offered, but in most if not all of these cases the effects on the subsequently seeded crops or adjacent plants could also be explained by the deleterious effects of root pathogenic fungi whose activity was promoted by glyphosate treatment of other plants growing at the same site. Lynch and Penn (19) have demonstrated under laboratory conditions that the damage they often observed in barley (*Hordeum vulgare*) after glyphosate treatment was caused by *Fusarium culmorum* that had colonized the rhizomes of quackgrass. Mielke (20) showed in greenhouse and field trials that the incidence of take-all (*Gaeumannomyces graminis*) in wheat was greater when quackgrass was controlled with Roundup than with cultivation. We were unable to detect any adverse effects of glyphosate used to control established weed seedlings on the emergence of several crop species under field conditions (18).

No effect was observed on spring seeded barley in field plots where volunteer barley and grassy weeds were killed with glyphosate in the previous fall. Substantially reduced emergence and seedling vigor occurred in barley that was planted within 3 to 7 days of treatment of volunteer barley with glyphosate in the spring, however. Populations of *Rhizoctonia* root rot were enhanced in the established barley plants treated with glyphosate, and *Rhizoctonia solani* AG-8 was the major cause of reduced emergence in the subsequently seeded crop (Cook, R.J., USDA-ARS, Washington State University, Pullman, WA, personal communication, 1989.).

Concluding Remarks

Industrialists and agriculturalists are proposing many new uses for glyphosate. These new uses could become economic if a marked decline in the price occurs when the use patents for glyphosate expire. Such expanded uses are not without potential biological risks. The question of host specificity of GSF is of critical importance in risk assessment. Glyphosate has potential for use in preharvest weed control and for crop desiccation (21). At present, this use is neither legal nor economic for cereals and oilseeds in Canada, but is considered by some to be a realistic possibility for the future (22). Some analysts predict that glyphosate used as a desiccant might be applied to 5 to 10 million hectares of prairie cropland. Clearly, critical growth and yield tests of the crops planted in the year following application of glyphosate for preharvest weed control and crop desiccation need to be done.

Glyphosate was applied to some 29,700 hectares of coniferous forest in British Columbia for control of competing vegetation in 1987 (23). Approximately 30% of this area was sprayed from the air. The selectivity of

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glyphosate applied in this manner on unshielded conifers depends on the relative tolerance of conifers to glyphosate when applied in late summer after the current year's needle growth has hardened (7). Substantially increased growth of conifers released from the effects of competing broadleaf spp. by such treatment with glyphosate has been observed in some cases (24). Given the scale of such applications, and the probability of expanded use in the future, one can only hope that the tests for tolerance in the conifers have been of sufficient duration and rigor to confirm the absence of possible long term effects from apparently 'safe' doses and modes of application of glyphosate.

The interaction of GSF and glyphosate is presently a poorly defined and understood phenomenon. It also appears to be rather general and unavoidable under field conditions, at least on herbaceous seedlings. As understanding of the phenomenon grows, particularly as regards the degree of plant 'host' specificity of GSF, applications for biological or integrated chemical - mycoherbicidal weed control will emerge. Possible applications include the use of glyphosate as a mycoherbicide synergist, the use of particular GSF in tank mixes to confer selectivity or enhanced activity to glyphosate, and the use of glyphosate treated plants as probes for the detection and study of 'low grade' root pathogens in soil.

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Chapter 15

Soilborne Fungi for Biological Control of Weeds

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Current methods of biological weed control with plant pathogens rely mainly upon foliar application of host-specific pathogens which infect an established weed population. This contrasts with chemical weed control where application can provide broad-spectrum pre-planting, pre-emergence or post-emergence control of potential weed populations. This limitation to the value of mycoherbicides, relative to chemical herbicides, could be overcome with the finding that the saprophytic, soilborne fungus *Gliocladium virens* Miller, Giddens & Foster can provide the needed broad-spectrum pre-emergence control of weeds. Application of *G. virens*, cultured on peat moss amended with sucrose and ammonium nitrate, reduced a broad range of weeds by at least 90 percent. Those seedlings which did emerge were severely stunted. Herbicidal activity was correlated with production of the steroidal phytotoxin viridiol. Viridiol caused a severe necrosis of roots but did not affect other tissues. Crop toxicity was avoided by directed application of the mycoherbicide above the root zone of crop seedlings. The use of *G. virens* and other soilborne fungi for weed control is discussed.

Soilborne fungi remain the least studied organisms for the biological control of weeds. This is surprising when one considers that the first fully registered commercially available microbial herbicide was the soilborne fungus *Phytophthora palmivora* Butl. (1,2). Marketed as DeVine (Abbott Laboratories, Chicago IL), *P. palmivora* is applied to soil around citrus trees for the control of stranglervine (*Morrenia odorata* Lindl.). It has proven highly

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effective in control of both seedlings and mature vines. Persistence of this mycoherbicide is highlighted by the 95 to 100 % vine control reported 6 years after application of the fungus (3).

Soilborne fungi reduce weed populations through the decay of seeds before emergence or by causing the death of seedlings shortly after emergence. Soilborne fungi can also cause severe root decay, girdling soil-line lesions and internal stem necrosis, each resulting in reduced competitive ability and decreased reproductive capacity of infected weeds. While this damage may be overlooked on a weed, it is readily documented when it causes similar damage to a crop. Plant pathologists are well aware of the damage caused to crops by soilborne fungi, however their main interest is protection of the crop, not the potential to inflict the same type of damage to weed species. A wealth of information exists on the ecology and physiology of soilborne phytopathogenic fungi. This information can provide a valuable resource for selection and optimization of soilborne phytopathogenic fungi as mycoherbicides.

Weed hosts are an important reservoir for pathogens of cultivated plant species, thus they are listed in many studies of soilborne crop pathogens. This documentation can be a valuable source of new mycoherbicides. It should be possible to employ a crop pathogen for control of a closely related weed species as long as the crop species is not present. One of the very few reported examples where this strategy has been applied was the attempt to control yellow starthistle (*Centaurea solstitialis* L.) with pathogens of the closely related crop safflower (*Carthamus tinctorius* L.) (4). The use of phytopathogenic soilborne fungi is obviously limited by the potential for exposure of susceptible crop species. This limitation can be reduced if the fungus has a very limited host range, although this limits its value as a broad-spectrum weed control. Exposure can occur through the subsequent planting of susceptible crops into mycoherbicide infested soil, or by dispersal into surrounding fields containing susceptible crops. If crop rotations are practiced over a sufficient length of time it may be possible to plant susceptible crops, but only for those cases where the soilborne fungus is known to be greatly reduced in population in the absence of a host. The possibility that a soilborne fungus would be dispersed to surrounding fields, while possible, is less likely than the possible dispersal of a foliar pathogen whose propagules would be available for wind and water dispersal.

There are numerous additional advantages in the use of soilborne fungi for weed control. One advantage of soilborne fungi is their greater independence from environmental conditions (moisture, temp.) which limit the use of foliar mycoherbicides. The extended periods of high relative humidity required for germination and infection by foliar mycoherbicides severely restricts the period of application. Soilborne fungi can be applied at the convenience of the grower when weed control is desired. Soilborne fungi can also be applied in various granular formulations which provide a substrate which favors proliferation of the added mycoherbicide.

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Fusaria as Mycoherbicides

A list of soilborne phytopathogens which have been studied as mycoherbicides is found in Table I. With the exception of *Sclerotinia sclerotiorum* (Lib.) de Bary, tested for control of Canada thistle [*Cirsium arvense* (L.) Scop.](5), and *P. palmivora*, the only soilborne plant pathogenic fungi currently being studied as mycoherbicides in crops belong to the genus Fusarium.

Table I. Soilborne Phytopathogenic Fungi Studied as Mycoherbicides

Fungus	Target Weed	Reference
Fusarium lateritium	prickly sida spurred anoda velvetleaf	(8)
Fusarium oxysporum f. sp. *cannabis*	hemp	(7)
Fusarium oxysporum f. sp. *carthami*	yellow starthistle	(4)
Fusarium roseum	hydrilla	(9)
Fusarium solani f. sp. *cucurbitae*	Texas gourd	(6)
Phytophthora palmivora	stranglervine	(1)
Rhizoctonia solani	water hyacinth	(10)
Sclerotinia sclerotiorum	Canada thistle	(5)

Fusarium is found in virtually all soils on a worldwide basis. Survival in soil is generally achieved by production of chlamydospores which arise through the thickening of hyphal or conidial cell walls. Species of Fusarium have been reported as parasites on virtually all cultivated crop species. Many fusaria are quite host specific, and are classified in formae specialis according to their specificity. For example, *Fusarium solani* (Mart.) Appel.& Wr. formae specialis (f. sp.) *cucurbitae*, applied for control of Texas gourd (*Cucurbita texana* Gray)(6), is limited to infection of cucurbits. This specialization provides a predictable host range, thus reducing the risk of infecting a plant species absent from host-range screening. In the absence of a host, these specialized pathogens do not survive beyond a few seasons so they should not pose a threat to future plantings of susceptible crops. An additional benefit of this specialization is that resistance genes are available in some crops, thus allowing use of the fungus with otherwise susceptible crops. The presence of resistance in the crop suggests that resistance may be selected for in the weed population after repeated application of host-specific fusarium-based mycoherbicides. Fusarium resistance was found during initial application of *F. oxysporum* Schlecht. f. sp. *cannabis* Noviello & Snyder for control of illicit hemp (*Cannabis sativa* L.) (7).

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One limitation in the use of Fusaria as mycoherbicides has been the inability to control more than one weed species. Unless the target weed is the major component of the competitive weed population it is uneconomical to apply the mycoherbicide. This limitation has been reduced somewhat through use of *Fusarium lateritium* Nees ex Fr., which is effective against spurred anoda [*Anoda cristata* (L.) Schlecht], velvetleaf (*Abutilon theophrasti* Medic.) and prickly sida (*Sida spinosa* L.) (8). These weeds are, however, all members of the Malvacae, thus control remains confined to closely related weeds. It may be possible to combine various Fusaria to obtain a broader range of weed control.

While there is some promise in the use of Fusaria as mycoherbicides, there may be difficulties in registration and commercialization of a crop pathogen for weed control. Growers, concerned about the potential for damage to their own and neighboring fields may be wary of mycoherbicides classified as plant pathogens. Another type of mycoherbicide, employing non-phytopathogenic soilborne fungi, can provide an alternative to the risks of applying pathogenic fungi for weed control. The non-phytopathogenic fungi act by producing phytotoxic compounds under certain nutrient regimes. Thus, while they do not infect plants their phytotoxic metabolites result in stunting and death of nearby plants. As phytotoxic levels of synthesis occur only with the addition of high levels of specific nutrients the duration of herbicidal activity can be regulated. In the absence of the added substrate the fungus exists in the soil simply as a saprophyte, causing no potential disease threat.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch015

Phytotoxin Production by *Gliocladium virens*

Broad-spectrum weed control has recently been achieved using the non-phytopathogenic soilborne fungus *Gliocladium virens* (11,12). Application of the fungus after culturing on nutrient-amended peat resulted in dramatic reductions in seedling emergence and dry weights as shown in Table II. All test seeds were first planted in soil in which 16% of the total soil volume was the fungus-peat mixture. The majority of test weed species failed to emerge at this level, the few which did were severely stunted. Those species which failed to emerge at the 16% concentration were planted in soil in which 8.7% of the total soil volume was the fungus-peat mixture. Those failing to emerge at this concentration were planted at lower concentrations until emergence levels at least 10% that of the control were achieved.

An important aspect in addition to the broad-spectrum nature of this mycoherbicide is that is posseses pre-emergence activity. The system is similar in many ways to weed control provided by pre-emergence chemical herbicides, which account for the majority of herbicide applications. While most mycoherbicides cause various degrees of physiological stress to target weeds, it is often only after they have become established in the crop. As the effects of weed competition are generally greatest early in crop stand establishment, this is the most effective time for chemical or mycoherbicide activity (13).

The activity of *G. virens* as a mycoherbicide is achieved through production of the steroid-like phytotoxin viridiol whose structure is shown in Figure 1. Viridiol production in soil was

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Table II. Effect of *G. virens*-peat mixture on crop and weed species after 14 days

Application rate[a]	Plant	Seedling emergence	Dry weight[b] Root Control	Root Treated	Shoot Control	Shoot Treated
(%)		(% of control)	(mg)			
16.0	Cotton	75	44.1	3.4	150.1	41.1
	Sunflower	69	31.6	15.4	105.4	48.4
	Lettuce	32	1.4	0.2	5.6	0.4
	Soybean	41	59.0	34.6	176.7	86.7
	Cucumber	44	16.5	3.5	66.7	11.4
	Cantaloupe	25	13.9	0.6	61.7	1.6
	Corn	83	103.6	64.1	92.6	36.0
	Sudangrass	71	15.5	3.7	32.8	5.4
	Mustard	33	2.2	0.3	11.9	0.9
	Tomato	52	2.7	0.1	12.4	0.5
	Wild Oat	89	30.7	17.8	13.7	6.8
8.7	Safflower	100	30.8	16.3	77.7	31.8
	Bean	100	189.6	143.1	423.3	257.1
	Alfalfa	75	6.3	2.1	17.6	3.2
	Sugarbeet	100	8.5	1.6	25.2	3.5
	Spinach	79	8.1	2.3	19.5	4.3
	Black nightshade	23	0.9	0.9*	2.9	1.3
	Field bindweed	67	12.0	3.7	27.7	9.5
	Annual bluegrass	40	1.3	0.5	2.2	0.4
	Yellow foxtail	53	6.8	1.5	15.6	2.5
	Canarygrass	29	1.8	1.2	2.1	1.9*
	Curly dock	20	0.6	0.2	2.3	0.9
	Buckhorn plantain	46	0.9	0.5	1.5	0.8

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch015

4.5	Spotted catsear	58	1.6	0.1	2.7	0.7
	Redroot pigweed	71	1.5	0.1	5.7	0.1
	Common purslane	67	1.0	0.1	2.5	0.2
	Common fiddleneck	57	0.9	0.6	5.3	1.7
	Lambsquarter	69	0.2	0.1	0.7	0.4
2.3	Dandelion	60	1.9	0.9	2.6	1.2
	Bristly oxtongue	62	1.8	1.0	4.1	2.4
	Groundsel	96	0.5	0.3	1.8	1.1
	Annual sowthistle	58	1.0	0.6	3.1	1.0

[a]Fungus-peat mixture as percentage of total soil volume. Values represent treatment levels at which at least 10% of seedlings emerged relative to the control. Species that failed to emerge to this level were seeded at the next lower concentration until emergence exceeded 10% of controls.

[b]Values represent mean of individual seedlings. Differences between treatment means for dry weight were all significant (P=0.05) using ANOVA-1, except where noted by asterisk.

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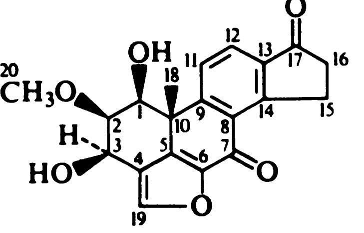

Figure 1. Steroidal phytotoxin produced by Gliocladium virens.

closely related with control of weed emergence, both lasting about two weeks. Production of phytotoxic levels of viridiol is dependent upon the high nutrient substrate on which the fungus is cultured. While numerous carbon and nitrogen sources support viridiol production, optimal levels are produced with high carbon:nitrogen ratios (14). It is important that the substrate be below pH 7.0 as viridiol is unstable in alkaline conditions. Application formulations are prepared by culturing *G. virens* on nutrient-amended peat in shallow trays for six days until the fungus has colonized the peat. The fungus-peat mixture is air dried then stored until needed. For application the dried mixture is incorporated in a band to the soil between the top of the crop seed and the soil surface.

Exposure to viridiol results in necrosis and death of emerging radicles. Root tips are affected in the same manner, whereas hypocotyls and foliage are unaffected. The organ specificity of viridiol allows it to be applied in the presence of sensitive crop species. Application is simply directed to the soil above the crop seed. As the crop seed germinates the emerging radicle and subsequent roots extend into the soil below the region of viridiol production. Viridiol production occurs only on the peat substrate, in contact with the added nutrients, thus synthesis does not occur beyond the substrate. We have found no evidence for movement of viridiol into the viridiol-sensitive root region of test crop seedlings. The region between the crop seed and the soil surface is where weed control can be seen. A weed seed which germinates at a depth greater than that which the fungus-peat mixture is applied will be unaffected. However, the majority of weed seeds germinate within the top few centimeters of soil therefore a high degree of weed control can be expected.

Numerous attributes make *G. virens* an ideal fungus for mycoherbicide formulations. It is readily cultured, grows rapidly and is long-lived in dried preparations. A unique feature of *G. virens* is the production of chemical compounds in addition to the phytotoxin viridiol. The two main compounds are the epipolythiodiketopiperazines gliotoxin and gliovirin (15,16). These compounds are strongly inhibitory towards *Pythium ultimum* Trow and *Rhizoctonia solani* Kuhn, two soilborne fungi responsible for the majority of crop seed and seedling decay. A closer examination of other phytotoxin-producing soilborne fungi may yield additional mycoherbicide candidates as effective as *G. virens*.

Screening for Phytotoxin-producing Soilborne Fungi

Various soilborne fungi have been reported to produce phytotoxins, some of which are listed in Table III. Certain phytotoxic compounds are known to have pronounced mycotoxic activity (eg. moniliformin). We have excluded those fungi which have the potential to colonize edible parts of crops and produce mycotoxic

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compounds as they would obviously be unsuitable for application. Very few phytopathogenic fungi are known to produce phytotoxins. The majority of phytotoxin-producing soilborne fungi are saprophytes.

