ACS SYMPOSIUM SERIES 557

Bioregulators for Crop Protection and Pest Control

Paul A. Hedin, EDITOR
Agricultural Research Service,
U.S. Department of Agriculture

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.fw001

Developed from a symposium sponsored
by the Division of Agrochemicals
at the 205th National Meeting
of the American Chemical Society,
Denver, Colorado,
March 28–April 2, 1993

American Chemical Society, Washington, DC 1994

SB 128 .B57 1994 Copy 1

Bioregulators for crop protection and pest control

Library of Congress Cataloging-in-Publication Data

Bioregulators for crop protection and pest control / Paul A. Hedin, editor

p. cm.—(ACS symposium series, ISSN 0097-6156; 557)

"Developed from a symposium sponsored by the Division of Agrochemicals at the 205th National Meeting of the American Chemical Society, Denver, Colorado, March 28–April 2, 1993."

Includes bibliographical references and indexes.

ISBN 0-8412-2918-X

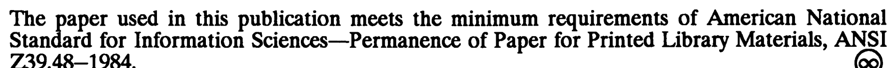
1. Plant regulators—Congresses.

I. Hedin, Paul A. (Paul Arthur), 1926– . II. American Chemical Society. Division of Agrochemicals. III. American Chemical Society. Meeting (205th: 1993: Denver, Colorado.) IV. Series.

SB128.B57 1994
631.8—dc20

94-14287
CIP

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.fw001



The paper used in this publication meets the minimum requirements of American National Standard for Information Sciences—Permanence of Paper for Printed Library Materials, ANSI Z39.48–1984. ♾

Copyright © 1994

American Chemical Society

All Rights Reserved. The appearance of the code at the bottom of the first page of each chapter in this volume indicates the copyright owner's consent that reprographic copies of the chapter may be made for personal or internal use or for the personal or internal use of specific clients. This consent is given on the condition, however, that the copier pay the stated per-copy fee through the Copyright Clearance Center, Inc., 27 Congress Street, Salem, MA 01970, for copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Law. This consent does not extend to copying or transmission by any means—graphic or electronic—for any other purpose, such as for general distribution, for advertising or promotional purposes, for creating a new collective work, for resale, or for information storage and retrieval systems. The copying fee for each chapter is indicated in the code at the bottom of the first page of the chapter.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

American Chemical Society
Library
1155 16th St., N.W.
Washington, D.C. 20036

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.fw001

1994 Advisory Board

ACS Symposium Series

M. Joan Comstock, *Series Editor*

Robert J. Alaimo
Procter & Gamble Pharmaceuticals

Mark Arnold
University of Iowa

David Baker
University of Tennessee

Arindam Bose
Pfizer Central Research

Robert F. Brady, Jr.
Naval Research Laboratory

Margaret A. Cavanaugh
National Science Foundation

Arthur B. Ellis
University of Wisconsin at Madison

Dennis W. Hess
Lehigh University

Hiroshi Ito
IBM Almaden Research Center

Madeleine M. Joullie
University of Pennsylvania

Lawrence P. Klemann
Nabisco Foods Group

Gretchen S. Kohl
Dow-Corning Corporation

Bonnie Lawlor
Institute for Scientific Information

Douglas R. Lloyd
The University of Texas at Austin

Cynthia A. Maryanoff
R. W. Johnson Pharmaceutical Research Institute

Julius J. Menn
Western Cotton Research Laboratory, U.S. Department of Agriculture

Roger A. Minear
University of Illinois at Urbana–Champaign

Vincent Pecoraro
University of Michigan

Marshall Phillips
Delmont Laboratories

George W. Roberts
North Carolina State University

A. Truman Schwartz
Macalaster College

John R. Shapley
University of Illinois at Urbana–Champaign

L. Somasundaram
DuPont

Michael D. Taylor
Parke-Davis Pharmaceutical Research

Peter Willett
University of Sheffield (England)

Foreword

THE ACS SYMPOSIUM SERIES was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of this series is to publish comprehensive books developed from symposia, which are usually "snapshots in time" of the current research being done on a topic, plus some review material on the topic. For this reason, it is necessary that the papers be published as quickly as possible.

Before a symposium-based book is put under contract, the proposed table of contents is reviewed for appropriateness to the topic and for comprehensiveness of the collection. Some papers are excluded at this point, and others are added to round out the scope of the volume. In addition, a draft of each paper is peer-reviewed prior to final acceptance or rejection. This anonymous review process is supervised by the organizer(s) of the symposium, who become the editor(s) of the book. The authors then revise their papers according to the recommendations of both the reviewers and the editors, prepare camera-ready copy, and submit the final papers to the editors, who check that all necessary revisions have been made.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

M. Joan Comstock
Series Editor

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.fw001

Preface

NATURAL AND SYNTHETIC PLANT GROWTH REGULATORS have been used for more than 50 years with increasing incidence to modify crop plants by changing the rate or pattern of their responses to the internal and external factors that govern development from germination through vegetative growth, reproductive development, maturity, senescence, and post-harvest preservation. The term "bioregulator" has been used to encompass the natural and synthetic compounds that regulate these various plant growth and developmental processes.

The commercial applications of bioregulators are increasing and now account for 5–10% of world agrochemical sales. Bioregulators have established a market niche for nursery and horticultural crops, fruits, sugarcane, grains, cotton, and oil seeds. Bioregulators have been subjected to the scrutiny associated with health and environmental issues, but the low levels of application and their generally limited adverse effects provide encouragement that many may be used safely. In fact, a number of them have been used effectively and safely at the commercial level for a number of years. We are rapidly moving into an era in which crops will be protected from a broad spectrum of pests, including plants, insects, and diseases, by new biotechnologies. The continued development of these agents along with genetically altered crops may provide the major new strategies by which crop production will be enhanced and crops protected from pests in future years.

This book includes a tutorial chapter on the development of plant growth regulators and sections on current applications of plant growth regulators to crop production, natural products as emerging bioregulators of crop production, and mechanisms of interactions of natural bioregulators and their hosts. The book will be of interest to industry, the academic and federal research sectors, and the agencies responsible for their regulation. I hope that it will contribute to a greater understanding of the opportunities for the use of bioregulators in crop production and the subsequent adoption of additional research strategies for their expanded use.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.pr001

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Acknowledgments

I thank the participants of the symposium and the contributors to this book, without whom the project would not have been possible, the Division of Agrochemicals for sponsoring the symposium, and Anne Wilson, acquisitions editor of ACS Books, for administrative and general support. Finally, I thank the Agricultural Research Service of the U.S. Department of Agriculture for providing me the opportunity and support to organize the symposium and compile the book.

PAUL A. HEDIN
Agricultural Research Service
Crop Science Research Laboratory
U.S. Department of Agriculture
P.O. Box 5367
Mississippi State, MS 39762–5367

February 2, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.pr001

Chapter 1

Plant Growth Regulators in Agriculture and Horticulture

Louis G. Nickell

Nickell Research, Inc., Hot Springs Village, AR 71909

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

Since the 1940's, natural and synthetic plant growth regulators have been used with increasing incidence to modify crop plants by changing the rate or pattern, or both, of their responses to the internal and external factors that govern development from germination through vegetative growth, reproductive development, maturity, senescence or aging, and postharvest preservation. This review deals with the history of plant growth regulators, regulation of plant metabolism, control of flowering, effects on fruit set and development, control of abscission, control of plant and organ size, recently developed plant growth regulators, and the perceived future for these compounds as related to agricultural needs and economics, the strategies for future searches for effective compounds, and the influence of regulatory issues on the development and availability of these compounds.

In December of 1982, CHEMRAWN (Chemical Research Applied to World Needs) II, sponsored by IUPAC (International Union of Pure and Applied Chemistry), was held in Manila, Philippines. The title of this meeting was "Chemistry and World Food Supplies: The New Frontiers". One of the presentations in the "Forward Edge" section was: "The role of growth regulators and hormones in enhancing food production" (1). The responsibility for summarizing the presentations and discussions, and for preparing the conclusions and recommendations for CHEMRAWN II was assigned to a Future Actions Committee. One of its recommendations was to hold periodic discussions, workshops, and research planning sessions throughout the world. At a subsequent workshop held in Buenos Aires, Argentina, in 1986, a paper "Plant Growth Regulators, Biotechnology, and World Food Production" was presented (2). This present chapter is an update of the thoughts presented on those two occasions.

0097–6156/94/0557–0001$08.00/0
© 1994 American Chemical Society

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

Uses of Growth Regulators

Plant growth regulators are used to modify a crop by changing the rate or pattern, or both, of its response to the internal and external factors that govern development from germination through vegetative growth, reproductive development, maturity, and senescence or aging, as well as postharvest preservation.

The regulation of plant growth (other than herbicidal action, which kills the plant) can be useful in numerous ways. Among others, it can: (1) regulate the chemical composition of the plant and/or the color of fruit, (2) initiate or terminate the dormancy of seeds, buds, and tubers, (3) promote rooting and propagation, (4) control plant or organ size, (5) promote, delay, or prevent flowering, (6) induce or prevent leaf and/or fruit drop (abscission), (7) control fruit set and further fruit development, (8) influence mineral uptake from the soil, (9) change the timing of crop development, (10) increase plant resistance to pests, (11) enhance plant resistance to such environmental factors as temperature, water, and air pollution, and (12) prevent postharvest spoilage.

History

The history of plant growth regulation goes back long before the time of Christ, when it was common practice in the Middle East to place a drop of olive oil on figs to promote development. It is now known that heat and time caused the breakdown of the oil, releasing ethylene, which in turn affected the development of the fig. It is said that the use of smoke to bring about floral initiation in pineapples was actually discovered in 1893 when a carpenter working in a greenhouse in the Azores accidently set fire to a pile of shavings. To the surprise of the grower who had thought his plants were ruined, they burst into flower instead of being damaged. By the 1920's, it was a recognized fact that pineapple could be forced to flower by smoke from fires (used during cold weather to prevent stoppage of growth) and that this effect was caused by the smoke's content of unsaturated gases such as ethylene. By the 1930's, ethylene was shown to accelerate flowering in pineapple, and soon acetylene gas was used commercially in Hawaii to force the initiation of flowering. During the 1940's, auxins were also shown to produce this effect, and naphthaleneacetic acid was the next forcing agent to become commercially used in pineapple.

There are different ways to summarize information on the role and effectiveness of plant growth regulators. The most common are: effects on plant function (3), by crop (4), by chemical or chemical class, and historically or chronologically.

One of the oldest commercial uses for plant growth regulators has been to initiate and/or accelerate the rooting of cuttings. Probably the best and most commonly used chemical for this purpose is indolebutyric acid (5). Considerable work has been conducted on promotion of rooting, resulting in thousands of publications and several books on the topic. Even though a number of compounds have

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

been found to be effective, it is interesting that with all this work we have not found one to date which is better than indolebutyric acid.

One of the best recognized uses of plant growth regulators is the suppression of sprouting of potatoes and onions by treatment with maleic hydrazide. This compound also was extensively used in the 1940's and 1950's to inhibit the growth of grasses in lawns, on golf courses, in parks and cemeteries, and along highways. For many years this was the most widely used plant growth regulator. Following the heyday of maleic hydrazide, the next most widely used regulator was CCC (chlormequat, Cycocel) which shortened the height of wheat without changing the size and quality of the ears. This decrease in height prevented lodging of the wheat plant after rain and wind, especially under high N fertilization. In this case, there is no increase in yield per se, but a prevention of loss.

By the early 1980's, the largest single market for plant growth regulators in the United States was as harvest aids for defoliating cotton. The next largest was growth regulators used to control suckers in tobacco in the United States. The largest uses for plant growth regulators outside the United States were ethephon on rubber throughout Malaysia and southeast Asia, and ripeners on sugarcane throughout the tropics.

Regulation of Plant Metabolism

The rationale for altering the internal metabolism of a given crop is to make it produce more sugar, more protein, more oil, more latex, or more or better fruit than it would under the best conditions without treatment. Enhancement of productivity of crops grown for processing could be achieved by selecting for high levels of processing components, e.g., insoluble solids, flavor, acidity, etc.

The use of ethephon, an ethylene-releasing agent, to boost the yield of latex from rubber trees has become standard plantation practice (6). This compound increases the length of time for the flow of latex between tappings and thereby increases by as much as 100% the commercial yield of dry rubber from commercially important tree varieties. The use of ethephon obviates frequent cutting of tree bark, thus helping preserve the life of the tree.

Rubber made from the guayule plant, which grows in the southwest United States and Mexico, is equal in every respect to that made from rubber trees. Inexpensive triethylamines when sprayed about 3 weeks before harvest increases yields 200-600%. Use of such chemicals could increase overall yields 30-35% and cut the growing time by 1-2 years (7). The oleoresins and tall-oil content of pine trees can be increased by treating the trees with the herbicide paraquat prior to harvesting (8). As much as 20-fold increases in the extractive content of treated pines have been reported.

One of the most important commercial developments in recent years of plant growth regulators has been the use of chemicals for the ripening of sugarcane (9-11). A wide variety of chemical structures have been shown to increase the sucrose content of cane at harvest. The first material that was considered seriously as a

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

ripener for increasing sugar yields was the dimethylamine salt of 2,3,6-trichlorobenzoic acid. Because of a number of technical, environmental, and legal problems, use of this material did not prove commercially feasible. However, it has served as a standard in screening tests directed to finding better, less expensive, and more acceptable compounds as ripeners. In 1980, only one product was registered in the United States as a sugarcane ripener -- glyphosine. Its monosubstituted derivative, the herbicide glyphosate, was registered later for use as a ripener and to replace glyphosine in much of the cane area now treated. The ethylene-producing compound ethephon is used commercially on sugarcane in South Africa and Zimbabwe. Numerous other compounds that have been registered for experimental testing include chlormequat, an endothal derivative, mefluidide, Fusilade. The increased sugar yield produced by treatment with ripeners is 5-25%, depending on the variety of cane treated and on prevailing weather and soil conditions. Changes in cultural conditions to adjust for the use of ripeners probably should increase sugar yields still further. The newest group of compounds which includes a number of esters of substituted pyridinyloxy-phenoxy propionic acids, is considerably more active than those originally considered as commercial candidates (11).

Growth regulators used for various other reasons also can effect fruit quality. Daminozide was introduced for application on apple trees in the mid-1960's. Applied during the summer, this growth regulator improves the fall coloration of red apples, reduces the development of water core and superficial scald on Red Delicious apples, and retains fruit firmness at harvest. Ethephon improves the red coloration of many apple cultivars and rapidly transforms green fruit to ripe fruit for early marketing (12). Both ethephon and daminozide have been used to advance the commercial harvesting of peaches (13). Similar positive results with these two compounds have been attained with cherries, grapes, and pears. Ethephon promotes the ripening of bananas, peppers, dates, and prunes.

Senescence in plants probably is initiated by a hormone-like substance which travels from the fruit to the leaves and other vegetative parts. Abscisic acid is a senescence promoter and a wide range of plant species are sensitive to it. Cytokinins are the most generally effective class of senescence-retarding growth regulators. Applications of auxins or gibberellins delay senescence in certain species, e.g., oranges. Rhizobitoxine analogues extend the vase life of cut flowers, e.g., carnations, iris, daffodils, chrysanthemum, and snapdragon, and the shelflife of broccoli.

Control of Flowering

The capability to induce flowering or, if flowering causes a decreased economic benefit, the ability to prevent flowering is necessary in many horticultural and agricultural crops. In the early 1930's, ethylene was shown to accelerate flowering in pineapple and, a few years later, acetylene gas was used commercially in Hawaii for this purpose. Later, auxins were shown

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

to produce this effect, and naphthaleneacetic acid was used commercially on pineapples. Although hydrazines commonly are thought of as growth retardants, several of them, particularly β -hydroxyethylhydrazine, induce flowering in Hawaiian pineapples. The latest group of compounds demonstrated to be effective forcing agents for pineapple are the haloethanephosphonic acids, e.g., ethephon--proper application of which produces total floral initiation (14).

In many fruit trees, especially apple, pear, and peach, the extent of floral induction is increased by spraying with inhibitors, e.g., daminozide and 2,3,5-triiodobenzoic acid. Many annual vegetables that normally flower only when days are long, e.g., lettuce, radish, mustard, and dill, can be made to flower early by treatment with gibberellins. Many biennial vegetables that require low temperatures to flower, e.g., beets, carrots, and cabbage, also do so after treatment with gibberellins. The effects of ethylene on flowering are promotion, inhibition, and sex reversal. Promotion of flowering is associated primarily with pineapples and other bromeliads, whereas inhibition of floral initiation occurs in many plant families. Growth regulators for sex reversal most often are related to the cucurbits.

In certain crops, including almond, peach, and tall-oil trees, delaying the onset of flowering may be desirable to avoid adverse weather conditions such as extremes in temperature and moisture. Appropriate delays also can bring two plant varieties with different flowering dates into synchronization for breeding purposes, e.g., almonds. Delay can be used to control the timing of flowering to coincide with important holidays when selling prices are higher, e.g., carnations and poinsettias.

Flowering decreases the yield of certain crops, e.g., sugarcane (15). Prevention of flowering in this crop can be effected by interrupting the night with light, lowering the temperature, trimming leaves and spindle, withdrawing water, or applying certain chemicals. Because the withdrawal of water is possible only on irrigated plantations - and even then there are operational problems - this practice has limitations. The first three approaches are labor intensive and not practical. The first potentially useful commercial chemical for the prevention of flowering in sugarcane was maleic hydrazide. Rapid developments led to the subsequent use of monuron, then diuron, and presently, diquat. Use of diquat is more cost effective than monuron or diuron. Proper application can provide 100% control.

Effects on Fruit Set and Development

Some plants, e.g., the cultivated banana, naval orange, oriental persimmon, and pineapple, as well as certain varieties of fig and pear, develop fruits in nature in the absence of pollen. Other plants, e.g., certain grape varieties, develop fruit from the stimulus provided by the presence of pollen alone without fertilization of the ovule, presumably because of its secretion of hormonal substances.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

Synthetic growth regulators that cause development of fruits in plants include 4-chlorophenoxyacetic acid and 2-naphthoxyacetic acid. These chemicals are most effective on fruits with many ovules, e.g., tomato, squash, eggplant, and fig. However, these chemicals usually are ineffective on peach, cherry, plum, and other stone fruit. Many fruits that can be set by such hormonal compounds also can be set by gibberellins. Gibberellins also can set fruit in some species that do not respond to the other growth regulators. An extensive study has shown gibberellic acid (GA_3) to be the growth regulator of choice to improve fruit set of citrus. Application of aqueous sprays of gibberellic acid to entire trees in full bloom increases the set and the yield of seedless fruit of five self-incompatible citrus cultivars. Seedless fruits, however, are more likely to drop after initial fruit set than seedy fruits produced by cross-pollination. Gibberellins have been used in the production of seedless Delaware grapes in Japan. One of the single largest uses of GA_3 in the United States is in the production of Thompson seedless grapes.

Yield improvement from cucumber plants is directly related to increased fruit set on individual plants. Generally, cucumbers do not set more than two fruits per plant at any one time because the earliest fruit that sets restricts the development of additional fruits. Certain growth regulators, e.g., methyl chlorflurenol, effectively overcome this limitation in fruit set. However, for chlorflurenol to be totally effective, the plant must have predominantly female flowers. Ethephon induces female flowering in the cucumber and enhances the potential for chlorflurenol to increase yield.

The ability to control the shape of fruits has practical implications. For example, apple cultivars known to have superior internal quality attract premium prices provided the consumer can identify the product by some characteristic feature of its external appearance. In the marketplace, the Red Delicious apple is identified primarily by the elongated appearance of the fruit and the presence of pronounced calyx lobes on the crown. A fruit having these attributes is called "typey". Commercial application of a growth regulator product that is a combination of gibberellins A_4 and A_7 with the cytokinin 6-benzyladenine causes improved typiness in Red Delicious apples. The period between full bloom and early petal fall is when the flower parts are most receptive to the treatment and capable of absorbing and transferring the regulators to the developing fruit. The success with apples has resulted in this product being tested for a number of different uses on a wide variety of crops.

<u>Control of Abscission</u>

The control of abscission, i.e., the separation or shedding of a plant part such as a leaf, flower, fruit, or stem from the parent plant, is extremely important in agriculture and horticulture. To ensure the most effective crop growth, leaves usually should be retained in a healthy, green state. On the other hand, to simplify the mechanical harvesting of certain crops, e.g., cotton, it is highly desirable to have the leaves removed. In tree crops that

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

have a large number of fruit started, it sometimes is desirable to thin the fruit by using an abscission-inducing compound, thereby increasing the size and quality of the remaining fruits. During crop growth, the fruits should be retained on the tree for maximum development and maturity. However, at harvest for many crops, e.g., citrus, the use of an abscission-inducing agent can be highly profitable by reducing labor requirements and costs.

A research program has been underway in Florida since the early 1960's to search for compounds that promote abscission of citrus fruit. It was found that an antifungal antibiotic, cycloheximide, loosens citrus fruit. Although cycloheximide is used commercially in Florida on most orange varieties, it is not used during the harvesting of Valencia oranges because it damages the flower and the immature fruit. A selective abscission material, 5-chloro-3-methyl-4-nitro-1H-pyrazole, was discovered and developed, and it now provides effective induction of abscission in mature Valencia oranges without damaging new twig growth and immature fruit.

Sweet cherries respond to daminozide in a number of ways, e.g., maturity and coloration are advanced and soluble solids are increased. The fruit-removal force is decreased, thereby promoting fruit abscission for machine harvesting.

Considerable research is being conducted in Mediterranean lands and in California to decrease the amount of labor needed for olive harvesting. Efforts to decrease labor consumption are directed toward a mechanical or a chemical solution. In the latter case, ethephon and 2-chloroethyltris (2-methoxyethoxy) silane have been found to be effective.

Selective removal of blossoms and small fruits in apples is achieved using plant growth regulators (12). Early thinning increases the size of the remaining fruits and improves other aspects of fruit quality in addition to providing a better fruit appearance. Another and often more important reason for flower and fruit thinning relates to the biennial-bearing tendency of many apple cultivars. Before 1949, biennial bearing was a national problem in the United States. After that year, it largely disappeared. Among the more widely used thinning agents are naphthaleneacetic acid, ethephon, naphthalene-acetamide, sodium 4,6-dinitro-o-cresylate, and the insecticide carbaryl. These materials are used alone or in combinations.

Results with peaches have not been nearly as satisfactory as those with apples. Ethephon both over- and under-thins peaches plus having a phytotoxic side effect. the simultaneous application of GA_3 with ethephon either eliminates or significantly reduces the undesirable side effects of ethephon without altering the thinning response.

Ethephon appears to do a more satisfactory job in the thinning of plums and cherries than it does for peaches. Ethephon has been used effectively in the spraying of macadamia trees in Australia to induce nut drop. In California, the same chemical has been effective in permitting an earlier harvest of walnuts.

Several types of chemical harvest aids are used on more than three-fourths of the cotton acreage in the United States. The degree of success of chemical harvest aids depends largely on the

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

relationship between the chemical and technique used, the conditions of the crop, and climate. The chemicals used include defoliants and desiccants, or other types of growth regulators. Harvest aid chemicals are important in the production of high quality fiber. Two of the most widely used defoliants are the organophosphorus compounds S,S,S-tributyl-phosphorotrithioate and tributyl-phosphorotrithioite. Defoliants are the preferred class of harvest aids to remove leaves without drying them. Abscized leaves are green and moist and, unlike dried leaves, tend to fall free of the lint in the open bolls. Harvesting usually can be precisely scheduled after a defoliant is applied. Defoliants are used primarily where cotton is harvested by spindle pickers, which cannot operate at peak efficiency if plants are rank and have an abundance of green succulent foliage. There have been questions as to the safety of these organophosphorus compounds, but several new promising compounds are now being evaluated in the field. Among other defoliants in use are sodium chlorate, which is a highly flammable material when dry and usually is formulated with a fire retardant, and magnesium chlorate which is a hygroscopic material, thereby offering fire retardant properties. There is little difference in effectiveness between these chlorate defoliants; they work best on fully matured leaves and have very little effect on young, immature leaves or on re-growth vegetation. Another commonly used defoliant is the arsenical cacodylic acid, formulated as the sodium salt. Its use is confined primarily to the western United States where cotton leaves are consistently more difficult to remove than those in the eastern states (16).

Control of Plant and Organ Size

The control of plant and organ size can be of great importance in agriculture. If maximum weight, length, or diameter affects final yield, then an increase in size is desirable. On the other hand, if it would be of commercial benefit, it may be important to be able to reduce the overall size of the plant.

The elongated "foolish seedling" effect in rice is caused by infection with the fungus *Gibberella* fuji-kuroi. A metabolite of the fungus was isolated in 1938 and was shown to be the causative agent of the disease. This work led to the discovery of a new class of plant hormones, the gibberellins. In most plants, the outstanding effect of the gibberellins is to elongate the primary stalk. This effect occurs in the young tissues and growth centers and is caused by an increase in cell length, an increase in the rate of cell division, or both. Gibberellins have a remarkable effect on many dwarf plants, including peas, corn, and beans. Gibberellins also may affect the extent to which the plant develops side branches, and they may increase the size of many young fruits, especially grapes. Because gibberellins induce the production of the enzyme amylase in barley, they commonly are used in the malting of this grain.

Although the gibberellins can induce flowering in many plant species, their greatest commercial uses have been in increasing the size of grapes and in stimulating the growth of sugarcane by

lengthening its primary stalks (15). Treatment of sugarcane with as little as 2 ounces/acre increases the output of cane and raises the yield of sucrose.

Extracts of rape (Brassica napus) pollen have novel growth-promoting effects. A specific growth promoter has been isolated from the extracts and identified as a steroid. This compound, which contains a 7-member B-ring lactone, promotes cell elongation and cell division in several bioassays. Active structural isomers of brassinolide have been synthesized. Preliminary field test results show yield increases occur with a variety of crops. Cost will certainly be a factor in determining its commercial potential.

There are circumstances where reducing the size of the overall plant can be of commercial benefit, e.g., the reduction of stem length in cereal crops by chlormequat chloride. This compound prevents or greatly reduces the probability that wheat will lodge or fall over in heavy winds and rain. The bulk of the wheat grown in Europe is treated with this compound.

Mowing turf grass is a time-consuming and costly maintenance procedure. Certain chemical growth retardants make possible reduction in mowing frequency and, therefore in management costs of such turf areas as highway roadsides, rights-of-way, parks, cemeteries, golf courses, and lawns. Maleic hydrazide has been utilized since the early 1960's for cool-season regulation of grass growth. Methyl chlorflurenol, fluoridamid, mefluidide, ethephon, and ancymidol have been evaluated for their effectiveness alone and in combination for the suppression of both vegetative growth of grasses and seed-head formation.

The development of growth retardants has had a considerable impact on the production of certain crops, especially floral crops, e.g., chrysanthemum, poinsettia, and other decorative flowers. The most commonly used growth retardants are daminozide and chlorphonium chloride. Ancymidol and dikegulac sodium have been extensively investigated, particularly for poinsettias in the case of ancymidol and for the prevention of shoot elongation in junipers by dikegulac sodium. Several triazoles are now used for this purpose.

The effect of retardants on stem growth occurs on the subapical region of the shoot tip where cell division and, to a lesser extent, cell elongation are inhibited. Thus, internodes of retardant-treated plants are shorter primarily because they possess fewer cells. Many growth retardants act by inhibiting a specific step in the synthesis of naturally-occurring gibberellins, which is necessary for the maintenance of subapical meristem activity. When such retardants are used, it is possible to reverse the inhibitory effect in intact plants by the application of an appropriate dose of GA_3.

Regardless of the amount of available water, the cotton plant tends to produce more leaf, stem, and flower material than is necessary for a given fiber yield. This tendency has a restrictive influence on the planting of a greater crop density, on the optimal use of fertilizer, and on the introduction of more efficient harvesting techniques. Only in regions with access to irrigation is the grower able to partially reduce cotton's undesirable rank

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

growth by regulating the amount and timing of the water supply. Mepiquat chloride causes the cotton stand to develop a healthy dark green color. The inhibition of cell elongation and node formation results in decreased growth in length and breadth. This inhibition of growth is particularly marked in the upper plant parts which are not as important for the production of fiber. Cotton plants so treated have a more compact, conical form and, thus, can be spaced closer with narrower rows and a higher plant population (16). The result is a boll set with considerably higher weight and greater yield at the first picking.

Plant Growth Regulators Developed in the 1980's

Among some newer compounds, or new uses for old compounds on different crops, one of the first which should be mentioned is paclobutrazol. This material has been referred to in the literature for quite some time as PP-333. It is a growth retardant which, when applied to the soil around the base of apple trees, controls the shoot growth for several seasons with little effect on fruit size. However, the yield of treated trees under some conditions can be increased because of the increased amount of sunlight which now can get to the fruiting spurs. By reducing excessive terminal growth of the shoots, tree efficiency is increased. The explanation is that many plants produce more leaves than are needed for maximum photosynthesis, and the shade from one or two leaves markedly reduces photosynthesis in those leaves beneath them which are shaded (17). The mode of action of paclobutrazol is by interfering with gibberellin synthesis at an early stage. It can be applied as a foliar spray, as a trunk drench to the soil, or in solution culture.

Other materials are Limit (amidochlor), a grass retardant, and the compound Pix (mepiquat chloride) which is used to reduce the size of cotton plants, allowing more of them to be grown per acre resulting in increased yields. Another material is a phenylurea cytokinin (CPPU) which, when applied to grapes and other plants pre-bloom or just at bloom, prevents abscission. When applied post-bloom in grapes, it increases the size of the berries significantly. This is an extremely active material having its major effects between 1 and 10 ppm of solution, which translates essentially into that number of grams per acre (18). Another newer phenylurea, Dropp (thidiazuron), is used as a defoliant on cotton. Still another compound of interest is the 3-chlorobenzyl-ester of dicamba, which increases the sugar content of grapes (18). Its use in connection with CPPU is a natural for table grapes as the latter increases the berry size only, not the sugar level. The dicamba ester also should prove useful to the wine industry as well as to other crops.

In the United States, the major percentage of potato production is used for processing (french fries, chips, dehydrated, frozen, etc.,) (19). The presence of sugars in raw potatoes is a major problem for processors (20). The major sugars found in potatoes are sucrose, glucose, and fructose. The latter two are reducing sugars and are responsible for the undesirable dark color which may develop in processed potato products. Heat applied during the

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

processing causes reducing sugars to react with amino acids and other organic compounds resulting in darkening. Recent studies have shown several chemicals which reduce the sugar content of raw potatoes and the color of processed potatoes when applied to the crop in the field. Outstanding among these active compounds are 5-bromosalicylic acid, 5-chlorosalicylic acid (21), aspirin (acetylsalicylic acid), and sulfanilamide (22).

The Future

With such a long history in the use of plant growth regulators, why are they not more widespread in their commercial uses, and why have we not heard more about their successes? There are a number of reasons for this, not the least of which was the discovery during the early 1940's by scientists at the Boyce Thompson Institute in the United States and ICI scientists in England that phenoxyacetic acids, when applied to mixed lawns, were found to have no adverse effect on grasses except to kill broadleaf plants. This was the beginning of what has now become the tremendous worldwide herbicide industry. The timing was exquisite. We were in the middle of a war, manpower was short, the use of these materials was shown to be effective, not only biologically but economically. Following the war, emphasis continued and has continued to the present time on herbicides which, in a sense, are but one offshoot of plant growth regulation--they just "regulate" the target plants to death.

Because of the financial success with these chemicals, the work directed toward plant growth regulation, which is much more difficult from almost every point of view, became an orphan. In fact, during the 1960's and 1970's most companies who said they were working on plant growth regulators really were not. What they actually did was to notice if anything unusual happened in their herbicide screening programs; then, if so, they would give it a minimum of attention. There were no, or very few, dedicated staff members working with plant growth regulation. Thus, because of the well established economic value of, the success with, and the relatively simple and straight forward methods of searching for herbicides, the weed-killing aspects of plant growth regulation have overshadowed its other uses.

However, because most crops have an array of herbicides which can be used with them, thus leaving only niches for new herbicides, and with the successful introduction of plant growth regulators for certain important crops, industry has increasingly turned its interest to the search for plant growth regulators within recent years. It should be emphasized that the task of discovering plant growth regulators is substantially more demanding than that involved in the search for a new herbicide. Estimates of the complexity and the costs connected with these searches are estimated to be many times greater for plant growth regulators than for herbicides (23). However, the absence of effective plant growth regulators for many of the major crops makes this field attractive with large potential payoffs. For success, long term commitments are necessary, both to the research and development programs and to the time periods now necessary for approval by regulatory bodies throughout the world.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

The differences between plant growth regulator and herbicide programs are numerous--some of which are major. For example, in a screening program for herbicidal activity, one can test the chemical and let the results of the screen suggest on what crop or crops the chemical might be used for the control of which weeds. There is no such screen as this for plant growth regulators. Much strategic thinking as well as gathering of marketing information needs to enter in decisions involving a plant growth regulator program. First, one must decide the crop for which to develop a regulator. In reaching this decision, a considerable amount of market research information is necessary covering such factors as (a) total worldwide value of the crop, (b) value per acre of the crop, (c) history of chemical use on the crop, (d) educational program necessary to convince the consumer to use a regulator on the crop, and (e) what specifically should be done to make the plant growth regulator of value to that crop. Next, when a target crop is chosen, one must know or should develop an extensive knowledge of the physiology and biochemistry of the crop. Following that, one must answer how the plant growth regulator is to enhance the value of the crop after its treatment, i.e., is it to change some metabolic process, change the physiognomy of the crop, or to increase its yield per se? If the latter, is the economic value of the crop found in its vegetative or in its reproductive state? Is this value in a developing organ such as fruit, or in the root, or in the vegetative shoot? Next, does one expect to apply the plant growth regulator as a foliar spray, directly to the soil, or through an irrigation system? To have its maximum economic effect, when should the chemical be applied?

The complexities in searching for plant growth regulators for a given crop have been amply presented by Archer and his colleagues (24) in their study of the opportunities for the use of plant growth regulators on maize. This study clearly demonstrates the need for knowledge of the life cycle of the crop involved and the need to determine the rate-limiting factor(s) and their interactions in order to determine when a plant growth regulator must be applied to have its maximum effect or, in some cases, to have any effect.

Having emphasized the problems involved, what advantages exist in making plant growth regulators worthwhile? Once they are available, the use of plant growth regulators will have an impact on the economic production of crops both because of their savings in energy and their savings in cash outlay as well as increased profitability. Increasing the yield of a crop for a given area of land means an incremental gain, the only output for which is the cost of the chemical and its cost of application. No extra land must be purchased, no extra land must be tilled, generally no extra fertilizer must be added, no extra weed control or other pest control measures must be taken, and no additional harvesting need be made. In fact, a further reduction in costs can be achieved for example by the synchronization of flowering, eliminating the need for several harvests. There will be extra processing costs in some instances because there will be either additional plant material or

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

additional economic products such as oil, protein, or sugar added to the crop by the proper use of the appropriate plant growth regulator (1).

Regulatory issues will continue to influence the development and availability of agricultural chemicals and future pesticide research directions. Registration requirements, together with the high cost of product development, will affect the availability of current pest control chemicals and the introduction of new products. In addition, future regulatory decisions will affect various segments of agriculture when products are withdrawn without a suitable alternative (25).

An outstanding case in point is the Alar (daminozide) debacle. As pointed out in the Detroit News editorial of February 25, 1990, (26), exactly one year before, CBS Television's "60 Minutes" aired a 15-minute segment on Alar, "a growth hormone sprayed on a large portion of America's apple crop to keep the apples from dropping from the trees prematurely. The program painted a terrifying picture of a threat to the health of American children from the apples and apple juice they consume in school and at home. The result was near panic, an immediate ban on Alar, and a new wave of concern about food and environmental safety".