Table III. Phytotoxin-producing Soilborne Fungi

Fungus	Phytotoxin	Reference
Actinomycete spp.	uncharacterized	(18,19)
Gliocladium deliquescens	desmethoxyviridiol	(14)
Gliocladium virens	viridiol	(11,12)
Macrophomina phaseolina	phaseolinone	(24)
Nodulisporium hinnuleum	desmethoxyviridiol	(25)
Penicillium charlesii	citreoviridin	(26)
Periconia circinata	PC toxin	(27)
Streptomyces sp.	anisomycin	(28)
Streptomyces saganonensis	herbimycins A,B	(29)
Streptomyces viridochromogenes	bialophos	(30)
Streptoverticillium sp.	cyclocarbimide A,B	(26)
Trichoderma harzianum	pentyl pyrones	(31)
Trichoderma viride	pentyl pyrones	(32)

Few studies have centered on the isolation of phytotoxin-producing soilborne fungi. The phytotoxic activity of fungal metabolites is generally found during routine screening of metabolites for overall biological activity (17). Screening of soilborne fungi specifically for the production of phytotoxins should yield numerous promising candidates for use as mycoherbicides. To date, efforts to identify phytotoxin-producing strains have been largely limited to *Streptomyces* and *Actinomycete* spp. (18,19).

Various methods are available to rapidly screen soilborne fungi for production of phytotoxic metabolites. One of the simplest involves placing a fungal culture on an agar plate containing seeds of the target weed. This is limited to fungal isolates which do not rapidly colonize the agar plate. Rapid colonization would obscure phytotoxin production. For those fungi of restricted colony size, one relies on the ability of the phytotoxin to diffuse from the colony to the susceptible target tissue. While this is adequate for some compounds, it may limit the detection of hydrophobic compounds which diffuse less readily through the agar. An alternative to direct screening of potential phytotoxin-producing fungi is the testing of crude metabolites produced in liquid culture by the fungi. The metabolites can be placed in wells cut into the agar next to the test weed seed or it may be incorporated directly into the agar. The metabolites may also be applied directly to seeds, calli or shoot cultures (20) to test for metabolite toxicity. Algae have been used as indicators of metabolite phytotoxicity. *Chlorella* is often used and is effective for detection of photosynthetic inhibitors (21). *Chlamydomonas* may also prove a useful algal model for detection of fungal

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phytotoxins. *Chlamydomonas* is sensitive to inhibitors of cell division and enlargement (22), thus it could provide a sensitive indicator of compounds inhibitory to seed germination and seedling growth where rapid cell division and enlargement is occuring. Inhibitors of seed germination and seedling growth would be particularily desirable activities for a mycoherbicide as they could eliminate weed competition at a very early stage. Cell cultures, callus and shoot cultures may be used in metabolite screening, however most cultures are currently derived from tobacco and it is not known how difficult it would be to culture cells from various weeds. Another interesting source of tissue for phytotoxicity studies would be *Agrobacterium rhizogenes*-transformed root cultures (23), as they maintain the cellular structure of intact roots. For both the algal and tissue culture systems it is not well known if they adequately represent the response of actual plants. While each of the previously mentioned methods for detecting phytotoxic metabolites may prove useful they should not replace testing of the actual seed or seedling of the target weed in soil. They may, however, provide a useful system for the initial screening of very large numbers of fungal isolates.

Factors Affecting Phytotoxin Production

Screening for phytotoxin production should be carried out with as many different media compositions as possible due to the variable level of metabolite synthesis on different media. The composition of the media significantly affects production of the phytotoxin viridiol by *G. virens*. In this case, it was not the presence of a specific carbon or nitrogen source but their relative ratio. The pH was also critical because viridiol was not stable in alkaline solution. Large scale screening of liquid media can be performed in disposable microfuge tubes. We have screened numerous media by inoculating *G. virens* conidia into 1.5 milliliter microfuge tubes containing one milliliter of medium. Following a five-day incubation, the fungal mycelium is pelleted by centrifugation and the supernatant transferred to another tube. Both the mycelium and the culture fluid can then be extracted and fractionated to isolate phytotoxic metabolites. Production of phytotoxins should be monitored over an extended time period since metabolites may be produced and then converted to another compound with a different level or spectrum of activity. This was the case for viridiol production. Viridiol was shown to be the product of a C-3 ketoreduction of the antibiotic compound viridin, which is produced by *G. virens* shortly before conversion to viridiol.

It is possible that a metabolite produced in liquid culture will not be produced at the same level on a solid substrate. Testing for metabolite production on solid medium is best achieved by application of the nutrients from the liquid medium to an inert solid, preferably one which could be used as a carrier for field application.

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Prospects for Soilborne Fungi as Mycoherbicides

To compete with the current arsenal of chemical herbicides, mycoherbicides must provide an effective means for controlling a broad spectrum of weeds, preferably early in weed seedling development. Among the phytopathogenic soilborne fungi, *Fusarium* spp. show the greatest promise, although their use will remain limited unless the problems previously outlined can be overcome. A more promising avenue for future mycoherbicide research may be found in the use of non-phytopathogenic soilborne fungi. The non - phytopathogenic fungi achieve weed control through production of phytotoxic compounds. Production of these compounds generally relies upon the availability of certain nutrients, thus phytotoxin synthesis can be controlled. Phytotoxic metabolites of some soilborne fungi have a sufficiently broad spectrum of activity to be effective in control of numerous members of a weed community.

Future progress will begin by screening a wide range of soils to isolate phytotoxin producing fungi. Screening methods are simple enough to accomodate large scale screening. Strain selection and improvement should yield more effective phytotoxin producers. We have found that viridiol production by *G. virens* varied many-fold between isolates obtained from the same field soil, thus there is still room for further enhancement of this mycoherbicide after more extensive selections.

After selection of optimal strains, further improvements can arise through protoplast fusions, genetic crosses or direct genetic manipulation by gene insertions. Protoplast fusions may be too unstable for general use, and genetic crosses are only possible if the sexual or teleomorph stage of the fungus is available. Application of genetic engineering techniques may yield the best results. While the genetic composition of strains arising from protoplast fusion and genetic crosses remain undefined, genetic engineering allows incorporation of defined genetic elements. For the majority of soilborne fungi, methods for direct genetic manipulation remain untested. This is not the case for *Streptomyces* spp., in which cloning and expression techniques are well documented. Gene clusters involved in antibiotic synthesis and resistance have been cloned and expressed in various species of *Streptomyces* (33,34). Isolation of gene cluster responsible for phytotoxin production should be no different. Multiple phytotoxin synthesis genes may be expressed by insertion into a single species of *Streptomycete* thus allowing a broader degree of weed control. Control of phytotoxin expression might be engineered by insertion of a promoter responsive to a specific substrate not readily available in the soil. The application of genetic techniques to strain improvement will require a long-term commitment to the project. For the near term, the previously mentioned screening methods should provide numerous soilborne phytotoxin-producing fungi which will prove valuable as future mycoherbicides and be available for more immediate field application.

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Chapter 16

Formulation and Application Technology for Microbial Weed Control

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Fungal weed pathogens (mycoherbicides) are difficult to formulate into effective products because, as living organisms, their viability must be preserved throughout processing and storage. Furthermore, the pathogens, as packaged in a final product, must be able to control weeds after application under natural environmental conditions of moisture and temperature. This overview of formulation and application technology for microbial weed control describes the current developmental status of experimental and commercial products. A brief review of combinations of mycoherbicides with chemical pesticides and beneficial insects is included.

There are two strategies commonly employed for the biological control of weeds. One is the classical in which the microbial herbicide, after release, is capable of self-dissemination and epidemic initiation. The other strategy, inundative biological control, requires instant introduction of large populations of a plant pathogen, and its application follows the same patterns as chemical herbicides (43). The inundative strategy also calls for formulation of a delivery system for the microorganism, since this type of strategy is not dependent on the microbial herbicide spreading rapidly on its own.

Formulation is a mixing of the active ingredient with appropriate adjuvants and carrier. Today, the formulation of chemical herbicides may be considered a science, whereas the formulation of microbial herbicides remains more of an art. A major difference between the two types of herbicides is that the life and virulence of the biological control organism must be maintained. To this end, the different additives and diluents of the formulation must be tested, singly

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and in various combinations, to insure the viability of the mycoherbicide. These adjuvants and diluents should also increase the efficiency of application, and particularly, the efficacy of the control agent.

Formulation also may involve integrating the pathogens into a weed management system. Researchers have found both beneficial and detrimental interactions between commonly used insecticides, herbicides, and weed pathogens (44, 45). In such a system the problems of application are magnified. Application can include new or conventional agricultural equipment and variables such as when, where, and how the formulation is applied. Of course, the researcher makes compromises in this complex matrix in an effort for maximum efficiency at minimum cost.

This chapter discusses microbial weed control agents and their experimental and commercial formulations. Because most microbial herbicides have a narrow host specificity, they are most likely to be used in an integrated pest management strategy with chemical herbicides, insecticides, plant growth regulators, and insect biocontrol agents. All of these research investigations are presented with emphasis on field studies, except for those agents in their developmental stage.

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Water and Surfactant-Based Formulations

Water has been the carrier of choice, especially since it is inexpensive, easy to handle, and necessary for maintaining the life of the weed pathogens. The most elementary delivery system is a simple mixture of the microbial agent and water. Such a system is usually the first used by the researcher in testing the biocontrol potential of the organism. Sometimes, this simplistic approach is bypassed in favor of a conidial suspension which contains a surfactant. Surfactants perform two functions: they help to disperse the spores or other fungal propagules in the tank mix and they serve as wetting agents to minimize runoff and the resulting loss of active ingredient from the target weed.

Table I lists research on water and surfactant-based mycoherbicides that has progressed to the greenhouse stage. In six of the eight investigations, a surfactant was used. The efficacy of *Protomyces gravidus* was highly dependent upon the strict environmental condition of a minimum of 48 hours of dew (2). These studies and others have shown that older plants require more spores/ml and/or longer dew periods for control. The investigations listed in Table I are based on the minimum spores/ml and minimum dew period to kill at least 80% of the target weed at the four (or fewer) true-leaf stage (young seedlings).

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Table I. Water and Surfactant-Based Formulations

Weed	Microbial Agent	Surfactant (conc.)	spores/ ml	Dew (hrs)	Ref.
Eastern black nightshade *Solanum ptycanthum* Dun.	*Colletotrichum coccodes* (Wallr.) Hughes (conidia)	Tween 80 (0.05%)	1×10^6	16	1
Giant ragweed *Ambrosia trifida* (L.)	*Protomyces gravidus* Davis (ascospores)		1×10^7	48	2
Horse purslane *Trianthema portulacastrum* (L.)	*Gibbago trianthemae* Simmons (conidia)	Tween 20 (0.02%)	1×10^5	16	3
Florida beggarweed *Desmodium tortuosum* (Sw.) DC.	*Colletotrichum truncatum* (Schw.) Andrus and Moore (condia)		1×10^5	14	4
Prickly sida *Sida spinosa* (L.)	*Fusarium lateritium* (Nees) emend. Snyder and Hans.	Tween 80 (0.02%)	1×10^5	18	5
Velvetleaf *Abutilon theophrasti* Medik.	"	Tween 20 (0.05%)	1×10^6	16	6
Spurred anoda *Anoda cristata* (L.) Schlecht.	*Alternaria macrospora* Zimm. *Fusarium lateritium* Nees ex Fr.	Tween 80	1×10^5	18	7
Common purslane *Portulaca oleracea* (L.)	*Dichotomophthora portulacae* Mehrlich and Fitz ex M.B. Ellis	Tween 20	5×10^5	5	8

Miscellaneous Adjuvant-Based Formulations

Certain adjuvants may stimulate the microbial agent to either germinate or infect the target weed more effectively. Table II lists adjuvants, one of which, sucrose (12), was reported to enhance disease development. For another adjuvant, gelatin, the function was not given, but gelatin could increase the viscosity of the formulation to reduce runoff or function as a humectant to improve spore survival. In those investigations with gelatin, only greenhouse results were reported. With the addition of Sorbo (64% sorbitol, Atkemix Inc., Brandford, Ontario) in the suspension, the number of viable spores of *Colletotrichum coccodes* recovered from inoculated leaves increased 20-fold. Also, three 9-hour dew periods on consecutive nights were as effective for disease incubation as one 18-hour dew period (10).

The length of the dew period usually correlates with success or failure in a majority of the work referenced in this review. Recently, it has been reported that invert (water-in-oil) emulsion carriers composed of paraffin wax, mineral (paraffin) oil, soybean oil, and lecithin retarded water evaporation (13). Thus, the pathogen has water available over a longer period of time for germination and infection of the target weed. Initially, the spores were applied in an aqueous carrier followed by an overspray of an invert emulsion applied with specialized air-assist atomizing nozzles (14). Greenhouse results using *Alternaria cassiae* against sicklepod were successful (88% mortality), but the volumes sprayed were too high for practicality (13). Further research with a one-step application of *A. cassiae* in invert emulsions showed that the quantity of paraffin wax and droplet deposit size determined the rate of water evaporation (Daigle, D. J.; Connick, W. J., Jr.; Quimby, P. C., Jr.; Evans, J. P.; Trask-Morrell, B.; Fulgham, F. E., USDA, ARS, New Orleans, LA, and Stoneville, MS, unpublished results). Therefore, invert emulsions could be both a successful delivery system and water source for *A. cassiae*. However, the lack of sufficient mortality at reasonable application rates indicates that the invert emulsion carrier needs other adjuvants or a more virulent pathogen strain for commercial success.

Another approach is to combine chemical and microbial herbicides. It was reported that the addition of sublethal rates of the herbicides, linuron [N′-(3,4-dichlorophenyl)-N-methoxy-N-methylurea], imazaquin {2-[4,5-dihydro-4-methyl-4(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-quinolinecarboxylic acid}, and lactofen {(±)2-ethoxy-1-methyl-2-oxoethyl-5-[2-chloro-4-(trifluoromethyl)phenoxyl]-2-nitrobenzoate} to an

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch016

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch016

Table II. Miscellaneous Adjuvant-Based Formulations

Weed	Microbial Agent	Adjuvants	spores/ml	Dew (hrs)	Ref.
Velvetleaf *Abutilon theophrasti* Medik.	*Colletotrichum coccodes* (Wallr.) Hughes	gelatin (0.75%) Sorbo	1×10^7	24	9 10
Field bindweed *Convolvulus arvensis* (L.)	*Phomopsis convolvus* Ormeno (conidia)	gelatin (0.1%)	5×10^6	12-24	11
Spurred anoda *Anoda cristata* (L.) Schlecht.	*Alternaria macrospora* Zimm. (conidia)	Nonoxynol 9 (0.02% v/v) sucrose (5% w/v)	2.5×10^5	0-24	12
Sicklepod *Cassia obtusifolia* (L.)	*Alternaria cassiae* Jurair and Khan	paraffin wax mineral oil soybean oil corn syrup lecithin (soybean oil)	5×10^6	0-16	13 14 15

invert emulsion containing A. cassiae significantly increased weed control without dew (15).

With center pivot irrigation simulator equipment, peanut and other vegetable oils were superior to paraffinic oils for applying A. cassiae spores at rates of 0.4-8 g spores/ha to sicklepod (Phatak, S. C., Univ. of Georgia, Tifton, GA, personal communication). Good infection occurred at rates of 0.8-1.6 L/ha of the oil/spore mixture even without dew. Sicklepod control was best when spores were applied in oil, compared with wettable powder or conidial suspension.

Formulations Based upon Solid Carriers

Although water has been used as a carrier of Phytophthora palmivora (Smith) Leonean, a soil-borne fungus, in the control of stranglervine (Morrenia odorata L.) (36-38), the potential of any microbial agent may be limited only by formulation and/or application techniques. With soil-borne pathogens, researchers are given the further option of using solids as carriers (Table III).

Initially, solid carriers were simple substrates on which the pathogen germinated, grew mycelia, and sporulated. One carrier was vermiculite which was mixed with mycelia of Alternaria macrospora Zimm. (24). After sporulation and air drying, this mixture was applied, both preemergence and postemergence, to spurred anoda (Anoda cristata Schlecht) and gave 75 to 95% control of seedlings. However, at least two 18-hour dew periods at 25°C were needed for this level of control. The fungicides, PCNB (pentachloronitrobenzene) and ETMT [5-ethoxyl-3-(trichloromethyl)-1,2,4-thiadiazole], did not significantly affect spurred anoda control with A. macrospora. Field results showed no difference between the application of A. macrospora as a foliar spray of spores, or as a granular formulation applied to the soil (25).

For many biocontrol applications, a solid matrix that could entrap or encapsulate microorganisms is advantageous. The use of a chemical reaction between sodium alginate (NaAlg) and calcium ions to make granules containing any of a wide variety of mycoherbicides was introduced in 1983 (46).

$$2\ NaAlg + Ca^{2+} \rightarrow Ca(Alg)_2\downarrow + 2\ Na^+$$

The alginate process for mycoherbicide formulation, which evolved from controlled-release herbicide work (47), is carried out in an aqueous system at ambient temperature and is gentle enough for use with living organisms (48). Uniform size, application using conventional equipment, biodegradation in the

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch016

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Table III. Formulations Based upon Solid Carriers

Weed	Microbial Agent	Carrier	Ref
Texas gourd *Cucurbita texana* (A.) Gray	*Fusarium solani* App. & Wr. f. sp. *cucurbitae* Snyd. & Hans.	cornmeal/sand	16
"	"	alginate-kaolin granules contg. 2% w/v soyflour	17
"	"	alginate-kaolin granules w/wo 2% w/v soy flour, ground oatmeal, cornmeal, or carboxymethyl cellulose	18 19
Velvetleaf *Abutilon theophrasti* Medik.	*Fusarium lateritium* Ness ex Fr.	alginate-kaolin granules	20
Prickly sida *Sida spinosa* (L.)	"	"	20
Canada thistle *Cirsium arvense* (L.) Scop.	*Sclerotinia sclerotiorum* (Lib.) de Bary	wheat kernels	21
Redroot pigweed *Amaranthus retroflexus* (L.)	*Gliocladium virens* Miller, Giddens & Foster	peat moss contg. sucrose and ammonium nitrate	22
"	"	long-grain rice	23
Spurred anoda *Anoda cristata* Schlecht.	*Alternaria macrospora* Zimm.	vermiculite	24 25

environment, and sustained activity are some positive attributes of alginate granules. Also, alginate granules containing kaolin (as filler) and mycoherbicide can be prepared and stored for up to 8 months before use (46).

A granular inoculation of Fusarium solani App. & Wr. f. sp. cucurbitae Synd & Hans., produced on a cornmeal/sand medium, effectively controlled (99% maximum) Cucurbita texana A. Gray (Texas gourd) seedlings (16). However, a liquid inoculum ($2x10^6$ spores/ml) sprayed to runoff achieved the same results. Recently, a series of papers utilizing the alginate process with this mycoherbicide to control Texas gourd have been published (17-19). The addition of nutritional amendments (at 2% w/v) such as ground oatmeal, cornmeal, or soy flour significantly increased conidial production on the surface of the granules and resulted in higher soil population than granular treatments without amendments or granules containing carboxymethyl cellulose (18). Granules amended with soy flour or ground oatmeal also provided greater control for a longer period than did remedial applications (19). These amended granules were applied up to 6 weeks preemergence and gave greater than 80% weed control (18).

Alginate granules containing Fusarium lateritium Ness ex Fr. were as effective as foliar applications of the fungus in controlling either velvetleaf (Abutilon theophrasti Medik.) or prickly sida (Sida spinsosa L.) in greenhouse studies (20). The conidial suspensions ($1.5x10^6$ spores/ml) that were sprayed contained Tween 80 (0.05% v/v) surfactant. In field tests, granules were slightly more effective against velvetleaf and slightly less effective against prickly sida than the conidial spray treatment.

Throughly colonized (infected) seeds are a commonly used carrier for biocontrol microorganisms. For example, inoculum consisting of Sclerotinia sclerotiorum-infested wheat kernels and sclerotia were hand-broadcast at five different locations to control Canada thistle [Cirsium arvense (L.) Scop.] (21). The mean percentage of dead shoots ranged from 20% to 80% which showed a dependence upon location and data collection times. However, at all five sites, the percentage of dead shoots was significantly higher in the treated than in the control plots.

Gliocladium virens grown on an autoclaved long-grained rice (Oryza sativa) medium produced a phytotoxin, viridiol, that caused necrosis of germinating cotton (Gossypium hirsutum L.) and redroot pigweed (Amaranthus retroflexus L.) seed in the laboratory (23). However, a dried and ground preparation of G. virens cultured on rice and worked into pigweed-infested soil above planted cotton seed produced viridiol in sufficient quantity and duration

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch016

to prevent emergence of pigweed without apparent harm to emerging cotton seedlings. Sucrose and ammonium nitrate on peat moss, a simple, more economical substrate, has recently replaced the rice grain (22). Peat moss, because of its acidic nature, enhances the stability of viridiol. Disadvantages of this system include high application rates and loss of persistence. Viridiol production peaks at five or six days after application and falls to undetectable levels by the end of two weeks. Because viridiol production by G. virens requires a high-nutrient substrate, a formulation such as alginate granules with appropriate amendments might produce and release viridiol more slowly and for a longer period of time.

Commercial Mycoherbicide Products

Cooperation among universities, government, and industry was essential in the commercialization of the mycoherbicides presented in Table IV. The formulation technology of these microbial agents constitutes proprietary information, but there are published reports of the utilization of these organisms in integrated pest management systems (31-36).

Collego (Ecogen Inc., Langhorn, PA) is a two-component product (26). One component is a water-suspendable dried spore preparation of the fungus *Colletotrichum gloeosporioides* (Penz) Sacc. f. sp. *aeschynomene* (CGA). The other is a rehydrating agent (a sugar) used for wetting the spores to improve germination. The dried spore component is packaged by the number of viable spores rather than by weight. Each package contains a minimum of 7.57×10^{11} viable spores.

Initial experiments with *C. gloeosporioides* showed that conidial suspensions sprayed until runoff gave good control of northern jointvetch (*Aeschynomene virginica* L., B.S.P.) (27). However, this biocontrol approach was slower than chemical herbicide control by 2,4,5-T [(2,4,5-trichlorophenoxy)acetic acid] because the incubation period of the fungus was 4 to 7 days, and the fungus did not kill the weeds for up to 5 weeks after treatment. Higher rates of inoculum application and humidity (warm, moist, cloudy weather and/or flooding of the rice fields) increased its effectiveness (28-29).

One of the commercial disadvantages of biological control is the host-specificity of the pathogen, i.e., only one weed is controlled per application. The fungus, *Colletotrichum gloeosporioides* (Penz) Sacc. f. sp. *jussiaea* (CGJ) was found to be highly specific for parasitism of winged waterprimrose (*Ludwigia decurrens* Walt.), a weed endemic in rice growing regions (30). CGJ was physiologically distinct from CGA as indicated by cross inoculation of their respective target weeds.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch016

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch016

Table IV. Commercial Microbial Agents

Weed	Microbial Agent	Commercial Name	Ref
Northern jointvetch *Aeschynomene virginica* (L.) B.S.P.	*Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *aeschynomene*	Collego	26-35
Stranglervine *Morrenia odorata* (Lindl.)	*Phytophthora palmivora* (Butl.) Butl. (chlamydospores)	DeVine	36-38
Round-leaved mallow *Malva pusilla* (Sm.)	*Colletotrichum gloeosporioides* f. sp. *malvae*	BioMal[a]	39
Waterhyacinth *Eichhornia crassipes* (Mart.) Solms	*Cercospora rodmanii* Conway	ABG-5003[b]	40-42
Sicklepod *Cassia obtusifolia* (L.)	*Alternaria cassiae* Jurair and Khan	MYD 751M (oil)[a] and MYX 104 (spores)	

[a] Nearing commercialization
[b] Experimental product - no longer available

A spore suspension mixture containing 2x10^6 spores/ml CGA and 1x10^6 spores/ml CGJ applied at 94 L/ha completely controlled both weeds, greatly enhancing the utility of this control method.

To increase the marketability of Collego, its compatibility with chemical pesticides has been investigated. Mixtures of CGA with propanil [N-(3,4-dichlorophenyl)propanamide], molinate [S-ethyl hexahydro-1H-azepine-1-carbothioate], 2,4,5-T, and benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] were detrimental to CGA's efficacy (31). If, however, propanil, 2,4,5-T, fentin hydroxide (triphenyltin hydroxide), pencycuron {N-[(4-chlorophenyl)methyl]-N-cyclopentyl-N'-phenylurea}, each at 0.56 kg ai/ha, and SN-84364 [3'-isopropoxy-2-(trifluoromethyl) benzanilide] (at 0.40 kg ai/ha) were applied after CGA treatment, disease and development were not inhibited (32). The herbicides, acifluorfen {5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid} (0.56 kg ai/ha) and bentazon [3-(1-methylethyl)-(1H)-2,1,3-benzothiadiazin-4(3H)-one 2,2-dioxide] (0.56 to 1.1 kg ai/ha), or the insecticides, malathion [diethyl(dimethoxyphosphinothioylthio)succinate] (0.56 kg ai/ha) and carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate), (0.56 kg ai/ha) could be applied with CGA from a single tank mixture (33-34).

Because acifluorfen applied postemergence effectively controls hemp sesbania [*Sesbania exaltata* (Raf.) Rydb. ex A. W. Hill] in soybeans (*Glycine max*), the combined application of the herbicide and CGA, respectively, controls hemp sesbania and northern jointvetch (35). The use of a low-volume (21.5 L/ha) controlled droplet applicator (CDA) (spinning-disc) with such a combination produced mixed results. If unfavorably dry environments existed when the CGA was used, infection was reduced resulting in unsatisfactory weed control. Benomyl (33) and propiconazol {2-[[2(2,4-dichlorophenyl)-4-propyl-1,3-dioxalan-2-yl]methyl]-1H-1,2,4-triazole} (32) applied sequentially 7 and 14 days after CGA suppressed the growth and development of the pathogen.

DeVine (Abbott Laboratories, North Chicago, IL) has as its active ingredient the live chlamydospores of *Phytophthora palmivora* (Butler) Butler. The commercial formulation is stable under refrigeration for only six weeks and must be applied to wet soil. The viability of DeVine may be reduced by the fungicides Aliett [aluminum tris(ethyl phosphonate)] and Ridomil [methyl N-(2-methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate]. If fungicides are to be used, application should occur 45-60 days prior to or after the DeVine treatment (36-38). Herbicides such as diuron [N'-(3,4-dichlorophenyl)-N,N-dimethylurea], glyphosate [N-(phosphonomethyl)glycine], and paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) also inhibited the efficacy of DeVine. The pathogen,

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch016

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch016

however, could be applied three weeks after spraying glyphosate (31).

The product, BioMal (PhilomBios, Saskatoon, Sask., Canada), which contains the mycoherbicide, *Colletotrichum gloeosporioides* f. sp. *malvae*, is nearing commercialization (Gantotti, B. V., PhilomBios, Saskatoon, Sask., Canada, personal communication). The fungus is a pathogen of round-leaved mallow (*Malva pusilla* Sm.), a common prairie weed in Canada that has become a problem in cultivated crops. An initial formulation of BioMal, using standard technology such as a silica gel carrier, has proven very effective in the field. The wettable powder formulation of this hydrophilic fungus disperses easily in water and is applied as a spray. However, for effective weed control, an overnight dew period is essential (39).

In spite of the efforts of several research groups over a 10-20 year period, there are no microbial agents now in commercial use for aquatic weed control. Abbott Laboratories had developed an experimental wettable powder formulation for *Cercospora rodmanii* Conway, a pathogen used to control waterhyacinth [*Eichhornia crassipes* (Mart.) Solms] (40). The formulation contained $4.54x10^8$ propagules per pound and the spray mixture called for Triton X-100 to be added by the user. One of the environmental obstacles to the efficacy of this pathogen was temperature. It must be applied when daytime temperatures are above 16°C and lower than 32°C. Also, when the host growth was rapid, long-term biocontrol with a single application was not obtained (41).

An approach now under evaluation is the use of combinations of *C. rodmanii* and insect biocontrol agents, or the pathogen plus sublethal rates of chemical herbicides (Charudattan, R., Univ. of Florida, Gainesville, FL, unpublished results). Experimental results confirmed that neither the pathogens nor the arthropods, mottled waterhyacinth weevil, *Neochetina eichorniae* Warner; chevroned waterhyacinth weevil, *N. bruchi* Hustache (both *Coleoptera*, *Curculionidae*); the moth, *Sameodes albiguttalis* (Warren) (*Leptidoptera*, *Pyralidae*); a mite, *Orthogalumna terebrantis* Wallwork (*Acarina*, *Galumnidae*); and the moth, *Arzama densa* Walker (*Lepidoptera*, *Noctuidae*), alone completely controlled waterhyacinth (60). By combining the pathogen with the arthropods, 99% control was achieved in 7 months. Limited experiments with sublethal rates of glyphosate, thidiazuron [1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea], ethephon (2-chloroethylphosphoric acid), 2,4-D [(2,4-dichlorophenoxy)acetic acid], and diquat (1,1'-ethylene-2,2'-dipyridylium ion), in combination with the pathogen did not enhance control of waterhyacinth.

An experimental formulation of *A. cassiae* to control sicklepod in soybeans and peanuts (*Arachis*

hypogea) has been developed by the Mycogen Corporation (San Diego, CA). It is a two-component product consisting of: (a) an emulsifiable paraffinic oil (designated MYD 751M), and (b) spores of A. cassiae (MYX 104, 100% ai). To prepare the spray mixture, the oil component is first emulsified in water at a 1% (v/v) concentration and then the spores are added and dispersed by stirring. This formulation is applied at a rate of about 0.4 lb spores/ha to sicklepod in the cotyledon to 2-leaf stage of development (Bannon, J. S., Mycogen Corp., Ruston, LA, personal communication).

Integrated Weed Management System with Microorganisms

In recent years, one priority in agricultural research to reduce the amount of herbicide applied. To do this, researchers have developed an integrated weed management system (IWMS) (49). Some examples of this approach have already been discussed. IWMS utilizes the different combinations and interactions between beneficial insects, chemical herbicides, microorganisms, and plant growth regulators to control a broad spectrum of weeds because no single agent gives optimum results.

The rust fungus, Puccinia canaliculata (Schw.) Lagerh., did not give complete biological control of yellow nutsedge (Cyprus esculentus L.) even under the best conditions for infection and dissemination (50). Urediniospore suspensions containing Triton B 1956 (0.1% v/v) gave only 60% control (53). However, application of the chemical herbicide, paraquat, after the rust epiphytotic was developed gave 99% control compared to 10% with paraquat alone. Preliminary results indicated that a beneficial interaction occurred when a sequential application of rust followed imazaquin (51-53). Enhanced control was also observed after using a mixture of the Maryland rust strain and bentazon (54).

Both insect-microorganism and chemical treatment-microorganism interactions have recently been studied in the control of velvetleaf by reduction of seed viability (55-57). Niesthea lousianica Sailer, a native scentless plant bug, has been found to reduce production of viable velvetleaf seed directly through feeding and promoting infection of seed with detrimental microorganisms. Three main fungal genera (Alternaria, Cladosporium, and Fusarium) and three bacterial genera (Pseudomonas, Eswinia, and Florobacterium) were consistently isolated from nonviable seed. Selection of the most effective microorganism for seed deterioration may improve this approach. Chemical treatment of the soil with ethephon, AC94377 [1-(3-chlorophthalimido)cyclohexane carboximide], butylate (S-ethyl di-isobutylthiocarbamate), or carbofuran plus Fusarium sp.

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resulted in nonviable seed heavily infected with mycelia (57). Ethephon or carbofuran in combination with Fusarium sp. also resulted in a reduction in emergence. The pathogen was delivered on a shredded wheat medium.

Another promising IWMS strategy to control velvetleaf involves the spray application of tank-mix combinations of the plant pathogenic fungus Colletotrichum coccodes (Wallr.) Hughes and the plant growth regulator thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-yl-urea) (58-59). The thidiazuron causes severe stress in velvetleaf by inhibiting stem elongation, leaf development, and flowering, and the Colletotrichum causes leaf necrosis and abscission. Use of this combination in field trials led to significant velvetleaf control and increased soybean yield.

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Epilogue

Researchers have taken different approaches in their efforts to improve germination of mycoherbicides and/or infection of target weeds. One approach has called for direct provision of water in carriers formulated to retard water evaporation. Except in those few cases where high humidity or dew is naturally present (in flooded rice fields, over ponds containing water hyacinths, or in wet soil), prediction of a dew fall is usually not adequate for reliable results. Preliminary investigation of water-holding formulations have shown promise, but weed mortality has not been sufficient or consistent. A second approach, the use of humectant-type adjuvants, e.g., a sugar, has been successful in the formulation of Collego. Sugars have also been used successfully in solid carrier formulations to promote growth of the pathogen (22).

A viable concept that has not been sufficiently exploited is the use of adjuvants that interact with the plant and/or microbial agent to promote the efficacy of the infection process. Because each disease and host biology combination is unique (see Chap. 11, 12), the formulator's challenge is formidable. Microbial interaction on plant surfaces should also be considered in the development of formulations for biological weed control. Researchers in integrated weed management studies have proposed that a variation of the concept is possible now with the addition of chemical herbicides and/or insects. The additional stress (chemical and physical injury) inflicted by these agents makes some microbial agents more efficacious. The promise of widespread use of microbial agents as herbicides may first be realized through integrated weed management systems that broaden the spectrum of weeds controlled by one application.

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Chapter 17

Bioherbicide Technology

An Industrial Perspective

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Technology which supports commercial development of new bioherbicides will continue to require integrated, multidisciplinary research efforts from public/private sectors for optimization of success. Discovery/isolation of stable, virulent bioherbicide candidates as well as strain improvement/long-term preservation form the foundation for commercial bioherbicide development. An economical, profitable means of propagule production is essential in determination of fermentation parameters, time of propagule harvest, and subsequent processing. Processing, which includes stabilization and formulation, is based on biological requirements of the organism for maintenance in a viable state prior to marketing and subsequent transition to a virulent state upon delivery to the weedy host. Upon delivery to the host, a major barrier to consistent performance of foliar-applied bioherbicides is the requirement for free moisture. Development of technology to circumvent this requirement will be tantamount to the successful commercialization of certain foliar-applied bioherbicides.