Some might consider the Alar episode a case of the regulatory system working the way it's supposed to: detecting a risk, moving swiftly to end it, and forcing industry to "clean up its act". Warren T. Brookes, a Detroit News columnist, believes "the episode suggests something very different: a regulatory system acting in collusion with an opportunistic media and overzealous lobbyists, to needlessly scare the public and drive an industry to the brink of ruin". The series of editorials and columns, titled "The Environment: Risk and Reality", published in the Detroit News from February 25 through March 11, 1990, makes interesting reading.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

Literature Cited

1. Nickell, L. G. In Chemistry and World Food Supplies: The New Frontiers; Shemilt, L. W., Ed.; CHEMRAWN II, Pergamon Press: Elmsford, NY, 1983, pp 601-606.
2. Nickell, L. G. In Rol de la quimica en el suministro de alimentos; Latin American Regional CHEMRAWN II Seminar, Buenos Aires, Argentina, May 28-30, 1986.
3. Nickell, L. G. Plant Growth Regulators - Agricultural Uses; Springer-Verlag: Berlin, 1982.
4. Nickell, L. G. Plant Growth Regulating Chemicals; CRC Press, Inc.: Boca Raton, FL, 1983, Vol. I and II.
5. Avery, G. S., Jr.; Johnson, E. F. Hormones and Agriculture; McGraw-Hill: New York, NY, 1947.
6. Bridge, K. In Plant Growth Regulating Chemicals; Nickell, L. G., Ed.; CRC Press, Inc.: Boca Raton, FL, 1983, Vol I; pp 41-58.
7. Yokoyama, H.; Hsu, W. J.; Hayman, E.; Poling, S. M. In Plant Growth Regulating Chemicals; Nickell, L. G., Ed.; CRC Press, Inc.: Boca Raton, FL, 1983, Vol. I; pp 59-70.

8. Schwarz, O. J. In Plant Growth Regulating Chemicals; Nickell, L. G., Ed.; CRC Press, Inc.: Boca Raton, FL, 1983, Vol. II; pp 79-97.
9. Nickell, L. G. Outlook on Agriculture, 1976, 9, pp 57-61.
10. Nickell, L. G. In Bioregulators: Chemistry and Uses; Ory, R. L.; Rittig, F. R., Eds.; ACS Symp. Series No. 257; American Chemical Society: Washington, DC, 1984; pp 101-112.
11. Nickell, L. G. Sugar Jour.; 1988, 50, pp 7-11.
12. Looney, N. E. In Plant Growth Regulating Chemicals; Nickell, L. G., Ed.; CRC Press, Inc.: Boca Raton, FL, 1983, Vol. I; pp 1-26.
13. Looney, N. E. In Plant Growth Regulating Chemicals; Nickell, L. G., Ed.; CRC Press, Inc.: Boca Raton, FL, 1983, Vol. I; pp 27-39.
14. Abeles, F. B. Ethylene in Plant Biology; Academic Press: New York, NY, 1973.
15. Nickell, L. G. In Plant Growth Regulating Chemicals; Nickell, L. G., Ed.; CRC Press, Inc.: Boca Raton, FL, 1983, Vol. I; pp 185-205.
16. Cathey, G. W. In Plant Growth Regulating Chemicals; Nickell, L. G., Ed.; CRC Press, Inc.: Boca Raton, FL, 1983, Vol. I; pp 233-252.
17. Williams, M. W. In Bioregulators: Chemistry and Uses; Ory, R. L.; Rittig, F. R., Eds.; ACS Symp. Series No. 257; American Chemical Society: Washington, DC, 1984; pp 93-99.
18. Nickell, L. G. Acta Hort. 1986, 179, pp 805-806.
19. Kunkel, R.; Thornton, R. E. Wash. State Univ., Coop. Ext. Bull. 0717, 1980, 15 pp.
20. Iritani, W. M.; Weller, L. D. Wash. State Univ., Scientific Paper No. 7267, 1986, 113 pp.
21. Nickell, L. G.; Vogt, G. E. Proc. 17th Ann. Mtg. Plant Growth Regulator Soc. Amer., 1990, pp 88-93.
22. Nickell, L. G. Proc. 18th Ann. Mtg. Plant Growth Regulator Soc. Amer., 1991, pp 115-120.
23. Sacher, R. M. In Chemical Manipulation of Crop Growth and Development; McLaren, J. S., Ed.; Butterworths: London, 1982, pp 13-15.
24. Archer, M. C.; Hansen, D. J.; Loussaert, D. F. In Chemical Manipulation of Crop Growth and Development; McLaren, J. S., Ed.; Butterworths: London, 1982, pp 253-265.
25. Menn, J. J.; Christy, A. L. In Fate of Pesticides in the Environment; Schnoor, J. L., Ed.; John Wiley and Sons, Inc.: New York, NY, 1992, pp 409-430.
26. Editorial; "The Risk Averse Society"; The Detroit News, Feb. 25, 1990.

RECEIVED December 6, 1993

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch001

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

Chapter 2

New Chemical Approaches for Control of Biennial Bearing of Apples

M. W. Williams

Agricultural Research Service, U.S. Department of Agriculture, 1104 North Western Avenue, Wenatchee, WA 98801

The chemicals sulfcarbamide (monocarbamide dihydrogen sulfate), pelargonic acid [$CH_3CH_2)_7CooH$] and endothallic acid [7,oxybicylo(2,2,2)heptane-2-3 dicarboxylic acid] are new, effective blossom thinning agents used to prevent alternate year cropping of apples. Early removal of potential fruit reduces competition for photosynthates and favors flower initiation for the next year's crop. Sulfcarbamide is nearing full registration for commercial use. Pelargonic acid and endothallic acid require further testing for commercial development. Early application of the post-bloom thinner carbaryl (1-naphthyl-n-methyl carbamate) at petal fall time is effective for reducing fruit set and provides an alternative approach to blossom thinning for preventing biennial bearing.

Apple trees (*Malus domestica* Borkh) that set more fruit than is needed for a desirable harvest go into a pattern of alternate year cropping which is referred to as biennial bearing. In addition, over cropping results in tree breakage and many small unmarketable fruit. It is an orchard management practice to interplant the main commercial cultivar with pollinator trees and to provide ample bee hives for cross pollination. The purpose of bee activity and cross pollination is to assure a crop under adverse climatic conditions. Under ideal fruit set conditions, heavy blooming trees can produce 3 or 4 times more fruit than can be matured to adequate size and quality. Under these conditions much of the photosynthates are utilized in fruit formation and few flower buds are formed for the next year's crop. Fruit thinning of apples by hand cannot be accomplished early enough to offset the effect of heavy blossoming and excessive fruit set on flower formation.

Chemical removal of apple fruit in commercial orchards has been practiced since the early 1940s when Elgetol (dinitro-ortho-cresol) was introduced as a blossom thinner and, NAA (naphthalene acetic acid), a plant hormone was

This chapter not subject to U.S. copyright
Published 1994 American Chemical Society

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

developed as a post-bloom thinner (*1*). In the 1960s carbaryl was introduced as a post-bloom thinner, and a combination of NAA and carbaryl is presently used in commercial orchards.

Economic Impact of Biennial Bearing

Biennial bearing or alternate year bearing of apples can be very expensive for the orchardist and the consumer. In the low cropping year the warehouse may not have enough fruit to operate efficiently, and the consumer often pays inflated prices for the available fruit. To maintain good markets it is necessary to consistently supply large quantities of uniform high quality fruit. The loss of market share in the "off" year makes "on" year marketing more difficult. During the "on" year the fruit size is smaller and fruit quality is inferior because of the heavy drain on the trees, and in the "off" year the fruit is too large and can be easily damaged during packing and transport.

The loss of income to the orchardist is serious, e.g. 'Fuji' a new popular cultivar sold on the export market can return more than 25 dollars a packed box, and a full bearing orchard can produce up to 3000 or more packed 20-kg boxes per hectare. When early fruit removal is not accomplished 'Fuji' becomes a very biennial cultivar . A crop of 'Fuji' every other year means the grower could lose thousands of dollars in the "off" year. On an industry wide basis the loss with all biennial cultivars can amount to millions of dollars.

Once a tree goes into biennial bearing the pattern will continue until the cycle is broken by reducing fruit competition soon after bloom. As apple trees get older and become less profitable, the cost of early hand thinning becomes prohibitive. To accomplish both hand blossom thinning and follow up post-bloom thinning by hand the cost can be over 2000 dollars per hectare, whereas a full chemical blossom and post-bloom thinning program costs less than 300 dollars per hectare, and the timeliness of early chemical removal of fruit promotes return bloom and annual cropping.

Physiological Basis for Biennial Bearing

During the flower initiation period, active cell division occurs in many parts of the tree. As new fruitlets develop new leaves are formed and active shoot growth begins. Because of the competitive "sink" effect at the flowering site, there is heavy competition for photosynthates. Therefore the period preceding and during flower initiation is a "critical time" in the reproductive cycle. Strong active vegetative growth and early fruit development can alter the endogenous growth regulator balance influencing flowering. Stress induced in the tree by climatic and cultural conditions or by chemical thinning agents during the "critical period" stimulates ethylene production and subsequent fruit abscision; thus competition is reduced and floral initiation is favored (6).

There is an antagonistic relationship between seed development and flowering; 'Spencer', a seedless cultivar, bears fruit annually, but trees carrying fruit with a high complement of seeds have less tendency to initiate flowers than those with fewer seeds (15). Seeds produce gibberellins (GA) which promote

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

vegetative and fruit growth and inhibit flower bud formation. Spur type 'Golden Delicious' trees with a moderate crop of fully seeded fruit produce more terminal shoot growth than similar trees without flowers and a seeded crop. This observation may suggest GA production stimulated by seed formation is utilized in shoot elongation which competes with flower initiation (*13*).

Chemical Thinning Strategy

A satisfactory chemical thinning program will remove enough fruit to assure good fruit size and an adequate return bloom the following season. Growers try to slightly over thin with chemicals to avoid excessive supplemental hand thinning. Some hand thinning is needed to remove poorly shaped and small fruit to improve fruit quality. The limited need for hand thinning after chemical thinning can be done over a relatively long period without affecting the next year's crop or reducing the size and quality of the current fruit crop.

On bearing trees, flowers are borne on spurs 2 years of age and older. To maintain consistent annual flowering and cropping it is necessary each year to have no more than 30-40% of the spurs flowering. Since most of the flower initiation for next year's crop is determined by 4 to 6 weeks after the current year's bloom, it is necessary to remove fruit competition by 4 weeks after bloom (*2-5*).

To assure adequate fruit thinning and return bloom on biennial cultivars, such as 'Golden Delicious' in Washington State, 2 or 3 chemical thinning sprays are often necessary. In the past a blossom spray of Elgetol was applied when 80% of the blossoms were open. At 7 to 14 days from bloom, 1-naphthaleneacetamide (NAAm) was applied and if necessary, a carbaryl spray was applied when the fruitlets were 10 to 15 mm in diameter. NAAm removed the small fruit on 1-year-old wood in the tops of the trees. Currently NAAm and 1-naphthyl-n-methyl carbamate (carbaryl) are used in combination as early post bloom sprays for thinning 'Golden Delicious'. Naphthalene acetic acid (NAA) at a rate of 3-5 ppm can be combined with carbaryl for consistent delayed post bloom thinning of 'Golden Delicious', 'Spur Red Delicious' and other biennial apple cultivars (*7*).

Need for New Chemicals

In 1989, Elgetol the DNOC spray registered as a blossom spray on apples was removed from the market by the manufacturer because of the high cost of reregistration. This action caused a renewed research effort to find viable new blossom thinners with action similar to Elgetol, in addition, ways to improve the efficacy of post bloom thinners were explored.

The action of Elgetol in reducing fruit set apparently was to damage the pollen tubes to prevent fertilization of the ovules (*8*). Elgetol was applied after the dominant blossoms "king bloom" were pollinated and fertilized but before the recently opened blossoms were fertilized. When the application was timed correctly Elgetol worked well as a blossom thinner. The disadvantage of

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

Elgetol was if rewetted by heavy dew or light rain with cold temperatures, too much chemical was absorbed, tissue damage was too extensive and over thinning occurred. Conversely when weather was hot and dry at application time chemical absorption was less and little fruit thinning occurred. Elgetol was most effective when the bloom occurred over a short period of time and temperature at bloom time was moderate. The commercial use of Elgetol was mostly in Washington State and other western states where ideal conditions for bloom and fruit set generally prevail.

New Chemicals for Blossom Thinning

Sulfcarbamide. Monocarbamide dihydrogen sulfate, sulfcarbamide, is a urea sulfuric acid complex which has a caustic effect on flower styles and pollen tubes. Sulfcarbamide breaks down quickly to urea and sulfates which are utilized as plant nutrients. Unlike Elgetol there is no further effect on fruit thinning after the spray is dry, thus no adverse affect of rain or cold on fruit set.

The first trials with sulfcarbamide were conducted by the author on Primrose flowers. A low concentration (0.25% v/v) damaged flower parts without affecting vegetative tissue. Sulfcarbamide was next tested on apples in Australia and in Washington State. After 6 seasons of testing from 1990 to 1993, 3 in Australia and 3 in Washington State, sulfcarbamide was marketed in 1993 on a limited basis as Wilthin in Washington, Oregon and Idaho under an experimental label. The manufacturer, Unocal, Sacramento, California, expects to have registration for full use in 1994.

In all experimental trials full bearing apple trees were selected for uniformity of growth and amount of bloom. Only trees with 85% or more of the growing points with flower clusters were used for treatment and data collection. Four single tree replicates with filler trees between rows and treatments were used and 3 limbs with uniform bloom were tagged and flower clusters were counted. Following treatment and after fruit drop was over, the number of fruit on each of the count limbs was counted and recorded. Results are expressed as number of fruit per 100 blossom clusters. Trees were sprayed to run off with a high pressure hand gun or a commercial air blast sprayer when 70% of bloom was open. The Waller-Duncan K-ratio t-test was used to test for differences among treatments and controls (*9*). The sulfcarbamide used was formulated product 79% A.I. A commercial rate of Elgetol at 0.25% v/v was used as a comparison with sulfcarbamide.

Typical results of the Washington trials are shown in Table I. At the 0.375% v/v rate sulfcarbamide was as effective as Elgetol on 'Delicious' and more effective than Elgetol on 'Fuji' and 'Gala' (*10*). Fruit marking which appears as spots or as russet when severe can occur when sulfcarbamide at the 0.375% v/v rate is applied too late (full bloom) or follows a mineral element spray such as Cu. The damage occurs when large droplets of spray dry on the young delicate fruit receptacles; the droplets dry, and the chemical becomes more concentrated (14). The addition of a non-ionic surfactant such as Regulaid at 0.125% v/v increases the wetting of the flower parts and decreases the

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

droplet size. The result is more fruit thinning activity and elimination of fruit marking Table II. On 'Delicious', 'Fuji', 'Gala', and 'Braeburn' the 0.25% v/v rate of sulfcarbamide plus 0.125% v/v Regulaid reduced fruit set more than the 0.375% v/v rate of sulfcarbamide used alone. The use of Regulaid with sulfcarbamide rates higher than 0.375% v/v may not reduce marking.

The sulfcarbamide trials in Australia were conducted the first year to determine optimum concentration for fruit reduction without excessive leaf and fruit marking. The optimum rates were 0.25% v/v to 0.375% v/v. The second season in Australia the effect of temperature on fruit and leaf injury at time of application was determined. Sprays were applied early morning or mid afternoon with a temperature spread from 13 to 25°C. There was no significant effect of these temperatures on fruit or leaf injury. Later comparison of night vs day spraying in Washington indicated more injury with a longer drying time at night. The third season trials in Australia were conducted to verify the fruit injury reduction effect by the addition of Regulaid to the 0.25% v/v rate of sulfcarbamide. Fruit set in the latter trial was poor due to adverse weather for pollination and fertilization so the effect on thinning could not be fairly evaluated. However, the effect on fruit marking confirmed the Washington data in Table II in that 0.25% v/v sulfcarbamide plus Regulaid eliminated fruit marking.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

Table I. Chemical Thinning of 'Fuji', 'Gala' and 'Delicious' Apples with Sulfcarbamide, Expressed as Number of Fruit per 100 Blossom Clusters, Washington, 1991

Treatment*	*Fuji*	*Gala*	*Delicious*
Control	232 a**	237 a	122 a
Elgetol 0.25% v/v	203 ab	189 b	80 c
Sulfcarbamide 0.25% v/v	171 bc	170 b	94 b
Sulfcarbamide 0.25% v/v twice	152 c	140 c	78 c
Sulfcarbamide 0.375% v/v	150 c	112 cd	72 c
Sulfcarbamide 0.5% v/v	175 bc	105 d	44 d

*Treatments applied at 70% bloom open.
**Treatments with a common letter are not different by Waller-Duncan, K-ratio, t-test, P = 0.05. Adapted from reference 10.

Pelargonic Acid. Pelargonic acid [$CH_3(CH_2)_7CooH$] is a natural component of plants and is used commercially in some foods. Mycogen Corp, San Diego, California, is presently developing a product for blossom thinning of apples. It presumably will be marketed as an organic fruit thinning agent. Pelargonic acid

has been tested in Washington State for two seasons. A rate of 0.25% v/v of a 60% A.I. formulated product provides consistent and adequate fruit reduction without causing fruit russeting. Unfortunately, higher rates cause fruit russetting on most cultivars. In limited trials it appears the addition of a surfactant can increase fruit thinning without russeting. The above proposal will be tested further in Australia in the 1993-94 season. A comparison of the thinning of pelargonic acid, endothall and sulfcarbamide on 'Delicious' is presented in Table III. With all three compounds the amount of fruit removal was adequate to prevent biennial bearing.

Table II. Number of Apples per 100 Blossom Clusters after Treatment with Sulfcarbamide and Regulaid, Washington, 1993

*Treatment**	*Delicious*	*Gala*	*Fuji*	*Granny Smith*	*Braeburn*
Control	86	75	98	89	52
Sulfcarbamide 0.375% v/v	82	68	63	52	38
Sulfcarbamide 0.25% v/v + Regulaid 0.125% v/v	68*	50*	29*	58*	19*

*Addition of Regulaid eliminated fruit marking.

Endothallic Acid. Endothall [7, oxybicylo(2,2,2)heptane-2-3 dicarboxcylic acid] is used as an aquatic herbicide and has a registered tolerance of 0.2 parts per million in potable water. At very dilute rates it is effective as a blossom thinner for apples. Trials conducted in Washington State indicate positive fruit thinning can be obtained on several apple cultivars (Table III). The product tested was the potassium salt of endothallic acid containing 0.5% A.I. (manufacturer is Elf Atochem N.A., Philadelphia, Pennsylvania). Fruit injury was not present in any of the endothall trials even when 3 times the acceptable thinning rate was used.

Some minor leaf necrosis occurred on the primary spur leaves. The leaf damage was not considered serious since the primary leaves are only a small part of the total foliage. New leaves formed after the application were not affected. Further tests with endothall will be conducted in Australia and New Zealand in 1993 and in Washington in 1994. Usually from 3 to 5 seasons or more of testing is necessary before a product can be recommended for commercial use.

To be commercially acceptable a blossom thinner compared to untreated controls, should reduce fruit set on full bloom trees by 25 to 50%. This is usually sufficient for return bloom and annual cropping. At blossom time the objective is to under-thin rather than over-thin because pollination and fertilization of flowers may be poor due to adverse weather. Also if frost occurs after blossom thinning, the trees may still have enough undamaged

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

flowers to bear a full crop of fruit. Only about 5% of the blossoms on a full bloom tree are necessary for a full crop.

Table III. Comparison of Blossom Thinners on Apples Expressed as Fruit per 100 Blossom Clusters, Washington, 1993

Treatment	*Delicious*	*Fuji*	*Granny Smith*
Control	86 a*	98 a	89 a
Sulfcarbamide 0.25% v/v + Regulaid 0.125% v/v	68 b	29 c	58 bc
Pelargonic acid 0.125% v/v	66 bc	95 a	46 c
Pelargonic acid 0.25% v/v	55 c	66 b	29 c
Endothall 0.125% v/v	67 bc	85 ab	48 c
Endothall 0.25% v/v	59 c	72 b	23 c

*Treatments with common letters are not different by Waller-Duncan, K-ratio, t-test, P = 0.05. Adapted from reference 10.

Improved Post Bloom Fruit Thinning

After chemical blossom thinning, post bloom fruit thinning sprays are necessary on most biennial bearing cultivars, at least one or two applications are needed to obtain adequate fruit size and quality. If only post bloom thinning is used, good fruit thinning can occur, but the tree may still go biennial. The latter occurs because a blossom thinner is not used or because the tree is low in vigor (N). Post bloom thinners are usually applied when the fruit are 10-15 mm in diameter and may not be effective in promoting return bloom because it is past the blossom initiation period, or cool conditions following post bloom applications result in poor thinning and return bloom.

The post bloom thinners registered for use on apples are carbaryl and NAA. Early work with NAA on 'Jonathan' apples indicated a spray applied at petal fall was more effective in reducing fruit set than when applied 16 days later (*11*). After the loss of Elgetol trials were conducted to determine if carbaryl was also effective as a petal fall post bloom thinner. Petal fall is the stage of bloom when 50% or more of the flower petals can be blown off the tree. The cultivars used were 'Delicious', 'Fuji' and 'Granny Smith'. The experimental design was the same as described above for the blossom thinning trials. The trees were sprayed to run off with high pressure hand guns or with a commercial air blast sprayer. The formulation of carbaryl used was XLR-plus flowable 50% A.I. supplied by Rhone-Poulenc, Research Triangle Park, North Carolina.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

In the 1992 trials petal fall sprays of carbaryl were more effective than NAA in reducing fruit set (Table IV) (*12*). The 10 ppm NAA sprays caused leaf curling and reduced terminal shoot growth, whereas carbaryl sprays had no effect on leaf shape or shoot growth. Average seed number per fruit was greater in the carbaryl treated fruit than in the NAA or control fruit (Table V). A possible reason for the latter is a single fruit per cluster remained on the carbaryl treated trees whereas 2 to 3 fruit per cluster remained on the NAA and control trees. The tendency for carbaryl to remove all but "king bloom" fruit is a very desirable feature. "King bloom" fruit are the largest and normally have a full seed count and optimum fruit shape. NAA-treated fruit had the lowest seed counts which suggests induced seed abortion. A full complement of seed is needed in an apple to obtain optimum fruit shape and growth. When seed are aborted on one side of a fruit it is often misshapen and is assigned to a lower grade. In 1993 carbaryl was applied at a rate of 0.178% v/v at petal fall and at 7 days past petal fall to determine if effective thinning occurred later than at petal fall. The degree of fruit thinning for 'Delicious' and 'Fuji' was the same for both spray times (Table VI). 'Granny Smith' fruit were thinned more at 7 days after petal fall.

A petal fall application of carbaryl is not intended for use after blossom thinning but is an attractive alternative if a blossom spray is not used or if bloom and weather conditions are less than optimum. In fruit growing regions where bloom is extended over a long period a petal fall spray would be effective in reducing crop load and biennial bearing. For heavy fruit setting cultivars such as 'Golden Delicious' and 'Fuji' carbaryl can be combined with NAAm post bloom sprays to increase fruit removal. In cool growing areas the combination of post bloom thinners such as NAA and carbaryl is practiced (*7*).

Table IV. Effect of Early Sprays of NAA or Carbaryl on Fruit Set of 'Delicious Apples', Washington, 1992

Treatment	*Application Time**	*Fruit Set per 100 Blossom Clusters*
Control		65 a**
NAA 5 ppm + Regulaid	P.F.	54 abc
NAA 10 ppm + Regulaid	P.F.	50 decf
Carbaryl 0.125% v/v	F.B.	52 bdc
Carbaryl 0.178% v/v	F.B.	43 decf
Carbaryl 0.125% v/v	P.F.	38 ef
Carbaryl 0.178% v/v	P.F.	35 f

*F.B. = full bloom spray; P.F. = Petal fall spray.
**Treatments with common letters are not different by Waller Duncan t-test.
Adapted from reference 10.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

Table V. Seed Number per Fruit after NAA or Carbaryl Petal Fall Sprays on 'Delicious' Apples, Washington, 1992

Treatment	*Application Time**	*Number of Seed per Fruit*	*Average Number of Fruit per Cluster*
Control		5.5 c*	3.5 cd**
NAA 5 ppm & Regulaid	P.F.	2.3 d	3.0 c
NAA 10 ppm + Regulaid	P.F.	3.5 e	2.8 bc
Carbaryl 0.178% v/v	F.B.	7.5 a	1.5 b
Carbaryl 0.178% v/v	P.F.	6.8 b	1.0 a

*Average for 100 fruit per treatment. Treatments with common letters are not different by Waller-Duncan, K-ratio, t-test, P = 0.05.
**Average number of fruit after "June drop". Adapted from reference 10.

Table VI. Effect of Petal Fall Sprays of Carbaryl on Fruit Set per 100 Blossom Clusters of Apples, Washington, 1993

Treatment	*Delicious*	*Granny Smith*	*Fuji*
Control	86 a*	84 a	98 a
Petal fall	44 b	62 c	51 b
Petal fall + 7 days	54 b	48 b	66 a

* Treatments with common letters are not different by Waller-Duncan, K-ratio, t-test, P = 0.05.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

Literature Cited

1. Williams, Max W.; Edgerton, Louis J. Fruit thinning of apple and pears with chemicals. **1981**. USDA. Info Bull. No. 289.
2. Williams, M.W. *Acta Hort.* **1972**, *34*, 167-174.
3. Wertheim, S.J. *Acta Hort.* **1972**, *34*, 321-332.
4. Edgerton, L.J. *Acta Hort.* **1972**, *34*, 333-352.
5. Williams, Max W. *Hort Review* **1979**, *1*, 272-302.
6. Williams, Max. W. *Acta Hort.* **1989**, *240*, 222-228.
7. Crop protection guide for tree fruits in Washington. **1993**. Washington State University Bull. EB0419.
8. Hildebrand, E.M. *Amer. Soc. Hort. Sci.* **1944**, *45*, 53-58.
9. SAS Institute Inc. **1987**, Cary, NC. 6 edition.
10. Williams, Max W. *HortTechnology.* **1993**, 3(4):428-429.

11. Donoho, Clive W. Jr. *Proc. Amer. Soc. Hort. Sci.* **1968**, 92-55-62.
12. Williams, Max W. *HortTechnology*. **1993**. *3*(3), 323-324.
13. Curry, E.A. and Looney, N.E. *HortScience* **1985**. *21*, 1015-1017
14. Curry, E.A. and Williams, M.W. *Proc. Plant Growth Reg. Soc. Amer.* **1992**, p. 168.
15. Luckwill, L.J. *Physiology of trees*. Acad. Press, NY. **1970**, p. 249.

RECEIVED February 1, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch002

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

Chapter 3

Bioregulator-Induced Changes in the Composition of Sugarcane

Effects of Tops on Processing

B. L. Legendre[1], M. A. Clarke[2], and M. A. Godshall[2]

[1]Sugarcane Research Unit, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 470, Houma, LA 70361
[2]Sugar Processing Research Institute, Inc., New Orleans, LA 70124

The bioregulator glyphosate (N-phosphonomethyl glycine) increased sucrose % cane and the yield of theoretical recoverable sugar per ton of sugarcane (TRS/TC). For glyphosate treated sugarcane, there was a reduction in juice purity and reducing sugars to ash ratio but an increase in total polysaccharide (TPS), dextran, and leucoanthocyanin when compared to untreated but mature sugarcane. Glyphosate had no apparent effect on reducing sugars, inorganic ash, fiber % cane, or starch content. The use of glyphosate does not come without potential adverse affects on processing, namely the decrease in the reducing sugars to ash ratio and a possible increase in TPS and dextran content which can ultimately contribute to sugar losses and lower sugar quality. However, these negative responses to the use of glyphosate can be partially offset by cultivar selection and/or removing the leaves and upper portion of the stalk by topping.

Through plant breeding and selection of sugarcane, cultivars which are capable of producing relatively high yields of sugar during a 7 to 9 month growing season have been developed in Louisiana (Legendre, B.L., Proc. Int. Soc. Sugarcane Technol., in press.); however, sugarcane growers still depend largely on natural environmental conditions to trigger the maturation process. As an alternative to reliance on climatic factors affecting natural ripening, and with cognizance of the need of Louisiana sugarcane farmers to harvest immature sugarcane, research was begun on the use of bioregulators as chemical ripening agents in the 1940s (*1, 2*). To date, hundreds of bioregulators have been evaluated world-wide for their ability to ripen sugarcane; however, until 1980 glyphosine [N,N-bis (phosphonomethyl) glycine] was the only compound registered for this use in the United States (*3*). In the autumn of 1980, glyphosate (N-phosphonomethyl glycine) was also registered as a ripener for sugarcane. Nickell (*3*) stated that glyphosate formulations improve the sucrose content over a wide range of climatic conditions, are less cultivar specific, and the ripening response induced in the sugarcane is more consistent and rapid than that obtained with glyphosine. In Louisiana and Hawaii, glyphosate has resulted in an average gain of over 0.4 and 1.2 tons of

0097–6156/94/0557–0026$08.00/0
© 1994 American Chemical Society

sugar per hectare and is used on over 30 and 90% of the harvested crops, respectively (Nickell, (*3*); Legendre, B.L., USDA-ARS, Houma, LA, unpublished data.). Prior to 1943, sugarcane in Louisiana was hand-cut and hand-stripped and yields of recoverable sugar per ton of cane nearly equalled theoretical yield based on the Java formula (*4*). However, by 1943, there was a considerable discrepancy between the actual and the theoretical yield of sugar. This discrepancy was largely due to the drop in juice extraction and boiling house efficiency brought about by the change to mechanical harvesting and the increase in cane trash, to include tops, delivered with the harvested cane. Even before the advent of the mechanical harvester, it was recognized that the level (severity) of topping had a considerable effect upon the quality of juice from harvested sugarcane (*5, 6*). Proper setting of the topper blade on the whole-stalk (soldier) Louisiana harvester has an important effect on the quality of harvested sugarcane (Legendre, B.L., Sugar J., in press.). When sugarcane stalks are topped too low by the harvester operator, mature joints on the taller stalks are left in the field resulting in a loss in the yield of cane. When topped too high, immature joints on the shorter stalks are delivered to the mill with a resulting loss of sugar yield (*7*). This relationship is most important early in the harvest season when relatively immature cane is delivered to the mill.

However, the response to glyphosate is primarily in the top one-third of the sugarcane stalk ((*8*); Legendre, B.L., USDA-ARS, Houma, LA, unpublished data.). Further, the growth retarding properties of the compound cause a reduction in overall cane yield; therefore, farmers today using glyphosate have a tendency to harvest cane without removing much, if any, of the top in order to maximize sugar yield. As early as 1983, there was concern that glyphosate also increased the level of total polysaccharide (TPS), notably dextran, in the juice of harvested cane. In preliminary studies conducted in 1985 and 1986, the results were inconclusive (Legendre, B.L., USDA-ARS, Houma, LA, unpublished data.). Further, the data indicated that dextran concentration (ppm on solids) was not influenced by removal of the top above the apical meristem (bud) of the cane with or without glyphosate. The present study was the first comprehensive study initiated to examine the effects of glyphosate and topping height on the composition of three sugarcane cultivars with specific emphasis on processing responses.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

Materials and Methods

Six field experiments, repeated twice, were conducted using first- or second-ratoon crops of three cultivars, CP 65-357, CP 70-321, and CP 72-370 (Table I). In three of the sampled fields, glyphosate was applied to each of the three cultivars aerially at 0.3 kg ha^{-1} in 46.7 L ha^{-1} spray mixture 30 to 35 days prior to harvest. In the remaining three fields, no glyphosate was applied prior to harvest. In order to compare the results at approximately the same level of maturity (glyphosate normally advances the maturity curve by approximately one month), samples from non-treated fields were harvested 3 to 5 weeks later than the glyphosate treated fields.

For each sampled field, three hundred stalks were serially selected along three rows (100 per row) of the field in standing cane, cut at the soil surface, stripped of all older leaves, and removed from the field with the green tops intact. Stalks were then divided at random into five, 60-stalk lots. The tops of the stalks in each lot were removed at one of five different heights (treatments): 1) no removal of tops; 2) 5 cm above the apical meristem (bud); 3) 10 cm below the bud; 4) 25 cm below the bud; and 5) 40 cm below the bud. Treatments were then divided at random into six subsamples (replications) containing 10 stalks each. Each subsample was weighed to calculate mean stalk weight (bundle weight divided by 10) prior to passing it through a shredder to prepare the sample for analyses according to the procedure described by Legendre (*4*). A 1000 g sample of the

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

Table I. Locations, dates, and mean stalk length of individual experiments

Experiment no.	Cultivar	Location	Harvest dates[1]	Mean stalk length[2] (cm)
		Glyphosate treated		
1a	CP 65-357	James Breaux	10-22-92	167
1b	CP 65-357	Laurel Valley	11-05-92	215
2a	CP 70-321	Little Texas	10-28-92	180
2b	CP 70-321	Laurel Valley	11-05-92	207
3a	CP 72-370	Boudreaux Bros.	10-23-92	185
3b	CP 72-370	Godfrey Knight	11-12-92	222
		Non-treated (no glyphosate)		
4a	CP 65-357	Ardoyne Farm	11-30-92	235
4b	CP 65-357	Ardoyne Farm	12-01-92	250
5a	CP 70-321	Ardoyne Farm	11-30-92	255
5b	CP 70-321	Ardoyne Farm	12-01-92	222
6a	CP 72-370	Ardoyne Farm	11-30-92	237
6b	CP 72-370	Ardoyne Farm	12-01-92	262

[1] Harvest dates were 30-35 days after application of glyphosate at 0.3 kg ha^{-1}.
[2] Mean stalk length of stalks from apical meristem (bud) to base of stalk: basis for standardizing topping in each experiment.

prepared cane was pressed in a hydraulic press at a pressure of 246 kg/cm^2 for 2.5 minutes. The hydraulic press separated the sample into juice (approximately 80% extraction) and residue (bagasse), both of which were analyzed, the former for Brix by refractometer and pol by saccharimeter and the latter only for moisture (by drying) for 24 hours at 150°C. From each sample of juice a 100 ml subsample was removed, frozen, and saved for additional analyses at the Sugar Processing Research Institute, Inc. (SPRI) laboratory in New Orleans, LA. The Brix, sucrose, purity (ratio of sucrose to Brix), and fiber % cane and yield of theoretical recoverable sugar per ton of cane (TRS/TC) were calculated from these analyses.

The invert sugars, glucose and fructose (reducing sugars) % juice, were determined by near infrared spectrophotometry. The correlation of conventional analytical data (HPLC and GLC methods) to NIR spectral data was accomplished by partial least squares.

Inorganic ash was determined as conductivity ash, using the International Commission for Uniform Methods of Sugar Analysis (ICUMSA) Method (*9*). Inorganic ash is reported as a percent of cane juice solids.

Total polysaccharide (TPS) was measured colorimetrically using phenol-sulfuric acid after precipitation from the sugarcane juice with 80% alcohol, by SPRI Method (*10*). TPS is reported as ppm on solids.

Dextran was measured after isolation as a copper complex using Association of Official Analytical Chemists (AOAC) Method (*11*). Dextran is reported as ppm on solids.

Starch was measured using iodine colorimetry according to the Sugar Milling Research Institute (SMRI) (South Africa) Method (*12*). Starch is reported as ppm on solids.

Leucoanthocyanin was determined by absorption at 485 λ after conversion to the colored anthocyanin moiety, during the TPS test. That is, in the TPS test, after polysaccharide is isolated as the 80% ethanol precipitate, it is briefly boiled with 1% sulfuric acid and brought to 200 ml volume. During the sulfuric acid treatment, the leucoanthocyanin is converted to the pigmented form. The sample is filtered on filter paper and absorbance measured. This test measures only the leucoanthocyanin associated with the polysaccharide and is reported as relative absorbance (X 1000) on cane juice.

Analyses of variance were performed and differences among means were tested using the least significant difference test ($LSD_{0.05}$).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

Results and Discussion

Sucrose % Cane. A non-significant glyphosate*variety effect indicated that differences in sucrose % cane between treated and non-treated cane were statistically similar with no difference in response for the three cultivars studied (Table II). Average sucrose % cane was significantly higher for glyphosate treated cane than cane not treated with glyphosate. It is well documented that glyphosate application 3 to 7 weeks before harvesting significantly increases sucrose content in sugarcane stalks (*3, 13-15*). Further, Osgood and Teshima (*16*) stated that recovery of sugar by the factory is greater from sugarcane containing a higher level of sucrose.