Natural antagonists have continued to suppress most pests including weeds for thousands of years (1). Weed predators such as insects and fish have accounted for successful biological control of prickly pear (*Opuntia* sp.), Klamath weed (*Hypericum perforatum* L.), and certain aquatic species (2-4). The use of perpetuating insect and fish species as well as obligate plant parasitic rusts (5,6) has been defined as the classical biological control tactic (7). It is interesting to note the documentation of the classical tactic to control Canada thistle [*Cirsium arvense* (L.) Scop.] in 1894 (8). Although the efficacy of the classical approach has been demonstrated, there is little or no profit incentive for industry. Therefore, research and development of this type of biocontrol strategy will be conducted primarily by the public sector.

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The bioherbicide approach is the other biocontrol tactic, as defined in several reviews (9-11). This approach provides the best opportunity for profitable commercial bioherbicide development as exemplified by DeVine [*Phytophthora palmivora* (Butl.)] (12) and Collego [*Colletotrichum gloeosporioides* (Penz.) Sacc. f. sp. *aeschynomene*] (13). DeVine is an efficacious bioherbicide for the control of stranglervine [*Morenia odorata* (H.&A.) Lindl.] in citrus. Since DeVine is efficacious within the soil matrix and kills seedling stranglervine plants before they become established, free moisture, i.e., dew/irrigation, is not a requirement to optimize activity of this bioherbicide. Moisture found in the soil matrix is sufficient for stranglervine seed germination, which subsequently causes an increase in *P. palmivora* levels, thereby controlling the weedy host (12). However, free moisture enhances efficacy of Collego (13,14) and other foliar epiphytic bioherbicides (15-19) on their respective hosts.

Free moisture is the most important environmental component of the disease triangle i.e. the interaction of the foliar epiphytic bioherbicide with its host and the environment. More importantly, an understanding of host-pathogen physiology/biochemistry as influenced by free moisture and other environmental factors will be more likely to yield solutions to bioherbicide efficacy problems caused by insufficient free moisture following host inoculation. Experiments described below examine host-pathogen physiology and identify factors that would improve bioherbicide efficacy and performance consistency with focus on free moisture interaction. Additionally, technology required to discover, maintain, and produce bioherbicides for commercialization is described below.

Bioherbicide Discovery

Since free moisture determines bioherbicide performance consistency and efficacy, it is expected that research and development strategies would be guided by this facet of the disease triangle. Bowers (20) has reviewed the role of industry in discovery of host-specific bioherbicides. The bioherbicide industry must continue to depend on the expertise of public sector scientists in diverse locations to discover host-specific bioherbicides. It is axiomatic that the requirement for free moisture will vary among bioherbicides. Thus, an assessment of this environmental variable should be foremost in preliminary studies on bioherbicide efficacy. The availability of free moisture in the target weed market and the response of the bioherbicide to free moisture are primary determinants in the decision to commercialize respective candidates. For example, the lawn and turf market would be more likely to augment natural free moisture through frequent irrigation than certain row crop markets. Thus, efficacy of epiphytic foliar bioherbicide candidates may be enhanced by matching the bioherbicide candidate with the appropriate market.

The survey method, which utilizes the weedy host to screen pathogens, has been utilized to discover most of the host-specific bioherbicide candidates currently under development. Again, the disease triangle, i.e., the host, bioherbicide pathogen, and the environment determine bioherbicide efficacy. Often, disease symptoms may not occur in epidemic proportions. As such, manifestation of disease symptoms may occur in a very narrow time span when all factors of the disease triangle interact. Thus, discovery of host-specific, foliar, epiphytic bioherbicide candidates is dependent on

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astute observation when symptoms are manifested on the host. Traditionally, scientists in the public sector have discovered most bioherbicide candidates because of proximity to field experiments, research stations, and more importantly, familiarity with plant diseases and symptomology. Utilization of the survey method to discover bioherbicide candidates by the biopesticide industry will continue albeit primarily through licensing efforts.

An alternative approach to discovery of bioherbicide candidates by the survey method is a genetic approach. In a recent review, Sands *et al.* (Chapter 10, this volume; Sands, D. C. *et al.* Weed Technol., in press) have stated that this approach increases the number of fungi useful as bioherbicides. Because broad host range pathogens are utilized in this approach, selective weed control must be achieved through mutation technology. Pathogen delimitation must occur to restrict host range and prevent spread and survival of this type of bioherbicide. Pathogen delimitation provides a method to control the amount of bioherbicide in the environment. Successful examples of pathogen delimitation of bioherbicide candidates have recently been reported (21; Miller, R. V. *et al.* Can. J. Microbiol., in press).

Successful discovery of efficacious bioherbicides will depend on implementation of an objective, balanced research strategy. Such a research strategy will depend on a cooperative research effort between public and private sector scientists. Focus on components of the disease triangle and target market characteristics will be tantamount to successful discovery of bioherbicides.

Bioherbicide Culture Maintenance

Maintenance of virulence and viability of bioherbicide cultures is a vital component of any commercially successful research and development effort. Several reviews on specific technology related to preservation and long-term storage of microorganisms have been written (22-25), and specific details will be omitted from this review.

Sensitivity of bioherbicide organisms to various preservation techniques will vary according to the specific organism. Selection of the optimum technique to preserve viability, virulence, and pathogenicity is guided by the type of propagule being preserved, pathogen species, and numerous other factors.

Alternaria cassiae Jurair and Khan is being developed as a bioherbicide to control sicklepod (*Cassia obtusifolia* L.). Sicklepod plants are inoculated with conidia of this bioherbicide. Thus, it is desirable to preserve viability, virulence, and pathogenicity of *A. cassiae* conidia. Accelerated ageing techniques are used routinely to predict viability of seed lots (26). Since fungal spores are similar to plant seed in certain aspects, an accelerated ageing technique was developed to rapidly assess *A. cassiae* conidia preservation methods. *A. cassiae* conidia were lyophilized according to established methodology. Lyophilized conidia were sealed in glass ampules and stored in an oven at 40° C for various time periods prior to bioassay on sicklepod plants. Non-lyophilized conidia were stored in open containers in the same oven at 40° C. Non-lyophilized conidia are normally stored in a desiccator at 5° C, and conidia were maintained in this manner as a control. Results of this study show that viability and virulence of *A. cassiae* are preserved best when conidia are lyophilized (Figure 1).

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch017

Thus, moisture removal is critical to preserve the integrity of *A. cassiae* bioherbicide propagules.

Maintenance of stock cultures for fermentation and production of bioherbicide candidates is another important aspect of bioherbicide culture maintenance (27). Uniformity and stability of stock cultures subsequently determine homogeneity of bioherbicide propagules. For example, virulence of *A. cassiae* declines after four culture transfers (Figure 2.). Production of homogeneous bioherbicide propagules is dependent upon a uniform source of inoculum.

Bioherbicide Propagule Production

Bioherbicide propagules may be spores, cells, mycelia, sclerotia, chlamydospores, etc. Propagules of the commercial bioherbicides, DeVine and Collego, are chlamydospores and spores, respectively. Production of bioherbicide propagules has been reviewed recently (27,28), and it is beyond the purpose of this report to elaborate the details of fermentation processes.

Bioherbicide propagules may be produced by either solid state or submerged fermentation processes. Both Collego and DeVine are produced by submerged fermentation process. Although solid state fermentations may approximate natural conditions for fungal growth (29), submerged fermentations may be more economical. *A. cassiae* conidia are produced by solid state fermentation (15).

The fermentation process and subsequent processing determine the efficacy of a bioherbicide propagule. A thorough understanding of the effects of fermentation and formulation on viability and virulence is required to guide these processes. For example, the carbon source in the fermentation medium has a profound effect on *Fusarium lateritium* Nees ex. Fr. efficacy (Figure 3). Additionally, carbon source and other components of the medium and its physical/chemical environment determine induction/yield of bioherbicide propagules.

After an economical yield level of bioherbicide propagule has been achieved in a fermentation process, formulation becomes a critical factor which influences product efficacy. Because the fermentation must be stopped at a point when virulence/viability are optimum, the live bioherbicide propagule must be stabilized, formulated, and packaged. Physiology of dehydration and rehydration must be examined if the stabilization process involves propagule drying. It has been stated that spore dehydration and rehydration research was the greatest challenge in the development of Collego (13). The chlamydospore formulation of DeVine required a refrigerated distribution system to maintain stability (12).

Host-Pathogen-Environment Interactions

Effects of Free Moisture. Availability of free moisture after host inoculation with a foliar, epiphytic bioherbicide is critical to the initiation of the infection process. Experiments on free moisture (dew) interactions with other environmental parameters and the host-pathogen system are usually conducted in temperature-controlled dew chambers. This type chamber is constructed such that the plant radiates energy to a heat sink which is the temperature-controlled wall of the chamber. The humid air in the chamber is also temperature controlled, and dew forms on the plant

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch017

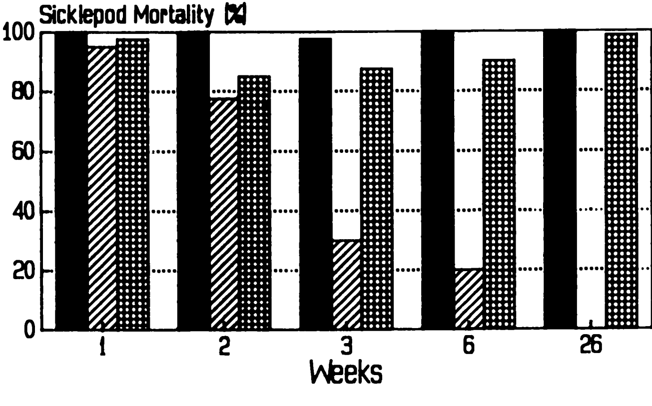

Figure 1. Effect of *Alternaria cassiae* spore storage environs on sicklepod mortality. *A. cassiae* spores stored in a desiccator refrigerated at 5° C was the control treatment. A sublot of these spores was stored similarly at 40° C in sealed glass ampoules at 40° C after lyophilization (L). Another sublot was stored in open containers at 40° C but not lyophilized.

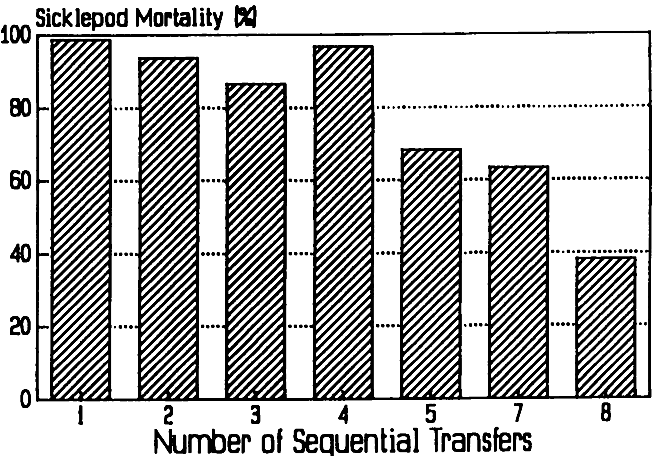

Figure 2. Effect of sequential, weekly culture transfer on sicklepod control by *Alternaria cassiae* grown on V-8 agar (52). Spores were harvested from plates in a solution of 0.02% (v/v) Sterox NJ surfactant. Sicklepod plants in the cotyledon to 1-leaf growth stage were inoculated with *A. cassiae* spores (10^5 spores/ml), then subjected to a 6-h dew period at 25° C. Sicklepod plants were evaluated 14 days after inoculation.

when the host plant temperature reaches or falls below the dew point of the air.

In the host establishment of plant pathogens for experimental purposes the incubation period frequently exceeds 24 h. In this case the period of host tissue wetness will approximate the actual incubation time. It is desirable for a bioherbicide to infect plant tissue with minimum dew requirements because (a) the period of host tissue wetness will vary considerably under natural field conditions, and (b) the unpredictable frequency of dew occurrence in nature. If the experimental dew period in a dew chamber is brief, the actual period of host tissue wetness may not correlate with the actual incubation time. This may be dependent on host species as well as interaction of the host species with the physical parameters of the dew chamber. A lengthy dew period may be sufficient to induce runoff and displace propagules from the host. A lengthy dew period may also give misleading results with host-pathogen-chemical herbicide interactions.

Incubation of the host tissue after inoculation with a plant pathogen or bioherbicide is the most important factor determining success (efficacy) of the inoculation (30). When velvetleaf is inoculated with *F. lateritium* spores (10^6 spores/ml) harvested from petri plates (31,32), incubation in an environment conducive to dew formation is required immediately after inoculation (Figure 4). A delay of 4 h from host inoculation until the occurrence of dew resulted in a 50% decline in efficacy of this bioherbicide.

"Predisposition" is a term used to describe the influence of environmental factors on host susceptibility. Environmental predisposition has been reviewed extensively by Colhoun (33). Research has shown that the decline in bioherbicide efficacy of *F. lateritium* caused by a delay in occurrence of dew (Figure 4) can be circumvented by predisposing the host with rain. Predisposition of velvetleaf with simulated rain (1.3 cm) resulted in nearly 100% kill after a 1-h delay in dew occurrence (Figure 5).

<u>Effect of Spore Dormancy</u>. Spore dormancy may contribute to a decline in bioherbicide efficacy. Spore dormancy in fungal spores may be maintained by chemicals as well as metabolic blocks (34). The presence of inhibitors may be indicated by an increase in efficacy upon addition of an adsorbent such as activated charcoal to the bioherbicide at the time of inoculation. This was the case when adsorbent was added to *F. lateritium* spores (10^6 spores/ml) and the occurrence of dew (20 h) was delayed for 8 h after inoculation (Figure 6). The agent causing spore dormancy may be synthesized by either the spore or host.

<u>Effect of Chemical Herbicides</u>. The interaction of chemical herbicides with bioherbicides has been reported as a means to improve the spectrum of weed control as well as efficacy of the bioherbicide (9,35). In the laboratory evaluation of chemical herbicide/bioherbicide combinations, it is important to study the efficacy of each component separately prior to evaluation of combination treatments. Thus, the activity of a given chemical herbicide must be evaluated in the same dew period regime as a given bioherbicide prior to evaluation of combination treatments. Rates of chemical herbicides are tested at less than optimum levels because of higher phytotoxicity observed under laboratory conditions. Chemical herbicide treatments to be combined with biological

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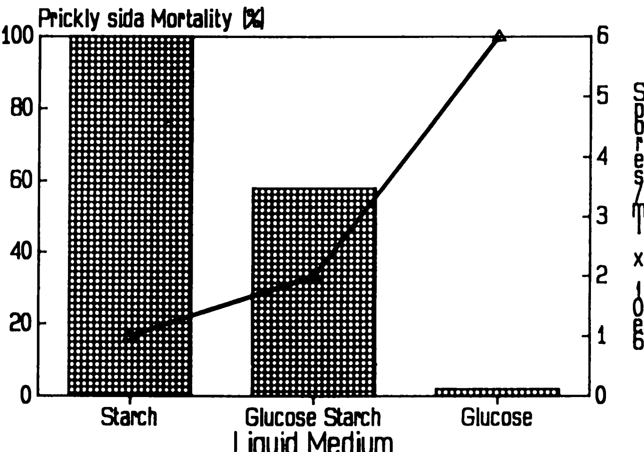

Figure 3. Effect of carbon source on efficacy and spore yield of *Fusarium lateritum*. Spore yield (Δ-Δ) was inversely related to virulence. Proprietary fermentation medium contained starch, glucose-starch, or glucose as the primary carbon source. Spores were harvested from the fermentor and immediately used at 10^6 spores/ml to inoculate prickly sida (*Sida spinosa* L.) in the primary leaf growth stage. Plants were subjected to a 20-h dew period at 25° C, removed to greenhouses for observation, and evaluated 14 days after inoculation.

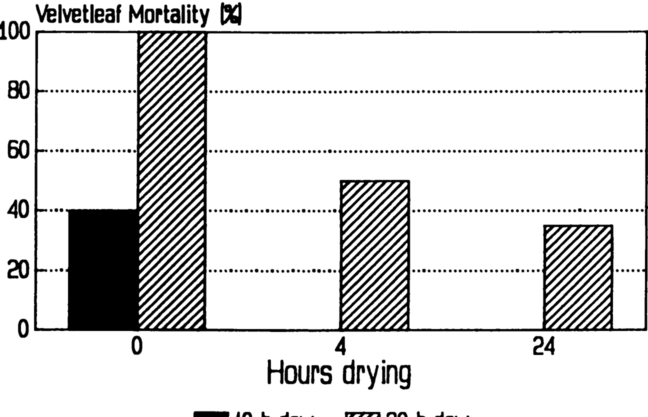

Figure 4. Effect of propagule drying time (dew-delay) prior to a 10-h or 20-h dew period on *Fusarium lateritium* efficacy on velvetleaf. Velvetleaf plants in cotyledon to 1-leaf growth stage were inoculated with *F. lateritum* spores (10^6 spores/ml) harvested from petri plates (31,32). Dew chamber (Percival Manufacturing Co.) air temperature was 25° C. Dew was delayed (drying time) for indicated times.

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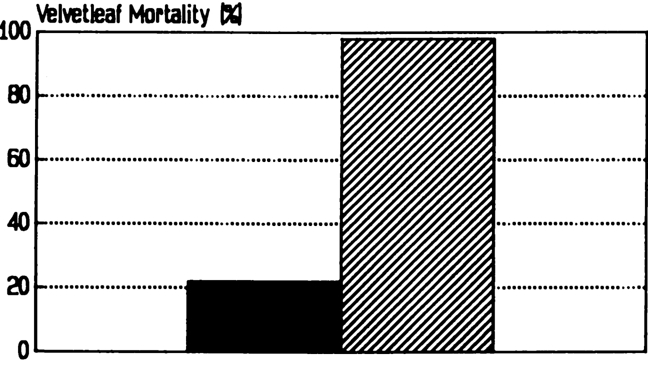

Figure 5. Effect of host (velvetleaf) predisposition with simulated rainfall (1.3 cm) prior to inoculation at cotyledon to 1-leaf growth stage with *Fusarium lateritium* (10^6 spores/ml). Incubation in a dew chamber at 25° C was delayed for 1 h after inoculation. Spores were produced and harvested as described previously (31,32).

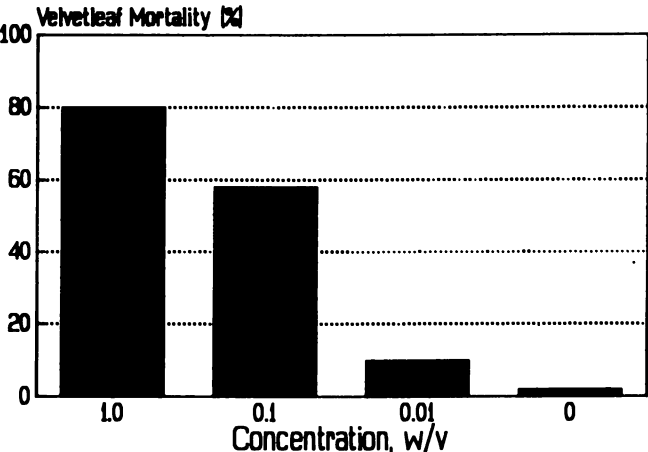

Figure 6. Effect of adsorbent (activated charcoal) concentration on *Fusarium lateritium* efficacy on velvetleaf inoculated at cotyledon to 1-leaf growth stage with 10^6 spores/ml. Spores were produced and harvested as described previously (31,32). Incubation in dew chamber was delayed for 8 h after inoculation.

herbicides may also include surfactants dependent upon the sensitivity of the bioherbicide to these substances.

Several chemical herbicides were examined for efficacy on velvetleaf (*Abutilon theophrasti* Medic.) after placement in a dew chamber for 20 h at 25° C. Because many chemical herbicides have a label restriction that defines a drying time prior to exposure of herbicide-treated weeds to rainfall, drying time was varied prior to placement in a dew chamber. Bentazon [3-(1-methylethyl)-(1H)-2,1,3-benzothiadiazin-4(3H)-one, 2,2-dioxide] activity on velvetleaf increased very slowly as drying time increased until 70% velvetleaf control was attained for those plants remaining dry for the entire experiment (Figure 7). Velvetleaf control by 2,4-DB [4-(2,4-dichlorophenoxy) butanoic acid] reached nearly 100% after 1 h drying time (Figure 7). In addition to suggesting differential uptake of 2,4-DB and bentazon by velvetleaf, this experiment clearly shows the importance of understanding response of chemical herbicides in the experimental systems used to evaluate bioherbicides. When acifluorfen [5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid] activity on velvetleaf was observed in a dew chamber environment after exposure to increasing drying time after application, herbicide activity reached a peak after 1 h drying, slowly declined, then reached another peak at the 24 h drying period (Figure 8). It is interesting to note that the label for acifluorfen specifies a 6-h drying period after application for minimizing effects of rainfall. Under conditions of these tests in which chemical herbicide was applied in 1871 L per hectare with a laboratory atomizer, treated 1-2 leaf velvetleaf plants were placed in a greenhouse for specified drying times then placed in a dew chamber for 20 h at 25° C; observations were made 14 days after treatment. Obviously, in order to optimize effects of both a chemical herbicide and bioherbicide as a combination treatment, the effects of drying time prior to an incubation period must be thoroughly understood for both the biological and chemical herbicide agents.

Effect of Spray Adjuvants. The effect of a surfactant with acifluorfen is also shown in Figure 8. The effects of chemical herbicides in the presence or absence of a surfactant must be determined in experimental systems used to evaluate bioherbicides. The effect of surfactants/adjuvants on chemical herbicides are many and varied and have been reviewed recently (36). It is axiomatic that many adjuvants are toxic to microorganisms (37-39), thus, the interaction of spray adjuvants with bioherbicides must be evaluated on a case-by-case basis.

The apparent hydrophobicity of *A. cassiae* spores prevents even dispersion in aqueous spray systems without the use of a surfactant. Although non-ionic surfactants such as Sterox NJ {nonoxynol (9 to 10 POE) [α-(*p*-nonylphenyl)-ω-hydroxypoly (oxyethylene)]} have been used to disperse *A. cassiae* spores (15), it was found that emulsified oils are superior to non-ionic surfactants for this purpose (40) (Figure 9). This may be attributed to the fact that binding of *A. cassiae* spores to the host is superior when emulsified oils 1% (v/v) are included in the spray solution. When drying time following host inoculation with *A. cassiae* spores is varied prior to 0.6 cm simulated rainfall, efficacy of this bioherbicide on sicklepod is increased in the presence of emulsified oils compared to non-ionic surfactants (Figure 10). The adhesive effect of oils, surfactants, and other spray adjuvants is known (41).

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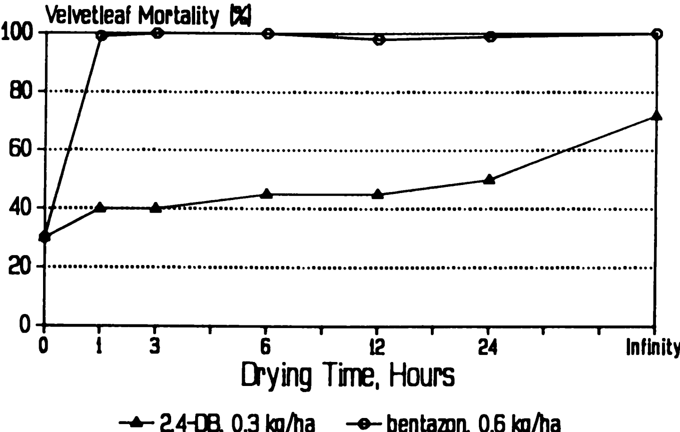

Figure 7. Effect of drying time prior to dew chamber incubation for 20 h at 25° C on 2,4-DB or bentazon efficacy on velvetleaf. Observations were made 14 days after treatment, and herbicides were applied at rates indicated in 1871 L/ha. Herbicide solutions contained X-77 surfactant, 0.25% (v/v).

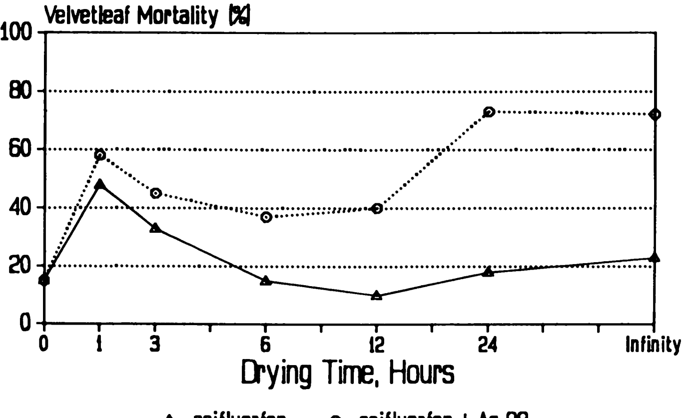

Figure 8. Effect of drying time prior to dew chamber incubation (20 h) on acifluorfen efficacy on velvetleaf. Triton AG-98 is a non-ionic surfactant, and it was added to the diluent at a rate of 0.25% (v/v) where indicated. Herbicides were applied to 1-2 leaf velvetleaf plants in 1871 L/ha and observations were recorded 14 days after treatment.

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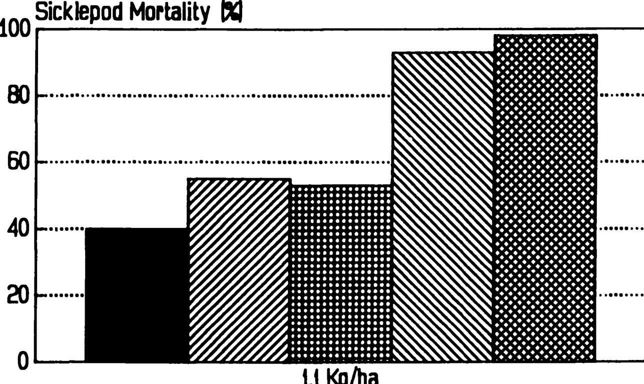

Figure 9. Effect of non-ionic surfactants 0.25% (v/v) (X-77, Sterox NJ, AG-98) or emulsified oils 1% (v/v) (Agri-Dex, Soy-Dex) on efficacy of *Alternaria cassiae* (1.1 kg/ha) under field conditions. Sicklepod plants in cotyledon to 1-leaf growth stage were inoculated with *A. cassiae* spores in 337 L/ha. No dew formation occurred for 24 h after inoculation. Overhead irrigation (0.6-1.3 cm) was applied to plots 24 h after inoculation. Observations were recorded 4 weeks after treatment. Spores were produced by a proprietary modification of the method of Walker (15).

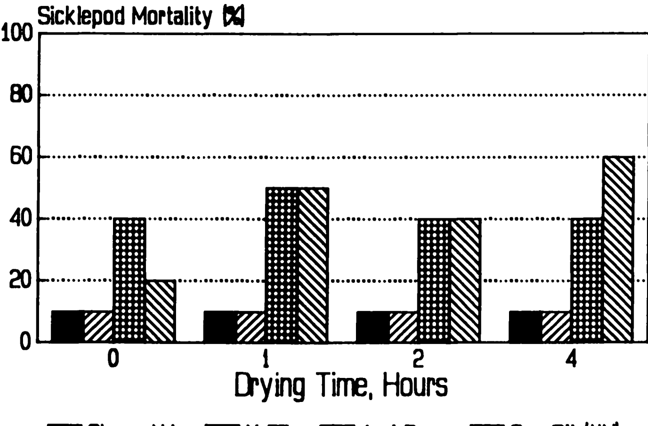

Figure 10. Effect of drying time prior to simulated rainfall x spray adjuvant interaction on efficacy of *Alternaria cassiae* (1.1 kg/ha). Spores were produced by a proprietary modification of the method of Walker (15). Sicklepod plants in cotyledon to 1-leaf growth stage were inoculated with *A. cassiae* spores in 1871 L/ha containing 0.25% (v/v) Sterox NJ or X-77; 1% (v/v) Agri-Dex or Sun Oil (11N). After indicated drying times inoculated plants were subjected to 0.6 cm simulated rainfall and placed in a dew chamber for 8 h at 25° C. Plants were removed to a greenhouse and observations were recorded 14 days after inoculation.

Recently, Quimby *et al.* (42) have shown that an invert emulsion greatly improves efficacy of a bioherbicide, *A. cassiae*, in the absence of free moisture. Further research is in progress to evaluate effects of invert emulsions and their formulation components on bioherbicide efficacy, especially in the absence of free moisture.

Effects of Temperature. Walker and Riley (15) characterized the efficacy of *A. cassiae* on sicklepod under different temperature regimes. Optimum efficacy was observed at 25° C, and efficacy declined significantly at 35° C. It would be advantageous for a bioherbicide to control its respective host(s) over a wide temperature range. Laboratory tests (unpublished data) have shown that *A. cassiae* spores germinate at 35° C on the host tissue but do not infect or kill the host. *A. cassiae* is a necrotrophic bioherbicide, which, conceptually, means that a toxin is produced in advance of the colonizing bioherbicide (43). The bioherbicide then lives on dead plant tissue as a saprophyte. The AK-toxin production characteristic of *Alternaria alternata* is inactivated by heat (44). Perhaps a similar phenomenon is occurring in *A. cassiae* although specific toxins responsible for observed sicklepod phytotoxicity have not been identified. It should be noted that Hradil *et al.* (45) recently identified four phytotoxins from *A. cassiae*, and all of these compounds exhibited a low level of phytotoxicity to sicklepod. Further research is required to understand metabolic control of processes relating to virulence and pathogenicity of bioherbicides.

Source-Sink Relationships. Cellular sugar levels often influence pathogenicity of certain diseases. Glucose inhibition of carbohydrase production by *Alternaria solani* (Ell. and G. Martin, L. R. Jones and Groust) was reported by Sands *et al.* (46). Carhohydrases have been reported to be involved in mechanisms of pathogenicity (47-50). The fact that cytokinins can simulate the accumulation of nutrients at the site of application is well-known by plant scientists (51). In order to simulate a sink effect, leaflets on one side of a mature sicklepod leaf were treated with a 30 µM solution of kinetin; whereas, leaflets immediately opposite were treated with the diluent only used to dissolve the kinetin. The leaf was then treated with a 1.1 kg/ha (10^5 spores/ml) rate of *A. cassiae* and incubated in a dew chamber for 8 h at 25° C. It was observed that the kinetin-treated leaflets failed to develop severe necrosis, whereas, the untreated opposite leaflets developed necrotic symptoms and senesced. A second experiment was initiated to examine the effects of a photosynthesis inhibitor to decrease sicklepod leaf sugar levels followed by an application of *A. cassiae* spores. When the photosynthesis-inhibitor, linuron, was applied to sicklepod and followed 3 to 5 days later with a 1.1 kg/ha application of *A. cassiae*, effective control of 3 to 5 leaf sicklepod was obtained (Table I). Normally, *A. cassiae* is effective only on cotyledon to 1-leaf sicklepod plants. Although leaf sugar levels were not analyzed, these data suggest that further research should be initiated to explore interactions between intermediary metabolites and enzyme systems which influence pathogenicity of bioherbicides.

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Table I. Effect of *Alternaria cassiae* (1.1 kg/ha) on Sicklepod Control in Simultaneous or Sequential Combination With Linuron (0.1 kg/ha)

Treatment	Rate, kg/ha	Sicklepod Mortality, %
A. cassiae[1]	1.1	10
linuron	0.13	20
linuron + *A. cassiae*[2]	0.13 + 1.1	40
linuron + *A. cassiae*[3]	0.13 + 1.1	85

[1] Sicklepod plants inoculated with *A. cassiae* spores suspended in water containing emulsified crop oil 1%, v/v (MYD 751, Mycogen Corporation proprietary). Diluent containing spores was applied at 1871 L/ha to sicklepod plants in the 3 to 5-leaf growth stage. Dew period immediately following inoculation was 6-h at 25° C.

[2] *A. cassiae* spores tank-mixed with linuron.

[3] *A. cassiae* spores applied as in footnote 1 but 3 days after linuron application.

Summary. Successful discovery and development of novel bioherbicides will continue to be dependent upon an integrated research strategy involving both public and private sectors. Development of products which perform consistently will be based on an understanding of the bioherbicide and its interaction with the host and its environment. Research to-date suggests that free moisture is the most important environmental variable influencing consistent bioherbicide efficacy. However, other factors which affect propagule production, stabilization, pathogenicity, and virulence must be examined to successfully develop bioherbicides. The influence of proper market focus should not be excluded in research/commercialization strategies.

Moisture is a key component of the plant disease triangle. As such, it influences propagule stability through physiology of rehydration and dehydration. Proper moisture removal affects long term propagule storage. Interaction of free moisture (dew) with the bioherbicide propagule determines herbicide efficacy on the host. Although other factors such as spray adjuvants, predisposition, physiological state of the host, and chemical herbicide interactions also influence bioherbicide efficacy, predominant effects on host-pathogen interactions are exhibited by moisture. A proper understanding of bioherbicide physiology and biochemistry as influenced by moisture and other environmental variables will result in more consistent, efficacious bioherbicides.

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Chapter 18

Weed Control with Pathogens

Future Needs and Directions

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An increasing emphasis on biological alternatives to chemicals for pest control is expected because of the persistent lack of confidence in synthetic chemical pesticides by a discerning society. Chemical residue contaminations of food, water, soil and the environment from use or misuse of pesticides are undeniably strong emotive issues that lead to an even more stringent regulatory climate and consequently to cost increases for chemical pesticide development. The trend toward biologicals is further strengthened by rapid development of pest resistance to the safer, metabolically specific chemical pesticides.
The shift from chemical based to biologically based pest control technology will require substantially more public and private sector input into target and agent biology at the ecosystem, organismal and molecular levels than is now committed, and significant re-direction of genetic engineering research efforts to strain improvement of potential biological pesticides is required.

Discovery and development of biological alternatives to chemical pesticides is an essential agenda for agricultural scientists faced with the daunting tasks of protecting crops and livestock on a large scale from the wide range and ever shifting spectrum of agricultural pests such as weeds, insects, diseases and nematodes. Chemical pesticides are excellent tools in today's crop protection technology but they are not the only nor necessarily the best means for every pest problem. Although chemicals have proven safe, effective and economical for control of most pests, heavy reliance upon a single control strategy has created formidable problems(1,2,3,4,5,6,7,8,9,10).

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Even with proper use, chemicals may be hazardous. Toxic residues can accumulate in food, soil, ground and surface water (1,3,4,5,6,9,10). Injury to non-target crops and endangered species can occur and toxic metabolites may occur in food chains (1,5). Resistance may develop in the target pests, requiring increased numbers of treatments at higher rates and resulting in increased exposure and pollution of the environment(8). Beneficial organisms are often eliminated, resulting in pest resurgence to more serious levels or previously minor pests becoming a major problem due to destruction of their natural enemies (2).