Sucrose % cane, relative to the severity of topping, remained consistent for the three cultivars (i.e. non-significant topping*cultivar effect) (Table II). Also, sucrose % cane, relative to severity of topping, was found to be independent of whether glyphosate was applied. Further, sucrose % cane increased with the severity of topping. However, the increase in sucrose content is more pronounced in the younger internodes (usually the upper third of the stalk) than in the mature ones (*8, 17*). As early as 1935, Arceneaux (*5*) reported that the sucrose content of the juice from factory cane grown under Louisiana conditions would increase

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

Table II. Glyphosate treatment and topping effects on cane composition

	Non-treated (no glyphosate)							Glyphosate treated[1]							
	Topping level[2]							Topping level							
	1	2	3	4	5	LSD (0.05)[3]	Mean[4]	1	2	3	4	5	LSD (0.05)[3]	Mean[4]	LSD (0.05)[5]
			Sucrose (%)							**Sucrose (%)**					
CP 65-357	15.7a	16.4b	16.5bc	16.8cd	17.0d	0.4	16.4A	16.0a	17.1b	17.3bc	17.4bc	17.6c	0.4	17.1A	0.8
CP 70-321	15.6a	15.6a	16.0b	16.3bc	16.6c	0.4	16.0A	16.9a	17.1ab	17.5cd	17.3bc	17.7d	0.4	17.3A	0.8
CP 72-370	15.4a	15.6ab	15.8b	15.9b	16.3c	0.4	15.8A	16.0a	16.2a	16.6b	16.7b	16.9b	0.4	16.5A	0.8
LSD(0.05)[6]	1.9	1.9	1.9	1.9	1.9			1.9	1.9	1.9	1.9	1.9			
Mean[7]	15.6a	15.8b	16.1c	16.3d	16.6e	0.2[8]	16.1	16.3a	16.8b	17.1c	17.1c	17.4d	0.2[8]	16.9	0.5[9]
			Purity (%)							**Purity (%)**					
CP 65-357	92.5a	92.8ab	93.0ab	93.8b	93.6b	0.9	93.1A	87.7a	90.6bc	90.5bc	90.1b	91.0c	0.9	90.0A	2.0
CP 70-321	92.1a	92.0a	93.0b	93.2b	93.8b	0.9	92.8A	93.2a	93.0ab	93.6bc	93.6bc	94.0c	0.9	93.5B	2.0
CP 72-370	91.6a	92.3a	92.1a	92.4a	93.3b	0.9	92.4A	86.4a	87.9b	88.7bc	88.4b	89.3c	0.9	88.2A	2.0
LSD(0.05)[6]	4.9	4.9	4.9	4.9	4.9			4.9	4.9	4.9	4.9	4.9			
Mean[7]	92.1a	92.4ab	92.7b	93.2c	93.6c	0.5[8]	92.8	89.1a	90.5b	90.9b	90.7b	91.4c	0.5[8]	90.5	1.2[9]
			Fiber (%)							**Fiber (%)**					
CP 65-357	12.8a	13.1b	12.6a	12.9ab	12.7a	0.4	12.8C	11.9b	11.7ab	11.4a	11.6a	11.4a	0.4	11.8A	0.6
CP 70-321	11.4b	10.7a	11.1b	10.6a	10.7a	0.4	10.9A	12.0b	11.0a	11.3a	11.2a	11.3a	0.4	11.4A	0.6
CP 72-370	12.0b	12.0b	11.7ab	11.7ab	11.6a	0.4	11.8B	12.9b	11.8a	11.5a	11.6a	11.6a	0.4	11.9A	0.6
LSD(0.05)[6]	1.6	1.6	1.6	1.6	1.6			1.6	1.6	1.6	1.6	1.6			
Mean[7]	12.1b	12.0b	11.8a	11.8a	11.7a	0.2[8]	11.8	12.6b	11.5a	11.4a	11.5a	11.4a	0.2[8]	11.7	0.4[9]
			TRS/TC (kg)							**TRS/TC (kg)**					
CP 65-357	139.6a	145.1b	146.6bc	149.6cd	151.6d	4.1	146.4A	138.6a	152.1b	153.6b	153.6b	156.1b	4.1	151.1AB	7.7
CP 70-321	139.6a	140.6ab	144.6bc	147.1cd	150.6d	4.1	144.6A	151.6a	154.1ab	157.6bc	156.6b	159.6c	4.1	156.1B	7.7
CP 72-370	137.1a	139.1ab	141.1ab	142.1b	146.6c	4.1	141.1A	137.6a	142.1bc	146.1cd	146.1cd	148.6d	4.1	144.1A	7.7
LSD(0.05)[6]	19.0	19.0	19.0	19.0	19.0			19.0	19.0	19.0	19.0	19.0			
Mean[7]	138.6a	141.6b	144.1c	146.1c	149.6d	2.4[8]	144.1	142.6a	149.6b	152.6c	152.1c	155.1d	2.4[8]	150.6	4.5[9]

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

Table II. Continued

	Non-treated (no glyphosate)							Glyphosate treated[1]							
	Topping level[2]							Topping level							
	1	2	3	4	5	LSD (0.05)[3]	Mean[4]	1	2	3	4	5	LSD (0.05)[3]	Mean[4]	LSD (0.05)[5]
			Reducing sugars (%)							**Reducing sugars (%)**					
CP 65-357	0.12a	0.15c	0.14bc	0.13ab	0.13ab	0.02	0.13B	0.09a	0.14c	0.13c	0.11b	0.11b	0.02	0.12A	0.01
CP 70-321	0.10a	0.12bc	0.13cd	0.14d	0.12bc	0.02	0.12A	0.12a	0.13a	0.13a	0.15b	0.15b	0.02	0.14B	0.01
CP 72-370	0.15d	0.10a	0.12bc	0.11ab	0.13c	0.02	0.12A	0.10a	0.09a	0.12b	0.14c	0.14c	0.02	0.12A	0.01
LSD(0.05)[6]	0.02	0.02	0.02	0.02	0.02			0.02	0.02	0.02	0.02	0.02			
Mean[7]	0.12a	0.12a	0.13a	0.13a	0.13a	0.02[8]	0.12	0.10a	0.12b	0.13b	0.13b	0.13b	0.02[8]	0.12	0.01[9]
			Inorganic ash (%)							**Inorganic ash (%)**					
CP 65-357	0.56a	0.46a	0.45a	0.45a	0.47a	0.14	0.48A	0.56b	0.54ab	0.45ab	0.42ab	0.41a	0.14	0.48B	0.07
CP 70-321	0.48a	0.48a	0.45a	0.43a	0.42a	0.14	0.45A	0.63b	0.52ab	0.52ab	0.48a	0.46a	0.14	0.52B	0.07
CP 72-370	0.48a	0.46a	0.45a	0.46a	0.40a	0.14	0.45A	0.48b	0.42ab	0.37ab	0.37ab	0.34a	0.14	0.40A	0.07
LSD(0.05)[6]	0.32	0.32	0.32	0.32	0.32			0.32	0.32	0.32	0.32	0.32			
Mean[7]	0.51b	0.47a	0.45a	0.44a	0.43a	0.08[8]	0.46	0.56c	0.49bc	0.45ab	0.42ab	0.40a	0.08[8]	0.47	0.04[9]
			Total polysaccharide (TPS) (ppm)							**Total polysaccharide (TPS) (ppm)**					
CP 65-357	4745a	4252a	3934a	5482a	5860a	2979	4855A	4235a	3738a	5519a	5069a	4742a	2979	4661A	1684
CP 70-321	3801a	3872a	3582a	3900a	3433a	2979	3718A	4338a	3726a	4023a	3539a	3577a	2979	3840A	1684
CP 72-370	6455a	6395a	6235a	5989a	5438a	2979	6103B	10835a	8940a	21750b	8538a	8886a	2979	1890B	1684
LSD(0.05)[6]	7047	7047	7047	7047	7047			7047	7047	7047	7047	7047			
Mean[7]	5000a	4840a	4584a	5124a	4910a	1720[8]	4892	6469a	5468a	10431b	5715a	5550a	1720[8]	6740	972[9]
			Dextran (ppm)							**Dextran (ppm)**					
CP 65-357	148a	92a	67a	246a	75a	720	126A	626a	281a	406a	670a	580a	720	512B	240
CP 70-321	129a	124a	143a	518a	263a	720	235AB	113a	44a	117a	103a	111a	720	105A	240
CP 72-370	470ab	297a	1187b	480ab	352a	720	418B	845a	2794b	1301a	688a	730a	720	1272C	240
LSD(0.05)[6]	1537	1537	1537	1537	1537			1537	1537	1537	1537	1537			
Mean[7]	249a	171a	466a	415a	230a	416[8]	306	552a	1040b	608a	510a	476a	416[8]	639	139[9]

Continued on next page

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

Table II. Continued

	Non-treated (no glyphosate)							Glyphosate treated[1]							
	Topping level[2]							Topping level							
	1	2	3	4	5	LSD (0.05)[3]	Mean[4]	1	2	3	4	5	LSD (0.05)[3]	Mean[4]	LSD (0.05)[5]
	Starch (ppm)							Starch (ppm)							
CP 65-357	570a	1165a	1105a	1159a	1365a	1140	1073B	1520a	923a	1466a	1281a	1521a	1140	1356B	394
CP 70-321	383a	230a	252a	215a	168a	1140	250A	475a	415a	488a	480a	461a	1140	466A	394
CP 72-370	1703a	2082a	1670a	2098a	1730a	1140	1856C	1252a	1909a	1398a	1504a	1314a	1140	1461B	394
LSD(0.05)[6]	2444	2444	2444	2444	2444			2444	2444	2444	2444	2444			
Mean[7]	886a	1158a	1009a	1157a	1087a	658[8]	1060	1093a	1183a	1117a	1089a	1099a	658[8]	1096	227[9]
	Leucoanthocyanin (Relative absorbance)							Leucoanthocyanin (Relative absorbance)							
CP 65-357	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CP 70-321	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
CP 72-370	81a	68a	74a	68a	73a	31	73	128a	98a	115a	121a	112a	31	115	11
LSD(0.05)[6]	--	--	--	--	--	--	--	--	--	--	--	--	--	--	
Mean[7]	81a	68a	74a	68a	73a	18	73	128c	98a	115ab	121b	112ab	18	115	6

[1]Glyphosate applied at 0.3 kg ha^{-1} 30-35 days prior to harvest. Lowercase letters are used to compare among topping and uppercase to compare among cultivars.
[2]1 = no topping; 2, 3, 4, and 5 = 5 cm above and 10, 25, and 40 cm below apical meristem (bud), respectively.
[3]LSD value (0.05) for comparing toppings for a given cultivar or for combined cultivars. Letters indicating significant topping differences (0.05) are shown for each cultivar.
[4]Mean values accompanied by different letters indicate a significant cultivar difference (0.05) for either no glyphosate or glyphosate when all toppings are combined.
[5]LSD value (0.05) for comparing cultivar means when all toppings are combined and for each level of glyphosate. Letters indicating significant mean differences are in upper case.
[6]LSD value (0.05) for comparing cultivars for a given topping and glyphosate level. Letters indicating significant mean differences are not shown.
[7]Mean values accompanied by different letters indicate a significant topping difference (0.05) for combined cultivars. Uses LSD value in footnote 8.
[8]LSD value (0.05) for comparing topping means combined over cultivars for no glyphosate and glyphosate.
[9]LSD value (0.05) for comparing overall means for no glyphosate and glyphosate.

within certain limits by lowering the topping height of immature cane. Arceneaux (*5*) further stated that the most advantageous benefits of lowering the topping height may be expected from comparatively immature cane, as judged by the relative increase in yield of sugar per ton of cane and by the amount of available sugar in portions of the stalk discarded. In the present study, with no topping, the increase in sucrose content between treated and non-treated cane, as an average of all cultivars, was approximately 5%. Maturity tests conducted at Houma in 1992 indicated that an above normal sucrose content for the three cultivars included in the present study as well as the difference in sampling dates of treated and non-treated cane may explain this marginal increase in sucrose content as a result of the glyphosate treatment (Legendre, B.L., USDA-ARS, Houma, LA, unpublished data.). When removing 40 cm of the stalk by topping in non-treated cane, an increase of 6.4% was noted in sucrose content; however, by removing 40 cm of the stalk in the treated cane as compared to no topping in the non-treated cane, the increase in sucrose content was over 11%.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

Purity % Cane. A non-significant glyphosate*cultivar effect indicated that differences in juice purity between glyphosate and no glyphosate were statistically similar for the three cultivars studied (Table II). Average juice purity was marginally different among cultivars; however, in the present study, the average juice purity was significantly lower for glyphosate treated cane than cane not treated with glyphosate. This is highly unusual, since glyphosate treated cane normally has higher purity than non-treated cane (*8, 16*); however, in relatively mature crops, chemical ripeners have less effect on juice purity and sucrose percent fresh weight of cane than on immature sugarcane having a low initial juice purity (*18*). In other studies conducted at Houma in 1992, the natural level of maturity of the three varieties included in the present study was unusually high (Legendre, B.L., USDA-ARS, Houma, LA, unpublished data.). Any increase in purity means that a higher percent of the dissolved solids (Brix) in the juice is sucrose. In processing, sugarcane having the same quantity of sucrose per unit weight will yield a higher quantity of sugar if the purity is higher (*19*). This is true provided the final molasses purity remains the same. Thus, for example, the percent of sucrose in juice recovered as sugar would be of the order of 5 units more for a purity of 85% compared with a purity of 80% at the same molasses purity of 40%.

Juice purity, relative to the severity of topping, remained consistent for the three cultivars (i.e. non-significant topping*cultivar effect) (Table II). Further, juice purity, relative to the severity of topping, was found to be independent of whether glyphosate was applied. According to Davidson (*7*), to obtain the highest recovery of sugar in the factory, the cane should be topped to exclude only those portions of the stalks in which the average juice purity is lower than the final molasses purity. Lower "average topping" would effectively increase the quality of the cane delivered to factories without an economical loss to the growers (*20*). Further, Coleman (*20*) stated that 15 or 22 cm of additional topping in relatively immature or low sucrose cultivars improves the quality of the cane to such an extent that its increase in sugar recovery is equal to that of the loss in weight due to the increased topping. In the present study, juice purity increased with the severity of topping with or without glyphosate. However, the present-day cultivars have a much higher level of sucrose with a correspondingly higher purity of juice and, in the present study, the lowest level of purity still exceeded 92%. On the other hand, there are other considerations that must be taken into consideration when processing tops which will be discussed later in this section.

Fiber % Cane. A non-significant glyphosate*cultivar effect indicated that differences in fiber % cane between treated and non-treated cane were statistically the same for the three cultivars studied (Table II). Average fiber % cane was

significantly different among cultivars, but average fiber % cane was not significant between treated and non-treated cane. In previous studies, though not significant, fiber trend was lower in treated cane (Legendre, B.L., J. Amer. Soc. Sugarcane Technol., in press.). Further, according to Nickell (*3*), glyphosate increased sugar content by increasing the partitioning of dry matter toward sucrose storage and away from fiber production. It is known that the quantity of fiber entering the factory determines the capacity of the milling tandem and, other conditions being equal, the amount of sucrose lost in the bagasse (*19*). Further, the more fiber per unit of cane milled, the lower will be the extraction (factory recovery) per unit sucrose in cane and the quantity of bagasse will be greater.

Fiber % cane, relative to severity of topping, remained consistent for the three cultivars (i.e. non-significant topping*cultivar effect); however, fiber % cane was dependent upon whether glyphosate was applied (Table II). Significant fiber % cane differences, relative to severity of topping, were complicated due to a glyphosate*topping interaction. Without topping, fiber % cane was higher in treated cane; however, once the top was removed at 5 cm above the bud and lower, there was a tendency for cane treated with glyphosate to have lower fiber content. Further, ripened cane is usually subject to a better burn and the amount of trash (leaves and tops) entering the factory is less (*19*). This, in most areas, is a very important factor for increased sugar recovery for dry leaves may have four times the percent fiber as mature cane, and tops up to two times as much.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

Theoretical Recoverable Sugar per Ton of Cane (TRS/TC). A non-significant glyphosate*cultivar effect indicated that differences in TRS/TC between treated and non-treated cane were statistically the same for the three cultivars studied (Table II). Average TRS/TC was not statistically different among cultivars. Average TRS/TC was higher for glyphosate treated cane than cane not treated with glyphosate. An increase in the sucrose % cane would give an equivalent increase in the yield of TRS/TC; other factors, such as juice purity and fiber content remaining constant (*19*).

TRS/TC, relative to the severity of topping, remained consistent for the three cultivars (i.e. non-significant topping*cultivar effect) and was found to be independent of whether glyphosate was applied (Table II). Further, TRS/TC also showed a significant increase with the severity of topping. As early as 1935, Arceneaux (*5*) noted the effect of removal of varying proportions of the immature portion of the stalk on the yields of both TRS/TC and yield of sugar per hectare. However, Davidson (*7*) stated that with lowering the topping height, reductions in net ton yields (trash-free tons) more than offset the gain from higher TRS/TC. Legendre (Sugar J., in press.) found that, in mature cane, cane tonnage, and its component, stalk weight, decreased an average of 4.9%, while the yield of TRS/TC increased only 2.5% with each 15 cm reduction in the topping height. Thus, the increase in TRS/TC and potential yield of sugar per unit area of land is greater with the use of glyphosate as it ripens the immature tops, thus reducing the need to top at all.

Invert Sugars % Juice. A non-significant glyphosate*cultivar effect indicated that differences in the invert sugars, glucose and fructose (reducing sugars) % juice, between glyphosate and no glyphosate were statistically the same for the three cultivars studied (Table II). Average reducing sugars were not statistically different among cultivars. Further, there was no significant difference in reducing sugars between treated and non-treated cane. In preliminary tests conducted in 1983 (Legendre, B.L., USDA-ARS, Houma, LA, unpublished data.), there was a significant decrease in both glucose and fructose % juice in cane treated with glyphosate; however, the decrease was not evident until 42 days after the glyphosate treatment. In the present study, no differences were detected at 30 to

35 days after treatment which was, undoubtedly, influenced by the excellent natural maturity of the non-treated cane. Baird and De Stefano (*8*) in Florida and Maretzki, et.al (*21*) in Hawaii noted that reducing sugars content was greatly decreased by the application of glyphosate at 42 and 35 days after treatment, respectively. According to Osgood and Teshima (*16*), the presence of other soluble carbohydrates such as glucose and fructose in immature or unripe cane decreases the purity and adversely affects the recovery of sucrose in the expressed juiced at the factory.

Reducing sugars, relative to severity of topping, was complicated by significant glyphosate*topping and cultivar*topping interactions. There was a significant increase in the level of reducing sugars with an increase in the severity of topping but only in cane treated with glyphosate (Table II). The level of reducing sugars was lower in cane treated with glyphosate with intact tops when compared to untreated cane with intact tops.

Inorganic Ash % Juice Solids. Significant differences in inorganic ash % juice solids relative to cultivars were complicated due to the glyphosate*cultivar interaction (Table II). Average inorganic ash % juice was statistically different among cultivars; however, average inorganic ash % juice was significantly higher for only two of the three cultivars treated with glyphosate when compared to the non-treated cane. Osgood and Teshima (*16*) speculated that another factor affecting sucrose recovery is the reducing sugars to ash ratio which may be adversely affected by the use of glyphosate. This adverse affect in the production of raw sugar is due to the fact that sucrose crystallization is increased by high ratios of reducing sugars to ash (*22*). If, however, the sucrose has been formed at the expense of reducing sugars, that is the quantity of reducing sugars has been decreased, then the recovery will be diminished by the loss of reducing sugars (*19*). This is because in final molasses, reducing sugars replace sucrose. In other words, the quantity of total sugars in the molasses is close to a constant, other factors being equal. In the present study, there was a tendency toward lower reducing sugars and higher inorganic ash, both of which would have had a lowering affect on the reducing sugars to ash ratio, thus further reducing overall sucrose yield.

Inorganic ash % juice, relative to severity of topping, remained consistent for all three cultivars (i.e. non-significant topping*cultivar effect) (Table II). Significant differences in inorganic ash % cane, relative to severity of topping, were complicated due to the glyphosate*topping interaction; however, there was higher inorganic ash % juice with no topping, regardless of the cultivar or whether the cane was treated with glyphosate or not.

Total Polysaccharide Concentration (TPS)(ppm on Solids). Significant differences in TPS were complicated due to glyphosate*cultivar interaction (Table II). Average TPS was statistically different among cultivars as well as significantly higher in treated cane than non-treated cane. These results were similar to results obtained in a preliminary study conducted in 1985 (Legendre, B.L., USDA-ARS, Houma, LA, unpublished data.). Polysaccharides are large carbohydrate molecules (>10,000 MW) which have several important functions in the sugarcane plant (Godshall, M.A.; Legendre, B.L.; Clarke, M.A.; Miranda, X.M.; Blanco, R.S., J. Amer. Soc. Sugarcane Technol., in press.). Further, polysaccharides are hard to remove and slow down crystallization process, go into the sugar crystal, and may contribute to color formation, thus adversely affecting sugar quality and increasing the cost of processing and refining. In the present and past studies, TPS are increased by glyphosate and may contribute to reduced sugar yield and quality.

TPS, relative to the severity of topping, differed among the three cultivars studied (i.e. significant topping*cultivar effect) (Table II). However, the spike in

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

TPS and dextran for CP 70-321 in glyphosate treated cane at a topping height of 10 cm below the bud was apparently due to a delay in processing of samples. Irvine (*23*) stated that TPS are less abundant in the millable stalk, and stalk segments showed a decreasing concentration from top to bottom of the stalk with the highest concentration found in the leaves, green or dead. TPS was found to be dependent on whether glyphosate was applied. Significant differences in TPS, relative to the severity of topping, were also complicated due to the glyphosate*topping interaction with the highest level found where no tops were removed.

Dextran Concentration (ppm on Solids). Significant differences in dextran concentration were complicated due to glyphosate*cultivar interaction (Table II). Average dextran concentration was statistically different among cultivars; however, the average dextran concentration was statistically higher for treated cane than non-treated cane. These results were similar to those found in a preliminary study conducted in 1985 (Legendre, B.L., USDA-ARS, Houma, LA, unpublished data.); however, in a follow-up study in 1986, no difference was found in dextran in cane harvested 56 days after treatment (Legendre, B.L., USDA-ARS, Houma, LA, unpublished data.). Dextran is a polysaccharide produced by microbiological contamination which is highly dextrorotatory (3X sucrose) causing false polarization (Godshall, M.A.; Legendre, B.L.; Clarke, M.A.; Miranda, X.M.; Blanco, R.S., J. Amer. Soc. Sugarcane Technol., in press.). Further, dextran increases viscosity, slows crystallization rate, lowers sugar yield and quality, and reduces quality throughout processing and carries over into the raw and refined sugar with a possible payment penalty. The results of this study indicate that the increased dextran found as a result of the glyphosate treatment may have an adverse affect on processing; however, the overall concentration found is believed low enough as not to cause serious problems in the factory or refinery.

Dextran concentration, relative to severity of topping, differed among the three cultivars studied (i.e. significant topping*cultivar effect) (Table II). Dextran concentration, relative to the severity of topping, was dependent on glyphosate application. However, it appeared that in the present and past studies, the concentration of dextran was reduced by topping, especially when that portion 5 cm above to 10 cm below the bud was removed. Significant differences in dextran concentration, relative to the severity of topping, were complicated due to the glyphosate*topping interaction.

Starch Concentration (ppm on Solids). Significant differences in starch concentration were complicated due to glyphosate*cultivar interaction (Table II). Average starch concentration was statistically different among cultivars while the average starch concentration was not significantly different for treated when compared to non-treated cane. Starch is a normal constituent of the sugarcane plant and is normally higher in immature cane (Godshall, M.A.; Legendre, B.L.; Clarke, M.A.; Miranda, X.M.; Blanco, R.S., J. Amer. Soc. Sugarcane Technol., in press.). In processing, when juice temperature exceeds 71°C, starch is solubilized, and rather than being eliminated in the mud in clarification, it remains in the clarified juice, concentrating as the juice is evaporated where it increases viscosity and inhibits crystallization (*23*).

Starch concentration, relative to the severity of topping, differed among the three cultivars studied (i.e. significant topping*cultivar effect); however, there were no significant differences in starch concentration due to the severity of topping (Table II). Irvine (*23*) stated that starch varied significantly with the time of day that the sample was harvested and that harvesting systems which do not effectively remove tops or green leaf blades add to the starch load in clarification. However, he further stated that cane tops harvested in the morning hours would have the

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

lowest starch content when compared to cane harvested in the afternoon or evening. In the present study, all cane was harvested in the morning which may have contributed to the lack of significance in starch content due to severity of topping.

Leucoanthocyanin (Relative Absorbance X 1000). There was a significant difference in leucoanthocyanin between cane treated when compared to non-treated cane for the cultivar CP 72-370 (Table II). There was also no association between leucoanthocyanin and the severity of topping. Leucoanthocyanin are related to plant pigments, turn red, pink, or orange under certain conditions, such as acidity, heat, and disease, and are associated with sugarcane polysaccharides (Godshall, M.A., Legendre, B.L., Clarke, M.A., Miranda, X.M.; Blanco, R.S., J. Amer. Soc. Sugarcane Technol., in press.). However, to date, leucoanthocyanin are not associated with processing difficulties.

Summary

The net effect of glyphosate was to increase the yield of TRS/TC; however, this increase does not come without potential adverse affects on processing, namely the decrease in the reducing sugars to ash ratio and a possible increase in TPS and dextran which can ultimately contribute to sugar losses and lower sugar quality. However, these negative responses to the use of glyphosate can be partially offset by cultivar selection and/or removing the leaves and upper portion of the stalk by topping.

Acknowledgments

The authors wish to thank Ms. R.S. Blanco and Mr. X.M. Miranda, Sugar Processing Research Institute, Inc., New Orleans, LA for analysis of the samples, Mr. C.K. Finger, USDA-ARS, Houma, LA for analysis of samples and analyzing the data, and Dr. B.T. Vinyard, USDA-ARS, New Orleans, LA for analyzing the data.

Literature Cited

1. Coleman, R. E.; Hebert, L. P. *Sugar Bull.* 1957, *vol. 35*, pp. 389-391.
2. Legendre, B. L.; Martin, F. A. *Proc. Amer. Soc. Sugar Cane Technol.*, 1977, *vol. 6*, pp. 62-64.
3. Nickell, L. G. *Proc. 14th Plant Growth Regulator Soc. of Amer.*, 1987, pp. 423-437.
4. Legendre, B. L. *Sugar J.*, 1992, *vol. 54*, No. 9, pp. 2-7.
5. Arceneaux, G. *U.S. Dept. of Agri., Circ. 368*, 1935, pp. 1-32.
6. Simon, E. C. *Report of the Contact Committee of the Amer. Sugar Cane League*, July, 1933.
7. Davidson, L. G. *Sugar Bull.*, 1965, *vol. 44*, No. 1, pp. 14-17.
8. Baird, D. D.; De Stefano, R. D. *Proc. Sugarcane Ripening Sem.*, 1977, pp. 95-107.
9. Schneider, F. In "*Sugar Analysis, ICUMSA Methods*"; ICUMSA Press: Peterborough, England, 1979, pp. 85-86.
10. Roberts, E. J. *Proc. 1980 Technical Session Cane Sugar Refining Research*, 1981, pp. 130-133.
11. *Association of Official Analytical Chemists.* Roberts Copper Method, AOAC Official Methods of Analysis, 1990, pp. 1020-1021.
12. Chen, J. C. P. In "*Meade-Chen Cane Sugar Handbook, 11th Edition*"; John Wiley & Sons: NY, 1985, pp. 911-913.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

13. Legendre, B. L. *Sugar Bull.*, 1983, *vol. 61(18)*, pp. 8-10.
14. Legendre, B. L.; Finger, C. K. *Proc. 14th Plant Growth Regulator Soc. of Amer.*, 1987, pp. 479-484.
15. Martin, F. A.; Legendre, B. L.; Dill, G. M.; Dimarco, G. J.; Steib, R. J. *Sugar J.*, 1981, *vol. 43(10)*, pp. 20-22.
16. Osgood, R. V.; Teshima, A. *Proc. Plant Growth Regul. Work. Group*, 1979, pp. 29-34.
17. Hilton, H. W.; Osgood, R. V.; Maretzki, A. *Proc. Int. Soc. Sugar Cane Technol.*, 1980, *vol. 17*, pp. 652-661.
18. Rostron, H. *The South African Sugar Journal*, 1974, pp. 74-84.
19. Payne, J. H. *Proc. Sugarcane Ripening Seminar*, 1977, pp. 92-94.
20. Coleman, R. E. *Proc. Amer. Soc. Sugar Cane Technol.*, 1963, pp. 12-23.
21. Maretzki, A.; Thom, M.; Moore, P. H. *Hawaiian Planters' Record*, 1976, *vol. 59(3)*, pp. 21-32.
22. Moritsugu, T.; Somera, B. J.; Sloane, G. E. *Proc. Int. Soc. Sugarcane Technol.*, 1974, *vol. 12*, pp. 1236-1245.
23. Irvine, J. E. *Proc. Amer. Soc. Sugar Cane Technol.*, 1977, *vol. 6*, pp. 53-57.

RECEIVED January 31, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch003

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

Chapter 4

Use of Gibberellic Acid To Reduce Citrus Fruit Susceptibility to Fruit Flies

P. D. Greany[1], R. E. McDonald[2], W. J. Schroeder[2], P. E. Shaw[3], M. Aluja[4], and A. Malavasi[5]

[1]Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 1700 Southwest 23rd Drive, Gainesville, FL 32608
[2]U.S. Horticultural Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, 2120 Camden Road, Orlando, FL 32803
[3]Citrus and Subtropical Products Laboratory, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 1909, Winter Haven, FL 33880
[4]Instituto de Ecologia, A.C., 91000 Xalapa, Veracruz, Mexico
[5]Department of Biology, University of São Paulo, São Paulo, Brazil

Citrus fruit are resistant to attack by tephritid fruit flies prior to the occurrence of peel senescence. We have shown that grapefruit and oranges sprayed with *ca* 10 ppm gibberellic acid (GA) and a suitable surfactant are significantly less susceptible than untreated fruit to attack by Caribbean, Mexican, and Mediterranean fruit flies. The treatment does not inhibit internal ripening of the fruit. In addition to remaining fruit fly resistant, GA-treated fruit also are less prone to late season abscission, exhibit desirable cosmetic peel properties for at least 3 months longer than untreated fruit, and are often less susceptible than untreated fruit to postharvest decay problems. GA use on citrus already is approved by the U.S. Environmental Protection Agency. This approach should provide a biorational addition to existing fruit fly control strategies.

The natural plant growth regulator gibberellic acid (GA_3) has been used for over 30 years to extend the postharvest shelf life of citrus fruit (*1*). For this, growers must apply GA to the fruit while still on the tree, prior to colorbreak (i.e., before the usual transformation of the fruit from green to orange or yellow). While the application of GA to the fruit inhibits senescence-related changes in the fruit peel, it does not significantly interfere with the internal ripening of the fruit (*1*). Not only is chlorophyll breakdown delayed in GA-treated citrus fruit, so that they retain a green caste, but peel firmness also is sustained (*1*).

0097–6156/94/0557–0039$08.00/0
© 1994 American Chemical Society

Application of Gibberellic Acid for Fruit Fly Prophylaxis

The ability of GA to delay senescence in citrus fruit prompted investigations into its ability to also sustain the resistance of citrus fruit to attack by tephritid fruit flies (*2*). Initial studies were conducted in Florida on the use of GA to reduce the susceptibility of grapefruit to the Caribbean fruit fly, *Anastrepha suspensa* (Loew) (*3-5*). These studies indicated that for the Caribbean fruit fly, it is possible to significantly reduce the likelihood of successful infestation of grapefruit by this pest. In field cage studies conducted in Florida during 1991-92 and 1992-93, fruit treated with GA were *ca.* 80-90% less susceptible to Caribbean fruit fly attack than untreated fruit during the period of December through March each year (Greany, P., *et al.*, unpublished data). Studies performed in Israel also showed that GA could be used to reduce the susceptibility of grapefruit and especially oranges to *Ceratitis capitata* (Wiedemann) (*6*). The bases for sustained resistance of GA-treated fruit include reduced visual attractiveness to foraging fruit fly females, reduced acceptance of these fruit for oviposition, and reduced larval survival. This is due to their prolonged green color and peel toughnness, as measured by sustained puncture resistance, which deters neonate larvae from quickly escaping the peel region, wherein lie the plant's resistance factors (*2-7*).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

New Fruit Fly Control Measures Needed. It is increasingly important to develop preharvest methods for fruit fly control since traditional approaches available for postharvest disinfestation of citrus fruit are dwindling (*8*). Use of ethylene dibromide (EDB) fumigation of citrus fruit was abolished by the U.S. Environmental Protection Agency (EPA) in 1984 (*9*). While methyl bromide fumigation is still permissible, it is anticipated that this pesticide also will be eliminated by the year 2000 (*10*). Recently, a cold treatment for disinfestation of grapefruit (*11*) has been declared undesirable by fruit importers in Japan because it can predispose grapefruit to decay (Jenkins, C., Florida Dept. of Agriculture and Consumer Services, Division of Plant Industry, personal communication, 1993). Heat treatments (vapor heat, hot air, and hot water immersion) have not yet proved entirely suitable for commercial use in disinfesting citrus fruit (*12*, *13*). There also is a fear that aerially applied bait sprays employing protein hydrolysate attractants plus malathion may be limited in the near future because of concern for the environment (*8*). Several studies (reviewed in *14*) have shown that malathion bait sprays can precipitate non-target pest outbreaks. The possibility of tephritid fruit flies developing malathion resistance also has been demonstrated in the laboratory (*15*), and the possibility exists that sufficient selection pressure could be applied from repeated applications of malathion (or other insecticides) to result in development of resistant strains (*16*).

No method is available to kill fruit fly eggs and/or larvae in the field (i.e., prior to harvest), other than by biological control using natural enemies (*17*). Use of GA to sustain the innate resistance of fruit to attack, and thereby to reduce fruit fly infestation, represents an entirely new means of intervention and control against immature stages of fruit flies. Because GA is non-toxic, only direct pests of the fruit should be affected, and non-target insects, such as those providing biological control of scale insects and other citrus pests, should not be adversely affected. Thus, GA applications should be compatible with biological control of tephritids and other citrus pests, as well as use of conventional chemical control agents and the sterile insect technique (*18*).

The encouraging results obtained using GA in Florida and Israel prompted initiation of similar research in Mexico and Brazil to combat tephritid fruit fly

species occurring in these locations, including the Mexican fruit fly, *A. ludens* (Loew) in Mexico, and the South American fruit fly, *A. fraterculus* (Wiedemann), and the Mediterranean fruit fly, *C. capitata*, in Brazil. We anticipate being able to achieve a number of entomological, horticultural, and economic goals from this approach, as outlined in Table I:

Table I. Potential Entomological, Horticultural and Economic Benefits from the Use of Gibberellic Acid on Commercial Citrus

	FLORIDA		MEXICO		BRAZIL[1]	
	Grapefruit	*Oranges*	*Grapefruit*	*Oranges*	*Oranges*	*Mandarins*
ENTOMOLOGICAL						
Reduced probability of infestation	+	+	+	+	+	+
Compatibility with:						
Biocontrol	+	+	+	+	+	+
Sterile insect technique	+	+	+	+	+[2]	+[2]
Conventional insecticides	+	+	+	+	+	+
HORTICULTURAL						
Delayed peel colorbreak	+	n/t[3]	+	+	+	+
Sustained peel firmness	+	n/t	+	+	+	+
Sustained high peel oil levels	+	n/t	n/t	n/t	n/t	n/t
Increased fruit weight/size	n/t	n/t	+	+	+	n/t
Reduced fruit abscission	+	n/t	+	+	+	+
ECONOMIC						
Fly-free certification	+	+	n/a[4]	n/a	n/a	n/a
Extended shelf life	+	+	+	+	+	+
Increased value of late-season fruit	+	+	+	+	+	+
Allows better fruit set regulation	n/a	n/a	n/t	n/t	+	n/t
Allows delayed harvest	+	+	+	+	+	+

[1]Grapefruit are not produced commercially in Brazil.
[2]SIT has not been used against fruit flies in Brazilian citrus.
[3]Not tested.
[4]Not applicable or intended.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

Florida Perspective. As can be seen from Table I, the goals are somewhat different in each location. In Florida, one goal is to help achieve fly-free certification of fruit by treatment with GA. Commercial Florida grapefruit are only rarely attacked by *A. suspensa*, and even then only when senescent (*2*). Consequently, the greatest need in Florida is not to reduce yield losses due to *A. suspensa*, but rather to diminish the likelihood of infestation, especially late in the season, so as to allow shipment of the fruit under the Caribbean Fruit Fly Fly-Free Protocol (*9*). GA would be used to delay grapefruit senescence and sustain fruit resistance to *A. suspensa.*

The Fly Free Protocol, developed after the cessation of EDB use, enables Florida growers to ship grapefruit to Japan, California, Arizona, and Texas if they meet certain provisions. One of the provisions is that preferred Caribbean fruit fly host plants must be removed up to a specified distance from the perimeter of the grove (host free buffer zone) (*9*). Early in the season (prior to December 20), while the fruit still are considered non-senesent and highly resistant to the Caribbean fruit fly, the host free buffer zone must be at least 300 feet (*ca* 91 meters). However, after December 20, when Florida grapefruit increase in susceptibility, the host plant free buffer zone must be 1/2 mile (*ca* 805 meters) to increase security. Many growers are unable to qualify for this late season distance requirement because of proximity to residential neighborhoods with backyard host fruits, and have employed methyl bromide fumigation or cold treatment disinfestation techniques (*11-13*) for fruit harvested after December 20 in order to ship their fruit to quarantine-sensitive locations such as Japan. Now that these postharvest treatments are being curtailed, many growers are unable to certify their fruit as being fly-free by any pre- or postharvest means after December 20.

GA applications may soon allow these Florida grapefruit growers to obtain an extension of the early-season Fly-Free Protocol certification procedure involving a 300 foot host free buffer zone (*9*). The Florida Department of Agriculture and Consumer Services, Division of Plant Industries (FDACS-DPI), plans to request approval by the USDA-APHIS (Animal and Plant Health Inspection Service) of a GA-based modification of the existing early-season Bait Spray Certification Protocol. For this, in addition to methods normally employed prior to December 20 to ensure fly-free fruit, GA treatments also would be required (Figure 1). Existing early-season Bait Spray Certification Protocol provisions include: *i*) periodic treatment with aerially-applied malathion bait sprays, *ii*) provision of a 300 foot host plant free buffer zone, and *iii*) "negative trapping" of adult Caribbean fruit flies in the groves ("negative trapping" refers to lack of capture of adult flies in McPhail traps placed in the grove). Use of GA to retain early-season grapefruit resistance properties would be required in addition to these measures. Verification that treated fruit exhibit sustained early-season fruit properties (in terms of peel color and firmness) also may be a requirement, as these criteria have routinely correlated well with resistance of the fruit to attack by the Caribbean fruit fly (*2-5*). Specific requirements for the GA treatment and verification regimen are now under consideration by cooperating researchers and FDACS-DPI officials.

This modification of the Protocol could provide a significant economic benefit to growers who are otherwise unable to qualify for the post-December 20 Fly-Free Protocol, as the value of fruit for the export market (principally Japan) is considerably greater than that of fruit destined for the domestic market, by as much

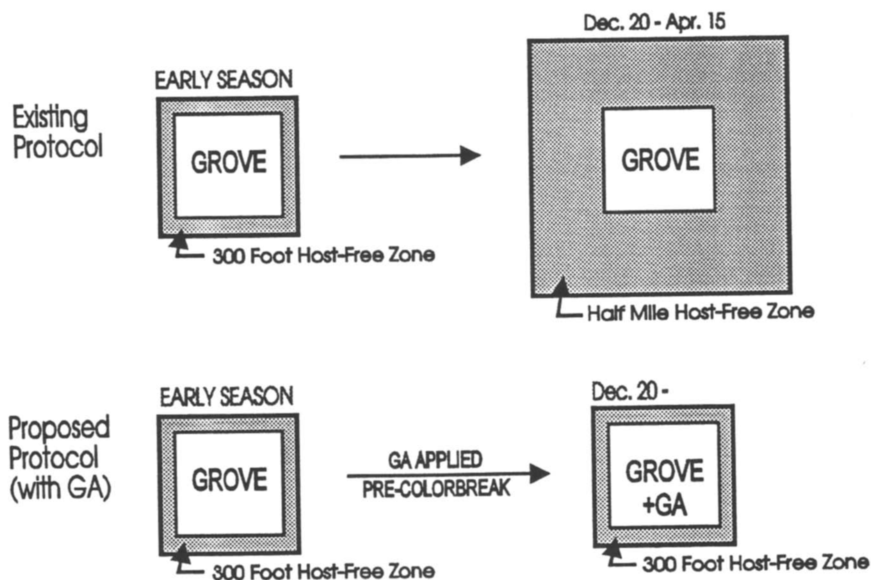


Figure 1. Proposed Modification of Bait Spray Fly-Free Protocol for Fruit to be Harvested in Florida after December 20.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

as $1,000 per acre (*ca* 0.4 ha) (Muraro, R., Univ. Florida, Dept. of Food & Resource Economics, personal communication, 1993).

In earlier tests, it was shown that GA-treated Florida grapefruit exhibited *i)* normal internal fruit quality (in terms of their sugar/acid ratio and sugar content), *ii)* tolerance to the approved cold treatment used to disinfest grapefruit (*19*), and iii) extended shelf life (*20*). The goals of GA tests in Florida for 1993-94 are to verify, using commercial speed sprayers, results obtained earlier using hand-held spray equipment. Tests are also being performed to determine the relative efficacy of GA treatments employing a non-ionic silicone polyether copolymer surfactant (Silwet L-77, OSi Specialties, Danbury, CT) *vs.* an aqueous solution of GA without any surfactant, using a commercial GA source (Pro-Gibb 4%, Abbott Laboratories, Chicago, IL). In earlier tests, inclusion of Silwet L-77 significantly improved the performance of GA over inclusion of Triton X-100 (*5*). Not only does Silwet L-77 facilitate coverage of the GA, but it also promotes rapid penetration of the hormone into the fruit (Policello, G., OSi Specialties, Tarrytown, NY, personal communication), thereby "rainproofing" the application. Use of Silwet L-77 also proved effective in promoting the action of GA on California 'Washington' navel oranges (*21*).

Mexico Perspective. The Mexican fruit fly, *A. ludens*, causes major damage to Mexican citrus, especially grapefruit and late-season oranges. Still, growers often retain fruit on the tree as long as possible in order to obtain the best price for their fruit. As a consequence, the fruit become quite susceptible to this pest as they lose their resistance due to senescence-related changes in the peel. Mexican growers may lose up to 40% of their fruit due to fruit fly attack and associated fruit drop. Control presently is attempted through use of malathion bait sprays, which do not always provide the sought-after degree of control. There is a great need to improve upon current methods for fruit fly control in Mexican citrus, especially in grapefruit, which are more susceptible than oranges to *A. ludens*. The principal goal is to increase the yield of fruit for the Mexican marketplace rather than to achieve fly-free certification to allow Mexican fruit to be shipped to quarantine-sensitive countries.

From data collected during the 1991-92 and 1992-93 seasons (Aluja, M., *et al.*, unpublished data, 1993) it appears that use of GA will be very helpful to Mexican citrus growers as a supplement to use of malathion bait sprays. GA alone has not provided adequate protection against the Mexican fruit fly when this well-adapted citrus pest is present at high population densities. This is especially of concern in the case of grapefruit. Tests are being conducted during the 1993-94 season on use of malathion bait sprays plus GA treatment to determine whether this combination can adequately protect late-season Mexican citrus fruit. It is anticipated that as fruit fly control capabilities improve generally, GA will provide a compatible technology that can be used along with non-insecticidal methods such as the sterile insect technique and biological control programs, which are currently being advocated by plant protection officials in Mexico.

In addition to reducing damage by fruit flies, GA treatment afforded a sharp reduction in spontaneous drop of GA-treated 'Valencia' oranges at the end of the season (Aluja, M., *et al.*, unpublished data, 1993). If not overwhelmed by large populations of *A. ludens*, late season GA-treated grapefruit also can be retained on the tree more successfully than untreated grapefruit, as GA helps prevent drop of grapefruit (*1*). As in tests conducted in Florida (19), no reduction in postharvest

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

fruit decay was detected when comparing GA-treated *vs.* untreated fruit (grapefruit & oranges) in Mexico, but the shelf life and favorable appearance of fruit that did not decay was extended (Aluja, M. *et al.*, unpublished data, 1993).

Results of the Mexican studies confirmed that GA treatment sustains the early season properties of both oranges and grapefruit in terms of peel color and puncture resistance, and GA-treated fruit showed a significant increase in fruit weight (as much as 17% greater). This could provide a marked benefit to growers, as they would derive a greater return per hectare for their fruit. In addition, they should be able to realize an increase in value because of the possibility of harvesting later than usual. Under current market conditions, late harvest (2 months after the normal harvest period) of oranges can increase profits of growers by *ca.* 30-40% (Webel, B., and Aluja, M. unpublished data, 1993) because fruit command a higher price late in the season.

Brazil Perspective. Due to fear of fruit fly attack (by *A. fraterculus* and *C. capitata*), orange producers in Brazil typically bait spray 12-17 times/season with sugar cane molasses & malathion. The sprays are used prophylactically, and typically are initiated just prior to fruit colorbreak without monitoring the fruit fly population. Tests conducted on 'Pera' oranges in Bebedouro, State of São Pãulo, during 1991-92 using GA at 20 ppm with 0.05% Silwet L-77 showed a 75% reduction in fruit fly infestation under field conditions (Malavasi, A., *et al.*, unpublished data, 1993). Tests in this grove during 1992-93, using 10 ppm GA with 0.1% Silwet L-77, showed a reduction in infestation of GA-treated fruit of 72 to 97% during the mid- to late season (Oct.-Dec.). Earlier in the season (Aug.-Sept.) neither untreated nor GA-treated oranges were susceptible to fruit fly attack. From these results, we believe the use of GA on 'Pera' and other varieties such as 'Valencia' and 'Natal' could reduce the need for bait sprays to only 3 or 4, and then only during the late season. Because the species of fruit flies threatening Brazilian citrus, including *A. fraterculus* and *C. capitata*, are well-adapted to citrus fruit, it is not anticipated that GA treatment alone would allow Brazilian citrus growers to obtain fly-free certification of their fruit.

From the horticultural standpoint, GA treatment shows potential benefits for nearly all important cultivars of citrus being cultivated in Brazil. The most promising are 'Pera', 'Valencia' and 'Natal' oranges, 'Murcott' and 'Ponkan' mandarins, and limes. Beneficial effects due to treatment include: *i*) reduced fruit drop among all 3 varieties of oranges tested throughout the season; *ii*) reduced fruit size variation; *iii*) greater flexibility in timing of harvests, allowing fruits to be stored on the tree for 3- 4 months after the normal harvest; and *iv*) sustained fruit quality (GA treatment had no effect on 'Pera' orange quality in terms of total sugar content, sugar/acid ratio, or taste) (Malavasi, A., unpublished data, 1993). For the fresh market, mainly for mandarins and limes, use of GA should extend shelf life. GA already is being used regularly in Brazil as a postharvest treatment to retain the green color of limes that are to be exported. In addition to these benefits, GA treatments may be used to increase synchrony in blooming and fruit set. This could be especially important for 'Pera' oranges, which otherwise exhibit multiple fruit sets.

These entomological and horticultural effects of GA application could significantly improve the economic situation for Brazilian growers. For example, late harvest of mandarins for fresh market consumption would increase profits of

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

growers because they can be sold at a greater price than those harvested during the usual season. In addition, the possibility of achieving greater control over harvest management for processed fruit (for orange juice production) would allow growers/processors to better manage and maximize their manpower and use of equipment.

Possible Drawbacks

Retained Green Color. In the U.S., because GA treatment delays the loss of chlorophyll (degreening) of treated fruit, fruit should be treated only if they are to be harvested during the mid- to late-season. However, they usually can be degreened artificially by use of ethylene if harvested at least 3-4 months after GA application at the rates envisioned. This is of concern only for fruit destined for the fresh market. For fruit that are to be processed (e.g., most Brazilian oranges), peel color is of no consequence. The delay in degreening is not a problem in Mexico, where green fruit harvested late in the season fetch a better price in the market than yellow or orange fruit because Mexican consumers are accustomed to externally green fruit being superior internally at this time.

Phytotoxicity. Minor (<10%) leaf drop has been noted after some GA treatments at some test locations in Florida (grapefruit), Mexico (grapefruit & oranges), and Brazil (oranges). Under normal conditions, it is believed that the leaves that drop may be old leaves that represent a photosynthetic drag on the trees (Menendez, R., Abbott Laboratories, personal communication, 1993). No concerns have been raised about the rate of leaf drop in Brazil from the use of GA on 'Pera', 'Natal', or 'Valencia' oranges, nor on 'Murcott' mandarins. In Florida (grapefruit) and Mexico (both grapefruit and oranges), GA-treated fruit have occasionally exhibited signs of cosmetic damage that were not seen in untreated fruit. Peel "burning" was most apparent on fruit from trees that were partially defoliated and on fruit that were exposed to the sun (exterior canopy). This phytotoxic effect of GA treatment was most likely to occur on stressed trees. GA treatments should not be applied to stressed trees, such as those suffering from lack of micronutrients, inadequate water balance (too much or too little), or which show symptoms of general stress. In addition to tree stress, other factors that may result in leaf drop and peel "burning" include: the rate of GA employed, the concentration and potency of any accompanying surfactant, and late application (after fruit colorbreak). Grapefruit rind blemish problems have been noted previously (*22*), and were attributed to an interaction between GA and adjuvant wetting agents.

Induced Pest Problems. There is some indication that GA may cause increased rust mite problems on oranges in Mexico. These mites seem to attack green fruit more seriously than orange-colored fruit. This is an anecdotal observation by cooperating growers, and should be evaluated scientifically. Similar problems have not been noted in Florida or Brazil.

Long Term Effects on Tree. There is no evidence that there should be any ill consequence for the trees when GA is employed in accordance with the manufacturer's recommended rate and if care is used in timing of the application. GA has been used in California (and elsewhere) for over 30 years to extend the postharvest shelf life of oranges (*1*).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

Treatment Cost. In Florida, studies are being conducted to optimize the GA-surfactant formulation and to reduce the spray volume required. It appears that the cost of treatment (materials, equipment, and labor) will be easily offset by the increased value of fruit for the export market along with a reduced propensity of the GA-treated fruit to drop and extended shelf life. The cost of materials alone (including GA and surfactant, but not equipment or labor) likely will be less than about $25 per acre. An economic analysis performed in Mexico (Webel, B., and Aluja, M., personal communication, 1993) showed that GA applications would be cost effective for growers who manage their groves at a moderate to excellent level of care, if market conditions are otherwise favorable. Under ideal conditions, the benefit/cost ratio could be as much as 15:1. Under Brazilian conditions, an economic study should be carried out to determine the benefit/cost ratio of the treatment. The relatively high cost of GA and surfactant in Brazil (primarily due to import duties) might hinder use of this technology there.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

Conclusions

Overall, GA applications should provide an effective means for significantly reducing fruit fly damage in citrus fruit. This approach is complementary to and compatible with other existing control strategies, and it should not cause harm to the environment. The reduced propensity of GA-treated fruit to drop should enable growers to better schedule their harvests. Finally, GA-treated fruit also generally exhibit extended shelf life. This combination of benefits should make the use of GA an attractive management tool for citrus growers in areas threatened by fruit flies. In so doing, the goals expressed by Fischer *et al.* (*23*), to enhance the ability of plants to produce their own "safer insecticides" will be realized. Ideally, the use of GA will be further enhanced or even supplanted by identifying the factor(s) possessed by lemons and limes that confer virtual immunity of these fruit to fruit flies (*7*). These could then be incorporated into grapefruit and oranges through conventional breeding or biotechnological methods to avoid attack by fruit flies altogether (*24*).

End Note

Mention of a commercial or proprietary product does not constitute endorsement by the U.S. Department of Agriculture.

Literature Cited

1. El-Otmani, M.; Coggins, C. W., Jr. *Acta Hort.* **1991,** *34*, 469-472.
2. Greany, P. D.; Shaw, P.E.; Davis, P. L.; Hatton, T. T. *Florida Entomol.* **1985,** *68*, 144-150.
3. Greany, P. D.; McDonald; R. E., Shaw, P. E.; Schroeder, W. J.; Howard, D. F. Hatton, T. T.; Davis, P. L.; Rasmussen, G. K. *Trop. Sci.* **1987,** *27*, 261-270.
4. McDonald, R. E.; Shaw, P. E.; Greany, P. D.; Hatton, T. T.; Wilson, C. W. *Trop. Sci.* **1987**, *27*, 17-22.
5. Greany, P. D.; McDonald, R. E.; Schroeder, W. J.; Shaw, P. E. *Florida Entomol.* **1991**, *74*, 570-580.

6. Rössler, Y.; Greany, P. D. *Entomol. exp. appl.* **1990,** *54*, 89-96.
7. Greany, P. D.; Styer, S. C.; Davis, P. L.; Shaw, P. E.; Chambers, D. L. Entomol. exp. appl. **1983,** *34*, 40-50.
8. Calkins, C. O. *Florida Entomol.* **1993,** *76*, 263-270.
9. Simpson, S. E. *Florida Entomol.* **1993,** *76*, 228-233.
10. Anonymous. *Methyl Bromide Substitutes and Alternatives: A Research Agenda for the 1990s.* USDA; **1993,** 28 pp.
11. Hatton, T. T; Cubbedge, R. H. *J. Amer. Soc. Hort. Sci.* 1982, *107*, 57-60.
12. Sharp, J. L. *Florida Entomol.* **1993,** *76*, 212-218.
13. McDonald, R. E.; Miller, W. R.; Mitcham, E. J. *Florida Entomol.* **1993,** *76*, 218-224.
14. Hoelmer, K. A.; Dahlsten, D. L *Environ. Entomol.* **1992,** *22,* 49-56.
15. Koren, B.; Yawetz, A.; Perry, A. S. *J. Econ. Entomol.* **1984,** *77*, 864-867.
16. Georghiou, G. P. In *Fruit Flies: Proceedings of the Second International Symposium*; Economopoulos, A. P., Ed.; Elsevier Science Publ. Co., New York, NY, **1987**; pp. 27-40.
17. Baranowski, R.; Glenn, H.; Sivinski, J. *Florida Entomol.* **1993,** *76,* 245-251.
18. Holler, T. C.; Harris, D. L. *Florida Entomol.* **1993,** *76,* 251-258
19. Miller, W. R.; McDonald, R. E. *Proc. Florida Hort. Soc.* **1992,** *105*, 116-119.
20. Ferguson, L.; Ismail, M. A.; Davies, F. S.; Wheaton, T. A. *Proc. Florida Hort. Soc.* **1982,** *95*, 242-245.
21. Coggins, C. W., Jr.; Henning, G. L.; Anthony, M. F. In *Adjuvants for Agrichemicals*; Foy, C. L., Ed.; CRC Press, Boca Raton, FL, **1992**; pp. 567-572.
22. Coggins, C. W., Jr.; Henning, G. L. In *Proc. Sixth Intl. Citrus Congress*; Goren, R.; Mendel, K., Eds.; Balaban Publ. Co., Philadelphia, PA, **1988**; pp. 333-338.
23. Fischer, D. C.; Kogan, M.; Greany, P. D. In *Safer Insecticides: Development and Use*; Hodgson, E.; Kuhr, R. J., Eds.; Marcel Dekker, Inc., New York, NY, **1990**; pp. 257-280.
24. Greany, P. D.; Shapiro, J. P. *Florida Entomol.* **1993,** *76*, 258-263.

RECEIVED March 3, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch004

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

Chapter 5

Bioregulator-Induced Effects on the Allelochemicals and Agronomic Traits of Cotton

Paul A. Hedin and Jack C. McCarty, Jr.

Crop Science Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 5367, Mississippi State, MS 39762

A number of naturally occurring and synthetic bioregulators were applied to the cotton plant *Gossypium* sp. to determine whether they elicited any effects on yield, agronomic traits, allelochemicals, or resident pests. Mepiquat chloride had the most dramatic effect, causing internode shortening. Mepiquat chloride and several other onium compounds also affected allelochemicals and yield to varying extents. Kinetin, with and without several adducts, tended to affect yield and gossypol favorably. There appears to be an opportunity to further improve these effects if time of application, concentration, and structure-activity relationships can be optimized.

Naturally occurring and synthetic bioregulators have an important role in the growth and development processes of plants. They may induce the biosynthesis of allelochemicals, secondary plant constituents that may protect the plant against infection and injury by phytophagous pests. Consequently, the chemistry and action of these bioregulators is of interest because of their abilities to improve yield and crop quality.

Agricultural scientists face the challenge of helping to provide food and fiber for a rapidly increasing world population with a limited amount of land. The development of cropping systems and more efficient agronomic procedures (including plant breeding) can contribute to increased production. Fertilizers have increased yields and pesticides have conserved yields, but these inputs of production are costly in some respects and have some environmental disadvantages.

New approaches to improving crop productivity are needed to keep pace with population growth. Perhaps the greatest hope for increased crop production is in the advent of genetically altered crop plants with the potential for improved yield, more efficient growth patterns, resistance to pests, and more desirable properties

This chapter not subject to U.S. copyright
Published 1994 American Chemical Society

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

for consumption. A somewhat associated development is the steadily increasing use of natural and synthetic bioregulators to improve or alter growing patterns and yield. In some instances, the bioregulator acts to modify plant gene expression, affecting levels of DNA, RNA, enzymes, and, finally, their products sas proteins, carbohydrates, lipids, and allelochemicals. In the control of pests, the biosynthesis of allelochemicals by the plant, relatively steady state or event induced, can protect yield by limiting or even eliminating damage to the economically important tissues, typically the fruit.

Some of the economically and environmentally important advantages of bioregulators are that they are active in small doses and generally have less impact on the environment than most pesticides. Their use is also under the control of the grower so that application can be made at the desired time. While some of the "natural" bioregulators (plant hormones) may be too expensive for commercial use, others such as gibberellic acid are used on some specialty crops. Studies of their functions have led to the identification of synthetic compounds (often structural analogues) with similar physiological action or, conversely, those that are antagonists or inhibitors of the natural bioregulators. Despite years of research and development by both private and government agencies, only a few products (other than herbicides) play a major role, and they amount to only about 5% of the total market of crop production chemicals (1). However, environmental initiatives may seriously decrease the use of herbicides and encourage the increased use of true yield-increasing plant growth regulators. In this review, the focus will be on those bioregulators which have had an effect on the cotton plant; however, the effects of bioregulators on pests that infest both cotton and other crops will be discussed when the activity could be a model (**Table I**).

Emergence of the Use of Bioregulators for Insect Control

Campbell et al. (2) found that most of the early reported effects of bioregulators on insects were unexpected side effects which mainly involved the application of 2,4-D (2,4-dichlorophenoxy-acetic acid) on the hosts. The application of 2,4-D may inadvertently alter the populations of herbivorous insects as the result of the effects of 2,4-D on natural enemies (parasites or predators) of these insects. For example, the increase in numbers of the sugarcane borer Diatraea saccharalis (Fabricius) on sugar cane was attributed by Ingram and co-workers (3) to the detrimental effect of 2,4-D on the parasitic wasp Trichogramma minutum Riley. Similarly, populations of a number of graminivorous aphids increased in oat fields treated with the herbicide. This was caused, according to Adams and Drew (4), by a reduction in the population of Hippodamia tridecimpunctata tibialis (Say), a predatory ladybird beetle of aphids. Retarded germination and reduced vigor of wheat by early application of 2,4-D was considered the major factor contributing to increased plant damage by the prairie grain wireworm Ctenicera aeripennis destructor Brown. The

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

direct toxicity of the herbicide was reportedly responsible for larval mortality of the wheat stem sawfly Cephus cinctus Norton (5).

Direct Effects of Plant Bioregulators in Diets on Insects

Campbell et al. (2) also identified several instances where there were direct (toxic) effects of bioregulators on insects. El-Ibrashy and Mansour (6) demonstrated that chlormequat chloride reduced larval growth and pupal weight of the black cutworm Agrotis ipsilon (Hufnagel). Antigrowth activity was also observed when the compound was injected into the larvae. Antifeeding activity of chlorphosphonium chloride on the cotton leafworm Prodenia litura Fabricius was reported by Tahori and co-workers (7) after application of this compound to cotton Gossypium sp. or Phaseolus sp. foliages (8).

Carlisle et al. (9) demonstrated that gibberellic acid is a necessary dietary constituent for normal maturation in the desert locust Schistocerca gregaria (Forskal). They further showed that artificial diets containing chlormequat chloride, a synthetic inhibitor of gibberellic acid biosynthesis, retarded and prevented sexual maturation of both the locust and the cotton stainer Dystercus cardinalalis Gerth. No significant effect was observed with abscisic acid, a natural antagonist of gibberellic acid.

Bioassays were conducted with several plant regulators against larvae of the corn earworm Helicoverpa zea (Boddie) by incorporating plant regulators into artificial diets. No significant activities were found with exceedingly high levels of the plant hormones gibberellic acid and 6-benzyladenosine. Also, no significant larval growth reduction was induced by the commercial bioregulators glyphosate, maleic hydrazide, and chlormequat chloride when these compounds were incorporated into diets at levels below those which normally caused phytotoxicity (2).

Effects of Bioregulator-Treated Plants on Insects

As previously discussed (2), in the early 1960s, several attempts were made to use the newly available plant growth regulator chlormequat chloride to modify and increase host plant resistance to aphids. It was suggested that the reduction in the growth, development, survival, and fecundity of the aphids was due to the reduction of free amino acids in the treated plants (10,11).

In addition to modifying the biochemical processes in the host plant, appropriately timed application of defoliants, desiccants, or growth-terminating plant growth regulators (PGRs) has been found to be a useful form of insect control. Kittock and co-workers (12) have found that chemical termination of late-season vegetative and reproductive growth in cotton Gossypium sp. has a significant effect on the population of the pink bollworm Pectinophora gossypiella (Saunders). It appears that timely removal of immature cotton bolls with plant regulators such as 3,4-dichloroisothiazole-5-carboxylic acid denies the late-season larvae adequate food to complete the full pupation required for

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

Table I. Assorted Bioregulators Affecting Cotton or Cotton Pests and Their Properties

Bioregulator/ Agent	Crop/ Tissue	Allelochemicals/ Phytoalexin	Effect/ Physiology	Ref.
Natural Plant Growth Hormones				
Gibberellic acid	Cotton	Tannins,Flavonoids	Increase tobacco budworm resistance	32,33
Indole-3-acetic acid	Tobacco callus	------	Disease resistance	34
Kinetin (Burst, Foliar Triggrr)	Cotton	Terpenes,Flavonoids	Affects yield, pests, allelo-chemicals	25,31
Kinetin plus $CaCl_2$, Na_2SeO_3, TGA, VA	Cotton	Terpenes,Flavonoids	Affects yield, pests, allelo-chemicals	31

Continued on next page

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

Table I. Continued.

Bioregulator/ Agent	Crop/ Tissue	Allelochemicals/ Phytoalexin	Effect/ Physiology	Ref.
Synthetic Bioregulators				
Mepiquat chloride (PIX)	Cotton	Gossypol, Tannin, Flavonoids	Growth rate of tobacco budworm	20,21, 24
Mepiquat chloride	Cotton	Terpenoids, Tannins	Decreases bollworm attack	20,21, 22,23
Mepiquat chloride	Cotton	Condensed Tannins	Decreases vegetative growth	18,19
Mepiquat chloride	Cotton	------	Increases nutrients	21
CCC	Cotton	Terpenes, Flavonoids	Affects yield, pests	22,25
BAS 105,109,110,111, 140-810	Cotton	Terpenes, Flavonoids	Affects yield, pests	22,25
2,4-dichlorophenoxy-acetic acid	several	------	Restricts food availability	2,3,4

their overwintering diapause. Similar techniques on cotton have also been reported for the possible control of the cotton bollworm H. zea and the tobacco budworm Heliothis virescens (13).

Other studies have shown that growth of the tomato fruitworm H. zea (L.) on tomato Lycopersicon sp. plants treated with bioregulators such as glycophosate and chlormequat chloride was reduced significantly (2). Mepiquat chloride at a higher concentration is also active against Helicoverpa larvae. Other growth regulators, such as gibberellic acid, 6-benzyladenosine, dinoseb, 1-naphthyl acetic acid, ethephon, and cycloheximide showed no dramatic activity in modifying the plant for larval growth suppression.

The biochemical mechanisms responsible for the induction of resistance to H. zea (an important cotton insect) in plant growth regulator-treated tomato plants is presently unknown. No significant correlation was made between the reduction of larval growth and changes in levels of simple carbohydrates (e.g., fructose, glucose, sucrose, and inositol) or insect growth inhibitors (e.g., tomatine, rutin, and chlorogenic acid) into tomato foliage (2). While both glyphosate and chlormequat chloride are generally recognized as plant growth retardants, their recognized modes of action are reported to be different (14).

In corn Zea mays (L.) after treatment with dinoseb, there was a dramatic reduction in earworm (H. zea [L.]) growth to approximately one half that of corn earworm reared on silks from untreated plants (2). There have been a number of observations that the activity of the PGR depends on the timing of the application (i.e., age of plant) and the genetic source of the plant. In turn, the activities of the plant-regulator treatment on tomato and corn against H. zea appear to be a result of the biochemical modification of host plants.

Gossypol has been considered to be the most important insect-resistance factor in cotton (15). However, condensed tannin and its monomeric flavonoid derivatives have also been shown to be factors important for the resistance of cotton to the cotton bollworm and tobacco budworm H. zea and H. virescens, respectively, and the pink bollworm P. gossypiella (16). Because the flavonoids and condensed tannins are derived from the phenylpropanoid biosynthetic pathway, it could be anticipated that the level of these compounds in cotton may be manipulated through bioregulation of phenylalanine ammonia-lyase (PAL), the key regulatory enzyme in this pathway (17). Therefore, it appeared appropriate to evaluate compounds from several classes of proven bioregulators for their effects on cotton plants and for their pests.

Onium Type Bioregulators Applied to Cotton

Among the first compounds applied to cotton were the onium compounds. PIX (1,1-dimethyl-piperidinium chloride, mepiquat chloride) was found to control undesirable vegetative growth and to promote boll set (18,19). PIX has now been extensively tested on cotton and is currently being used commercially. Its major effect, internode shortening, is visibly apparent, often about 25%, and the result is a more compact, darker green plant (18). Its effect on

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

yield has varied from season to season with both increases and decreases observed, evidently because of differences in environment (20,21,22,23). There have been a number of reports about the effects of PIX on insect pests of cotton. Zummo et al. (20) reported less plant damage, decreased bollworm [Heliothis zea (Boddie)] growth, and 10-20% increased terpenoids, tannins, and astringency (biological tannin) in Texas. Ganyard (24) in North Carolina, observed a 23% decrease in bollworm damage in PIX treated cotton.

Gossypol levels in buds, but not in the leaves, increased significantly four weeks after PIX treatment of the plants with the 3.6 l/hect. rate. The average concentration of gossypol, over time, was also significantly increased by this treatment with PIX. Leaf tannin and flavonoids were significantly decreased four weeks after applying PIX. Except for a small decrease of anthocyanins in leaves and buds at the highest level of treatment, there was no significant effect of PIX on the anthocyanin level at lower levels (21,22,23).

The increase in larval growth rate of tobacco budworm may also be attributed to increases in cotton terminal nutrients, perhaps coupled with the decrease of flavonoids in leaves. Increases were obtained for minerals (26%), protein (14%), and lipids (42%). Decreases were obtained for nitrogen free extract (9%), and more notably for crude fiber (16%) (21).

Increases were also recorded in PIX treated cotton leaves for larval growth rate, protein, lipid, minerals, and gossypol in buds; there were decreases in leaf flavonoids and tannins, crude fiber, and internode distance, but no effect on anthocyanins. In that growing year, 1982, there was more rainfall than average, so the cotton was not drought stressed. The higher rate of tobacco budworm larval growth may have been partially attributable to the higher nutrient concentrations and, perhaps, to the lower flavonoid level in PIX treated plants (21). The previously mentioned decreased larval growth rates observed in Texas by Zummo et al. (20) and in North Carolina by Ganyard (24) were obtained during adverse growing seasons when the cotton was stressed.

Several other onium compounds have also been evaluated for bioregulator activity on cotton plants (25). They include chlormequat chloride, cycocel, CCC (2-choroethyl)trimethylammonium chloride, BAS 105 00 W, LAB 13338, 4-chloro-5-(dimethylamino)-2-phenylpyridazin-3-one, BAS 109 00 W, all-cis-8-(4-chlorophenyl)-3,4,8-triazatetracyclo [4.3.1.$0^{2,5}0^{7,9}$]dec-3-ene, BAS 110 (2,[2,4-dichlorophenyl-3-methoxy-3-[1,2,4-triazolyl-1]-propan-2-ol), BAS 111 (1-phenoxy-5,5-dimethyl-3-[1,2,4-triazolyl-1]-pentan-4-ol), and BAS 140-810 (N-[2-propenyl]-N-[2-(2,4,6-trichlorophenoxy)-ethyl]-piperdinium chloride).

In a test of onium compounds which also contained some naturally occurring plant growth regulators for comparison, results showed that in uninfested cotton, BAS 105 significantly decreased the yield of seed cotton at either one or both of the levels. With CCC and PIX, the trend was downward, but failed significance. In infested cotton, PIX, BAS 105, and BAS 109 (low level only) significantly decreased yield. Only kinetin at both levels

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

significantly increased yield. A compound showing trends toward protection against these insects (though not statistically significant) was BAS 109 (25).

CCC decreased flavonoids by 19% in squares. GA significantly decreased tannins in the square by 6%, while BAS 109 significantly increased tannins in the leaf by 27%. GA increased anthocyanins in the leaf by 28 and 39%, respectively. This suggests that flavonoids generally are increased where yields are (or appear to be) increased, while flavonoids generally are decreased where yields appear to be decreased (22,25).

Lint yield was significantly affected by the low treatment rate of PIX. All the other plant growth regulators tested resulted in moderate increases or decreases in yield; however, they were not statistically significant. Lint percent was significantly increased by BAS 110 but two compounds, kinetin and Burst, reduced lint percent. The PIX treatment resulted in larger bolls and larger seed. In previous research with PIX treatment, there were increases in seed size. In general, the effect of the bioregulators on agronomic traits was minimal, with only a few significant changes detected (25).

The gossypol content of the terminal leaves and anthocyanin content of squares were the allelochemicals most often affected by the bioregulators. BAS 110 and BAS 111 also increased the levels of gossypol in squares. Seed gossypol was reduced by three bioregulators and increased by one. Tannin content of leaves was reduced by the PIX treatments, this being the only significant effect noted. The tannin content of squares was increased by PIX, Triggrr, and PIX plus Triggrr (25). The bioregulators did not have an effect on the anthocyanin level in leaves; however, several produced significant increases in squares.

Kinetin Compounds and Adducts

Kinetin is understood to influence many aspects of plant growth and development. Preliminary results from several field tests showed that kinetin and two commercial kinetin formulations, Burst (Burst Agritech, Overland, KS) and Foliar Triggrr (Westbridge Agricultural Products, San Diego, CA), tended to increase yield of cotton, pest resistance, and four allelochemicals: gossypol, condensed tannins, flavonoids, and anthocyanins (22,25,26). Kinetin riboside had no effects on allelochemicals or lint yield in these tests (25).