Widespread use of chemical pesticides in the home and in agriculture has led inevitably to greater misuse and greater risks to users and consumers, like pesticide-treated grain misused as hog feed, and contamination of a regional milk supply (6,9,10). Contamination of human food supply on a national scale was the case recently when California watermelons were contaminated by unapproved treatment with a systemic nematicide/insecticide (9,10).

Significant environmental and human health hazards have arisen at specific non-agricultural sites as a result of our requirement for synthetic chemical pesticides (4,5). Contamination of ground and surface water supplies from toxic-waste dumps or accidental spills has affected many regions remote from areas of pesticide use. Storage, transport and disposal of toxic materials and waste products are an increasing risk to the country as our dependence upon chemical pesticides grows. The social and economic consequences can be neither known precisely nor predicted with certainty, but the risks are substantial and increasingly recognized as analytical techniques improve and our understanding grows about the fate in the environment of synthetic chemical, their manufacturing contaminants and their breakdown products. The risks are of concern to pesticide producers, users, and government regulators and to the public in general. Alternative pest control measures are clearly needed. Prudence requires that we find and employ safer types of pesticides, agents whose mode of action is something other than innate toxicity (1).

Biological control of pests with their natural enemies can reduce our dependence upon toxic synthetic chemicals. In this presentation I will give an overview of biological control of pests: 1. Strategies used in biological control, its safety and barriers for development; 2. Research opportunities for biological herbicides in weed control and selection of improved strains of biological control agents; and 3. Future needs and direction in biological control of weeds.

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Biological Control Strategies, Safety and Barriers for Development.

The strategy of biological control of pests with their natural enemies is done by use of the following tactics:

a. The Classical Biological Control Tactic - Importation of exotic natural enemies for release, dissemination, and self-perpetuation on the target pest.

b. The Integrated Pest Management Tactic (IPM) - Conservation and enhancement of indigenous natural enemies.

c. The Biological Pesticide Tactic - Augmentation of

indigenous natural enemies with reared or cultured agents by application to pest populations.

Each tactic is scientifically sound and has been demonstrated to work. Unlike toxic synthetic chemicals, biological tactics have had no known nontarget effects (1,11,12,13). They all are dependent on fundamental knowledge of biological interactions at the organismal and ecosystem levels. Intensive, usually long-term research is required to find and understand the unique biology of specific agent/target combinations, evaluate potential of a candidate agent and determine empirically its utility in managed ecosystems. The expense of this research must usually be borne by the public sector. There is no profit incentive for the private sector to research and develop most biocontrol tactics, with the exception of certain agents used as biological pesticides in large markets.

We are thus faced with the paradox: biological control, although environmentally sound and advantageous generally, is not economically attractive to the private sector. In the past we opted for chemical control, a short-term economic solution at the expense of long-term environmental and human safety considerations -- thus our too-heavy dependence upon chemical pesticides. In the future we must invest more public funds in biological control research and development to ensure greater utilization of this environmentally compatible pest control technology and to bring biological pest control up to appropriate levels in our pest control efforts. The need is intensified by the fact that fewer and fewer chemical pesticides are available from the private sector. The cost of research and development of a new chemical pesticide, including cost of registration and liability, is currently estimated to be approximately 28.1 million dollars (14). Such costs mandate that only pests that represent large market potential be targeted for development by the private sector. Consequently, many pest problems are currently left unaddressed.

Economic benefits from public investment in biological control research accrue directly to the producer and ultimately to the consumer as pest control expense is diminished or eliminated from cost of production. The public also benefits indirectly by reduction in cost of regulating and monitoring for pesticide residues in fresh and processed food, ground water, and habitats. Public investment in biological control research and development is not only essential for safety and environmental considerations but also economically sound from a national perspective. There is a substantial body of knowledge about pests and their natural enemies, and the theory and practice of classical biological control, integrated pest management, and biological pesticides sufficient to ensure that greater public investment in this research effort would be productive and expand effectiveness of non-chemical pest control.

Barriers to the more general development of biopesticides are chiefly economic, not human hazard or environmental risks. The private sector is not willing to invest in research to discover and develop these environmentally compatible pesticides because their high pest specificity usually limits market potential. Public sector research is required to overcome the disincentives to commercialization, to demonstrate for each pest class that

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biological agents are available, effective, economical and consistent with the national interest to improve environmental quality and lessen human health hazards.

Research Opportunities - Biological Herbicides for Weed Control.

Natural enemies of weeds that may be used as biological pesticides include fungi, bacteria, viruses, nematodes, and insects. Fungi have the greatest potential because they can gain entry into the host plant without the assistance of a vector, and many may be mass produced in fermentation tanks with existing technology then dried and marketed just as bread yeast or brewing yeast.

Fungal diseases of weeds are common, but until recently they have not received much research attention: plant pathologists have emphasized research to control diseases of economic crops. Weed diseases have often been noted by weed scientists as confounding factors in weed research plots and by growers faced with severe weed infestations in crops for which chemical herbicides are either unavailable or inadequate to control a particular weed.

Only recently has research focused on use of fungal pathogens as biological pesticides for weed control. Three fungi, mycoherbicides, are currently used in the U.S., and two others are expected to be approved for commercial use in the near future. One is available in The People's Republic of China, and active projects are known to be underway in 14 other countries. Sufficient exploration has been conducted in the U.S. and abroad to illustrate that, although there are numerous fungal pathogens of weeds with potential, they are not uniformly distributed with the weed hosts and they do not all have equivalent potential as biological herbicides. Some have fastidious nutritional requirements for growth and reproduction in artificial culture, and some are widely distributed and commonly encountered but exhibit low virulence even when applied as inundative inoculum to young plants. Some have strict environmental requirements that limit their use to particular times or within geographic limits that are impractical in comprehensive pest control systems.

Strain Selection and Improvement Research

Current research on herbicide potential of fungi has revealed many opportunities for strain improvement research (15,16,17). For example, many weed-damaging fungi have fastidious nutritional requirements for growth and reproduction or particular physical requisites for reproduction in artificial culture. Orange rust and white rust of morningglory,(Ipomomea spp.), exemplify opportunities in fungal nutrition research. These two diseases routinely devastate mature populations of weedy morningglory in grower fields, too late, however, to benefit the growing crop. Effective use of these two fungal pathogens as biological pesticides depends upon having a supply of artificially cultured inoculum for application to young seedlings before they compete with the crop. Neither fungal pathogen can be grown in artificial culture with existing technology, so research is needed to overcome this barrier and make possible the use of these two ubiquitous fungi as mycoherbicides.

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch018

Extension of such new culture technology to other orange and white rust pathogens would enable the development of countless other rust species to control their specific weed hosts.

The development of certain weed pathogens as biological herbicides is constrained by innate low virulence or loss of virulence in artificial culture (15,16). Species of the common fungal pathogens, Alternaria, Cercospora, and Helminthosporium, for example, generally are too weakly virulent to kill their weed host even when applied as inundative inoculum to seedling plants. Infected plants may be diseased but not debilitated enough to remove them from competition with the crop. Research to understand and overcome this constraint would permit development of mycoherbiciees for some of the serious weed problems now only marginally controlled by chemical herbicides or those not currently controlled by chemicals at all.

Many fungal pathogens of weeds are not developed as mycoherbicides because they lose virulence in artificial culture and will not infect their specific hosts when inoculated. The fungal pathogen of persimmon wilt disease, for example, can be used to rid pastures and rangelands of weed persimmon, (Diospyros virginiana L.), only after limited subculture in the laboratory, yet it is non-infectious when culture conditions are scaled-up for large-scale trials. Infectivity can be restored by re-isolation of cultures from natural infections or passage of weak isolates through the host -- procedures too impractical for large-scale production needed for control of this widespread intransigent weed problem. This type of virulence loss is commonly encountered among fungal pathogens of plants, insects, or nematodes; therefore, research to understand the physiological or genetic basis for it, so that virulence can be stabilized, would enhance the biological control potential of many specific fungi for control of several classes of pests.

In some instances the potential of specific fungal strains is diminished by strict requirements for particular environmental conditions or sequences for infection, colonization, or reproduction in the host plant. The strain thus may be limited to use in narrowly circumscribed geographic locations or at particular times in the season. The weed killing fungus, Colletotrichum malvarum, for example, can kill its host plant if inoculated when daily maximum temperatures are below 32° C. Unfortunately the weed host, prickly sida, (Sida spinosa L.), emerges and becomes weedy throughout most of its range when maximum temperatures are above this limit; therefore, use of the fungus is severely restricted and impractical. Efforts to collect strains of the fungus throughout the range of its host have not revealed any with sufficiently broad environmental adaptation. Physiological and genetic research to understand the molecular basis for this type of environmental restraint on pathogens and means of overcoming it could accelerate efforts to utilize fungal pathogens for all types of biological pesticides.

Molecular genetic techniques now available hold great potential for improving strains for biological pesticide development. For example, low-virulence strains may be enhanced to improve efficacy. Species difficult to grow or that reproduce poorly in artificial culture may be genetically altered to make them available in large

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quantities and practical for field use. Unstable strains that lose virulence in artificial culture may be genetically stabilized and thus available for widespread use. While much of what can be envisioned is speculative at the moment, the power of the new technology is not.

Technology is now available for the physical isolation of genes from bacteria and fungi, for recombination of these genes in the laboratory, and for inserting them into the organism from which they came or into other organisms (17). The research challenge is to understand host-parasite interaction at the molecular level sufficiently so that molecular biological methods can be used to address strain improvement of biological control methods. For example, the enzymes, cutinase, pectin lyase, and polygalacturonase have been found to be operative during infection, colonization, and symptom development by Colletotrichum, an important fungal pathogen with demonstrated mycoherbicide capability (18). Identification of the gene that controls production or activity of these enzymes might be used to transform other Colletotrichum species or even other fungi that are specific for, but non-lethal to, important weed hosts and thus enhance mycoherbicide potential of these fungi. Genetic vectors are available and have been used to transform Colletotrichum with antibiotic and nutritional marker genes; therefore, transformation of this fungus with genes that are influential in pathogenesis is plausible.

A similar example can be cited for genes that control appressorium formation, a structure essential in many fungi for host penetration. Molecular genetic probes have been used to demonstrate that these genes become operative upon initiation of this organ in a rust fungus (19). Isolation of these genes and transformation of certain fungi with them would improve penetration efficacy of strains and thus mycoherbicide potential.

A less advanced but perhaps even more plausible example is transformation of fungal strains with genes that control production of highly specific pathotoxins. Many fungi are known to produce biochemically specific, low molecular weight metabolites in the disease development process. Enhancement of strains with genes that control production or activity of these metabolites is a clear researchable goal that would improve mycoherbicide potential of many non-lethal, host-specific fungi (17).

Future Needs and Directions

Greater research emphasis needs to be placed on exploration, both domestic and foreign, to find new fungi on weeds and new strains of known weed pathogens. Surveys should include plants now known to be weedy but also plants that have potential of becoming weedy by succession because of their tolerance to selective chemical herbicides now in use. Specimens of these fungi and isolates in culture should be maintained and renewed periodically to assure maintenance of virulence and reproductivity and to assure that a broad spectrum of the genetic diversity available in the organism is well represented in the collection.

New developments and prospects in biotechnology give cause for optimism also. It can be expected that powerful new genetic

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch018

engineering tools can be used to enhance existing biocontrol tactics, improve our ability to manipulate the agent practically or make possible entirely new tactics based on understanding molecular level interaction between natural enemies and pests that can be manipulated to advantage. Some of these developments have profit potential and are attractive to the private sector. Public investment in biotechnology with biological control agents is required, however, to ensure full utilization of these powerful new techniques in our effort to develop biological control as the foundation of pest control in the U.S.

A significant shift away from chemical pesticides to greater reliance upon biological control methods is restrained by lack of knowledge. More information is required at the organismal and molecular levels. The uniqueness of the biology of each agent/target combination plus the complexity of their interaction with other organisms in the ecosystem requires intensive specialized research plus complementary interdisciplinary teamwork. Molecular interactions are equally complex and require multidisciplinary teams of well-trained scientists. Such teams include geneticists, biochemists, molecular biologists, microbiologists, physiologists, plant pathologists, entomologists and ecologists. A comprehensive nationwide, pluralistic research effort on biological control is needed to remove restraints to development and make greater use of this pest control resource. A pluralistic approach is essential because the agents and mechanisms occur naturally and may be discovered in localized geographic areas, yet they can be operative and manageable in entirely separate regions and ecosystems (1,12).

Research on molecular biology must be broad based also. Fundamental understanding of biological interactions at the molecular level permits utilization of genes and their biologically active products in biological control tactics in ways quite remote from their natural occurrence. Genes and their biologically active products can be identified and isolated in one living entity and then used to transform and enhance biological control potential of another more suitable species. There is optimism that the many new techniques in biotechnological research will be the key to development of biological control as the primary pest control method in the United States (1).

New knowledge about potential biopesticides in required to make this biocontrol tactic more generally available. Candidates may be exotic or indigenous microorganisms, specific natural enemies of particular introduced or indigenous pest species. These natural enemies normally occur at insignificant levels but may be increased in fermentation or rearing facilities, applied as sprays, and integrated into pest management systems. Those not reaching their natural pest hosts die and deteriorate in the natural cycle. Biological pesticides are considered the most nearly perfect pesticides. No environmental perturbations, beyond removal of the pest host, have been detected nor would be expected from temporary elevation of the natural enemy population.

Biological pesticide research and development is most efficient and productive when conducted by teams of scientists: a research specialist on the target pest, a research specialist on the natural enemy, and a research specialist on fermentation or rearing the

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch018

class of organism to be developed. A specialist knowledgeable of the target pest, its life history and behavior is most likely to discover natural enemies with potential, understand the pest's most vulnerable growth stages and know how to evaluate field efficacy of a natural enemy for its potential as a biological pesticide. A research specialist knowledgeable about the natural enemy is able to assess the strengths and weaknesses of a potential biological pesticide in controlled environments and precisely define the environmental optima and limits to guide effective field assessment of an agent. A specialist knowledgeable about large-scale production of various classes of organisms is able to determine the nutritional and physical requirements for large-scale production of an agent and means for formulating it into a consistent, stable preparation and assess economic feasibility of production, formulation and storage on a commercial scale. The successful collaboration of such a team -- critical assessment of an agents' potential from perspectives of three scientific disciplines--permits rapid and effective technology transfer to private enterprise for production scale-up, EPA registration, formulation, packaging, shipping, and marketing of a biological pesticide.

Agents deemed to be impractical by such a collaborating team, ineffective because of biotic or other constraints, become candidates for strain improvement. Genetic enhancement of candidate potential may be by either classic methods or new genetic transformation techniques.

A few biopesticides have been commercially successful and serve as models for development of others (15,16). The diversity of natural systems, however, provides a wealth of potential biopesticides and requires adjustment of researchable goals appropriate for particular classes of candidate agents, either fungi, bacteria, viruses, or nematodes. The biosystematics of all these classes and the pests they attack need to be researched. Regional, national and international surveys need to be conducted to develop or update catalogues and collections of existing natural enemies and the precise host association and geographic range of each, apparent variability or stability of host or natural enemy, and particular climatic or other constraints that appear operative under natural conditions. Biosystematic research thus provides agents and some insight into their potential for specialists who evaluate them in controlled conditions.

Specialists of each class use the collection for strain selection and strain improvement research. Supplies of the prospective agents must be evaluated in laboratory culture; then epidemiological parameters must be researched in controlled environments of growth chambers and greenhouses. Promising candidates move to field plot tests in collaboration with scientists specializing in the target pests, preferably those biosystematists responsible for original collection of the potential agent. Other candidates are subjects for strain improvement research.

Agents that are found to be constrained by fastidious culture requirements, too strict epidemiological parameters, or inadequate virulence or that are too labile must be the subject of more nutritional, genetic or physiological research to enhance strain potential. Understanding the molecular basis for these sorts of

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch018

inadequacies and the genes that control these mechanisms presents opportunities for transforming the organism genetically to overcome deficiencies and achieve successful biological pesticides with many more agents than is now possible.

Final Comments

Biological control can be used to substantially reduce our dependence upon chemical pesticides and achieve pest control without negative non-target effect. To achieve significant transition to biological pest control, however, will require new knowledge of pests and their natural enemies and realignment of research and development responsibilities for pest control among public and private sectors. The major responsibility for biological control development must be with the public sector rather than with the private sector, which has advanced chemical technology. Biological control agents are specific and, when used in the classical or IPM tactic, self-perpetuating, thus having no profit potential for private enterprise. When agents are used as biological pesticides, their market potential may be limited by strict specificity to a single pest that generally represents small markets. Thus the very trait that makes biological control so desirable from environmental and human safety perspectives, specificity for the target pest, is the exact characteristic that eliminates economic potential of biologicals for private enterprise.

New biotechnological techniques open new opportunities for genetic improvement of biological control agents, especially in the bio-pesticide tactic, and provide some incentive for private-sector involvement in biological control. An extensive, pluralistic, public-sector research effort is required to find and understand the biology of potential biocontrol agents, select and improve strains for further development, and engage in technology transfer with private enterprise when candidates with potential for commercialization are developed. The challenge is to substantially enhance public-sector research enough to enable biological control to become the primary method of pest control in the United States.