The work of Sinha and Hait (27,28) demonstrated that seed and foliar treatments with a wide variety of inorganic and organic chemicals can sensitize plants so that their defensive responses to fungal pathogens are more intense, with the end result that the disease reaction of an otherwise susceptible variety comes to resemble that of a resistant variety. Because there is evidence that insect pests, like fungal pathogens, trigger the expression of resistance mechanisms such as the accumulation of allelochemicals, sensitizing treatments may enhance the resistance of plants to insects as well as fungi.

Keen and Bruegger (29) reported that Hg^{2+}, Cu^{2+}, and NaN_3 elicited various phytoalexins in several plants, imparting disease resistance. Sinha and Hait (27) and Hait and Sinha (30) reported

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

that cysteine, thioglycollic acid, sodium selenite, p-chloromercuribenzoate, lithium sulfate, mercuric chloride, cupric chloride, calcium chloride, barium chloride, sodium sulfite, and sodium malonate elicited phytoalexins, including phenols in rice and/or wheat, inhibiting brown spot disease in rice seedlings and spore germination of Helminthosporium sativum in wheat.

To test whether some of these compounds had an effect on allelochemicals or agronomic traits of cotton, they were applied along with kinetin, which had appeared to have a degree of promise in tests conducted in previous years. The compounds were tested as foliar applications. Accordingly, twelve candidate plant growth regulator formulations were applied twice at two levels to fruiting cotton. Leaves and squares were collected for analysis of allelochemicals (gossypol, tannin, anthocyanin, flavonoids) at least three and five weeks after the first treatment. The plots were machine harvested one time to determine yield. Seeds were delinted and analyzed for agronomic traits and gossypol (31).

Leaf gossypol and square gossypol were the categories most frequently increased by the bioregulators. Kinetin and kinetin plus $CaCl_2$ or Na_2SeO_3 and mepiquat chloride (PIX) alone or with a commercial cytokinin preparation (Foliar Triggrr) all increased gossypol and one or more of the other allelochemicals significantly (31).

Increased gossypol levels (statistically significant at the 5% level) from the zero treatment were observed at one or both levels at either collection data in leaves or squares as a result of the following treatments: kinetin, kinetin + $CaCl_2$, kinetin + Na_2SeO_3, PIX, PIX + FT, and PIX + urea. Similar significant increases of tannins were obtained with kinetin + Na_2SeO_3, kinetin + thioglycolic acid (TGA), and kinetin + vanillic acid (VA). Similar increases (10%) of anthocyanins were found as the result of treatments with urea, PIX, and PIX + FT. Finally, similar increases (10%) of flavonoids were found as the result of treatments with kinetin + IAA, kinetin + TGA, and kinetin + $CaCl_2$. In a few instances, significant decreases were observed (31).

Significant increases in yield occurred only following treatments with PIX + urea, but not with the kinetin tests. Significant decreases in yield were found following treatments with kinetin and kinetin + IAA, $CaCl_2$, and Na_2SeO_3. There were several statistically significant but limited changes in other agronomic traits (lint %, boll size, seed index) (31).

The prospects for inducing resistance to insects by compounds such as those that were evaluated, with or without added kinetin may be subject to the same limitations in efficacy that hold for any genetic resistance short of immunity. Thus, where pest populations are high and/or environmental conditions are especially favorable for pest development (or unfavorable for effective host response), induced resistance is likely to give less than satisfactory protection against yield loss. But a resistance-inducing (sensitizing) treatment may be an inexpensive way to retard the increase in pest populations, either reducing or eliminating the need for application of conventional pesticides.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

Also, because induced resistance impacts a wide range of pests (inlcuding bacteria, fungi, viruses, and insects), it may be effective against plant-parasitic nematodes.

Other Naturally Occurring Plant Growth Hormones

While there are numerous reports of the effects of gibberellic acid in promoting cell division, thus increasing growth rates and yield, etc., in only two cases were increases in allelochemicals reported. Coggins et al. (32), and Greany (33) found increased leaf anthocyanin in cotton, but the effect on insect resistance was not apparent because yield was not increased. Similarly, only two reports could be found where indole-3-acetic acid affected pest resistance. Haberlach et al. (34) found that tobacco (Tobacco sp.) callus was protected from "diseases" by IAA, but kinetin and benzylaminopurine reversed the protection. Iwata et al. (35) reported that IAA and ethylene suppressed rice blast, but that ABA counteracted the effect.

Responses to Bioregulators at the Genetic Level

There may be some similarities in the mechanisms of responses to bioregulators across plant species. Induction of phytoalexins, lytic enzymes, and wall reinforcement involves transcriptional activation of defense genes, in some cases within 2 to 3 min of an elicitation signal. Rapid-response genes include those encoding chitinase and phenylpropanoid biosynthetic enzymes for production of phytoalexins and lignin perturbation. Several hydroxyproline-rich glycoproteins are induced more slowly and at a distance from the initial perturbation, in response to several distinct endogenous intercellular stress signals. The *cis*-acting nucleotide sequences and *trans*-acting factors involved in these complex patterns of defense-gene activation have also been characterized (36).

Albersheim et al. (37) have exhaustively studied the role of polysaccharides in the activation of defense responses in plants. They have found that oligoglucoside fragments of a major structural polysaccharide of fungal cell walls elicit plant cells to accumulate phytoalexins, a plant response that appears to be a general defense mechanism against potential pathogens. Bacteria elicit the accumulation of phytoalexins in plant tissues by secreting enzymes that release an oligogalacturonide fragment of the plant cell wall. The heptaglucoside and oligogalacturonide elicitors, applied simultaneously to plant tissues, have a combined elicitor activity as much as 35-fold higher than the sum of the responses when assayed separately. That elicitors act as signals of tissue damage is supported by reports that oligogalacturonide and glucan elicitors can activate other defense responses of plants, like accumulation of lignin, hydroxyproline-rich glycoproteins, and protease inhibitors. Another mechanism by which plants resist microbial invasion is the hypersensitive response, the rapid death of plant cells at the point of attempted infection. Oligosaccharide fragments of cell walls applied to plant cells in liquid culture cause cell death. Evidence was also

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

obtained that by incubating tobacco thin-cell layers on liquid media in the absence or presence of plant cell-wall fragments, the fragments are able to regulate organogenesis.

Summary

Bioregulators are finding increased uses in improving yield, altering growing patterns, improving harvesting conditions, enhancing plant resistance to pests, and mitigating adverse environmental conditions. Most of the early reported effects of bioregulators on insect pests were unexpected side effects which mainly involved the application of herbicides on the host plant. With the advent of the "onium"-type bioregulators such as CCC and PIX which were important because they altered mainly growing patterns, thereby improving recoverable yield, some directed studies of the effects on pest resistance were carried out and some effects were observed. These bioregulators were also shown to stimulate biosynthesis of known allelochemicals.

Other studies have shown that a number of biological, physical, and chemical stimuli can elicit the biosynthesis of phytoalexins, counteracting various disease organisms in plants. This has led to the recognition that a wide variety of substances can elicit processes in plants that lead to resistance to insects and diseases. These include not only the natural plant growth hormones and their synthetic analogues and inhibitors, but also a relatively large number of commonly occurring inorganic and organic compounds. Moreover, components in mixtures may either synergize, amplify, or alternatively inhibit the activity of the other(s).

Much of what is known about the mechanisms of action has come from studies of the elicitation of phytoalexins and their subsequent effects on plant infection. Induction of phytoalexins involves transcriptional activation of defense genes encoding for enzymes that produce the resistance factors. Auxins and abscisic acid have been shown to act as signals to the genes, initiating the processes leading to plant resistance to pests.

Literature Cited

1. Ory, R. L.; Rittig, F. R. In Bioregulators: Chemistry and Uses; Ory, R. L.; Rittig, F. R., Eds.; ACS Symp. Ser. No. 257; American Chemical Society: Washington, DC, 1984, pp 1-8.
2. Campbell, B. C.; Chan, B. G.; Creasy, L. L.; Dreyer, D. L.; Rabin, L. B.; Waiss, A. C., Jr. In Bioregulators: Chemistry and Uses; Ory, R. L.; Rittig, F. R., Eds.; ACS Symp. Ser. No. 257; American Chemical Society: Washington, DC, 1984, pp 193-204.
3. Ingram, J. W.; Bynum, E. K.; Charpentier, L. J. J. Econ. Entomol., 1947, 40, 745-746.
4. Adams, J. B.; Drew, M. E. Can. J. Zool., 1965, 43, 789-794.
5. Gall, A.; Dogger, J. R. J. Econ. Entomol., 1967, 60, 75-77.
6. El-Ibrashy, M. T.; Mansour, M. H. Experientia, 1970, 26, 1095-1096.
7. Tahori, A. S.; Zeidler, G.; Halevy, A. H. J. Sci. Food Agric., 1965, 16, 570-572.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

8. Bhalla, O. R.; Robinson, A. G. J. Econ. Entomol., 1968, 61, 552-555.
9. Carlisle, D. B.; Ellis, P. E.; Osborne, D. J. J. Sci. Food Agric., 1969, 20, 391-393.
10. Maxwell, R. C.; Harwood, R. F. Ann. Entomol. Soc. Am., 1960, 53, 199-205.
11. Van Emden, H. F. J. Sci. Food Agric., 1969, 20, 385-387.
12. Kittock, D. L.; Arle, H. F.; Henneberry, T. J.; Bariola, L. A.; Walhood, V. T. Crop Sci., 1980, 20, 330-333.
13. Thomas, R. O.; Cleveland, T. C.; Cathey, G. W. Crop Sci., 1979, 19, 861-863.
14. Hoagland, R. E.; Duke, S. O. In Biochemical Responses Induced by Herbicides; Moreland, D. E.; St. John, J.; Hess, P. D., Eds.; ACS Symp. Ser. No. 181; American Chemical Society: Washington, DC, 1982, 175-205.
15. Bottger, G. T.; Sheenhan, E. T.; Lukefahr, M. J. J. Econ. Entomol., 1964, 57, 283-285.
16. Chan, B. G.; Waiss, A. C., Jr.; Binder, R. G.; Elliger, C. A. Entomol. Exp. Appl., 1978, 24, 294-300.
17. Creasy, L. L.; Zucker, M.; Wong, P. P. Phytochemistry, 1974, 13, 2117-2124.
18. Namken, L. N.; Gausman, H. W. Agricultural Research, US Dept. of Agriculture: Washington, DC, 1978, 27, pp 11-12.
19. Gausman, H. W.; Rittig, F. R.; Namken, L. N.; Rodriguez, R. R.; Escobar, D. E.; Garza, M. V. Proc. Fifth Ann. Mtg., Plant Growth Regulator Workshop Group, Blacksburg, VA, 1978, June 25-29, pp 137-145.
20. Zummo, G. R.; Benedict, J. H.; Segers, J. C. Proc. Beltwide Cotton Prod. Res. Conf., 1983, Jan. 2-6, San Antonio, TX, 73-74.
21. Hedin, P. A.; Jenkins, J. N.; McCarty, J. C., Jr.; Mulrooney, J. E.; Parrott, W. L.; Borazjani, A.; Graves, C. H., Jr.; Fuller, T. H. In Bioregulators: Chemistry and Uses; Ory, R. L.; Rittig, F. R., Eds.; ACS Symp. Ser. No. 257; American Chemical Society: Washington, DC, 1984, pp 171-191.
22. Hedin, P. A.; Jenkins, J. N.; Thompson, A. C.; McCarty, J. C., Jr.; Smith, D. H.; Parrott, W. L.; Shepherd, R. L. J. Agric. Food Chem., 1988, 36, 1055-1061.
23. Hedin, P. A.; McCarty, J. C., Jr.; Thompson, A. C.; Jenkins, J. N.; Smith, D. H.; Shepherd, R. L.; Parrott, W. L. J. Agric. Food Chem., 1988, 36, 742-745.
24. Ganyard, M. C. Successful Farming, 1982, p. 17.
25. McCarty, J. C., Jr.; Hedin, P. A. Miss. Agric. For. Exp. Sta. Res. Rep.; 1989, 14(16), pp 1-5.
26. McCarty, J. C., Jr.; Jenkins, J. N.; Hedin, P. A.; Shepherd, R. L.; Parrott, W. L. Miss. Agric. For. Exp. Sta. Res. Rep.; 1987, 12(13), pp 1-4.
27. Sinha, A. K.; Hait, G. N. Trans. Br. Mycol. Soc., 1982, 79, 213-219.
28. Hait, G. N.; Sinha, A. K. J. Plant Dis. Prot., 1987, 94, 360-368.
29. Keen, N. T.; Bruegger, B. In Host Plant Resistance to Pests. ACS Symp. Ser. No. 62; Hedin, P. A., Ed.; American Chemical Society: Washington, DC, 1977, pp 1-26.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

30. Hait, G. N.; Sinha, A. K. J. Phytopathol., 1986, 115, 97-107.
31. Hedin, P. A.; McCarty, J. C., Jr. J. Agric. Food Chem., 1991, 39, 549-553.
32. Coggins, C. W.; Scora, R. W.; Lewis, L. N.; Knapp, J. C. F. J. Agric. Food Chem., 1986, 17, 807-809.
33. Greany, P. L. Agric. Res., 1978, 27, 3.
34. Haberlach, G. T.; Budde, A. D.; Sequeira, L.; Helgeson, J. P. Plant Physiol., 1978, 62, 522-525.
35. Iwata, M.; Sekizawa, Y.; Iwamatsu, H.; Suzuki, Y.; Watamabe, T. Ann. Phytopathol. Soc. Jpn., 1981, 47, 646-653.
36. Lamb, C. J. In Proc. 2nd Int. Congr. Plant Molecular Biology, Jerusalem, Nov. 13-18, 1988, Abstr. 7.
37. Albersheim, P.; Augur, C.; Bucheli, P.; Cervone, F.; Darvill, A. G. In Proc. 2nd Int. Congr. Plant Molecular Biology, Jerusalem, Nov. 13-18, 1988, Abstr. 208.

RECEIVED March 3, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch005

Chapter 6

Endophytic Fungi of Pacific Yew *(Taxus brevifolia)* as a Source of Taxol, Taxanes, and Other Pharmacophores

Andrea Stierle[1,2], Donald Stierle[2], Gary Strobel[1], Gary Bignami[3], and Paul Grothaus[3]

[1]Department of Plant Pathology, Montana State University, Bozeman, MT 59717
[2]Department of Chemistry, Montana College of Mineral Science and Technology, Butte, MT 59701
[3]Hawaii Biotechnology Group Inc., Aiea, HI 96701

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

Investigation of the bioactive components of the endophytic microbes associated with the Pacific yew tree, Taxus brevifolia yielded a novel, hyphomycetous fungus that produces taxol and taxanes when grown in semi-synthetic liquid media. Taxomyces andreanae was isolated from the phloem (inner bark) of a Pacific yew tree, the traditional source of naturally occurring taxol. The presence of taxol in the fungal extract was confirmed by fast atom bombardment and electrospray mass spectrometry, comparative chromatographic behavior with yew taxol, reactivity with monoclonal antibodies specific for taxol, and 9KB cytotoxicity studies. Both acetate-1-^{14}C and phenylalanine UL-^{14}C served as precursors of taxol-^{14}C in fungal culture labeling studies, confirming the de novo synthesis of taxol by the fungus. Immunoassay techniques are currently being used to screen extracts of Taxomyces andreanae for new taxanes, and to determine if other endophytic fungi are taxol producers.

Cancer is the leading cause of death in the Western world, and the incidence of cancer continues to climb annually (1). Chemotherapeutic agents are instrumental in the fight against this dreaded disease. Effective anticancer agents, particularly those that are effective against refractory tumors, are critical objectives in western medicine. With its unique mode of action and its particular efficacy against refractory ovarian and breast cancer tumors, taxol has been the focus of international attention (2). Unfortunately, taxol has been traditionally isolated in extremely small quantities from the bark of the Pacific yew tree, a relatively rare understory shrub. There are simply not enough yew trees growing in North America to satisfy projected needs of this drug over the next twenty years (3). To ameliorate this problem, we tried to find a new source organism of taxol in the microbial flora of the yew tree.

0097–6156/94/0557–0064$08.00/0
© 1994 American Chemical Society

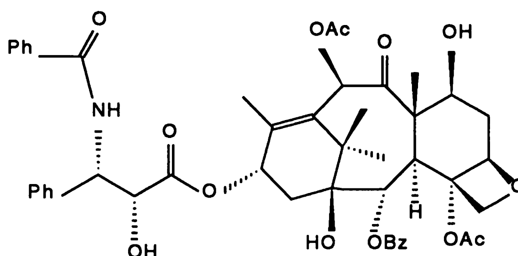
Over the last two years we have isolated over 300 fungi from the bark and needles of yew trees in Montana, Washington, Idaho, and Oregon. Of particular interest is the ability of a microbe to produce taxol or taxanes (taxoids) in culture. Promising taxol producers have been studied using a variety of different techniques, including chromatography, mass spectrometry and antibody based immunoassays. Immunoassay is proving an effective tool not only in assessing the presence of taxol and taxanes in crude extracts, but also in providing an efficient fractionation guide.

Both the organic and aqueous extracts of these fungi were examined not only for their ability to produce taxol or taxanes, but also for other pharmacophores as well. Particular attention was paid to compounds with either antifungal or anticancer potential. Fungi already provide a number of important antibiotics, including the penicillins and cephalosporins (4). Endophytic fungi, however, particularly those isolated from conifers, are an untapped reservoir of compounds with pharmaceutical potential. Fungi associated with medicinal plants are a reasonable starting point of an innovative approach to the discovery of new pharmacophores.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

Background

Taxol **1**, is one of the most promising anticancer agents currently under study by the National Cancer Institute and Bristol Myers-Squibb (2). This highly functionalized diterpene is isolated primarily from the inner bark of the relatively rare and slow growing Pacific yew tree, Taxus brevifolia, and a few related species, in extremely small yields (< .02% dry weight) (5).



1

Although initially isolated and characterized in 1971, taxol did not achieve notoriety until 1977, when its strong activity against human tumor xenograph systems and murine B16 melanoma cells prompted its development towards clinical trials (3). Interest in taxol intensified when its unique mode of antimicrotubule cytotoxicity was published in 1979 (6,7). Microtubules are among the most strategic of the subcellular chemotherapeutic targets. Antimicrotubule

agents are extremely potent, requiring only a few molecules to disrupt the microtubular structure of cancer cells (2). These compounds, which include the vinca alkaloids, are among the most important anticancer drugs currently employed (2).

Taxol: the Supply Dilemma. The emergence of taxol as an effective anticancer agent has created a dilemma long anticipated by the natural products community: how to insure an adequate supply of a compound of non-microbial origin. Advanced preclinical and phase I clinical development of taxol required several collections ranging in size from 5000 to 15000 pounds of dry bark. A mature Pacific yew (100 years old) yields approximately 10 lb. of dry bark, so each collection required the sacrifice of 500 to 1500 trees (3). As the efficacy of the compound became more apparent, the demand for additional taxol increased (3).

In 1987-1988 a 60,000 lb bark collection was undertaken with little controversy. The need for a second 60,000 lb bark collection in 1989, however, sparked concern about the impact such collection sizes may have on the continued existence of the yew tree. Although no accurate inventories of the tree have ever been undertaken, the Fish and Wildlife Service of the U.S. Department of the Interior states that "Much of the range of the yew has not been subject to statistical inventories, especially the northern portion (i.e., Alaska and British Columbia). Nonetheless, based on stand information, together with satellite imagery, the U.S. Forest Service estimates that 130 million yew trees occur on 1,778,000 acres of National Forest in the Washington and Oregon Cascades, and Oregon Coast Range" (3).

Even with this estimate, however, it is clear that additional sources of taxol must be found. A single course of clinical treatment is 125-300 mg of taxol, and typical treatments may extend for 10 or more courses. Treatment of the 12,000 women who die annually of ovarian cancer alone would consume as much as 36 kg of the drug (3). With current isolation methodologies, 1 kg of taxol is isolated from 25,000 lb of dried bark, or the bark of 2500 yew trees. Therefore, simply treating ovarian cancer over a one year period would consume 90,000 mature yew trees (3). Should taxol prove as effective against other refractory cancers as clinical trials have indicated, it is not unreasonable to assume that the demand for taxol may exceed 300 kg, or 750,000 trees per year (3). This represents an enormous demand on a limited resource.

Microbial Source for Taxol

The pharmaceutical potential of taxol has elevated the status of the yew tree from a nuisance weed suitable only for burning, to a precious commodity and natural resource. Unfortunately, this change in status does not alter the underlying dilemma - there are simply not enough yew trees to supply the growing demands for taxol (3). Several different strategies are being explored by laboratories around the world to ameliorate this problem. These include semi-synthesis, total

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

synthesis, and tissue culture. We attempted to isolate a new source of taxol, a microbial source. It is important to understand the significance of finding a microorganism capable of producing taxol. Such a discovery would represent a paradigm shift in the search for effective pharmaceutical agents.

Advantages of Microbial Source. From a practical viewpoint, microbial fermentation as a means of producing bioactive substances has several advantages (8).

1. Industrial production of a bioactive substance like taxol requires reproducible, dependable productivity. If a microbe is the source organism, it can be grown in tank fermentors as needed, producing a virtually inexhaustible supply of taxol (8).

2. Microorganisms typically respond favorably to routine culture techniques. Cultivation of macroorganisms (tissue culture) is considerably more challenging, requiring either specialized techniques or months of growth before harvesting is feasible (8).

3. Productivity amplification is relatively easy in microorganisms. In the case of penicillin, improved culture conditions and genetic manipulation of producing strains of Penicillium increased drug yield from a few micrograms per milliliter to thousands of micrograms per milliliter (4,9). With macroorganisms, larger collection sizes are the most reasonable option for improved productivity. In the case of taxol, larger collection sizes will lead to the eradication of the source organism within a few years if all of the demands for it are to be met.

4. Different bioactive compounds can be produced by altering culture conditions. The antibiotic aplasmomycins were produced by Streptomyces griseus SS-20 only after NaCl was added to the medium (10). Directed changes in culture conditions can be explored indefinitely as a method of optimizing various biosynthetic pathways, that may lead to even more effective derivatives of taxol (8).

What all of this means is that a **microbial source of taxol could provide an inexhaustible supply of taxol.**

The Gibberellins: Precedence for Microbial Source of Taxol. The search for a taxol producing fungus was prompted not only by the advantages inherent in a microbial source, but also by the fact that endophytic microorganisms can produce all of the known phytohormones. In fact, the gibberellins, also highly functionalized diterpenes, were first discovered in the phytopathogenic fungus Gibberella fujukuroi the causative agent of "foolish rice seedling disease" in Japan (11). Since then, it has been established that the pathways of gibberellin biosynthesis in the fungus and the higher plant are identical up to gibberellic acid $_{12}$ (12). This suggests the possibility of intergeneric-genetic exchange between higher plant and fungus. The search for a taxol producing microorganism should commence in the tissues of Taxus spp., particularly in the portion of the tree in which taxol is isolated (1).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

Search for a Taxol Producing Microorganism

Yew bark, needle, and root samples were collected in several national forests throughout Washington, Oregon, Idaho and Montana. Samples were taken from both healthy and diseased specimens. Each sample was placed on water agar, and developing microbial colonies were transferred to mycological agar as they appeared. Microbes were established in pure culture using standard methodology. Each microbe was grown in liquid medium (100 Ml) following purification. Fungi were grown in mycological broth to which 2% yew needle broth was added. Bacteria were grown in tryptic soy broth with the same amendment. Endophytic microbes often cease production of secondary metabolites when removed from the host organism. Yew needle broth may serve either as a critical precursor reservoir or as a genetic promoter for the biosynthetic mechanism of taxol production. It must be noted, however, that **yew needle broth is added only in the first fermentation**. Subsequent fermentations of promising microbes use strictly defined synthetic or semi-synthetic media.

Chemical Analysis of Fungal Extract. All microbial extracts were subjected to first-order examination, which consists of chemical extraction, thin layer chromatography, and nuclear magnetic resonance. Following this initial examination, promising microbial extracts were grown in 1L cultures **without the addition of yew broth**. These cultures were processed as before, and then subjected to second order examination, which consists of a chromatographic step followed by mass spectrometry. Third order examination involves purification of the potential taxol fraction followed by both intramural and extramural mass spectral analysis, extramural monoclonal antibody immunoassay and 9KB cytotoxicity determinations.

Twenty-one day cultures were filtered through cheesecloth. The residue (mycelia) was macerated and extracted thoroughly with methylene chloride-methanol (1:1). The filtrate was extracted with methylene chloride. The two organic extracts were examined by thin-layer chromatography on Whatman silica gel plates (0.5 mm, 5x10cm) with yew taxol as the standard, using three different solvent systems (13). The plates were visualized both by ultraviolet light and by using sulfuric acid-vanillin spray reagent. The ^{1}H NMR spectra of these extracts were also compared to that of yew taxol. Extracts with TLC spots reminiscent of taxol were prepared for second order analysis.

This preparation step consists of flash silica gel chromatography using acetonitrile as the solvent, followed by high performance liquid chromatography in 3:1 hexane-isopropanol (Rainin Dynamax-60A 8μm Cyano, 4.6x250mm). The appropriate fraction was analyzed by electron impact mass spectrometry. EIMS does not give the molecular ion but it does yield the important fragment peaks at m/z 509 and 569 amu.

Taxomyces andreanae, a Fungal Source of Taxol

Throughout our investigation we have examined microorganisms isolated from

more than twenty-five trees from more than twenty locations. Of the three hundred microbes screened to date, only one fungus has progressed to third-order analysis. This previously undescribed fungus, which we named Taxomyces andreanae, has demonstrated unequivocally the ability to produce taxol (13). It was isolated from the bark of a single yew tree in a unique location in an old growth cedar forest in northern Montana. Despite extensive searches, T. andreanae has not been found in any other yew tree examined to date.

Confirmation of Taxol Production by T. andreanae. The complete third order analysis of T. andreanae combined several different protocols, each of which was critical to confirmation. We will outline the entire analytical process in some detail to provide convincing evidence of the ability of T. andreanae to produce taxol.

Fermentation Procedure. T. andreanae was established in pure culture via hyphal tip transfer from water agar, on which the bark pieces had been placed, to mycological agar (DIFCO). The growing mycelium was then serially transferred three to six times, to fresh mycological agar. This eliminated the possibility that fungal hyphae carried a taxol or taxane "contaminant" from the source yew tree. T. andreanae grows well on mycological agar, and is a good source of inoculum for broth cultures. The transfers are made from mycelia 3-7 days after inoculation; older mycelia do not grow as well. T. andreanae has been successfully transferred from 2 month old M-1-D agar cultures, which is used as our maintenance medium.

Since the conidia of T. andreanae do not germinate, pieces of agar block (5x5mm) impregnated with mycelia are added to each autoclaved flask containing either modified mycological or S-7 media (11). Optimum conditions for taxol production appear to be 21 day still culture, at 25°C, with a surface:volume ratio of 1.3 (cm^2:Ml).

Fungal Taxol Isolation Protocol. At the end of the incubation period, the culture was filtered through 8 layers of cheesecloth. The filtrate was extracted with methylene chloride. The aqueous phase was lyophilized and extracted with methylene chloride-methanol (1:1). The mycelium was macerated and thoroughly extracted with methylene chloride-methanol (1:1). The solvent was removed from the organic extracts by rotary evaporation at 30°-35°C. Thin layer chromatography of all three organic extracts in three solvent systems (14) indicated that the taxol-like metabolite was concentrated in the methylene chloride extract of the filtrate.

This organic extract was dissolved in 2 ml of chloroform and placed on a 1 cm x 5 cm column of silica gel (60-200 mesh). The column was thoroughly rinsed with chloroform and then eluted with 20 ml of acetonitrile. The resulting fraction was dried and the residual oil was chromatographed on a preparative

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

Merck thin layer chromatography (TLC) silica gel plate (0.5 mm silica gel) and developed in chloroform-acetonitrile 7:3 v/v. The region at the R_F of taxol-baccatin at ca. 0.17 - 0.30 was removed by scraping, and eluted with acetonitrile.

The eluant was subjected to HPLC (silica gel 1.5 x 25 cm column) using chloroform-acetonitrile 7:3 v/v in an isocratic mode. The peak eluting with the same retention time as taxol was collected, dried and subjected to the final preparative TLC on a prewashed Merck silica gel plate (0.25 mm) in ethyl acetate-isopropanol 95:5 v/v. The area with the identical R_F to taxol was eluted with acetonitrile and dried. Various modifications of this extraction and purification method were also successful in yielding fungal taxol.

The compound isolated from T. andreanae had identical R_F values as authentic taxol in three different TLC solvent systems (14). It reacted positively with vanillin-sulfuric acid spray reagent, yielding a blue spot which turned brown after 12-24 hr (15). Fungal taxol has the same retention time (5 min.) on HPLC as authentic taxol on a 1.5 x 2.5 cm silica column using chloroform-acetonitrile 7:3 v/v as the solvent system. It also has the same retention time using analytical cyanopropyl bonded-phase HPLC with different solvent systems. In addition, the UV spectrum of fungal taxol is superimposable on that of authentic taxol, with two maxima at 273 nm and 235 nm (1).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

Mass Spectral Analysis of Fungal Taxol. Fungal taxol was examined by several mass spectral techniques. Electron impact mass spectrometry exhibited strong fragment peaks at m/z 509 and m/z 569, but the molecular ion at m/z 854 was not apparent (16). EIMS of authentic taxol exhibited a virtually identical fragmentation pattern. Electrospray mass spectrometry of the fungal taxol fraction primarily yielded peaks at m/z 854 and 876.5 (16). These masses represent the M^+ + H of taxol and the M^+ + Na of its sodiated adduct, respectively. Authentic taxol yielded the identical spectrum as fungal taxol following sodiation. In addition, the fast atom bombardment (FAB) spectrum of fungal taxol yielded the M^+ + H of taxol (854.3) with peaks characteristic of taxol at 509 and 569 when compared to authentic taxol (16,17). These data have been corroborated at least 5 times on different fungal preparations. Evidence was also obtained by liquid chromatography-mass spectrometry for the presence of baccatin III in T. andreanae, the parent peak at m/z 604 is consistent with M^+ + NH_4 of this compound.

Immunoassay Techniques as a Screening Tool. The selection and monitoring of fungal strains like Taxomyces andreanae for secondary metabolite production can be expedited if sensitive, specific, rapid screening methods are available for compounds of interest. Antibody based immunoassays provide an alternative to chromatographic or spectroscopic techniques, and often provide the advantages of greater sensitivity, simpler sample preparation and high sample throughput (18). Because the sensitivity and specificity of an immunoassay reflects the binding properties of the antibodies utilized, production of high affinity antibodies to the target analyte is a critical, initial step in assay development. Most organic compounds less than 2500 amu, which includes taxol

and its congeners, will not stimulate the antibody response in animals. It is necessary to covalently link such small molecules (haptens) to an immunogenic macromolecule, usually a protein. Serum taken from an animal immunized with hapten-conjugates will contain polyclonal antibodies with a wide range of specificity and affinity for the different components of the immunogen, including the hapten, the carrier protein, and the hapten-carrier complex (19). Immortal cell lines producing homogeneous monoclonal antibodies (mAbs) can be derived from individual antibody-producing lymphocytes by the hybridoma technique. Both monoclonal and polyclonal antibodies can be used for developing immunoassays as long as high affinity antibodies with reactivity to unconjugated hapten are present.

Development of Monoclonal Antibodies Specific to Taxol and its Congeners. A hybridoma cell line derived from a mouse immunized with keyhole limpet hemocyanin-7-succinyltaxol conjugate produces a high affinity mAb to taxol and its C-7 derivatives (16,20). This Mab, 3C6, is twenty-fold less reactive with cephalomannine and is virtually unreactive with baccatin III. Mab 8A10 was derived from a mouse immunized with succinylbaccatin III: it cross reacts with taxol, cephalomannine, baccatin III, and 10-deacetylbaccatin III. It did not react, however, with an analog lacking the C-20 oxetane ring, 20-acetoxy-4-deacetyl-5-epi-20,O-secotaxol. Thus Mab 8A10 appears to bind a determinant common to the intact tetracyclic diterpenoid ring structure common to many natural taxanes.

Indirect Competitive Inhibition Enzyme Immunoassay (CIEIA). Competitive inhibition enzyme immunoassays have been developed utilizing Mab 3C6 and Mab 8A10 to quantitate the amount of taxol vs total taxanes in crude sample extracts according to the method shown in Figure 1. The assay is performed in 10% methanol to facilitate dissolution and processing. The CIEIA is conducted in 96-well microtiter plates coated with 100µL of a bovine serum albumin (BSA) conjugate of the hapten, either 7-succinyltaxol for Mab 3C6 or 7-succinylbaccatin III for Mab 8A10. Additional BSA (200µL) is then added to prevent non-specific antibody binding to the solid phase. To generate standard curves, analytical standards are serially diluted between 0.5 and 300 Nm in phosphate buffered saline containing 0.25% BSA, 0.05% Tween-20 and 20% methanol, and 50 µL is combined with an equal volume of optimally diluted antibody in phosphate buffered saline containing 0.25% BSA and 0.05% Tween-20. Dried extracts of fungal cultures are suspended in 0.2 Ml MeOH, and diluted 1:4 with phosphate buffered saline containing 0.25% BSA and 0.05% Tween-20. The suspended extract is serially diluted in phosphate buffered saline containing 0.25% BSA, 0.05% Tween-20, and 20% MeOH and combined with antibody as described for analytical standards. Analyte present in standard or test wells competitively inhibits antibody binding to the solid-phase BSA-hapten conjugate. The bound antibody is detected indirectly using an anti-mouse immunoglobulin enzyme-conjugate and appropriate chromogenic substrate, to generate a

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

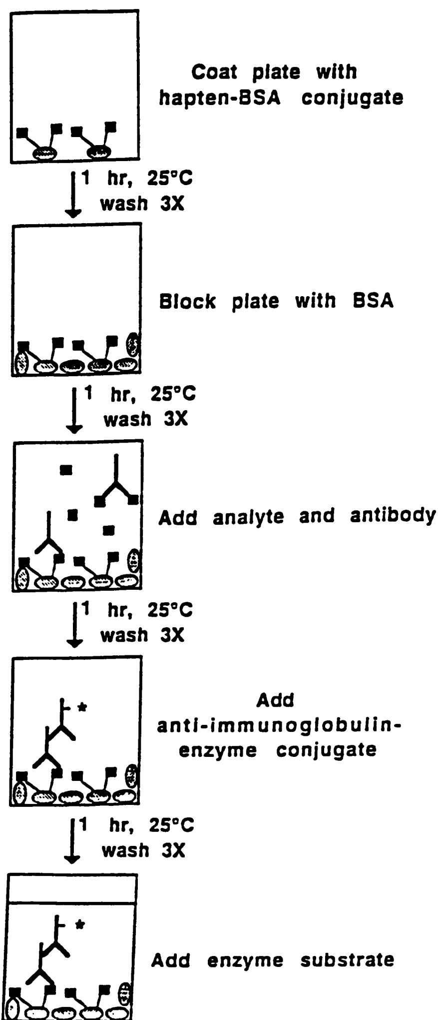


Figure 1. Schematic representation of the CIEIA method.

colorimetric assay endpoint which is inversely proportional to the analyte concentration (Figure 2).

CIEIA methods using these antibodies confirmed the presence of taxol in partially and totally purified preparations of fungal taxol. Extracts from ten different fermentations of T. andreanae have been tested by CIEIA with consistent levels of taxol and taxane in each test. Furthermore, a quantitative comparison between the CIEIA's using monoclonal antibodies specific to taxol with a CIEIA using monoclonal antibodies class specific to taxanes in general revealed that taxol comprises only 15-20% of the total taxanes present in the semi-purified fungal extract (1st TLC step in purification) (16).

Fungal taxol was isolated from 3 week old culture fluids of this fungus and identified by mass spectrometry, immunochemistry and chromatographic methods. T. andreanae also makes baccatin III and other taxanes, but cephalomannine has never been detected in the fungal extracts. In all yew tree bark extracts, however, taxol and cephalomannine are isolated together, and are difficult to separate using silica gel HPLC. The fungus and the tree may exploit biosynthetic pathways that differ to some degree, which may account for the absence of cephalomannine. An important corollary to this premise is the potential of the fungus to produce unique taxanes. Taxol is not the perfect drug. Its poor water solubility poses delivery problems that have not been adequately resolved. T. andreanae may produce related, more polar compounds with activity approaching that of taxol. We have already discovered taxanes with unique mass spectral profiles. These findings open the possibility for an unlimited source of taxol via fermentation technology.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

Radioisotopic Labeling Studies. To demonstrate that the taxol found in T. andreanae is actually produced by this fungus, we performed radiolabeling experiments with acetate-1-^{14}C and other radioisotopically labeled precursors (6). Each precursor was added to a 20 day old culture and then incubated for 4 days at 25° C. Phenylalanine-UL-^{14}C was the best precursor for fungal taxol-^{14}C, followed by acetate-1-^{14}C. ^{14}C was also found in baccatin III (Table I). Neither benzoate-7-^{14}C, nor leucine-UL-^{14}C yielded any taxol-^{14}C, although leucine is a very effective taxol precursor in Taxus brevifolia (21). Confirmation of the identity of taxol-^{14}C in the fungal preparations was done by 2 dimensional (TLC) co-chromatography with authentic taxol (16). The size, shape, and location of the vanillin/sulfuric acid and UV absorbing spot on the TLC plate was identical to the exposed single-spot on the x-ray film. As a control, ethanol (60%) killed mycelium was incubated with acetate-1-^{14}C and the culture medium processed in the identical manner. No radioactivity appeared in the area coincident with taxol (Table I).

Table I. Incorporation of ^{14}C into taxol and baccatin III by Taxomyces andreanae from potential precursors of taxanes

Precursor	taxol (dpm)	baccatin III (dpm)
Na Acetate-1-^{14}C	261 ± 32	128 ± 20
Na Acetate-1-^{14}C (mycelium pretreated with 70% EtoH)	0	0
Phenylalanine-UL-^{14}C	1241 ± 40	268 ± 25
Na benzoate-7-^{14}C	0	0
Leucine-UL-^{14}C	0	0

Fungal cultures were extracted, processed, and subjected to a series of TLC systems as described. The data are all normalized on the basis of 100 μCi administered per 2 g dry weight of fungal mycelium.

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Controlled Experiments. Several control tests were performed throughout the course of these studies. These tests eliminated the possibility that the detected taxol was either a carry over from the tree or an accidental contaminant of our fungal cultures. The entire volume of a given culture medium (5L) inoculated with agar blocks of fungal mycelium, yielded no detectable taxol at time zero. Likewise, it was impossible to detect taxol in the agar blocks of mycelium (inoculum alone). The presence of 1 mg/liter of chlorocholine chloride in the S-7 medium (22) completely abolished taxol production. This compound is also an effective inhibitor of gibberellin production in G. fujikuroi (11,12), although it stimulates petasol (sesquiterpenoid) production in Drechslera gigantea (23). We can reasonably conclude that the taxol isolated from cultures of T. andreanae is actually a product of the metabolism of this organism.

Taxol Yield as a Result of Media Amendments. The amounts of taxol (taxanes) produced by T. andreanae are disappointingly low, which is also reflected by the low incorporation of ^{14}C-precursors to taxol (Table 1). Estimates made by 2 different methods, electrospray mass spectrometry, and the quantitative monoclonal antibody (CIEIA) technique, indicate that 24-50 ng of taxol are produced per liter. We have observed, however, that many plant associated fungi require one or more plant metabolites to activate pathways critical to secondary product formation (24). In controlled experiments, the water soluble components of yew needles and shoot tips seem to encourage taxol production by T. andreanae

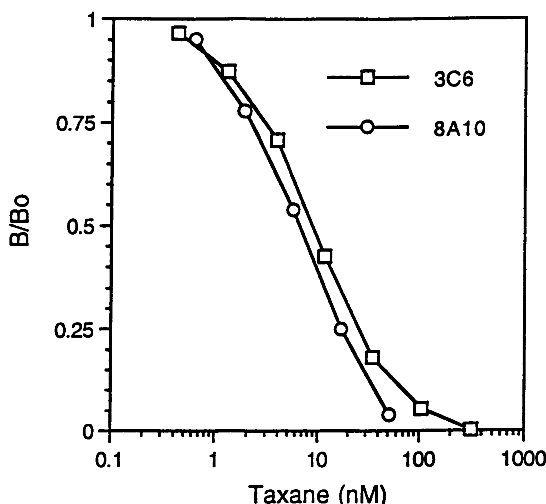


Figure 2. Standard curves for taxol (3C6) and taxane (8A10) indirect CIEIA's. B/B_0 values for each dilution were calculated by dividing the mean OD of a given set of replicates containing taxane inhibitor (B) by the mean OD of all the wells containing no inhibitor. Unknown taxane concentrations in test samples were calculated from the B/B_0 of the sample dilution(s) that fell within the log-linear portion of the standard curve. Taxol was used as the standard for 3C6 and baccatin III for 8A10.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

(16). Of course, because the purpose of this investigation was to prove that fungal taxol is a product of T. andreanae, no plant extracts were included in any growth media used to verify taxol production by the fungus. However, such water extracts may indeed augment taxol production. Improved culturing techniques, the addition of "activators", and the application of genetic engineering methods may ultimately permit the commercialization of T. andreanae for taxane production. Such improvements would not be unlike the course taken for virtually all microbes that have been subject to fermentation for industrial microbiological purposes (9).

The Search for Unrelated Pharmacophores from this Fungal Collection.

Each fungal extract was examined for its biological activity following determination of its taxol production potential. Although standard disk bioassays were utilized to determine activity against both fungi and Gram + and Gram - bacteria, special attention was paid to microbes whose organic extracts showed promise as anticancer or antitumor agents. Intramural screening of large numbers of extracts for these activities can be problematic. Simple in-house assays have been devised and perfected by a number of scientists to facilitate such activity assessments. Galsky and Ferrigni have promoted two assays, the crown gall tumor assay on potato discs and brine shrimp assay that can model the traditional 9KB and P388 in vivo mouse leukemic systems (25-27). These assays will facilitate bioassay guided fractionation. All pure compounds with activity in our in-house screens will be sent to the National Cancer Institute for rigorous testing.

Our preliminary investigations show promise. We have found the endophytic fungi isolated from Taxus brevifolia to be a rich source of potential pharmacophores. The fungi with significant antifungal activity or cytotoxicity will be grown in large volume cultures and the bioactive components will be isolated and characterized.

Fungal Culture and Chemical Analysis. The designated fungi were grown in 1 L broth cultures (same media as plates) at room temperature with 12 hour light/dark cycles. After 21 days each culture was killed by the addition of 50 Ml of methanol. Fungal cultures were filtered through cheesecloth to separate the mycelia from the filtrate. The fungal filtrates were rotoevaporated to remove the methanol, and lyophilized. The dried residue were thoroughly extracted first with methanol, then with methanol-chloroform (1:1). Mycelial mats were extracted in a similar manner. The organic extracts of each microorganism were combined.

Both aqueous and organic extracts were retested to confirm desired activity, and subjected to bioassay guided fractionation. Bioassays include antifungal standard disc assays, antibacterial standard disc assays, brine shrimp toxicity assay, an effective method for predicting cytotoxicity (27), and crown gall potato disc assay (25,26). Extracts will also be sent to our pharmaceutical collaborators for additional testing.

Preliminary work on the endophytic fungi associated with the yew has yielded over 300 isolated fungi. Our in-house antimicrobial assays on the organic, freeze dried organic and freeze dried water extracts of these fungi showed the following results:

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

Total fungi- 300
Percentage of organic extracts with antimicrobial activity in standard disk assays:

Bacillus subtilis	21%
Staphylococcus aureus	13%
Escherichia coli	9%
Pseudomonas aeruginosa	0.3%
Vibrio harveyii	5%
Candida albicans	4%
Helminthosporium sativum	0.6%

This data shows us that only a small percentage of these endophytic fungi synthesize compounds with antifungal activities. We are in the process of growing these bioactive fungi (particularly the Candida active) in larger amounts and isolating and identifying the bioactive compounds. The cytotoxic assay using brine shrimp has been run on all of the freeze dried organic extracts. Sixteen percent of these extracts show strong activities. We are currently following up these active extracts with the crown gall assay in the hopes to identify potentially new anticancer agents.

Acknowledgments

We would like to thank Bristol Myers Squibb Co. and NCI for yew taxol and baccatin III; Dr. L. Joseph Sears of the Chemistry Department, Montana State University for the mass spectrometry data in this report; Mr. Jeff Blackburn, Mr. Brett Niedens and Mr. Royce Wilkinson for assistance on fungal culturing; and Mr. Kent Harada and Ms. Carol Read for CIEIA data. Supported in part with assistance from the American Cancer Society, Montana Science and Technology Alliance, NSF grant CHE-9206803, and NIH.

Literature Cited

1. Munro, M.H.G.; Luibrand, R.T.and Blunt, J.W. In "Bioorganic Marine Chemistry"; Springer-Verlag: Berlin, 1987, Vol. 1, pp 94-96.
2. Rowinsky, E.K.; Cazenave, L.A. and Donehower, R.C. J. Natl. Cancer Inst. 1990, 82(15), 1247-1259.
3. Cragg, G.M. and Snader, K.M. Cancer Cells 1990, 3, 233.
4. "Antibiotics, Chemotherapeutics, and Antibacterial Agents for Disease Control"; Grayson, M., Ed.; John Wiley & Sons: New York, 1982.
5. Wani, M.C.; Taylor, H.C.; Wall, M.E.; Coggon, P. and McPhail, A.T. J. Am. Chem. Soc. 1971, 93, 2325.
6. Schiff, P.B.; Fant, J. and Horowitz, S.B. Nature 1979, 277, 665.
7. Schiff, P.B. and Horowitz, S.B. Proc. Natl. Acad. Sci. 1980, 77, 1561.
8. Okami, Y. Microbial Ecology 1986, 12, 67-78.
9. Demain, A.L. Science 1981, 214, 987-995.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

10. Nakamura, H.;Iitaka, Y.; Kitahara, T.; Okazaki, T. and Okami, Y. J. Antib. 1977, 30, 714-719.
11. Stowe, B.B. and Yamaki, T. Ann. Rev. Plant. Physiol. 1957, 8, 181.
12. Lang, A. Ann. Rev. Plant Physiol. 1970, 21, 537.
13. Strobel, G.A.; Stierle, A.; Stierle, D. and Hess, W.M. Mycotaxon 1993, 47, 71.
14. All thin layer chromatography (TLC) was done on 5x10 Merck silica gel plates, 0.25 mm in the following solvent systems: chloroform-acetonitrile 7:3 v/v; chloroform-methanol 7:1 v/v; dichloromethane-tetrahydrofuran 6:2 v/v, ethylacetate- isopropanol 95:5 v/v; and hexane-acetone 1:1 v/v.
15. Cardellina, J.H. J. Liquid Chromatography 1991, 14, 659.
16. Stierle, A.; Strobel, G. And Stierle, D. Science 1993, 260, 214-216.
17. McClure, T.D.; Schram, K.H. and Reimer, M.L.J. J. Am. Soc. Mass Spec 1992, 3, 672.
18. Robins, R.J. In "Modern Methods of Plant Analysis. New Series, Vol.4: Immunology in Plant Sciences"Linskens, H.F and Jackson, J.F., Eds.; Springer-Verlag: Berlin, 1986, pp.86-141.
19. Vanderlaan, M.; Stanker, L. and Watkins, B. In "Immunoassays for Trace Chemical Analysis"; Vanderlaan, M. Stanker, L.; Watkins, B.E. and Roberts, D.W., Eds.; ACS Symposium Series 451; American Chemical Society: Washington, D.C. 1990, pp.2-13.
20. Bignami, G.S., Grothaus, P.G.; O'Malley, S.; Harada, K.E.; Byrnes, J.B.; Waller, D.F.; Rowinsky, E.K.; McGuire, M.T.; Alvarado, A.B. and Raybould, T.J.G., manuscript in preparation.
21. Strobel, G.A.; Stierle, A. and Van Kuijk, T.J.G.M. Plant Science, 1992, 84, 65.
22. The S-7 medium consists of 1 g glucose, 3 g fructose, 6 g sucrose, 1 g Na Acetate, 1 g soytone per liter plus 1 mg thiamine, 1 mg biotin, 1 mg of pyridoxal, 1 mg Ca pantothenate, 3.6 mg $MgSO_4$, 6.5 mg $CaNO_3$, 1 mg $Cu(NO_3)_2$, 2.5 mg $ZnSO_4$, 5 mg $MnCl_2$, 2 mg $FeCl_3$, 5 mg phenlalanine, 100 mg of Na benzoate, and 1 ml of 1 M KH_2PO_4 (pH 6.8) per liter. The ratio of sugars used is identical to that occurring in the inner bark of Pacific yew. Modified mycological agar consists of 10 g bacto-soytone, 40 g glucose, 15 g bacto-agar, 1 g sodium acetate, and 50 mg sodium benzoate per liter.
23. Bunkers, G.J.; Kenfield, D. and Strobel, G.A. Mycol. Res. 1991, 95, 347.
24. Pinkerton, F. and Strobel, G.A. Proc. Natl. Acad. Sci. USA 1976, 73, 4007.
25. Galsky, A.G. and Wilsey, J.P. Plant Physiol. 1980, 65, 184-185.
26. Galsky, A.G., Kozimor, R., Piotrowski, D. and Powell, R.G. J. NCI 1981, 67:3, 689-692.
27. Ferrigni, N.R., McLaughlin, J.L., Powell, R.G., and Smith,Jr.,C.R. J. Nat. Prod. 1984, 47:2, 347-352.

RECEIVED January 15, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch006

Chapter 7

Effects of Neem and Azadirachtin on Aphids and Their Natural Enemies

D. T. Lowery[1] and M. B. Isman

Department of Plant Science, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

Applications of formulated neem seed (expeller) oil (NSO) effectively controlled several species of aphids infesting plants in the laboratory and in the field. Laboratory investigations involving NSO or its most active ingredient, the limonoid azadirachtin (AZA), indicated that feeding deterrency does not contribute significantly to the control of aphid populations. Antifeedant activity to the strawberry aphid, *Chaetosiphon fragaefolii* (Cockerell), for example, was lost within 24 hours following applications to strawberry in the greenhouse. Control instead results from the inhibition of adult reproduction and from the failure of nymphs to molt. Efficacy is variable, however, depending on the species of aphid, instar, and host plant. Populations of predacious and parasitic insects were not adversely affected by sprays of NSO or neem seed extract (NSE) in the field, but, based on laboratory trials, natural enemies of aphids are moderately susceptible to the insect growth regulating effects of neem. In comparison to synthetic insecticides, neem-based botanicals appear to be relatively benign to beneficial insects and are suitable for inclusion in integrated pest management programs.

Extracts from seeds and leaves of the Indian neem tree (syn. Indian lilac, margosa tree, nim), *Azadirachta indica* A. Juss. (Meliaceae), have traditionally been used in Asia and Africa to protect crops, stored products, and livestock from the ravages of insects (*1-3*). Modern entomological investigation of the insecticidal properties of neem and its principle active ingredient, the tetranortriterpenoid (limonoid) azadirachtin (AZA), began with the discovery of their potent antifeedant effect toward the desert locust, *Schistocerca gregaria* Forsk. (*4*). Shortly thereafter, extracts from the seeds of neem and AZA were

[1]Current address: Agriculture Canada Research Station, Summerland, British Columbia V0H 1Z0, Canada

0097–6156/94/0557–0078$08.00/0
© 1994 American Chemical Society

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

shown to possess multiple biological activities toward insects from several orders (see *1-3*, for historical accounts). These activities include feeding and ovipositional deterrence, repellency, growth disruption, reduced fitness and sterility. The modes-of-action of neem can be conveniently divided into behavioral (antifeedant/repellent) and physiological (changes to the ecdysteroid-mediated control of insect growth, development, and reproduction) effects.

Studies of the effects of neem on aphids could help answer several questions pertaining to the potential usefulness of this plant-based pesticide. Empirical studies are required to widen the spectrum of neem-sensitive agricultural pests. Certain pest groups have largely been neglected in the past, specifically those with piercing-sucking mouthparts such as aphids (*5,6*).

The efficacy of neem may be influenced by the host plant of the insect (*7*). It has been demonstrated previously that control of whiteflies with foliar applications of neem is influenced by the host plant. Soil drenches of Margosan-O, a neem-based insecticide, reduced the number of leafhoppers on marigold and chrysanthemum, but not on zinnia (*8*). Neem has systemic action in some plants (*9,10*), and penetration of the leaf cuticle or translocation of the active component within plants likely varies with the plant species. Differences in systemic activity will be a particularly important factor for the control of phloem-feeding insects such as aphids.

Finally, aphids serve as a food source for numerous predatory and parasitic insects, and field and laboratory studies of the effects of neem on natural enemies of aphids could determine the potential usefulness of neem in integrated pest management (IPM) or biological control programs. According to Schmutterer (*6*) much research aimed at the integration of neem products into IPM systems remains to be conducted. Based on a handful of reports, neem materials appear to be only slightly harmful to natural enemies of pest insects (*7*). In a field trial, parasitism of larval rice leaffolders, *Cnaphalocrocis medinalis* (Guenee), was higher in fields of rice sprayed weekly with neem oil than in unsprayed fields, and adult parasitoids emerged normally (*5*).

Evaluation of Neem for the Control of Aphids

Aphids are economically important pests which are difficult to control because of their mobility, tremendous reproductive ability, and resistance to many synthetic pesticides. Studies directed against several species of aphids have indicated that neem-based insecticides are potentially efficacious natural control agents (*11,12*) that may be suitable for inclusion in integrated pest management programs (*13,2*).

Control of Aphids in the Laboratory. In the laboratory, mortalities of second instar bean aphids, *Aphis fabae* Scop., placed on broadbean sprayed with Margosan-O (3,000 ppm AZA) at concentrations of 0.05 to 2% was dose-dependent, ranging from 42.1 to 92.6% after four days (*14*). Neem-Azal S, containing 3,500 ppm AZA, resulted in mortalities ranging from 73.7 to 100% over the same concentrations. Similarly, Schauer (*11*) demonstrated that neem seed extracts containing undetermined amounts of AZA were toxic to first and

third instar *A. fabae* and pea aphid, *Acyrthosiphon pisum* Harr., confined on treated broadbean. In our own trials, neem seed oil (NSO) containing approximately 2,000 ppm AZA effectively reduced numbers of aphids on pepper, lettuce, rutabaga, and strawberry, in a dose-dependent manner (*15*). The effective concentration of NSO resulting in a 50% reduction in aphid numbers relative to controls ranged from as low as 0.2% for the green peach aphid, *Myzus persicae* (Sulzer), on pepper, to 1.4% for *Chaetosiphon fragaefolii (Cockerell)* on strawberry.

Control of Aphids in the Field. Field investigations of the efficacy of neem for the control of aphids have been few and fragmentary. It is well known that good or even excellent control of insects in the laboratory, particularly with insect growth regulators (IGR's), does not guarantee effective control under field conditions (*16*). In the Sudan, several pests of potato were effectively controlled with weekly sprays of aqueous neem seed extracts, resulting in yield increases of 0.5 tonnes per hectare (*17*). Cotton or melon aphid, *Aphis gossypii* Glover, populations were reduced by 26 to 75%.

During 1989 and 1990, at Vancouver, Canada, we treated plots of lettuce, cabbage, strawberry, and pepper, two or three times at weekly intervals with 1% NSO (20 ppm AZA) or emulsifier only as a control (*18*). Plants were sprayed to incipient runoff using commercial garden sprayers. Plots were separated from adjacent plots by untreated guard rows. One week after the final spray, plants were carefully harvested, weighed, and aphid populations assessed.

Combining the results from two trials on each crop, a 1% NSO treatment reduced aphid numbers per plant from approximately 40 to 98%, relative to numbers in the control plots (Table I). Generally, neem reduced aphid numbers in a dose-dependent manner (*15*). Mixtures of NSO with pyrethrum were no more effective than NSO alone.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

Table I. Control of aphids on plants in the field following foliar applications of 1% neem seed oil (NSO)

Crop	Aphid Species	No. Aphids/Plant Control	No. Aphids/Plant 1% NSO	% Reduction
Cabbage	*M. persicae*	7.3	0.9	87.7**
	B. brassicae	14.4	0.3	97.9**
Pepper	*M. persicae*	17.5	3.4	80.6**
	A. gossypii	58.6	11.3	80.7**
Strawberry	*C. fragaefolii*	16.7	7.5	55.1**
	F. fimbriata	7.9	2.1	73.4**
Lettuce	*N. ribisnigri*	15.2	9.2	39.5*

* Significantly different at $P<0.05$, ** significantly different at $P<0.001$.

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

To ensure that results are reliable and to allow for comparisons between studies, there is a need for greater standardization of neem products (*16,19*). The AZA content of neem oils and extracts varies tremendously, and, at the very least, concentrations of this limonoid should be reported in all future studies (*20,21*). Failure to control aphids and other insects in the past may have resulted, in part, from the use of poorly formulated materials having little or no active ingredients.

Research to improve formulations and application methods is required to optimize control of phloem-feeding insects, as the active components of neem must enter the plant to be fully effective. For example, Schauer (*11*) increased the toxicity of a neem seed kernel extract to *A. fabae* and *A. pisum* with the addition of sesame oil, lecithin, and dimethyl-sulfoxide; most likely from increased penetration of the plant cuticle.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

Antifeedant Activity of Neem to Aphids

Pradhan *et al.* (*4*) were among the first to report that extracts from seeds of neem were phagodeterrent to the desert locust, *Schistocerca gregaria* Forsk., and a bioassay based on the antifeedant activity of neem to locusts led to the isolation and determination of the most active ingredient, AZA (*22*). Subsequently, the antifeedant activity of neem extracts or AZA has been reported for numerous insect pests (*23*), including the variegated cutworm, *Peridroma saucia* Hubner (*21*). However, AZA does not appear to be a general inhibitor of insect feeding (*22*) and some insects are undeterred by neem or AZA.

Neem extracts have been shown to deter or repel several homopteran pests, including leafhoppers and planthoppers on rice (*5,24*), and sweetpotato whitefly, *Bemisia tabaci* (Gennadius), on cotton (*25*). Studies of neem's antifeedant activity to aphids have produced conflicting results. Neem extracts or AZA were reportedly antifeedant to *A. pisum* on broadbean (*26*), *M. persicae* on *Nicotiana clevelandii* Gray (*27*), and the bird cherry-oat aphid, *Rhopalosiphum padi* (L.), and English grain aphid, *Sitobion avenae* (F.), on barley (*28*). Contrary to these findings, two separate studies determined that neem was not deterrent to *M. persicae* (*29,30*).

In our own investigations (*31*), NSO applied to leaf disks at concentrations up to 2% was deterrent to *A. pisum* and *C. fragaefolii,* but not to *M. persicae*, the currant-lettuce aphid, *Nasonovia ribisnigri* (Mosley), and *Fimbriaphis fimbriata* Richards. Furthermore, deterrency of NSO to *C. fragaefolii* was not related to the AZA content of the oil and activity was rapidly lost, within 24 hours, following application to strawberry in the greenhouse. Based on these findings, the antifeedant activity of neem would not be expected to contribute significantly to the control of aphids in the field.

Effect of Neem on Aphid Growth and Development

Ruscoe (*32*) was one of the first to report that, in addition to its antifeedant properties, AZA was a potent inhibitor of insect growth and development. Combined oral and topical treatment of larval diamondback moth, *Plutella*

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

xylostella (L.), and tobacco budworm, *Heliothis virescens* (F.), and nymphal cotton stainer, *Dysdercus fasciatus* Signoret, inhibited molting. Although susceptibility varies among species, AZA is considered to be a general inhibitor of insect growth and development (*23*) and neem has affected the growth and development of most insects tested to date. It is this property of neem that is considered to be the most economically important (*2,33*).

AZA often disrupts insect development at very low concentrations. For example, topical or oral treatment of migratory locusts, *Locusta migratoria* (L.), with AZA at a rate of 2μg/g insect weight completely suppressed molting (*34*). AZA interferes with the hormonal regulation of the molting process by disrupting normal titres of hemolymph ecdysteroid (*35,36*). Based largely on histological studies, neem has also been shown to interfere with the neurosecretory function of the brain (*34,37,38*). Immature insects often die during failed attempts to molt due to improper apolysis and ecdysis (*7,39*).

Because mortality occurs primarily during molting applications of neem or AZA do not result in rapid death of insects as occurs with synthetic neurotoxins. Exposure of second instar *N. ribisnigri* for three days to AZA (40ppm) or 1% NSO applied to leaf disks of lettuce resulted in complete mortality within 7 days, but nymphs lived an average of 2.4 and 2.5 days, respectively, compared to 6.7 days for controls (Table II). At 17°C, up to 9 days may be required for the complete expression of the IGR effects (*40*). NSO and AZA were not toxic to adult *N. ribisnigri*, and percent survival and days of survival did not differ from controls ($P > 0.05$).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

Table II. Percent survival and days of survival for second and fourth instar and adult *N. ribisnigri* exposed to 1% NSO or AZA (40ppm) applied to leaf disks of lettuce

	Percent Survival			Days of Survival[a]		
Treatment	2nd	4th	adult	2nd	4th	adult
Control	66.7a	100.0a	75.0a	6.7a	12.0a	5.0a
NSO 1.0%	0.0b	0.0b	83.3a	2.5b	3.8a	5.4a
AZA 40ppm	0.0b	16.7b	87.5a	2.4b	6.0a	5.1a

[a]Average days of survival recorded over 7, 12, and 6 days for second, fourth, and adult instars, respectively.

Means within a column followed by the same letter are not significantly different at $P = 0.05$.

Schauer (*11*) demonstrated that, while attempting to molt, the exocuticle of *A. pisum* exposed to neem extracts detached from the body but could not be ruptured, or if it ruptured it could not be completely cast off. In our studies, nymphal aphids were also observed to swell (apolysis) in preparation for ecdysis, but they either failed to molt or could not escape the exuvium (*40*).

AZA is considered to be the primary bio-active principle of neem (20,41), but extracts contain numerous other active components (*42,43*). Survival of second and fourth instar *N. ribisnigri* did not differ ($P>0.05$) for AZA treatment compared to 1% NSO containing approximately the same amount of AZA (Table II), indicating that AZA accounts for most of the observed mortality to this species.

Comparative studies of the susceptibility of a closely related group of insects to neem have seldom been conducted. Therefore, the effective concentration of AZA resulting in 50% mortality (EC_{50}) was determined for several species of aphids. Mortalities after nine days, with an initial three days on treated leaf disks, were recorded for second instar nymphs on disks dipped in AZA, or for nymphs confined to the lower surfaces of disks treated on the upper surface with AZA (=translaminar exposure).

EC_{50} values for aphids on leaf disks dipped in AZA ranged from 2.4 ppm for *M. persicae* on pepper to 635 ppm for *C. fragaefolii* on strawberry (Table III). Variability in these values may have resulted from interspecific differences among aphids or from differences in the systemic movement of AZA into or within the various host plants.

Table III. Effective concentrations of AZA resulting in 50% mortality (EC_{50}) after nine days for aphids on leaf disks dipped in solutions of AZA, or confined to the opposite surface of treated disks (translaminar)

		EC_{50} (ppm AZA)	
Aphid Species	Host Plant	Dipped	Translaminar
N. ribisnigri	Lettuce	3.1	7.2
M. persicae	Pepper	2.4	8.8
A. pisum	Broadbean	44.7	11.9
R. padi	Corn	87.9	454.0
C. fragaefolii	Strawberry	635.0	629.1

The translaminar movement of NSO appears to be very good, as mortality rates for *N. ribisnigri* confined to leaf disk surfaces treated with 1% NSO did not differ from rates for aphids confined to the opposite untreated surface (*18*). Effective concentrations of AZA applied in a local systemic manner resulting in a 50% reduction in aphid survival (EC_{50}) ranged from 7.2 to 629.1 ppm, which are generally comparable to the values for leaf disks dipped in AZA (Table III).

Although AZA appears to move readily within leaves, additional studies are required to assess movement of AZA across the cuticle and epidermis of leaves (*33*). According to Larew (*9*), neem moves readily into leaves, but the degree of movement is influenced by the species of plant. Differential penetration of leaves may help explain some of the observed variability in the toxicity of neem to aphids.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

Toxicity of AZA to *M. persicae* is clearly influenced by the host plant (Table IV). Concentrations of AZA required to reduce survival of nymphs by 50% ranged from 1.8 ppm for nymphs on mustard cabbage to 43.3 ppm for sweet corn, a more than twenty-fold difference.

Table IV. Effective concentrations of AZA applied to leaf disks of various host plants resulting in 50% mortality (EC_{50}) after nine days for second instar *M. persicae*

Host Plant	EC_{50} (ppm AZA)	(95% C.I.)	R^2
Mustard cabbage	1.8	(±0.5)	0.870
Pepper	2.4	(±0.5)	0.878
Lettuce	10.4	(±1.4)	0.940
Broadbean	15.4	(±1.9)	0.945
Sweet corn	43.3	(±5.0)	0.838

The systemic activity of neem is still poorly understood (*13,33*) and penetration of leaf surfaces, translaminar movement, and translocation of the active components of neem, requires additional study. A better understanding of these processes might help explain the variable levels of control observed in the past for phloem-feeding insects, and efficacy may be improved substantially by adding formulants which facilitate penetration of plant cuticles.

Effect of Neem on Aphid Reproduction

Neem extracts and AZA have been shown to negatively influence the reproduction of female insects from several orders (*1,38*). The documented antifeedant activity could indirectly reduce fecundity, but inhibition primarily results from interference with the hormonal control of reproduction (*37*). Insects treated with AZA have degenerate or improperly developed ovaries and fat bodies (*39,44*), oocytes are resorbed, minimal amounts of yolk are deposited, and chorionic surfaces of eggs are often abnormal (*1,38,45*).

Exposure of *N. ribisnigri* to AZA or NSO as adults or fourth instars resulted in significantly fewer offspring compared to controls (*18*). Fecundity of *N. ribisnigri* exposed as adults to 1% NSO and 40 ppm AZA was 0.5 and 1.01 live offspring/aphid/day, respectively, compared to 1.48 offspring/aphid/day for controls. Treatment of fourth instar nymphs was more disruptive, resulting in reproductive rates of 0.14, 0.10, and 1.04 offspring/aphid/day for 1% NSO, 40ppm AZA, and controls, respectively.

We determined the effect of AZA on the fecundity of *N. ribisnigri* by confining adults to treated leaf disks for three days followed by a further three days on untreated disks, when numbers of dead and live offspring per aphid per day were recorded.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

AZA applied to leaf disks of lettuce reduced the fecundity of adult *N. ribisnigri* in a linear dose-dependent manner (Table V). The estimated concentration resulting in 50% fewer live offspring/female/day compared to controls was 14.4 ppm AZA. Aphids exposed to AZA produced a large number of fully-developed but non-viable embryos which were dark in color and had appendages which closely adhered to the body. The number of dead (embryonic) offspring produced by *N. ribisnigri* increased with increasing concentrations of AZA. A greater number of dead offspring does not account for all of the reduction in numbers of live offspring, however, as the total number of offspring (live and dead) also declined in a dose-dependent manner (Table V). Dissection of aphid ovaries demonstrated that neem inhibits reproduction due to both a decrease in the maturation of oocytes and death of mature embryos (*18*).

Table V. Fecundity of adult *N. ribisnigri* following three days of exposure to AZA applied to leaf disks of lettuce

Treatment	Offspring/Aphid/Day		
(AZA ppm)	Living	Dead	Total
0.0	1.18a	0.00	1.18
6.25	1.05ab	0.00	1.05
12.5	0.70bc	0.09	0.79
25.0	0.44cd	0.11	0.55
50.0	0.41cd	0.23	0.64
100.0	0.12d	0.37	0.49
EC_{50}	14.4	---	---
R_2	0.904	---	---

Treatment means followed by the same letter are not significantly different (P>0.05, Tukey's test for separation of means).

According to Schmutterer (*7*), the fecundity of homopterous insects is strongly influenced by exposure to neem or AZA. Reproduction of *A. pisum* placed as first instar nymphs on broadbean sprayed with a 0.002% methanolic neem seed extract produced 1.6 offspring/female/day compared to 7.1 offspring/female/day for controls (*11*). Similarly, sweetpotato whitefly, *Bemisia tabaci* (Gennadius), confined on cotton treated with 2% neem seed extract deposited more than 80% fewer eggs compared to controls up to seven days post-treatment (*25*).

Our studies have demonstrated that NSO and AZA effectively inhibit aphid reproduction. As for the growth regulating effects, the effect of AZA on aphid fecundity was variable, with EC_{50} values ranging from as low as 14.4 ppm for *N. ribisnigri* on lettuce to 616.4 ppm for *R. padi* on sweet corn (*18*). Decreased rates of reproduction would contribute directly to the control of

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

aphids in the field, and even a modest decrease in aphid population growth rates might allow natural enemies to effectively maintain aphid numbers below economic injury levels.

Effect of Neem on Natural Enemies of Aphids

The widespread use of synthetic neurotoxins has resulted in insecticide resistance, pest resurgences, and secondary pest outbreaks. These problems can be attributed partly to the disruption of natural enemy populations. Due to these and other concerns, there is a need for effective, bio-degradable pesticides with greater selectivity (*46*). The current level of interest in neem's insecticidal properties is partly due to its reported selectivity toward phytophagous insects (*47*). Neem-based materials are thought to be relatively harmless to mammals, earthworms, predators, parasitoids, and honeybees (*13,46,48*). However, a review of the available literature reveals that few studies are available regarding the impact of neem on beneficial insects. Since aphids are a food source for very many predatory and parasitic insects, the effect of neem on non-target organisms is particularly important for the management of these pests.

During our 1990 field trials for the control of aphids with foliar applications of neem, numbers of larval predators (syrphids, neuropterans, coccinellids, cecidomyiids) and parasitized aphids (mummies) per plant were assessed during final sampling. Based on these counts, 1% NSO significantly reduced the absolute number of predators per plant by 56%, but numbers relative to aphid populations did not differ from controls. Numbers of mummies on plants treated with 1% NSO did not differ from controls and mummies per 1,000 aphids, a reflection of the parasitism rate, was nearly four times higher on NSO-treated plants compared to controls (*18*). These results suggest that neem sprays are not detrimental to natural enemy populations under field conditions, particularly in comparison to synthetic insecticides.

In the greenhouse, parasitism of the cotton aphid, *Aphis gossypii* (Glover), and greenhouse whitefly, *Trialeurodes vaporarium* (Westwood), on plants treated with a neem-based pesticide was comparable to controls and significantly higher than synthetic pesticide treatments (*47*). Predacious coccinellids survived applications of NSO to sorghum in the field that successfully controlled corn leaf aphids, *Rhopalosiphum maidis* (Fitch), and sugarcane aphids, *Melanaphis sacchari* (Zehntner) (*49*), while NSO applied to field plots of rice controlled leafhoppers and planthoppers, but did not affect numbers of predatory spiders or mirids (*2,5*).

Our laboratory studies revealed that neem is not without effects on natural enemies of aphids. Topical treatment of early second instar eleven-spot ladybeetle, *Coccinella undecimpunctata* (L.), and hoverfly, *Eupeodes fumipennis* (Thompson), larvae with 1% NSO did not result in reduced pupation or emergence of adults compared to controls (Table VI). However, NSO applied to canola infested with *M. persicae* negatively affected the survival of these predators. For *E. fumipennis*, pupation and adult emergence was approximately 50 and 93% lower, respect-ively, than controls (Table VI). *C. undecimpunctata*

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

was even more sensitive to neem, and no larvae on plants treated with 1% NSO pupated successfully. In the laboratory, NSO applied to mustard cabbage had little effect on parasitism rates, but emergence of adult parasitoids was reduced in a dose-dependent manner (*18*).