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Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ch018

Author Index

Altman, Jack, 240
Andrews, J. H., 192
Atkins, M., 305
Bannon, James S., 305
Baragona, J., 305
Cardellina, John, 53
Charudattan, R., 132
Connick, William J., Jr., 288
Crowley, R. H., 305
Cutler, Horace G., 72
Daigle, Donald J., 288
Durbin, Richard D., 63
Ford, E. J., 184
Hancock, Joseph G., 276
Hoagland, Robert E., 1,87
Johal, Guri S., 260
Jones, Richard W., 276
Joye, Gary F., 155
Keen, N. T., 114
Kenerley, C. M., 192
Lévesque, C. André, 260
Long, D., 305
Miller, R. V., 184
Neate, Stephen, 240
Nicholson, Ralph L., 218
Park, Sang Ho, 53
Rahe, James E., 260
Riley, J. A., 305
Rovira, Albert D., 240
Sands, D. C., 184
Stierle, Andrea, 53
Strobel, Gary, 53
TeBeest, D. O., 176
Templeton, George E., 320
Weidemann, G. J., 176
White, James C., 305

Affiliation Index

Colorado State University, 240
Commonwealth Scientific and Industrial Research Organization, 240
Montana State University, 53,184
Mycogen Corporation, 305,184
Purdue University, 218
Simon Fraser University, 260
Texas A&M University, 192
U.S. Army Engineer Waterways Experiment Station, 155
U.S. Department of Agriculture, 1,63,72,87,288
University of Arkansas, 176,320
University of California—Berkeley, 276
University of California—Riverside, 114
University of Florida, 132
University of Wisconsin—Madison, 63,192

Subject Index

A

AAL toxin
  biological activity, 14
  isolation, 14
  structure, 16
Abiotic elicitors, function, 119
Abscisic acid
  applications, 73–74
  development as agricultural chemical, 74–75
  isolation of metabolites in fungi, 75–78,80
  poor transport across leaf cuticles, 74
Abscisic acid—*Continued*
  presence in mammalian brains, 73
  structure, 73,76*f*
  synthesis, 73
  synthetic products, 78–82
Abscisic acid analogues
  practical applications, 81,82*f*,83,84*f*
  structure, 79,82*f*
(±)-Abscisic acid, effect on growth of etiolated wheat coleoptiles, 83,84*f*
Abscisin II, *See* Abscisic acid
Aceria chondrillae Canestrini, use for weed control, 136

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix001

Author Index

Altman, Jack, 240
Andrews, J. H., 192
Atkins, M., 305
Bannon, James S., 305
Baragona, J., 305
Cardellina, John, 53
Charudattan, R., 132
Connick, William J., Jr., 288
Crowley, R. H., 305
Cutler, Horace G., 72
Daigle, Donald J., 288
Durbin, Richard D., 63
Ford, E. J., 184
Hancock, Joseph G., 276
Hoagland, Robert E., 1,87
Johal, Guri S., 260
Jones, Richard W., 276
Joye, Gary F., 155
Keen, N. T., 114
Kenerley, C. M., 192
Lévesque, C. André, 260
Long, D., 305
Miller, R. V., 184
Neate, Stephen, 240
Nicholson, Ralph L., 218
Park, Sang Ho, 53
Rahe, James E., 260
Riley, J. A., 305
Rovira, Albert D., 240
Sands, D. C., 184
Stierle, Andrea, 53
Strobel, Gary, 53
TeBeest, D. O., 176
Templeton, George E., 320
Weidemann, G. J., 176
White, James C., 305

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Affiliation Index

Colorado State University, 240
Commonwealth Scientific and Industrial Research Organization, 240
Montana State University, 53,184
Mycogen Corporation, 305,184
Purdue University, 218
Simon Fraser University, 260
Texas A&M University, 192
U.S. Army Engineer Waterways Experiment Station, 155
U.S. Department of Agriculture, 1,63,72,87,288
University of Arkansas, 176,320
University of California—Berkeley, 276
University of California—Riverside, 114
University of Florida, 132
University of Wisconsin—Madison, 63,192

Subject Index

A

AAL toxin
- biological activity, 14
- isolation, 14
- structure, 16

Abiotic elicitors, function, 119
Abscisic acid
- applications, 73–74
- development as agricultural chemical, 74–75
- isolation of metabolites in fungi, 75–78,80
- poor transport across leaf cuticles, 74

Abscisic acid—*Continued*
- presence in mammalian brains, 73
- structure, 73,76*f*
- synthesis, 73
- synthetic products, 78–82

Abscisic acid analogues
- practical applications, 81,82*f*,83,84*f*
- structure, 79,82*f*

(±)-Abscisic acid, effect on growth of etiolated wheat coleoptiles, 83,84*f*
Abscisin II, *See* Abscisic acid
Aceria chondrillae Canestrini, use for weed control, 136

Acetylaranotin
biological activity, 15
isolation, 15
structure, 16
Acifluorfen
effect on phenylalanine ammonia-lyase activity, 102
structure, 102,105f
Adhesion of fungi to host
composition of adhesives, 228–230
extracellular mucilage of *Colletotrichum graminicola*, 230–235
functional significance, 219–220
mechanism, 219
preformed adhesives, 223
preparation of infection court by fungal pathogens, 224–228
prevention of displacement, 219–220
requirement of firm anchor, 220,221f
water insolubility, 220
zoospore encystment in oomycetes, 220,222–223
Adjuvant-based formulations for weed control
examples, 291,292t
function of adjuvant, 291,293
Agrochemical interactions with pathogens, weed control examples, 10
AK toxin
biological activity, 15
isolation, 15
structure, 17
Alligatorweed
control with plant pathogens, 170,172f
photograph, 170,171f
Alternaria alternanterae Holcomb & Antonopoulos, use for control of alligatorweed, 170,172f
Alternaria alternata
isolation, 54
phytotoxins, 54–61
Alternaria cassiae Jurair and Khan
culture maintenance technique, 307–308,309f
host–pathogen–environment interactions, 308,310–317
use for weed control, 140,141t
Altersolanol A
biological activity, 15
structure, 17
L-α-Aminooxy-β-phenylpropionic acid, inhibition of phenylalanine ammonia-lyase, 98–99,100f
(1-Amino-2-phenylethyl)phosphonic acid, inhibition of phenylalanine ammonia-lyase, 98–99,100f
AM toxin
biological activity, 15
isolation, 15
structure, 17
Anisomycin
biological activity, 15
isolation, 15
structure, 17
Anthocyanin, effect on phenylalanine ammonia-lyase activity, 102,104f
Antibiosis, role in biocontrol of weeds, 211
Appressoria, development, 197
Aquatic weeds
biological control with plant pathogens, 157–172
causes of problems, 156–157
economic devastation, 156
problems caused by weeds, 156
Arthrobotrys oligospora, adhesion to host, 223–224
Attachment of fungal pathogens, occurrence, 219
Avirulence genes
application, 126
cloning from bacterial pathogens, 122
definition, 120
purification, 123
role as race-specific elicitors, 122

B

β inhibitor, presence in sycamore buds, 72–73
Bacterial phytoxins from crop and weed pathogens
coronatine, 34–35
cyanobacterin, 34–35
phaseolotoxin, 34–35
rhizobitoxine, 34–35
stigmatellin, 35–36
tabtoxin, 35–37
tagetitoxin, 36–37
Bacterial toxins, synthesis, 65
Benzadox
biological activity, 27
isolation, 27
structure, 28
Bialaphos
biological activity, 18
biotechnology and biochemistry of production, 21,22f
description, 15,18
isolation, 15,18
structure, 18,19f
Bioassay techniques
examples, 9–10
importance, 9–10
Biochemical responses of plants to pathogens
enzyme inhibitors to manipulate inflection, 97–99
prospects, 106–107
role of enzymes in plant defense, 88–99

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Biochemical responses of plants
to pathogens—*Continued*
xenobiotic compounds that alter secondary
plant products, 99–106
Bioherbicide(s)
definition, 8
host–pathogen–environment interactions,
308,310–317
Bioherbicide control strategy, example of
DeVine, 306
Bioherbicide culture maintenance
effect of culture transfer on weed
control, 308,309*f*
effect of spore storage on mortality,
307–308,309*f*
sensitivity to preservation
techniques, 307
Bioherbicide discovery
genetic method, 307
survey method, 306–307
Bioherbicide propagule(s), examples, 308
Bioherbicide propagule production
effect of carbon source on efficacy and
spore yield, 308,311*f*
technique, 308
Bioherbicide targets, examples, 38,39*f*
Biological alternatives to chemical
pesticides, importance of discovery and
development, 320
Biological weed control
advantages of fungi use, 241
approaches, 241,244
development, 241
economic benefits and barriers, 322–323
research opportunities, 323
safety, 322
soil-borne fungi, 276–285
strategies, 288,321–322
use of herbicides
future needs and directions, 325–327
research opportunities, 323
strain selection and improvement research,
323–325
use of plant pathogens for aquatic weeds
advantages, 156
approach to research, 157
control of alligatorweed, 170,171–172*f*
control of blue-green algae, 170,172*f*
control of duckweeds, 168,169*f*,170,171*f*
control of eurasian watermilfoil,
165,166–166*f*,168
control of hydrilla, 161–166
control of waterhyacinth, 159,160*f*,161
control of waterlettuce, 168,169*f*
development, 156
obstacles, 157–158
BioMal
description, 299
use for weed control, 139
Biosynthetic pathway activation, use of
elicitors, 125
Biotechnological approaches to weed control
with pathogens
coinduction of suicidal gene with another
gene, 187,189*f*
genetically engineered containment
systems, 187
incorporation of recombinant genes into
suicide vector, 187
natural containment systems, 184–185
nonengineered approach to containment,
185,186*f*,187,188*f*
reasons against containment, 190
reasons for containment, 190
splitting of gene expression among
different loci, 187,189*f*
Blue-green algae
control with plant pathogens, 170
photograph, 170
Botrydienal
biological activity, 23
isolation, 23
structure, 24
Breeding line selection, use of
elicitors, 125
Broad-spectrum mycoherbicides, examples of
potential pathogens, 142,143–144*t*

C

Carbamate herbicides, effect on
mycoherbicide action, 253
Casst, use for weed control, 139
Cephalosporium diospyri Crandell, use for
weed control, 146
Cercospora rodmanii Conway, use for control
of waterhyacinth, 159,160*f*,161
Cercosporin
biological activity, 23
isolation, 23
structure, 24
Chaetoglobosins
biological activity, 23
isolation, 23
structure, 24
Chemical pesticides
advantages and disadvantages, 320–321
environmental and human health
hazards, 321
misuse, 321
Chemical weed control
chemical names and structures,
240–241,242–243*t*
development, 240
Chitin, role in disease defense, 119
Chitinase
inhibition, 97–98

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Chitinase—*Continued*
role in plant defense mechanism, 89,90*f*,91
Chitosan, role in disease defense, 119
Chlamydomonas, detection of fungal phytotoxins, 283–284
Chlorella, detection of photosynthetic inhibitors, 283
Chlorsulfuron
effect on pathogen–plant interaction, 249–250
effect on phenylalanine ammonia-lyase activity, 106
structure, 102,105*f*
Chondrostereum purpureum (Pers.:Fr.) Pouzar, use for weed control, 146
Citreoviridin
biological activity, 23
isolation, 23
structure, 24
Cladosporin
biological activity, 23
isolation, 23
structure, 24
Classical biocontrol agents, *See* Inoculative agents
Classical biological control strategy
applications, 241
definition, 305
description, 241
examples, 241
lack of profit incentive for industry, 305
method description, 288
Collego
compatibility with chemical pesticides, 298
description, 245,296
development, 245
discovery and development, 139–140
effect of genetic variability on use, 181–182
examples of weed control, 10,139
Colletotrichin
biological activity, 23,25
isolation, 23
structure, 24
Colletotrichum gloeosporioides (Penz.) Sacc. f. sp. *clidemiae*, use for weed control, 146
Colletotrichum gloeosporioides (Penz.) Sacc. f. sp. *malvae*, use for weed control, 140
Colletotrichum graminicola
application, 232,235
extracellular mucilage, 230–235
Commercial mycoherbicide products, examples, 296–300
Compatible host–pathogen combinations, definition, 115
Composition of fungal adhesives
glycoprotein–carbohydrate matrix, 228
lectins, 228–230
Concep II, effect on plant diseases, 254*t*,255
Conservation tillage, effect of herbicide use, 250*t*,251
Coronatine
biological activity, 35
isolation, 35
structure, 34
Curvulins
biological activity, 11
isolation, 11
structures, 12
Cuticle
description, 193,196
function, 196
Cyanobacterin
biological activity, 35
isolation, 35
structure, 34
Cyclocarbamides A and B
biological activity, 25
isolation, 25
structure, 24
Cyclopenin
biological activity, 25
isolation, 25
structure, 24
Cystiphora schmidti Rubsaamen, 136

D

Defense chemicals, examples, 88
Defense gene activation, use of elicitors, 125
(+)-Deoxyabscisic acid isomers, structure, 79,82*f*
Deoxyradicinin
biological activity, 31
isolation, 31
structure, 32
Desmethoxyviridiol
biological activity, 25
isolation, 25
structure, 26
DeVine
description, 244,298–299
discovery and development, 139
example of bioherbicide control strategy, 306
problems, 244–245
use for weed control, 139
2,4-Dichlorophenoxyacetic acid
effect on phenylalanine ammonia-lyase activity, 102
structure, 102,105*f*

Dihydropergillin
biological activity, 25
isolation, 25
structure, 26
(+)-(2Z,4E)-*trans*-1,4'-Dihydroxy-γ-ionylideneacetic acid
isolation, 75
structure, 75,77*f*
Diketopiperazines
identification, 57
isolation, 54,56*t*
phytotoxicity, 57
structure, 54–55
structure–activity relationship, 57
Dilophospora alopecuri, adhesion to *Anguina agrostis*, 223
1',4'-*trans*-Diol of abscisic acid
isolation, 78
structure, 78,80*f*
Disease defense in plants, levels of pathogen recognition and exclusion, 115
Disease on nontarget hosts, effect of herbicides, 246–247,248*t*
Disease resistance genes, definition, 120
Disease response genes, definition, 120
Duckweed(s), photographs, 168,169*f*,171*f*
Duckweed bioassay system, advantages, 10

E

Economics of weed problems
annual costs, 3*t*
annual losses from weeds in commodity groups, 3,4*t*
environmental concerns of pesticide use, 3
estimates of pesticide use in United States, 3,5*f*
relative percentage of global pesticide sales, 3,5*f*
Elicitor(s)
applications, 124,125*t*,126
initiation of phytoalexin production, 123–124
Elicitor–receptor model, description, 120
Enhancers of phytoalexins, function, 120
Entyloma compositarum Farlow, use for weed control, 137
3-Epideoxyradicinol
biological activity, 31
isolation, 31
structure, 32
Epidermis, function, 196
Eremophilanes
biological activity, 11,13
isolation, 11
structures, 12
Erysiphe, cuticular erosion, 225
Erysiphe graminis
contact stimulation of esterase release from conidia, 226,227*f*,228
release of liquid, 226
Ethylene
function in plants, 96
role in plant disease resistance, 96–97
Eurasian watermilfoil
control with plant pathogens, 165,168
distribution in United States, 165,167*f*
photograph, 165,166*f*
Excised oat mesocotyl bioassay, advantages, 10
Excised wheat coleoptile test, advantages, 9–10
Exploitative competition, description, 209
Extracellular mucilage of *Colletotrichum graminicola*
composition, 230,232
enzyme content, 232
high-performance LC separation of phenolic components of leachate, 232,233*f*
phytoalexin structures, 232,234*f*
scanning electron micrograph of fungi, 230,231*f*
scanning electron micrograph of surface of conidia, 232,234*f*

F

Formulation
definition, 288
description, 288–289
Formulations for weed control based upon solid carriers
examples, 293,294*t*,295–296
mechanism, 293,295
Free moisture, component of disease triangle, 306
Fungal adhesion, *See* Adhesion of fungi to host
Fungal pathogens, genetic variability, 178
Fungal pathogens as biological pesticides for weed control
development, 323
developmental constraints, 324
strain selection and improvement research, 323–325
use of molecular genetic techniques for strain improvement, 324–325
Fungal plant pathogen
dependence of weed control on genetic composition, 176
effect of genetic variability on host–range limitations, 181*t*
Fungal toxins, synthesis, 65
Fungal weed pathogens, production of phytotoxic compounds, 11–14

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Fungi
  advantages for use in biological weed control, 241
  classification schemes, 205–206
Fusaria as mycoherbicides
  disadvantage, 279
  examples, 278*t*
  host specificity, 278
  limitation, 279
  resistance, 278
  survival in soil, 278
Fusarium solani f. sp. *cucurbitae*, use for control of Texan gourd, 245
Fusicoccin
  biological activity, 25
  isolation, 25
  structure, 26