Table VI. Percent survival to pupation and to adult emergence for second instar syrphid, *Eupeodes fumipennis* (Thompson), and coccinellid, *Coccinella undecimpunctata* (L.), larvae topically treated with NSO, or reared on canola infested with *M. persicae* and treated with NSO

	Topical Treatment		Treated Canola	
	Control	1% NSO	Control	1% NSO
E. fumipennis				
% pupation	87.0a	69.6a	66.7a	33.3b
% emergence	82.6a	69.6a	58.3a	4.2b
C. undecimpunctata				
% pupation	88.5a	53.8a	50.0a	0.0b
% emergence	69.2a	42.3a	41.7a	0.0b

For each species, treatment means followed by the same letter are not significantly different ($P > 0.05$, chi-square analysis).

Laboratory experiments may help determine the long-term or sub-lethal effects of neem on predatory or parasitic insects, but these results may differ substantially from those obtained in the field. For example, coccinellid and neuropteran larvae reared in the laboratory on prey treated with Margosan-O had lower rates of survival, but the same material was essentially non-toxic to predators and parasitoids of *A. gossypii* and the sweetpotato whitefly on plants in the greenhouse (*47*).

Studies conducted under test conditions proposed by the Pesticides and Beneficial Organisms Working Group, International Organization for Biological Control, determined that neem extracts were not harmful to eggs, larvae, or adults of the predatory lacewing, *Chrysoperla carnea* (Steph.) and seven-spot ladybeetle, *Coccinella septumpunctata* L. (*50*). However, the effect of neem on beneficial insects has not been investigated in any detail, and results should be interpreted cautiously. As Schmutterer (*1*) points out, in contrast to bioassays with conventional pesticides, bioassays involving neem require a relatively long time, sometimes weeks, due to the delayed action of AZA and related compounds. In addition to direct mortality, the possible long-term effects of neem on the fitness and behavior of predators and parasitoids needs to be studied in greater detail (*47*). In spite of these concerns, neem appears to be suitable for inclusion in integrated pest management programs.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

General Summary

Control of aphids and the viruses they transmit has depended largely on the use of broad-spectrum synthetic insecticides. Because of the widespread and often indiscriminate use of these chemicals, at least 20 species of aphids are now resistant to at least one class of insecticide (*51*). Our studies have demonstrated that neem-based materials are potentially efficacious as aphicides, and their use appears to be compatible with other control strategies, including the use of biological agents.

Based on our studies, control of aphids with foliar applications of neem results from nymphal mortality during molting and reduced fecundity of adults, whereas antifeedant or repellent activity is of minor importance. Efficacy of neem to aphids is variable, however, likely due to differences in susceptibility between aphid species and variabiltiy in systemic action within the host plants.

Exposure to neem also causes several sub-lethal and fitness-reducing effects, such as physical abnormalities to wings, that may be important for the control of aphid populations over longer time periods. Because effects on insects are often subtle and delayed, novel approaches are required to properly evaluate neem materials. Acute short-term bioassays previously utilized for studies of synthetic neurotoxins are inappropriate for investigations of neem. Botanical insecticides based on neem offer an effective means of aphid control that is potentially compatible with other control strategies, while causing minimal damage to the environment.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

Acknowledgments

We thank Safer Ltd., Victoria, B.C., for providing the formulated neem materials, and J.T. Arnason, University of Ottawa, Ottawa, ON, for the pure azadirachtin. C.K. Chan, Agriculture Canada, Vancouver, B.C., provided the original source of aphids. Technical assistance was provided by N.L. Brard and K.G. Craig, University of British Columbia, Vancouver, B.C. Supported by a NSERC Co-op R&D grant (39317) and operating grant (OGP2729) to M.B.I., a Science Council of British Columbia Great Award (#72) to D.T.L. and M.B.I., and a NSERC postgraduate scholarship to D.T.L.

Literature Cited

1. Schmutterer, H. In *CRC Handbook of Natural Pesticides. Vol. III Insect Growth Regulators Part B*; Morgan, E.D.; Mandava, M.B. Eds.; CRC Press, Inc.: Boca Raton, Fl., 1987; pp. 119-170.
2. Saxena, R.C. In *Insecticides of Plant Origin*; Arnason, J.T.; Philogene, B.J.R.; Morand, P., Eds.; ACS Symposium Series No. 387; Amer. Chem. Soc.: Washington, D.C., 1989; pp 110-135.

3. Koul, O.; Isman, M.B.; Ketkar, C.M. *Can. J. Bot.* **1990,** 68, 1-11.
4. Pradhan, S.; Jotwani, M.G.; Rai, B.K. *Indian Farming* **1962,** 12, 7-11.
5. Saxena, R.C.; Liquido, N.J.; Justo, H.D. In *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss.);* Schmutterer, H.; Ascher, K.R.S.; Rembold, H., Eds.; Proc. 1st Int. Neem Conf.; GTZ GmbH: Eschborn, Germany, 1981; pp. 171-188.
6. Schmutterer, H. In *Neem's Potential in Pest Management Programs*; Locke, J.C.; Lawson, R.H., Eds.; Proc. USDA Neem Workshop; USDA ARS-86; 1990; pp. 15-22.
7. Schmutterer, H. *Annu. Rev. Entomol.* **1990,** 35, 271-297.
8. Jacobson, M. In *Neem's Potential in Pest Management Programs*; Locke, J.C.; Lawson, R.H., Eds.; Proc. USDA Neem Workshop; USDA ARS-86; 1990; pp. 4-14.
9. Larew, H.G. *J. Econ. Entomol.* **1988,** 81(2), 593-598.
10. Arpaia, S.; van Loon, J.J.A. *Entomol. exp. appl.* **1993,** 66, 39-45.
11. Schauer, M. In *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss.) and other Tropical Plants;* Schmutterer, H.; Ascher, K.R.S., Eds.; Proc. 2nd Int. Neem Conf.; GTZ GmbH: Eschborn, Germany, 1984; pp. 141-150.
12. Patel, M.B.; Srivastava, K.P. *Neem Newsletter* **1989,** 6(4), 41-44.
13. Schmutterer, H. *J. Insect Physiol.* **1988,** 34(7), 713-719.
14. Dimetry, N.Z.; Schmidt, G.H. *Anz. Schädlingskde., Pflanzenschutz, Umwelttschutz* **1992,** 65, 75-79.
15. Lowery, D.T.; Isman, M.B; Brard, N.L. *J. Econ. Entomol.* **1993,** 86(3), 864-870.
16. Schmutterer, H.; Hellpap, C. In *Focus on Phytochemical Pesticides, Vol. I: The Neem Tree*; Jacobson, M., Ed.; CRC Press, Inc.: Boca Raton, Fl., 1989; pp. 69-86.
17. Siddig, S.A. In *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss) and other Tropical Plants*; Schmutterer, H., Ed.; Proc. 3rd. Int. Neem Conf.; GTZ GmbH: Eschborn, Germany, 1987; pp. 449-459.
18. Lowery, D.T. *Effects of Extracts from Neem, Azadirachta indica (A. Juss.), on Aphids (Homoptera: Aphididae) with Respect to Their Control.* Ph.D. Thesis, University of British Columbia, Vancouver, B.C., Canada. 1992.
19. Isman, M.B.; Koul, O.; Lowery, D.T.; Arnason, J.T.; Gagnon, D.; Stewart, J.G.; Salloum, G.S. In *Neem's Potential in Pest Management Programs*; Locke, J.C.; Lawson, R.H., Eds.; Proc. USDA Neem Workshop; USDA ARS-86; 1990; pp. 32-39.
20. Ermel, K., Pahlich, E.; Schmutterer, H. In *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss) and other Tropical Plants*; Schmutterer, H., Ed.; Proc. 3rd. Int. Neem Conf.; GTZ GmbH: Eschborn, Germany, 1987; pp. 171-184.
21. Isman, M.B.; Koul, O.; Luczynski, A.; Kaminsky, J. *J. Agric. and Food Chem.* **1990,** 38, 1406-1411.
22. Butterworth, J.H.; Morgan, E.D. *J. Insect Physiol.* **1971,** 17, 969-977.
23. Warthen, J.D. Jr. *Proc. Entomol. Soc. Wash.* **1989,** 91, 367-388.
24. Saxena, R.C.; Khan, Z.R. *J. Econ. Entomol.* **1985,** 78, 222-226.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

25. Coudriet, D.L.; Prabhaker, N.; Meyerdirk, D.E. *Environ. Entomol.* **1985,** 14, 776-779.
27. Woodford, J.A.T.; Nisbet, A.J.; Strang, R.H.C.; Connolly, J.D. In *Aphid-Plant Interactions: Population to Molecules*; Peters, D.C.; Webster, J.A.; Chlouber, C.S., Eds.; USDA/ARS and Oklahoma State Univ.: Stillwater, Ok., 1991; pp. 305.
28. West, A.J.; Mordue, A.J. *Entomol. Exp. Appl.* **1992,** 62, 75-79.
29. Griffiths, D.C.; Pickett, J.A.; Smart, L.E.; Woodcock, C.M. *Pestic. Sci.* **1989,** 27, 269-276.
30. Braker, E.; Toscano, N.; Reuter, L.; Perring, T. Poster Presentation. Ann. Gen. Meet., Ent. Soc. Amer., Reno, NV., 1991.
31. Lowery, D.T.; Isman, M.B. *J. Chem. Ecol.* **1993,** 19(8), 1761-1773.
32. Ruscoe, C.N.E. *Nature* **1972,** 236, 159-160.
33. Wood, T. In *Neem's Potential in Pest Management Programs*; Locke, J.C.; Lawson, R.H., Eds.; Proc. USDA Neem Workshop; USDA ARS-86; 1990; pp. 76-84.
34. Sieber, K.P.; Rembold, H. *J. Insect Physiol.* **1983,** 29, 523-527.
35. Marco, M-.P.; Pascual, N.; Belles, X.; Camps, F.; Messeguer, A. *Pesticide Biochem. Physiol.* **1990,** 38, 60-65.
36. Barnby, M.A.; Klocke, J.A. *J. Insect Physiol.* **1990,** 36, 125-131.
37. Rembold, H.; Forster, H.; Czoppelt, Ch. In *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss) and other Tropical Plants*; Schmutterer, H., Ed.; Proc. 3rd. Int. Neem Conf.; GTZ GmbH: Eschborn, Germany, 1987; pp. 149-160.
38. Rembold, H. In *Focus on Phytochemical Pesticides, Vol. I: The Neem Tree*; Jacobson, M., Ed.; CRC Press, Inc.: Boca Raton, Fl., 1989; pp. 47-68.
39. Schluter, U.; Schulz, W.D. In *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss.) and other Tropical Plants;* Schmutterer, H.; Ascher, K.R.S., Eds.; Proc. 2nd Int. Neem Conf.; GTZ GmbH: Eschborn, Germany, 1984; pp. 227-236.
40. Lowery, D.T.; Isman, M.B. *Entomol. Exp. appl.* **1993,** (in press).
41. Isman, M.B.; Koul, O.; Arnason, J.T.; Stewart, J.G.; Salloum, G.S. *Mem. Ent. Soc. Can.* **1991,** 159, 39-47.
42. Balandrin, M.F.; Lee, S.M.; Klocke, J.A. *J. Agric. Food Chem.* **1988,** 36, 1048-1054.
43. Klocke, J.A.; Balandrin, M.F.; Barnby, M.A.; Yamasaki, R.B. In *Insecticides of Plant Origin*; Arnason, J.T.; Philogene, B.J.R.; Morand, P., Eds.; ACS Symposium Series No. 387; Amer. Chem. Soc.: Washington, D.C., 1989; pp. 136-149.
44. Schluter, U. In *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss) and other Tropical Plants*; Schmutterer, H., Ed.; Proc. 3rd. Int. Neem Conf.; GTZ GmbH: Eschborn, Germany, 1987; pp. 331-348.
45. Schulz, W.D.; Schluter, U. In *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss.) and other Tropical Plants;* Schmutterer, H.; Ascher, K.R.S., Eds.; Proc. 2nd Int. Neem Conf.; GTZ GmbH: Eschborn, Germany, 1984; pp. 237-252.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

46. Saxena, R.C. In *Natural Pesticides from the Neem Tree (Azadirachta indica A. Juss) and other Tropical Plants*; Schmutterer, H., Ed.; Proc. 3rd. Int. Neem Conf.; GTZ GmbH: Eschborn, Germany, 1987; pp. 81-93.
47. Hoelmer, K.A.; Osborne, L.S.; Yokomi, R.K. In *Neem's Potential in Pest Management Programs*; Locke, J.C.; Lawson, R.H., Eds.; Proc. USDA Neem Workshop; USDA ARS-86; 1990; pp. 100-105.
48. Larson, R.O. In *Neem's Potential in Pest Management Programs*; Locke, J.C.; Lawson, R.H., Eds.; Proc. USDA Neem Workshop; USDA ARS-86; 1990; pp. 23-28.
49. Srivastava, S.K.; Parmar, B.S. *Neem Newsletter* **1985**, 2, 7.
50. Kaethner, M. von. *Anz. Schädlingskde., Pflanzenschutz, Umweltschutz.* **1991**, 64, 97-99.
51. Van Lenteren, J.C. In *Aphid-Plant Interactions: Population to Molecules*; Peters, D.C.; Webster, J.A.; Chlouber, C.S., Eds.; USDA/ARS and Oklahoma State Univ.; Stillwater, Ok., 1991; pp. 3-28.

RECEIVED December 6, 1993

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch007

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

Chapter 8

Structural Requirements for Monoterpenoid Activity against Insects

Pamela J. Rice and Joel R. Coats

Pesticide Toxicology Laboratory, Department of Entomology, Iowa State University, Ames, IA 50011

The topical, fumigant and ovicidal activity of fourteen monoterpenoids and thirty-one monoterpenoid derivatives were evaluated using the house fly, *Musca domestica*. The toxicity data of acyclic, monocyclic, and bicyclic phenols, alcohols, and ketones were compared to determine structure-activity relationships involving the monoterpenoids' structural shape, type of functional group, and degree of saturation. Monoterpenoid acetate, propionate, pivalate, trichloroacetate, and trifluoroacetate derivatives were synthesized and their insecticidal activities were evaluated. The toxicities of the monoterpenoid acetate and haloacetate derivatives were compared with each other and the parent alcohols or phenols to evaluate the influence of derivatization on toxicity. Monoterpenoid ketones were more insecticidal than alcohols in the topical and ovicidal bioassays. Pivalate and acetate derivatives were more toxic than the haloacetate derivatives in both the topical and ovicidal bioassays. Thymyl trifluoroacetate was the most effective fumigant followed by menthol and fenchone. Thymol and geranyl acetate were the most insecticidal monoterpenoid and monoterpenoid derivative in the topical bioassays. Geraniol, geranyl propionate, terpineol, carvacrol and menthone were as ovicidal as the pyrethrin standard.

Plants produce secondary metabolites or allelochemicals that include a wide range of chemical compounds. The structural classes that are encompassed by the secondary compounds include terpenoids (mono, sesqui-, and diterpenoids), amines (the insecticide pellitorine), phenolic compounds (flavonoids including hydroquinones, tannins, and rotenone), alkaloids (nicotine), and nitriles (*1*). Secondary compounds that are directly utilized as insecticides are classified as botanicals. Several examples of botanicals include the contact stomach poison rotenone (extracted from *Derris elliptica*, *Derris malaccensis, Lonchocarpus utilis* and *Lonchocarpus urucu*), pyrethrum (found in the flowers of plants from the genus *Chrysanthemum*), and nicotine (extracted from *Nicotiana rustica* and *Nicotiana tabacum*) (*2*).

0097–6156/94/0557–0092$08.00/0
© 1994 American Chemical Society

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Presently, synthetic pesticides are utilized more commonly than botanical insecticides in agriculture. Several previously used synthetic insecticides were proven problematic due to their persistence in the environment and their toxicity to non-target organisms. This is illustrated with the chlorinated hydrocarbon DDT, its metabolite DDE (*2*), and the fumigant insecticide ethylene dibromide (EDB) (*3*). In addition, several insects have become resistant to synthetic insecticides (*4*, *5*). In light of these problems, alternative classes of synthetic compounds have been developed and alternative means of pest control are being explored. One approach that may be taken is the development of [more natural,] biodegradable pesticides based on natural products that are chemically stable, yet responsive to degradation by microorganisms and to photolysis. Derivatization of degradable natural products, rendering them relatively more stable and more toxic to the target organism, may be a viable strategy toward a more biorational approach to pest control. This has been demonstrated with the natural insecticide pyrethrum and the more photostable and more toxic synthetic pyrethroids resmethrin, permethrin, cypermethrin, and deltamethrin (*2*) and in the development of the herbicide cinmethylin that is structurally based on the monoterpenoid 1,8-cineole (*6*).

Monoterpenoids characteristically are toxic to insects, safe to mammals and readily obtainable (abundant in plants and easily synthesized in the laboratory) which renders them ideal candidates in the development of more environmentally safe insecticides. Several monoterpenoids are presently used in commercial pest control products. Citronellal is a major component of insect-repellent candles. Limonene and linalool are used in flea shampoos (*7*). Linalool is also used in insecticidal sprays for house plants, while menthol is utilized as a fumigant for tracheal mites in honey bees (*8*). Although monoterpenoids have shown toxicity to insects, most monoterpenoids are less toxic to mammals. In fact several monoterpenoids are included on the generally recognized as safe (GRAS) list and they are used as artificial flavorings in foods, as fragrances in cosmetics, and as pharmaceuticals. 1,8-Cineole a component of eucalyptus oil, is found in over-the-counter formulations of nasal and bronchial decongestants, inhalational expectorants, and external analgesics (*9*). Essential oils have also been employed as preservatives and paint solvents (*10*).

Natural Occurrence

Terpenoids are produced by many living organisms including microorganisms, higher plants, and a few animals (*11*). In plants, essential oils containing terpenoids are synthesized and stored in globules within cells, in specialized ducts and glands, or in dead cells (*1*). At one time it was believed that terpenoids with the exception of carotenoids, sterols, prenols, gibberellins, dormin, abscisin, phytol, and prenols were metabolic waste. However, radiotracer studies have shown terpenoids to be rapidly synthesized even in young tissue (*11*). Terpenoids are believed to aid plants in chemical defense against phytophagous insects, bacteria and fungi. This chemical defense strategy is illustrated in the distribution of terpenoids within the plant and the physical structures associated with these secondary substances that adorn various plant surfaces. Synthesis and distribution of the toxin are often restricted to tissues that are the most susceptible to attack (*12*). Typically young lateral leaves contain higher concentrations of monoterpenoids than mainstem leaves. Bottom mainstem leaves often contain the lowest concentrations (*13*). Physical structures associated with secondary plant substances may include glandular hairs and leaf glands. They secrete a protective

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

substance that repels or is toxic to the plant's enemies (*1*). The plant's age and tissue type influence its ability to synthesize and store secondary compounds. The quantity of secondary plant compounds produced is affected by climatic and edaphic conditions. Although defense against the plant's enemies is believed to be a primary role of terpenoids, it has been theorized that terpenoids may also play a part in maintaining the activity of the plant's enzyme systems during dormancy (*10*). In fact monoterpenoids are believed to aid in maintaining the respiratory coenzymes in a reduced form (*11*).

Insect herbivores often use chemical cues containing terpenoids for communication, attraction or defense. The components of these chemical signals may be sequestered dietary compounds, conversion products of the host terpenoids or manufactured *de novo* by the insect (*14*). Two species of the scentless plant bug, *Niesthrea louisianica* and *Jadera haematoloma* (Hemiptera: Rhopalidae), produce and secrete a mixture of the aromatic monoterpenoid thymol and the monoterpene hydrocarbons limonene and β-pinene from their metathoracic glands (*15*). Several monoterpenoids have been identified in the sex and aggregation pheromones of bark beetles. In *Ips typographus* verbenol is an attractant while verbenone acts as a repellent (*16*). Neral and geranial have been detected in the mandibular gland secretions of several ant species that are associated with alarm-defense and attraction-trail (*17*). *d*-Limonene, *l*-limonene, α-pinene, β-pinene, and β-myrcene were found in the poison gland secretions of *Myrmicaria natalensis* (Hymenoptera: Formicidae) (18).

Toxicity of Monoterpenoids

Most terpenes are lipophilic, depending on their state of oxidation and glycosylation. They are capable of interfering with insect herbivores' biochemical and physiological functions. Typically, plant allelochemicals are not acutely toxic to mammals or insect herbivores. A few exceptions include the terpenoids grayanotoxin and ergosterol which are toxic to mammals (*19*) and a few insecticidal plant allelochemicals including the natural pyrethrins, tobacco alkaloids, rotenoids, and steroidal alkaloids (*20*). Monoterpenoids that are acutely toxic to insects include citral, an active fumigant against house flies, *Musca domestica*, (*21*) and *d*-limonene, toxic to rice weevils, *Sitophilus oryzae*, German cockroaches, *Blattella germanica* (*22*), and *Dendroctonus* pine beetles (*23*, *24*).

Plant monoterpenoids' sublethal or chronic effects appear to be more important in the plant's defense than any acute toxic effects. The plant allelochemical's ability to repel insects and act as a feeding deterrent are the plant's first defense against polyphagous insects. The familiar yellow citronellal candle, a common repellent approach, contains citronellal a monoterpenoid from lemon grass. Cineole repels the American cockroach, *Periplaneta americana* (*25*), verbenone is a deterrent to spruce bark beetles, *Ips typographus* (*26*), and geraniol is repulsive to red flour beetles, *Tribolium castaneum* (*27*). Linalool, (+)-isopulegol, (+)-pulegol, (+)-pulegone, (-)-carvone (*28*), and *d*-limonene repel the German cockroach, *Blattella germanica* (*22*). Feeding deterrents interfere with the insect's ability to ingest and utilize food, which leads to reduced growth and prolonged development. *d*-Limonene acts as a feeding deterrent to cat fleas, *Ctenocephalides felis*, (*29*) and pulegone acts as a strong feeding deterrent to the sixth instar fall armyworm, *Spodoptera frugiperda* (*20*).

Monoterpenoids may also interfere with the developmental processes of insects beginning with embryogenesis, and continuing into molting, pupation,

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

metamorphosis, and adult emergence (*30*). *d*-Limonene inhibits embryonic development in the cat flea, *C. felis* (*7*) and pulegone decreases larval growth of the southern armyworm, *Spodoptera eridania*, when it is continually ingested from the fourth instar to pupation (*30*).

Plant allelochemicals may also reduce reproduction success of insects by acting as an ovipositional repellent and by reducing mating success, egg production, and egg viability. Cineole indirectly affects reproductive success of the leafhopper, *Amrasca devastans* by interfering with the sonic communication between the sexes (*31*). In addition, it is a feeding repellent and an ovipositional repellent against adult yellow fever mosquitoes, *Aedes aegypti* (*9*). *d*-Limonene inhibits egg hatch of exposed western corn rootworm eggs, *Diabrotica virgifera virgifera* LeConte (*22*).

Studies on Mode of Action

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

It has been demonstrated that the monoterpenoids can induce several different types of bioactivities. Modes of action for the various effects have not been determined, but there have been studies that provide some clues to their possible specific mechanisms of action.

The acute symptomology elicited in insects is typified by a remarkably fast onset of tremors, followed by rapid knockdown and eventually, death. Neurotoxic activity has been documented using electrophysiological recording techniques (*32*). The noninvasive recordings of limonene-induced neurotoxicity in the earthworm, *Eisenia foetida*, yielded a distinctive set of symptoms. The primary effects were reduced conduction velocity, spontaneous activity, rebounding of nerve potentials back up the medial giant fiber, and finally, blocking of all neural activity in the medial and lateral giant fibers. Effects were fully reversible if the concentration of the toxin was less than the lethal level. Several of the symptoms were similar to those demonstrated by dieldrin in earlier experiments using the same neurotoxicity test system (*33*). Four other monoterpenoids (pulegone, myrcene, α-terpineol and linalool) also exhibited the same neurotoxic effects in the *Eisenia fetida* assay (*34*). While that non-invasive assay did not allow determination of the exact site or mechanism of action, these studies provide circumstantial evidence that the monoterpenoids act in a manner similar to the cyclodienes. The action of the chlorinated cyclodienes and lindane is acknowledged to occur at the picrotoxinin site of the GABA (γ-amino butyric acid) receptor-ionophore complex, and as an antagonist of GABA, therefore inhibiting chloride uptake into the neuron through the chloride channel (*35*, *36*). Polyhalogenated monoterpenoids have been isolated from a marine alga, and they have been shown to elicit lindane-like GABA-antagonist effects (*37*). Acetylcholinesterase inhibition has also been reported as a possible mode of action for this class of natural products (*38*).

The repellency activity of the monoterpenoids, often species-specific and compound-specific, obviously affects the insects' sensory receptors. There are indications that coevolutionary processes have resulted in plant-insect interactions such that a monoterpenoid in a plant may be repellent to many insects, but be attractive to certain host-specific species. There are also cases in which one of the chemicals is attractive to an insect at low concentrations but repellent at higher levels. Specific studies using monoterpenoids could help elucidate their mechanisms of action as repellents.

Although monoterpenoids have definite effects on insect growth, development,

and reproduction, the specific mechanisms are not well understood. Their biosynthesis via the isoprenoid pathway is in common with juvenile hormone biosynthesis. Juvenoid activity, as characterized by an extended period in juvenile forms or additional juvenile stages, has not been observed. In contrast, there have been cases of more rapid development to the adult forms in cockroaches (*39*) and mosquitoes (unpublished). Precocenes elicit anti-juvenile hormone effects that result in precocious, unfit adult forms (*40*). The rapid-development effect does not seem to be similar to that caused by precocenes.

Research Objectives

The overall objectives of the current investigation were to evaluate the toxicity of monoterpenoids and their derivatives and to begin a systematic examination of their structural requirements for bioactivity against insects. To accomplish these goals, the following studies were carried out...

1.) The topical, fumigant, and ovicidal toxicity of fourteen monoterpenoids were evaluated using house flies, *M. domestica*. Comparisons were made between aromatic, acyclic, monocyclic, and bicyclic phenols, alcohols, and ketones to determine toxicity differences involving the monoterpenoids' skeletal structures, amount of saturation, and associated functional groups.

2.) Monoterpenoid derivatives were synthesized from their alcohols or phenols and their toxicities were evaluated with topical, fumigant, and ovicidal house fly bioassays. The toxicities of the monoterpenoid derivatives were compared with the toxicities of the parent alcohols or phenols and related derivatives to determine the influence of derivatization.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

Experimental Methods

Monoterpenoids. The monoterpenoids evaluated in the house fly, *M. domestica*, topical, fumigant, and ovicidal bioassays include carvacrol, (-)carveol, geraniol, linalool, *l*-menthol, menthone, pulegone, α-terpineol, thujone, thymol, (S)-*cis* verbenol, verbenone (Aldrich Chemical Company, Milwaukee, WI), *d*-carvone, and *l*-fenchone (Pfaltz and Bauer, Waterbury, CT) (Figures 1 and 2). The purity of the monoterpenoids ranged from 85-99%. The standards for comparison included chlorpyrifos (DowElanco, Indianapolis, IN), 20% pyrethrins (Pet Chemicals, Miami Springs, FL), and dichlorvos (Chem Service Inc., West Chester, PA).

Derivative Synthesis. Monoterpenoid derivatives were synthesized by reacting the parent alcohol or phenol (1 mol) with one of the acetic anhydrides (2-3 mol of acetic anhydride, trimethylacetic anhydride, trichloroacetic anhydride, or trifluoroacetic anhydride) or an acetyl chloride (2-3 mol of propionyl chloride) in the presence of methylene chloride (30 ml) and pyridine (8-20 drops) (Figure 3) (*41*). Reaction products were purified by preparatory thin layer chromatography (254 nm fluorescent-indicator silica gel with 9:1 hexane:ethyl acetate) and/or column chromatography (silica gel and 9:1 hexane:ethyl acetate). The identity of the purified samples was confirmed by comparison of their nuclear magnetic resonance (NMR) spectra with those of the standards (*42*).

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

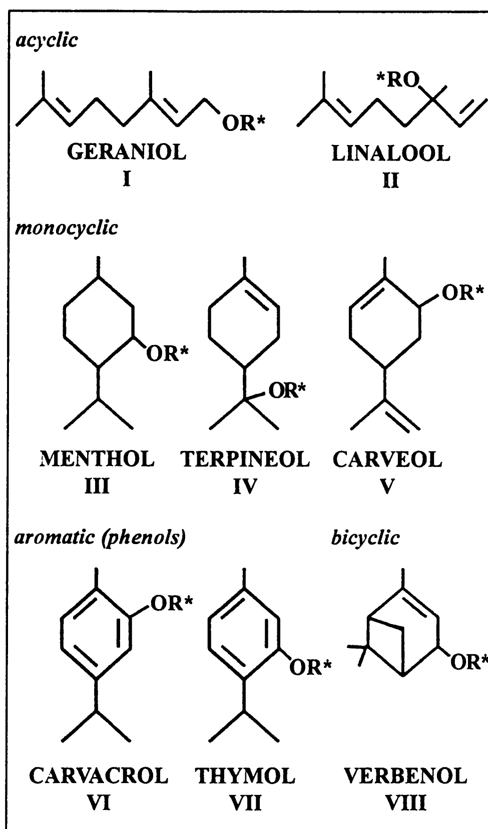


Figure 1. Monoterpenoid alcohols. *R = H

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

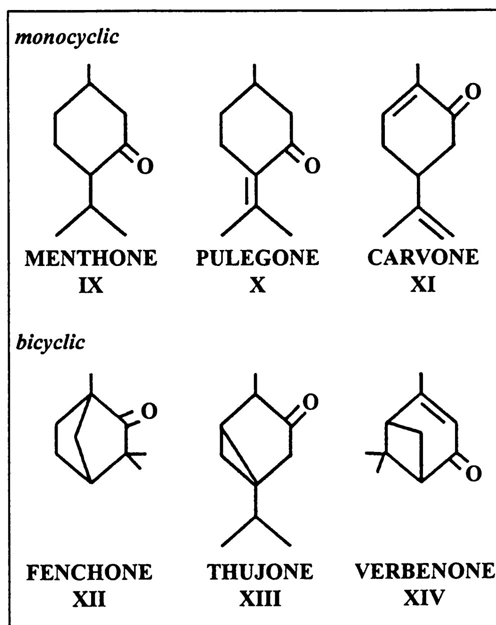


Figure 2. Monoterpenoid ketones.

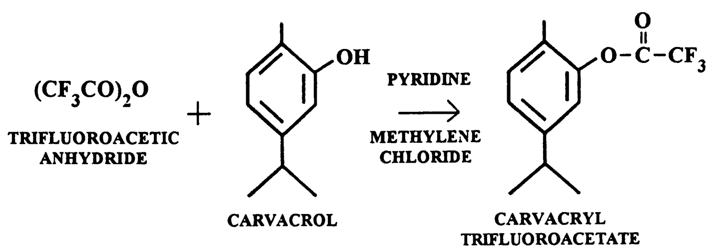


Figure 3. Typical synthesis of acyl monoterpenoid derivatives.

Biological Assays

Evaluation of Topical Toxicity. The acute topical toxicity of the monoterpenoid alcohols, ketones, and derivatives were evaluated using house flies, *M. domestica*. One microliter of the monoterpenoid or monoterpenoid derivative in acetone was applied to the pronotum of an anesthetized fly (10 days post-eclosion) with an electric microapplicator. Range-finding bioassays determined the appropriate testing concentrations. Chlorpyrifos and pyrethrins served as the standards for comparison. Acetone was utilized as the control. Each compound was evaluated at four concentrations. A minimum of three replications with ten flies per replication were obtained for each of the concentrations in the final bioassays. Mortality was assessed at 24 h. The trimmed Spearman-Karber method was used to determine the LD_{50} (*43*). Additional replications were added as necessary to narrow the confidence intervals.

Evaluation of Fumigant Toxicity. Fumigant activity of the monoterpenoids and monoterpenoid derivatives were evaluated using adult *M. domestica*. Ten house flies were placed inside a small cage that was suspended in a test chamber containing a monoterpenoid, a derivative or the dichlorvos standard (*42*). The fumigant activity of each compound was evaluated at four concentration levels. Each test was replicated at least three times. Mortality was assessed at 14 h and the LC_{50} [μg of compound/cm^3 (volume of test chamber)] was calculated using the trimmed Spearman-Karber method (*43*).

Evaluation of Ovicidal Toxicity. Approximately 50 *M. domestica* eggs (less than 12-h old) were wetted in 1500 μl of the 833 μg/ml treatment, standard (pyrethrins), or control (corn oil) solutions (*42*). The bioassays were terminated when the control eggs hatched (no less than 4 days). At least four replications were tested for each of the compounds. The total number of treated eggs and the number of hatched larvae were recorded. Percent inhibition of egg hatch was calculated using the formula described by Sharma and Saxena (*44*). Significance was determined using Chi-square analysis (*45*).

Structural Comparisons. The topical, fumigant and ovicidal toxicity data of the monoterpenoids and their derivatives were compared to determine structure-activity relationships. Acyclic aliphatic, monocyclic aliphatic, bicyclic aliphatic, and monocyclic aromatic monoterpenoids were compared to evaluate the affect of structural shape on toxicity. The influence of the type of functional groups and the degrees of saturation were evaluated in comparisons between structurally similar alcohols and ketones and structurally similar saturated and unsaturated monoterpenoids. The toxicities of structurally similar monoterpenoid acetate, propionate, pivalate, trichloroacetate, and trifluoroacetate derivatives were compared with each other and their parent alcohols or phenols to determine the effect of derivatization on toxicity (Figure 4). In the following tables, figures, and discussion, monocyclic aliphatic compounds and monocyclic aromatic compounds are referred to as monocyclic and aromatic (phenolic) compounds, respectively.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

	R*
ALCOHOL/PHENOL (PARENT)	$-H$
ACETATE	$-C(=O)-CH_3$
PROPIONATE	$-C(=O)-CH_2CH_3$
PIVALATE	$-C(=O)-C(CH_3)_3$
TRICHLOROACETATE	$-C(=O)-CCl_3$
TRIFLUOROACETATE	$-C(=O)-CF_3$

Figure 4. Types of acyl monoterpenoid derivatives synthesized. *Refers to the R in Figure 1.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

Results

Monoterpenoids: Aromatic vs. Acyclic vs. Monocyclic vs. Bicyclic. In the topical bioassay the phenols, carvacrol (Table I and Figure 1, compound number VI) and thymol (VII), were significantly more effective than the other monoterpenoids tested, according to the trimmed Spearman-Karber analysis. The acyclic alcohol geraniol (I) was more insecticidal than the monocyclic (III, IV, V) and the bicyclic (VIII) alcohols. Acyclic alcohols (I, II) were more ovicidal than monocyclic (III, V) and bicyclic (VIII) alcohols, with the exception of α-terpineol (IV). There were no apparent structure-activity relationships noted between the phenols and the acyclic, monocyclic, and bicyclic alcohols (VI, VII, I, II, III, IV, V, VIII), in the fumigant bioassay.

Among the monoterpenoid ketones tested, monocyclic ketones (IX, X, XI) were significantly more insecticidal than bicyclic ketones (XII, XIII, XIV) in 7 of 9 topical bioassay comparisons. Vapors of bicyclic ketones (XII, XIII, XIV) were significantly more active fumigants than monocyclic ketones (IX, X, XI) in 6 of 9 comparisons. There were no distinct trends noted involving the ovicidal activity of the monocyclic (IX, X, XI) and bicyclic (XII, XIII, XIV) ketones.

Monoterpenoids: Alcohols vs. Ketones. The topical, fumigant and ovicidal toxicity of structurally similar alcohols and ketones were compared. Ketones were significantly more toxic than alcohols (XI vs. V; IX vs. III; XIV vs. VIII) in two of three topical and ovicidal comparisons. There was no clear trend associated with the fumigant activity of the ketones and alcohols tested.

Monoterpenoids: Saturated vs. Unsaturated. The phenols carvacrol (VI) and thymol (VII) were significantly more insecticidal than the saturated (III), mono-unsaturated (IV) and di-unsaturated (V) monocyclic alcohols when they were topically applied to adult house flies. Menthol (III), the saturated monocyclic alcohol, was the most effective fumigant and one of the least effective ovicides relative to the other monocyclic alcohols evaluated (IV, V, VI, VII).