G

Gabaculine
  biological activity, 27
  isolation, 27
  structure, 26
Geldanamycin
  biological activity, 27
  isolation, 27
  structure, 26
General resistance, definition, 115
Genetically engineered containment systems
  coinduction of suicidal gene with another gene, 187,189*f*
  incorporation of recombinant genes into suicide vector, 187
  splitting of gene expression among different loci, 187,189*f*
Genetic method, use for discovery of bioherbicides, 307
Genetic technology, advantages and disadvantages, 184
Genetic variability of fungal pathogens, effect of reproductive systems, 178
Genetic variability of fungal pathogens and weed hosts
  benefits, 180,181*t*,182
  potential for genetic exchange through somatic cell fusion and heterokaryosis, 180*t*
  potential for genetic exchange with related wild-type strains, 179–180
  problems, 179,180*t*
  regulatory requirements, 179
  stability of commercial formulation, 179
Genetic variability of weeds
  effect of ecological factors, 176–177
  effect of reproductive system, 176–177
  effect of selective pressure exerted by pathogen population, 176–177
  example of control by endemic pathogen, 177,178*t*
Gliocladium virens
  advantages as mycoherbicide, 282
  phytotoxin production, 279,280*t*,281*t*,*f*,282
Global food production
  balance between surplus and shortage, 3
  importance of pest control, 3
  surpluses, 2–3
1,3-β-Glucanase
  inhibition, 97–98
  role in plant defense mechanism, 89,90*f*,91
Glucan elicitors, role in disease defense, 117,119
Glutamine synthetase
  inhibition by L-methionine sulfoximine, 21
  inhibition by phosphinothricin, 18,20*f*,21
  role in ammonia assimilation–reassimilation, 18
β-Glycosidases
  inhibition, 97–98
  role in plant disease resistance, 94,96
Glyphosate
  cause of plant death, 261,263
  description, 261
  effect on mycoherbicide action, 253
  effect on pathogen–plant interaction, 249
  effect on phenylalanine ammonia-lyase activity, 102
  metabolic target, 261,262*f*
  structure, 102,105*f*
  symptoms of phytotoxicity, 261
  synergistic role of soil fungi, 263–273
Gostatin
  biological activity, 27
  structure, 26
Growth regulators, effect on mycoherbicide action, 253

H

HC toxin
  biological activity, 27
  isolation, 27
  structure, 28
Helminthosporium oryzae, cuticular erosion and wax degradation, 225
Herbiace, *See* Bialaphos
Herbicidal efficacy of glyphosate, synergistic role of soil fungi, 260–273
Herbicide(s)
  effect of use on conservation tillage, 250*t*,251
  effect on disease on nontarget hosts, 246–247,248*t*
  effect on mycoherbicide action, 251–253
  effect on pathogen–plant interaction, 247

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Herbicide(s)—*Continued*
effect on phenylalanine ammonia-lyase, 102–106
rational-based design, 38–39
Herbicide safeners, effect on plant diseases, 254t,255
Herbicide targets, examples, 37–38,39f
Herbicidins
biological activity, 27
isolation, 27
structure, 28
Host–pathogen–environment interactions for bioherbicides
effect of chemical herbicides, 310,313,314f
effect of free moisture, 308,310,311–312f
effect of spore dormancy, 310,312f
effect of spray adjuvants, 313,315f,316
effect of temperature, 316
source–sink relationships, 316,317f
Host-specific toxins, definition, 9
Hydrilla
control with plant pathogens, 161,164,166f
distribution in United States, 161,163f
photograph, 161,162f
3′-Hydroxy-γ-ionylideneacetic acid, structure, 79,80f
(+)-(1′S,3′S)-(2Z,4E)-3′-Hydroxy-γ-ionylideneacetic acid, structure, 79,80f
(+)-4′-Hydroxy-α-ionylideneacetic acid
structure, 75,77f
synthesis, 75
(2Z,4E)-(+)-4′-Hydroxy-γ-ionylideneacetic
isolation, 78
structure, 78,80f
Hydroxyphenol, effect on phenylalanine ammonia-lyase activity, 102,103f
Hydroxyterphenyllin
biological activity, 31
isolation, 31
structure, 32
Hymenoscyphus ericae, adhesion to host, 224
Hypersensitive response, components, 115

I

Incompatible plant–pathogen combinations, definition, 115
Inoculative agents
examples, 136–137,138t
requirements for use, 133,136
Integrated weed management system with microorganisms
description, 300
examples, 300–301
Interference competition, description, 211
Inundative agents
examples, 139–149
requirements for use, 137,139
Inundative biological control of weeds, method description, 288
β-Ionone
effect on *Aspergillus flavus*, 81,83
effect on growth of etiolated wheat coleoptiles, 83,84f
structure, 81,82f
(+)-(2Z,4E)-α-Ionylideneacetic acid
isolation, 75
structure, 75,77f
(2Z,4E)-γ-Ionylideneacetic acid
isolation, 78
structure, 78,80f
(2Z,4E)-γ-Ionylideneethanol
isolation, 78
structure, 78,80f
Irpexil
biological activity, 27
isolation, 27
structure, 28

K

Koch's postulates, description, 8

L

Leaf surfaces
anatomy, 193–197
chemicals, 197,198t,199,200f
classes of compounds in waxes, 193,195f
microbial interactions, 208–213
microbial populations, 205–208
microclimate, 199,201–204
outer covering of land plants, 193,194f
Leaf wax, inhibitory effect against pathogens, 193
Lectins, component of fungal adhesives, 228–230
Lettuce seed–seedling bioassay, advantages, 10
Lytic enzyme, role in plant–pathogen interactions, 88,90f,91

M

Macrocyclic trichothecenes
biological activity, 33
isolation, 33
structure, 34
Macrolides
biological activity, 27,29
isolation, 27
structures, 28,30

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Macrophomina phaseolina, control of hydrilla, 164,166*f*
Maculosins
binding to knapweed, 60
biological activity, 13
conversion after application to knapweed, 60–61
host specificity screening, 58,59*t*
identification, 57
isolation, 13,54
mode of action, 58–60
structure, 12,54
structure–activity relationship, 57
Meria coniospora, adhesion to *Magnaporthe grisea*, 223
L-Methionine sulfoximine
inhibition of glutamine synthetase, 21
structure, 19*f*
synthesis, 21
Methoxyphenone
development, 15
structure, 17
Metribuzin, effect on pathogen–plant interaction, 249
Mevinolin
biological activity, 29
isolation, 29
structure, 30
Microbial herbicide(s)
applications, 244
biochemical–chemical considerations, 40
commercial examples, 244–245
criteria, 38
description, 244
examples, 244
genetic and strain selection, 40
problems, 38
Microbial interactions on leaf surfaces
evidence, 208
relationship with bioherbicide development, 212–213
role of exploitative competition, 209
role of interference competition, 208
role of nutrient depletion, 209–211
Microbial phytotoxin(s) as herbicides
discovery, 9
screening, 9
Microbial phytotoxin targets, examples, 38,39*f*
Microbial populations of leaf surface
diurnal patterns, 207–208
qualitative data, 205–206
quantitative data, 205–206
seasonal patterns, 207–208
spatial distribution, 206–207
Microbial products as herbicides
criteria, 38
problems, 38
Microclimate of leaf surfaces
air temperature, 202
description, 199,201
effect on pathogen development, 201
leaf temperature, 201–202
radiation, 203–204
relative humidity, 202–203
wind speed, 204
Molecular genetic techniques, use in improving strains for biological pesticide development, 324–325
Moniliformin
biological activity, 29
isolation, 29
structure, 30
Mycoherbicides
definition, 8
effect of growth regulators on action, 253
effect of herbicides on action, 251–253
fusaria, 278
improved activity through herbicidal action, 245
low-input technology, examples, 146*t*
niche markets, examples, 147,148*t*
problems in practical use, 147,149
selection of herbicide, 252
Mycoleptodiscus terrestris, use for control of eurasian watermilfoil, 165,168
Mycoparasites, role in biocontrol of weeds, 211–212

N

NADPH oxidase, role in plant disease resistance, 96
Naturally produced phytotoxins as herbicides, use for weed control, 255–256
Nigerazines A and B
biological activity, 29
isolation, 29
structure, 30
Nigericin
biological activity, 29
isolation, 29
structure, 30
Non-host-selective phytotoxins, biochemistry, 63–70
Nonspecific elicitors
function, 117
physiological role, 119
structure, 117,118*f*,119
types, 117

O

Oosporein
biological activity, 29
isolation, 29

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Oosporein—*Continued*
structure, 30
Ophiobolins
biological activity, 13
isolation, 13
structure, 12
Orlandin
biological activity, 31
isolation, 31
structure, 30
Oxetin
inhibition of glutamine synthetase, 21
structure, 19f
(+)-(2Z,4E)-4'-Oxo-α-ionylideneacetic acid, structure, 75,76f

P

Parasitism, importance of infection process, 219
Pathogen(s)
agrochemical interactions, 10
biochemical responses of plants, 87–107
biotechnological approaches to weed control, 184–190
discovery, 8
evasion of plant defense mechanisms, 87–88
host-range tests, 8
interactions on plant leaf surfaces, 192–213
screening, 8
Pathogenic fungi, response to leaf topography in infection initiation, 197
Pathogen(s) of economically attractive weeds, examples, 142,143–144t
Pathogen–plant interaction, effect of herbicides, 247,249–250
Pathogens with potential for weed control
advantages, 133
examples of weed control, 133,134–135t
inoculative agents, 133,136–137,138t
inundative agents, 137,139–149
progress toward practical use, 133
Perioxidase
inhibition, 97–98
physiological role, 91
role in plant disease resistance, 91t,92
Perylenequinones
biological activity, 14
isolation, 14
structures, 14–16
toxicity to knapweed, 61
Pesticide use
environmental concerns, 3–4
estimates in United States, 3,5f
relative percentage of global pesticide sales, 3,5f
(–)-Phaseic acid, structure, 81,82f
Phaseolotoxin
biological activity, 35
isolation, 35
structure, 34
Phenylalanine ammonia-lyase
chemical structures of inhibitors, 98,100f
function, 92,93f
inhibition, 98–99
role in plant disease resistance, 92,94,95f
xenobiotics that alter activity, 102–106
Phosalacine
biological activity, 18
isolation, 18
structure, 18,19f
Phosphinothricins
biological activity, 18
biotechnology and biochemistry of production, 21,22f
inhibition of glutamine synthetase, 18,20f,21
isolation, 18
L-N^5-Phosphonomethionine *S*-sulfoximine
inhibition of glutamine synthetase, 21
structure, 19f
Phragmidium violaceum (Schultz) Wint., use for weed control, 136
Phytoalexin
description, 115
elicitors, 117,118f,119
enhancers, 120
evidence for role in resistance, 116–117
importance, 114
initiation of production by elicitors, 123–124
isolation, 115
race-specific elicitors, 120,121f,122
role in hypersensitive response, 115
structures, 115–116,118f
suppressors, 120
Phytoalexin elicitors, examples, 117,118f,119
Phytophthora palmivora Butl., use as mycoherbicide, 276–277
Phytotoxic compounds produced by fungal pathogens of crops and nonpathogenic fungi, 14–34
Phytotoxic compounds produced by fungal weed pathogens,11–14
Phytotoxic compounds produced by microorganisms
examples from fungal crop pathogens and nonpathogenic fungi, 14–34
examples from fungal weed pathogens, 11–14
examples of bacterial phytotoxins, 34–37
Phytotoxin(s)
advantages, 70
biosynthetic pathways, 65

Phytotoxin(s)—*Continued*
characteristics as secondary metabolites, 65
classification scheme based on site of action, 63,64*t*
disease production, 70
nonselectivity, 63
properties, 65
symptoms elicited in host, 64
See also Herbicides
Phytotoxin(s) from weed pathogens
future work, 61
maculosin, 54–61
occurrence, 53–54
perylenequinones, 53
properties, 53–54
tetramic acids, 61
Phytotoxin-producing soil-borne fungi, screening, 282,283*t*
Phytotoxin production by *Gliocladium virens*
effect on crop and weed species, 279,280–281*t*
mechanism of herbicidal activity, 282
preemergence activity, 279
structure, 279,281*f*
Plant defense
enzyme inhibitors to manipulate infection, 97–99
enzymes involved in reactions, 88–97
examples of enzymes involved in mechanisms, 88,89*t*
plant growth regulator interactions, 88
problems in defense factor assessment, 97
xenobiotic compounds that alter secondary plant products, 99–106
Plant diseases
effect of herbicide safeners, 254*t*,255
effect of soil exudates, 251
Plant growth regulator interactions, role in plant defense, 106
Plant pathogens, biological control of aquatic weeds, 155–172
Polyphenol oxidase
inhibition, 97–98
role in plant disease resistance, 94
Poorly disseminating pathogens, description, 133
Prehelminthosporal
biological activity, 31
isolation, 31
structure, 32
Proteases, role in plant disease resistance, 96
Puccinia canaliculata (Schw.) Lagh., use for weed control, 146–147
Puccinia carduorum Jacky, use for weed control, 136–137
Puccinia chondrillina Bubak. & Syd., use for weed control, 136
Pythium myriotylum, use for control of duckweeds, 168,170

R

Race-specific elicitors
applications, 125–126
avirulence genes, 122–123
definition, 120
evidence for existence, 120–121
isolation, 121*f*
Radianthin
biological activity, 31
isolation, 31
structure, 32
Radicinin
biological activity, 31
isolation, 31
structure, 32
Rhizobitoxine
biological activity, 35
isolation, 35
structure, 34
Root colonization
description, 268
pathogenicity of root colonizers, 269
rate for glyphosate-treated plants, 268–269,270*f*
specificity of root colonizers, 269,271*t*
Rust fungi, perpendicular orientation of germling growth, 196–197

S

Sclerotia, definition, 187
Sclerotinia sclerotiorum mutants
effect of external cytosine on virulence, 185,186*f*
host ranges, 184,185*t*
radial growth response to pyrimidine supplements, 185,186*f*
virulence of non-sclerotia-forming mutants, 187,188*f*
Self-disseminating pathogens, description, 133
Shikimic acid pathway, steps, 261,262*f*
Simple trichothecenes
biological activity, 33
isolation, 33
structure, 34
Soilborne fungi
advantages for use in weed control, 277
factors affecting phytotoxin production, 284
fusaria as mycoherbicides, 278*t*,279
mechanisms of weed control, 277
phytotoxin production by *Gliocladium virens*, 279,280*t*,281*t*,*f*,282

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Publication Date: September 25, 1990 | doi: 10.1021/bk-1990-0439.ix002

Soil-borne fungi—*Continued*
prospects as mycoherbicides, 285
screening for phytotoxin production, 282,283*t*,284
use for biological control of weeds, 276
Soil exudates, effect on plant disease, 251
Soil fungi, synergistic role in herbicidal efficacy of glyphosate, 260–273
Specific resistance, definition, 115
Spotted knapweed
economic importance, 58
seed diversity, 58
toxicity to maculosin, 54–61
Stigmatellin
biological activity, 35
isolation, 35
structure, 36
Suppressors of phytoalexin, function, 120
Surfactant-based formulations for weed control
examples, 289,290*t*
functions of surfactants, 289
Survey method, use for discovery of bioherbicides, 306–307
Synergistic role of soil fungi in herbicidal efficacy of glyphosate
contribution of fungi to efficacy, 271–272
determination using root colonization, 268–269,270*f*,271*t*
effect of autoclaving of soil extracts on mortality of glyphosate-treated seedlings, 263*t*
effect of heat treatment of soil on LD_{50} values for glyphosate, 266*t*,267*f*,268*t*
effect on emergence and growth in subsequent crops, 272–273
evidence, 263
glyphosate LD_{50} values in heat-treated and raw soils, 264,265*f*
indirect effects of elevated activity of fungi on untreated plants, 271–272
logistic regression model for LD_{50} estimation, 264,267*f*
Synthetic herbicides
discovery, 7
screening, 7–8

T

Tabtoxin
biological activity, 35,37
isolation, 35
structure, 36
Tabtoxinine β-lactam
glutamine synthetase inhibition, 66–67
inhibition of glutamine synthetase, 21
structure, 19*f*,65*f*,66
synthesis, 66
Tagetitoxin
biological activity, 37
chlorosis production, 69
isolation, 37
mechanism of action, 69
structure, 36,68*f*,69
Tentoxin
biological activity, 31
existence of analogue, 68
isolation, 31
mechanism for chlorosis production, 67
reactions of *Nicotiana* hybrid seedlings, 67,68*t*
structure, 32,67*f*
Tenuazonic acid
biological activity, 31
isolation, 31
structure, 32
Terphenyllin
biological activity, 31
isolation, 31
structure, 32
Tetramic acids, toxicity to knapweed, 61
Thidiazuron, effect on mycoherbicide action, 253
Thiolactomycin
biological activity, 33
isolation, 33
structure, 32
Tobacco mosaic virus, host range specificity, 122
Toxins, advantages, 69–70
Toyocamycins
biological activity, 33
isolation, 33
structures, 32
Trichoderma, surface erosion, 225–226
Trichomes, function, 196
Trichothecenes
biological activity, 33
isolation, 33
structures, 33–34
Trifluralin
effect on mycoherbicide action, 253
effect on pathogen–plant interaction, 247,249–250
Triticones
biological activity, 33
isolation, 33
structure, 34
Trytophol
biological activity, 14
isolation, 14
structure, 16

U

Uromyces appendiculatus, preparation of infection court of *Phaseolus vulgaris*, 224–225

V

Victorin
application, 126
function, 121–122
Viridiol
production by *Gliocladium virens*, 279,282
structure, 26,279,281f

W

Water-based formulations for weed control, examples, 289,290t
Waterhyacinth
control with plant pathogens, 159,160f,161
photograph, 159,160f
Waterlettuce
control with plant pathogens, 168
photograph, 168,169f
Weed(s)
biological control, 241,244
characteristics of successful weed control programs, 240
chemical control, 240–241,242–243t
economic cost, 240
genetic variability, 176–177,178t
Weed control
biotechnological approaches with pathogens, 184–190
historical perspectives, 4,6t,7
Weed–pathogen interactions, importance, 88

X

Xenobiotic compounds that alter secondary plant products
alteration of extractable phenylalanine ammonia-lyase activity, 102–106
examples, 99,100–101t

Y

Yellow starthistle, control with phytopathogenic soil-borne fungi, 277

Z

Zinniol
isolation, 14
structure, 16
Zoospore encystment in oomycetes, example of adhesion, 220,222–223

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