The monocyclic mono-unsaturated ketone pulegone (X) was more insecticidal than either the saturated or the and di-unsaturated monocyclic ketones (IX, XI) in the topical bioassays. The saturated monocyclic ketone menthone (IX) inhibited egg hatch more so than the structurally similar unsaturated ketones (X, XI). The vapors of menthone (IX) and pulegone (X) were more insecticidal to adult house flies than the monocyclic di-unsaturated ketone *d*-carvone (XI).

Monoterpenoids: Comparison with Standards. In the topical bioassay, the substituted phenol thymol (VII) was the most effective monoterpenoid (LD_{50}=33 µg/insect). However, by comparison the commercially available pyrethrin (XVII) and chlorpyrifos (XV) standards were one to three orders of magnitude more effective. The commercial fumigant, dichlorvos (XVI), was at least two orders of magnitude more effective than any of the monoterpenoids evaluated. However, four monoterpenoid alcohols and a single monoterpenoid ketone inhibited egg hatch as well as the pyrethrin standard (I, II, IV, VI and IX vs. XVII).

Monoterpenoid Derivatives: Acetates vs. Propionates. Monoterpenoid acetate derivatives were more insecticidal than their related propionate derivatives in 80% (4 of 5) of the topical bioassays (Ia vs. Ib; IIa vs. IIb; Va vs. Vb; VIa vs. VIb; VIIa

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

vs. **VIIb**) (Table II and Figure 4), and 100% of the fumigant (**IIa** vs. **IIb**; **VIa** vs. **VIb**) bioassay comparisons.

Monoterpenoid Derivatives: Acetates vs. Pivalates. The topically applied monocyclic pivalate derivatives were significantly more effective than their corresponding acetates in two of three comparisons (**IIIc** vs. **IIIa**, **Vc** vs. **Va**, **VIIc** vs. **VIIa**). In contrast the acyclic and bicyclic monoterpenoid acetate derivatives were more effective than the pivalates (**Ia** vs. **Ic**, **VIIIa** vs. **VIIIc**) when they were topically applied to adult house flies. There were no trends associated with the acetate and pivalate comparisons in the fumigant bioassay (**Ia** vs. **Ic**; **IIa** vs. **IIc**; **IIIa** vs. **IIIc**; **VIa** vs. **VIc**; **VIIa** vs. **VIIc**; **VIIIa** vs. **VIIIc**). Both the acetates and the pivalates were more effective fumigants than related propionate derivatives (**IIa**, **IIc** vs. **IIb**; **VIa**, **VIc** vs. **VIb**).

Monoterpenoid Derivatives: Trichloroacetates vs. Trifluoroacetates. Trifluoroacetates were more effective fumigants than the trichloroacetates (**IIe** vs. **IId**; **IIIe** vs. **IIId**; **VIe** vs. **VId**; **VIIe** vs. **VIId**). However, the acyclic trichloroacetates were more insecticidal than the acyclic trifluoroacetates (**Id** vs. **Ie**; **IId** vs. **IIe**) when they were topically administered to adult house flies.

Monoterpenoid Derivatives - Acetates vs. Trichloroacetates and Trifluoroacetates. The acetates were significantly more insecticidal than their corresponding trichloroacetates and/or trifluoroacetates in the topical and ovicidal bioassays. The exceptions to this trend were those involving thymyl trifluoroacetate and linalyl and verbenyl acetates and haloacetates in the ovicidal bioassay, and carvacryl trifluoroacetate in the topical bioassay (**Ia** vs. **Id**, **Ie**; **IIa** vs. **IId**, **IIe**; **Va** vs. **Vd**; **VIa** vs. **VId**, **VIe**; **VIIa** vs. **VIId**; **VIIIa** vs. **VIIIe**). In the fumigant bioassay, the vapors of the geranyl, menthyl, carvacryl and thymyl trifluoroacetates were more toxic to adult house flies than their acetates and trichloroacetates (**Ie** vs. **Id**, **Ia**; **IIIe** vs. **IIIa**, **IIId**; **VIe** vs. **VIa**, **VId**; **VIIe** vs. **VIIa**, **VIId**). In contrast, linalyl and verbenyl acetates were more effective fumigants than their related haloacetate derivatives (**IIa** vs. **IIe**, **IId**; **VIIIa** vs. **VIIIe**).

Monoterpenoid Derivatives: Pivalates vs. Trichloroacetates and Trifluoroacetates. Comparisons among the pivalates and the haloacetates proved to be similar to the acetate-haloacetate comparisons (see above) because the pivalates were more toxic than their related trichloroacetates and/or trifluoroacetates in the topical and ovicidal bioassays. The exception to this trend was observed in the ovicidal activity of geranyl trichloroacetate and pivalate and carvacryl pivalate and haloacetates (**Ic** vs. **Id**, **Ie**; **Vc** vs. **Vd**; **VIc** vs. **VId**, **VIe**; **VIIc** vs. **VIId**, **VIIe**; **VIIIc** vs. **VIIIe**). The vapors of linalyl and verbenyl pivalates were more potent fumigants than their trifluoroacetates or trichloroacetates (**IIc** vs. **IIe**, **IId**; **VIIIc** vs. **VIIIe**). Geranyl trifluoroacetate and menthyl trifluoroacetate were more toxic than their pivalates and/or trichloroacetates (**Ie** vs. **Ic**; **IIIe** vs. **IIIc**, **IIId**) in the fumigant bioassay.

Monoterpenoid Derivatives: Derivatives vs. Parent Alcohol. Several monoterpenoid derivatives gave enhanced insecticidal activity relative to their parent alcohol or phenol. (-)Carvyl acetate, propionate, and pivalate (**Va**, **Vb**, **Vc**), menthyl acetate and pivalate (**IIIa**, **IIIc**), geranyl acetate (**Ia**), and verbenyl acetate (**VIIIa**) are more toxic than (-)carveol (**V**), menthol (**III**), geraniol (**I**), and

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

verbenol (**VIII**) in the topical bioassay. The vapors of linalyl acetate (**IIa**), thymyl acetate and trifluoroacetate (**VIIa, VIIe**), and geranyl pivalate and trifluoroacetate (**Ic, Ie**) were more active fumigants than linalool (**II**), thymol (**VII**), and geraniol (**I**), respectively. (-)Carvyl pivalate (**Vc**), and verbenyl pivalate and trifluoroacetate (**VIIIc, VIIIe**) were more ovicidal than (-)carveol (**V**) or verbenol (**VIII**). Although several monoterpenoid derivatives displayed enhanced insecticidal activity, they were not as effective as the commercially available standards in the topical and fumigant bioassays. Geranyl propionate (**Ib**) inhibited egg hatch as effectively as the pyrethrins standard (**XVII**).

Discussion

Monoterpenoids are insecticidal to a variety of insects. Although their specific modes of action are unknown, acute, subacute and sublethal effects have been reported in insects and acute neurotoxic activity has been measured electrophysiologically in earthworms *Eisenia foetida* (*32*). In addition monoterpenoids may act as attractants or repellents (*22*, *28*) and they affect enzyme titers, reproduction, growth and development (*19*, *39*). The structural characteristics of monoterpenoids and their derivatives can influence their insecticidal properties. Their shape, type of functional group, degree of saturation, and type of derivatization can influence their ability to penetrate the insect cuticle, move to, and interact with the active site, and influence their degradation.

The shape, type of functional group, and degree of saturation were the structural characteristics used to evaluate structure-activity relationships of the monoterpenoids. Phenols and acyclic alcohols were more insecticidal than monocyclic and bicyclic alcohols in the topical bioassays. Similar trends were noted in the evaluation of monoterpenoids' nematicidal activity (*46*).

Ketones proved to be more insecticidal than structurally similar alcohols as an ovicide and when they were topically applied to adult house flies. This may be related to their differential susceptibility to metabolism since applications of the mixed-function oxidase inhibitor, piperonyl butoxide, resulted in greater toxicity of alkenes and alcohols (*22*). The ketone/monocyclic alcohol toxicity differences appear to play a role in the defense strategy of the peppermint plant, *Mentha piperita* (L.), against the variegated cutworm, *Peridroma saucia* (Hubner). The more toxic monoterpenoid ketones, pulegone and menthone, predominate in the younger more vulnerable leaves while the less toxic monoterpenoid alcohols and aldehydes appear in the more mature leaves (*47*).

The degree of saturation appears to influence the toxicity of the monoterpenoids considerably. The monoterpenoid phenols were topically more insecticidal than the more saturated alcohols. In the fumigant bioassay the saturated alcohol and saturated monocyclic ketone were more toxic than less saturated alcohols and ketones, respectively. In addition to the degree of saturation, the monoterpenoids' volatility is a major factor involving their fumigant toxicity in that the more volatile monoterpenoids were the more effective fumigants.

Derivatization of the monoterpenoids and evaluation of their toxicity revealed several informative trends. Acetates were more toxic than propionates in the topical and fumigant bioassays and they were more toxic than haloacetates in the topical and ovicidal bioassays. Trifluoroacetates were more effective fumigants than the acetates. This contradicted our expectations of greater toxicity with unnatural haloacetates relative to more natural acetates. The acetates' greater

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

Table I. Topical, fumigant, and ovicidal activity of monoterpenoids to the house fly, *Musca domestica,* adults and eggs

Compound		Insecticidal activity				
		Topical		Fumigant		Ovicidal
		LD_{50} (95% CI)[a] (μg/insect)		LC_{50} (95%CI)[a] ($\mu g/cm^3$)		% inhibition of hatch[b]
monoterpenoid alcohols and phenols						
I	geraniol	103	(95-112)	>1780	-----	99 j,k
II	linalool	189	(178-200)	6.8	(6.6 - 6.9)	87 g-j
III	menthol	193	(171-217)	3.6	(2.5 - 5.2)	47 b-e
IV	terpineol	199	(189-211)	74.5	(64.2 - 86.5)	99 j,k
V	carveol	282	(250-318)	1122	(972 - 1290)	56 c-f
VI	carvacrol	63	(60- 65)	27.4	(23.4 - 32.0)	99 j,k
VII	thymol	33	(30- 36)	142	(95 - 214)	78 e-h
VIII	verbenol	229	(220-238)	6.3	(4.6 - 8.8)	0 a
monoterpenoid ketones						
IX	menthone	148	(136-162)	13.7	(12.5 - 15.0)	100 j,k
X	pulegone	78	(77- 80)	9.2	(8.2 - 10.4)	78 e-h
XI	carvone	157	(147-168)	19.0	(15.5 - 23.2)	64 c-g
XII	fenchone	295	(282-310)	3.8	(3.6 - 3.9)	74 e-h
XIII	thujone	198	(181-217)	11.9	(9.5 - 14.8)	70 d-g
XIV	verbenone	176	(162-192)	7.7	(7.0 - 8.4)	65 c-g
standards						
XV	chlorpyrifos	0.08	(0.07-0.10)	-----	-----	-----
XVI	dichlorvos	-----	-----	0.01	(0.009 - .012)	-----
XVII	pyrethrins[c]	0.94	(0.78-1.1)	-----	-----	100 j,k

[a]95% confidence intervals (CI) were not adjusted for multiple inferences. The monoterpenoids' activity is considered significantly different when the 95% CI fail to overlap. Trimmed Spearman-Karber analysis was used to determine LD_{50}, LC_{50} and 95% CI (*43*).
[b]% inhibition of egg hatching = 100(X-Y)/X, X= control % hatch, Y = treated % hatch (*44*). Chi-square analysis was used to determine significance at 5% (*45*). Compounds that do not share a common letter are significantly different.
[c]adjusted for 20% active ingredient

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

Table II. Insecticidal Activity of Monoterpenoid Derivatives to the House Fly, *Musca domestica*

Monoterpenoid derivatives		Insecticidal activity				
		Topical LD_{50} (95% CI)[a] (μg/insect)		Fumigant LC_{50} (95% CI)[a] ($\mu g/cm^3$)		Ovicidal % inhibition of hatch[b]
Ia	geranyl acetate	55	(50 - 60)	>91	-----	89 b,s,u
Ib	geranyl propionate	309	(295 - 324)	-----	-----	100 a
Ic	geranyl pivalate	93	(88 - 99)	52	(42 - 65)	77 j,l,m,n,u
Id	geranyl trichloroacetate	135	(133 - 136)	-----	-----	44 e-h,k,m,o,r,t
Ie	geranyl trifluoroacetate	>500	-----	11	(3 - 38)	0 c,d
IIa	linalyl acetate	245	(234 - 256)	4.8	(4.1 - 5.7)	0 c,d
IIb	linalyl propionate	>1000	-----	66	(39 - 114)	-----
IIc	linalyl pivalate	-----	-----	10	(9 - 11)	-----
IId	linalyl trichloroacetate	333	(304 - 365)	>209	-----	0 c,d
IIe	linalyl trifluoroacetate	663	(605 - 727)	50	(44 - 58)	0 c,d
IIIa	menthyl acetate	147	(137 - 158)	43	(42 - 44)	52 e-k,m,o,p
IIIc	menthyl pivalate	85	(73 - 98)	56	(48 - 65)	23 d-f,q,r,t
IIId	menthyl trichloroacetate	------	-----	>108	-----	-----
IIIe	menthyl trifluoroacetate	-----	-----	35	(32 - 39)	-----
Va	carvyl acetate	111	(104 - 119)	-----	-----	73 j,l,m,n,p
Vb	carvyl propionate	206	(192 - 220)	-----	-----	52 e-k,m,o,p
Vc	carvyl pivalate	88	(83 - 94)	-----	-----	88 b,n,s,u
Vd	carvyl trichloroacetate	>700	-----	-----	-----	24 d-f,q,r,t
VIa	carvacryl acetate	107	(96 - 118)	48	(39 - 58)	93 b,s,u
VIb	carvacryl propionate	134	(131 - 137)	>188	-----	3 d,q,r,t
VIc	carvacryl pivalate	-----	-----	28	(25 - 31)	0 c,d
VId	carvacryl trichloroacetate	139	(128 - 151)	>114	-----	0 c,d
VIe	carvacryl trifluoroacetate	114	(100 - 131)	27	(22 - 34)	0 c,d
VIIa	thymyl acetate	94	(84 - 104)	40	(19 - 87)	57 f-k,m,o,p
VIIb	thymyl propionate	101	(81 - 127)	-----	-----	-----
VIIc	thymyl pivalate	80	(65 - 99)	90	(69 - 120)	-----
VIId	thymyl trichloroacetate	184	(166 - 204)	208	(202 - 423)	0 c,d
VIIe	thymyl trifluoroacetate	119	(111 - 129)	2.2	(1.4 - 3.4)	44 e-h,k,m,o,r,t
VIIIa	verbenyl acetate	117	(104 - 130)	32	(27 - 40)	0 c,d
VIIIc	verbenyl pivalate	213	(181 - 251)	8.9	(7.4 - 10)	71 h-j,l,m-p
VIIIe	verbenyl trifluoroacetate	>600	-----	>83	-----	93 b,s,u

[a]95% confidence intervals (CI) were not adjusted for multiple inferences. The monoterpenoids' activity is considered significantly different when the 95% CI fail to overlap. Trimmed Spearman-Karber analysis was used to determine LD_{50}, LC_{50} and 95% CI (*43*).
[b]%inhibition of egg hatching = 100(X-Y)/X, X = control % hatch, Y = treated % hatch (*44*). Chi-square analysis was used to determine significance at 5% (*45*). Compounds that do not share a common letter are significantly different.

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

activity in the topical and ovicidal bioassays may be explained by their greater stabilities than those of the trichloroacetates and trifluoroacetates. In fact, several of the haloacetate derivatives even degraded during cold storage (8°C). The trifluoroacetate derivatives were more potent fumigants than most of the related acetate, propionate and trichloroacetate derivatives tested. This is believed to be associated with the enhanced volatilities of the trifluoroacetate derivatives. The addition of methyl, chlorine, or fluorine substituents may influence the volatility and stability of the monoterpenoids, affect their rate of penetration into the insect cuticle, or inhibit the insect's ability to degrade the compound once it penetrates.

Conclusions

The results of the studies provide some indications that minor structural variations (ketones vs. alcohols, phenols vs. acyclic aliphatic, monocyclic aliphatic and bicyclic aliphatic alcohols) can elicit major differences in the monoterpenoid's toxicity. These results are supported by previous research that noted a difference in response of insects to isomers of the same compound (*23*, *19*), and to monoterpenoids that differ in molecular configuration and position of functional groups (*28*). Clearly, the basic carbon skeleton, the type of functional group, the degrees of saturation, volatility, and type of derivatization all influence insecticidal activity of the monoterpenoids and monoterpenoid derivatives evaluated here. Thymol was the most effective monoterpenoid/monoterpenoid derivative tested in the topical bioassays while its derivative thymyl trifluoroacetate was the most effective fumigant. Geraniol, geranyl acetate, terpineol, carvacrol, and menthone were the most ovicidal monoterpenoid/monoterpenoid derivatives tested.

The data collected from this investigation was used to begin the development of a systematic examination of the structural requirements for monoterpenoid and monoterpenoid derivatives' bioactivity against insects. Synthesis of additional derivatives, together with applied spectrum-of-activity research, mode of action studies, and a more systematic quantitative structure-activity relationship (QSAR) approach are needed in order to fully understand the critical physiochemical properties that contribute to monoterpenoid and monoterpenoid derivatives' bioactivity in insects. This information would be valuable in our selection and development of analogs that could be novel biodegradable insecticides.

Acknowledgments

We thank Stephanie Peterson and Mike Ward for their efforts in insect rearing, Mike Johnson, Adrienne Lyles and Greg VanOrsow for their assistance in synthesis and purification, and Cheryl Jensen and Wendy Hedding for their assistance with the bioassays. This article is Journal Paper number J-15693 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project no. 3187.

Literature Cited

1. Whittaker, R. H. *In The Biochemical Ecology of Higher Plants*; Sondheimer E.; Simeone, J. B, Eds.; Academic Press: New York, NY, 1970; pp 43-70.
2. Matsumura, F. *Toxicology of Insecticides*; Plenum Press: New York, NY, 1985: 2nd edn, pp 95-456.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

3. Morrison, G. *Analyt. Chem.* **1984**, *56*, 573-574.
4. Kerns, D. L.; Gaylor M. J. *J Econ. Entomol.* **1992**, *85*, 1-8.
5. Mink, J. S.; Boethel, D. J. *J Econ. Entomol.* **1992**, *85*, 1056-1062.
6. DiTomaso, J. M.; Duke, S. O. *Pestic. Biochem. Physiol.* **1991**, *39*, 158-167.
7. Hink, W. F. ; Fee, B. J. *J. Med. Entomol.* **1986**, *23*, 400-404.
8. Moffett, J. O.; Cox, R. L.; Ellis, M.; Rivera, R.; Wilson, W. T.; Cardoso, T. D.; Vargas,C. *J. Southwestern Entomol.* **1989**, *14*, 57-65.
9. Klocke, J. A.; Darlington, M. V.; Balandrin, M. F. *J. Chem. Ecol.* **1987**, *13*, 2131-2141.
10. Templeton, W. *An Introduction to the Chemistry of Terpenoids and Steroids*; Butterworth and Co. Ltd.: London, England, 1969; pp 1-70.
11. Banthorpe, D. V.; Charlwood, B. V. In *Chemistry of Terpenes and Terpenoids*; Newman, A. A., Ed.; Academic Press: New York, NY, 1972; pp 337-411.
12. Harborne, J. B. In *Bioactive Compounds from Plants*; Chadwick, D. J.; March, J.; Eds., John Wiley and Sons: New York, NY, 1990; pp 126-139.
13. Larson, K. C.; Berry, R. E. *Environ. Entomol.* **1984**, *13*, 282-285.
14. Gough, A. J. E.; Hamilton, J. G. C.; Games, D. E.; Staddon, B. W. *J. Chem. Ecol.* **1985**, *11*, 343-352.
15. Aldrich, J. R.; Blum, M. S.; Lloyd, H. A.; Evans, P. H.; Burkhard, D. R. *Entomol. Exp. Appl.* **1979**, *26*, 323-331.
16. Francke, W.; Vite, J. P. *Z. Angew. Entomol.* **1983**, *96*, 146-156.
17. Tengo, J.; Bergstrom, G. *Comp. Biochem. Physiol*, **1976**, *55B*, 179-188.
18. Brand, J. M.; Blum, M. S.; Lloyd, H. A.; Fletcher, D. J. C. *Ann. Entomol. Soc. Am.* **1974**, *67*, 525-526.
19. Brattsten, L. B. In *Plant Resistance to Insects*; Hedin, P. A., Ed.; ACS Symposium Series 208; American Chemical Society: Washington, D.C., 1983; pp 173-195.
20. Gunderson, C. A.; Brattsten, L. B.; Fleming, J. T. *Pestic. Biochem. Physiol.* **1986**, *26*, 238-249.
21. Rani, P. U.; Osmani, F. *Pestic.* **1980**, *8*, 6-8.
22. Karr, L. L.; Coats, J. R. *J. Pestic. Sci.* **1988**, *13*, 287-290.
23. Coyne, J. F.; Lott, L. H. *J. Ga. Entomol. Soc.* **1976**, *11*, 301-305.
24. Smith, R. H. *J. Econ. Entomol.* **1965**, *58*, 509-510.
25. Scriven, R.; Meloan, C. E. *Ohio J. Sci.* **1984**, *84*, 85-88.
26. Bakke, A. *Scand. J. For. Res.* **1987**, *2*, 179-185.
27. Saim, N.; Meloan, C. E. *J. Stored Prod. Res.* **1986**, *22*, 141-144.
28. Inazuka, S. *J. Pestic. Sci.* **1983**, *8*, 293-299.
29. Collart, M. G.; Hink, W. F. *Entomol. Exp. Appl.* **1986**, *42*, 225-229.
30. Gunderson, C. A.; Samuelian, J. H.; Evans, C. K.; Brattsten, L. B. *Environ. Entomol.* **1985**, *14*, 859-863.
31. Saxena, K. N.; Kumar, H. *J. Chem. Ecol.* **1984**, *10*, 1521-1531.
32. Karr, L. L.; Drewes, C. D.; Coats, J. R. *Pestic. Biochem. Physiol.* **1990**, *36*, 175-186.
33. Drewes, C. D.; Vining, E. P. *Pestic. Biochem. Physiol.* **1984**, *22*, 93-103.
34. Coats, J. R.; Karr, L. L.; Drewes, C. D. In *Naturally Occurring Pest Bioregulators*; Hedin, P. A., Ed.; ACS Symposium Series 449; American Chemical Society: Washington, DC., 1991; pp 305-316.
35. Cole, L. M.; Casida, J. E.; *Pestic. Biochem. Physiol.* **1992**, *44*, 1-8.
36. Coats, J. R.; *Environ. Health Perspect.* **1990**, *87*, 255-262.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

37. Watanabe, K.; Umeda, K.; Kurita, Y.; Takayama, C.; Miyakado, M.; *Pestic. Biochem. Physiol.* **1990**, *37*, 275-286.
38. Ryan, M. F.; Byrne, O.; *J. Chem. Ecol.* **1988**, *14*, 1965-1975.
39. Karr, L. L.; Coats, J. R. *J. Econ. Entomol.* **1992**, *85*, 424-429.
40. Binder, B. F.; Bowers, W. S.; *Arch. Insect Biochem. Physiol.* **1992**, *19*, 237-246.
41. Furniss, B. S.; Hannaford, A. J.; Rogers, V.; Smith, P. W. G.; Tatchell, A. R. *Vogel's Textbook of Practical Organic Chemistry*; Longman Group Ltd: London, England, 1978: 4th edn, pp 843-846.
42. Rice, P. J.; Coats, J. R. *Pestic. Sci.* **1994**, (in press).
43. Hamilton, M. A.; Russo, R. C.; Thurston, R. V. *Environ. Sci. Technol.* **1977**, *11*, 714-719.
44. Sharma, R. N.; Saxena, K. N. *J. Med. Entomol.* **1974**, *11*, 617-621.
45. Steel, R. G. D.; Torrie, J. H., *Principles and Procedures of Statistics: a Biometrical Approach*; McGraw-Hill: New York, NY, 1980; 2nd ed.
46. Sangwan, N. K.; Verma, B. S.: Verma, K. K.; Dhindsa, K. S. *Pestic. Sci.* **1990**, *28*, 331-335.
47. Harwood, S. H., Moldenke, A. F.; Berry, R. E. *J. Econ. Entomol.* **1990**, *83*, 1761-1767.

RECEIVED March 8, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch008

Chapter 9

Characterization of Natural Pesticide from *Nicotiana gossei*

Ray F. Severson[1], Orestes T. Chortyk[1], Michael G. Stephenson[2], David H. Akey[3], John W. Neal, Jr.[4], George W. Pittarelli[5], D. Michael Jackson[6], and Verne A. Sisson[6]

[1]Phytochemical Research Unit, Richard B. Russell Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 5677, Athens, GA 30613
[2]Nematodes, Weeds and Crops Research, Coastal Plain Experiment Station, Agricultural Research Service, U.S. Department of Agriculture, Tifton, GA 31793
[3]Western Cotton Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Phoenix, AZ 85040
[4]Floral & Nursery Plants Research Unit and [5]Soybean & Alfalfa Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705–2350
[6]Crops Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Oxford, NC 27565–1168

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

Field and greenhouse studies have shown that *Nicotiana gossei* Domin plants are resistant to attacks by the tobacco aphid, *Myzus nicotianae* Blackman, and the sweetpotato whitefly (SPWF), *Bemisia tabaci* (Gennadius). Fractionation of the methylene chloride cuticular extract of *N. gossei* by solvent partitioning, followed by Sephadex LH-20 liquid chromatography, produced a sugar ester isolate, which was toxic, in laboratory and field assays, to the sweetpotato whitefly. The sugar ester isolate consists of two major types of glucose esters (1-O-acetyl-2,3-di-O-acylglucose and 2,3-di-O-acylglucose) and two major types of sucrose esters (2,3-di-O-acyl-1'-O-acetylsucrose and 2,3-di-O-acyl-1',6'-di-O-acetylsucrose). The predominant acid moieties were 5-methylhexanoic and 5-methylheptanoic acids. Separation methodology, GC/MS data for the different sugar esters, and insect assay results for the *N. gossei* sugar esters will be discussed.

This chapter not subject to U.S. copyright
Published 1994 American Chemical Society

Most *Nicotiana* species have leaf hairs or trichomes. The trichomes have a single glandular cell or have a cluster of cells or glands on their tips, that may exude a complex chemical mixture. The exudates on *N. tabacum* types may consist of diterpenes [duvanes, α- and ß-4,8,13-duvatrien-1-ols (DVT-ols) and the α- and ß-4,8,13-duvatriene-1,3-diols (DVT-diols), and/or labdanes, *cis*-abienol and (13-E)-labda-13-ene-8α,15-diol] and/or sucrose esters (SE) (*1,2*).

Many of the cuticular compounds from *N. tabacum* modify the behavior of insects. The DVT-ols, *cis*-abienol, and SE are topically toxic to the tobacco aphid (*3*), whereas the DVT-diols and SE are ovipositional stimulants for the tobacco budworm moth (*4-7*).

Investigations into the composition of cuticular extracts of various *Nicotiana* species found sugar ester fractions with both SE and glucose esters (GE). The sugar esters consist of aliphatic acids esterified to the various free hydroxyl groups of the sugar. The SE from *N. tabacum,* specifically 6-O-acetyl-2,3,4-tri-O-acylsucrose has an acetate group on the number six carbon of glucose and three other C_3 to C_8 aliphatic acids, termed "acyl groups", on the 2, 3,and 4 carbons of glucose (*8*). The SE isolated from cuticular extracts from other *N.* species may lack a C6 -acetyl group and/or possess only two C_3-C_8 acyl moieties and may have from one to four acetyl groups on fructose. About 19 different structural types of SE and 5 different GE have been reported for *Nicotiana* species (*7,9-19*). (Glucose carbon atoms are designated C1 to C6, while fructose carbons are C1' to C6'). For any sugar ester type, a series of homologs are found due to the different acyl groups. The major acyl moieties are generally iso- and anteiso-methyl branched C_4 to C_8 isomers.

During the 1980's, the *Nicotiana* species were evaluated in the greenhouse (Beltsville, MD) and in the field (Oxford, NC and Tifton, GA) for resistance to pests present in the different environments. In the field, *N. gossei* was very resistant to aphid damage (Jackson, D. M., unpublished data) and in the greenhouse to the greenhouse whitefly (GHWF) *Trialeurodes vaporariorum* (Westwood) (*20*). Beltsville greenhouse studies determined that the "toxic factor" was present in the cuticular extract, more specifically in the sugar ester fraction (*13,21*). Several chromatographic fractionations isolated two types of SE, 2,3-di-O-acyl-6'-O-acetylsucrose and 2,3-di-O-acyl-1',6'-di-O-acetylsucrose, which alone or in combination were topically toxic to the GHWF (Westwood). A 0.1% concentration of the SE, emulsified in water and sprayed onto GHWF or SPWF nymphs on tomato leaves, produced 99+% mortality. (Neal, J. W., Jr.; Buta, J. G.; Pittarelli, G. W.; Lusby, W. R.; Bentz, J. A. *J. Ecol Entomol* in press).

Because both aphids and whiteflies are becoming more difficult to control, it was of interest to ARS to investigate the activity of the *N.gossei* compounds as a biorational alternative to conventional pesticides. To meet this goal, a team of ARS scientists was assembled to obtain sufficient material for field evaluation on cotton and vegetables.

In this paper we will discuss the methodology developed to extract and isolate *N. gossei* sugar esters for field assays and will provide a more complete characterization of the sugar ester isolate.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

EXPERIMENTAL

Production of *N. gossei*. The *N. gossei* plants used to isolate cuticular leaf chemicals were grown under agronomic conditions normally used for the production of flue-cured tobacco at the University of Georgia Coastal Plain Experiment Station, Tifton, GA. Plant-bed-produced plants were transplanted to a one acre plot on April 20, 1992 and to a 0.5 acre on May 6, 1992. At the dates shown below, plant material was harvested by cutting off the top portion, about 10 cm above the ground. Fresh weights of plant material obtained at each cutting were: May 27, 124 kg; June 11, 236 kg; June 30, 44 kg; July 6, 245 kg; July 21, 254 kg; and August 11, 13 kg. After each cutting the field was cultivated, hoed between plants within the row, fertilized and watered. About 10% of the plants were lost after each cutting.

Extraction of Cuticular Components. *N. gossei* leaves and stems were loosely packed into extended steel mesh baskets (76 cm x 23 cm x 30 cm), which were then placed into 16 gauge aluminum extraction containers (88 cm x 30 cm x 80 cm) containing 40-50 liter of methylene chloride. The basket was agitated for about 30 sec, lifted out of the solvent, turned onto its side to drain and then placed into a second tank of solvent and treated as above. After the extraction of 50-60 kg of plant material, the cuticular extract from the first tank was filtered through cheese cloth into solvent cans. Soil and other solids remaining in the tank were removed. After the addition of solvent, this tank became the second extraction tank and the process was repeated.

Isolation of *N. gossei* Biorational (NGB). The extracts were transported to Athens, GA. and the solvent was removed on a rotary-evaporator at reduced pressure and 40° C. The *N. gossei* cuticular extract (CE) was fractionated as shown in Figure 1.

Characterization of the *N. gossei* Sugar Esters. Samples of the cuticular extract and fractions were treated with BSTFA to convert free hydroxyl moieties to trimethylsilylether (TMS) derivatives to permit analyses by capillary gas chromatography (GC) and GC/mass spectrometry (GC\MS) (*1,8,16*). Also, the sugar ester isolates were saponified and the liberated acids were converted to butylesters and analyzed by GC (*8,16,22*). The NGB fraction was chromatographed on a Sephadex LH-20 system (*8,16*) using chloroform (640 ml) and 10% methanol-chloroform (200 ml) eluent and five ml fractions (F) were collected. Three sugar ester fractions were separately collected. A GE-I fraction containing 1-O-acetyl-2,3-di-O-acylglucoses was found in F24-F30. Chromatography fractions F55-F100 contained a series of SE-I (2,3-di-O-acyl-1',6'-di-O-acetylsucrose) and F136-F163 produced a mixture of GE-II(2,3-di-O-acylglucose) and a SE-II (2,3-di-O-acyl-1'-acetylsucrose).

Bioassay of *N. gossei* Biorational. The NGB was bioassayed against the GHWF

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

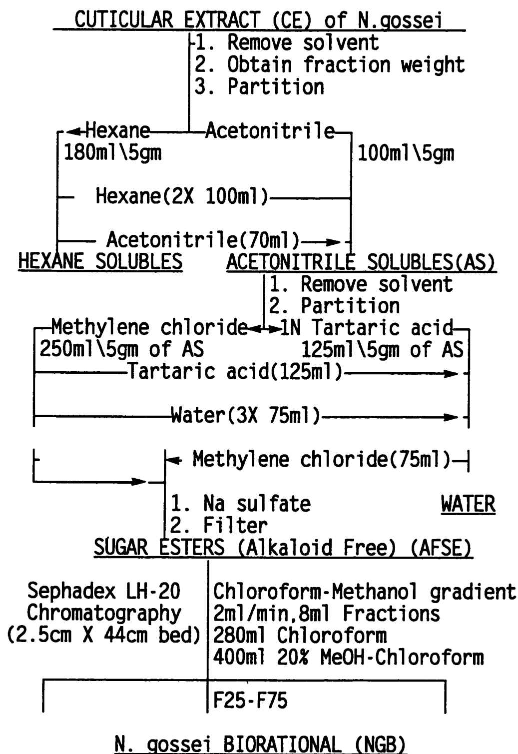


Figure 1. *N. gossei* Biorational Isolation Scheme.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

on tomato in the greenhouse as described by Neal et al (*J. Econ. Ent.*, in press) and on field grown cotton against the sweetpotato whitefly, *Bemisia tabaci* (Gennadius), as described by Akey et al. (*23*). The NGB was bioassayed topically on tobacco aphid. Thirty apterous aphids were each topically treated with 5 ug of NGB in one ul of 3:1 acetone:water.

RESULTS and DISCUSSION

GC and GC\MS analyses of the *N. gossei* CE showed that the major components were nicotine, two types of GE, a series of hydrocarbons, and two types of SE (Figure 2). The sugar ester (GE & SE) biorational was isolated as described in Figure 1. The solvent was removed from the methylene chloride extract to yield the CE which was then partitioned between hexane and acetonitrile. The cuticular hydrocarbons, fatty alcohols, wax esters and other non-polar components were transferred to the hexane fraction (*24*) and the polar sugar esters and alkaloids (primarily nicotine) to the Acetonitrile Solubles (AS) as shown in Figure 3. The alkaloids were removed by partitioning the AS between 1N tartaric acid and methylene chloride. The methylene chloride solubles were washed until the water fraction was neutral. The methylene chloride fraction was dried over sodium sulfate, filtered and the solvent removed to yield the alkaloid-free sugar ester fraction (AFSE) (Figure 4), which was then chromatographed on a preparative Sephadex LH-20 column to yield the *N. gossei* biorational. As shown in Figure 5, NGB consisted of about equal portions of GE and SE. Based on *N. gossei* fresh weight, the average yield of the cuticular components and fractions were; CE, 0.67 g\kg (range of 0.46-0.88 g\kg); AS, 0.29g\kg (range 0.10-0.43 g\kg); AFSE, 0.18 g\kg (range 0.07-0.31 g\kg) and NGB, 0.17 g\kg (range of 0.07-0.30 g\kg).

As in previous sugar ester characterizations (*6,8,16,21*), the next step, after the initial GC\MS of the trimethylsilyl (TMS) derivatives, involved the saponification of NGB with 1N methanolic KOH. The liberated acyl moieties were converted to their butyl esters and quantitated by GC on an SE-54 capillary column. The results of this analysis are given in Table I. Based on relative mole percent, acetic (100%), 5-methylheptanoic (81.8%), and 5-methylhexanoic (30.6%) acids were the major acyl moieties. (Table I). This indicated that the sugar esters of *N. gossei* contained mainly C_7- and C_8-acids, with one or more acetate groups per sugar molecule.

We have successfully employed MS data to characterize the sugar esters of the *Nicotiana* species (*8,16,25*). Electron Impact mass spectrometry yields ions, characteristic of the attached aliphatic acids and of the cleaved fructose and glucose ions, that are very helpful in deducing the SE structures. The C_7-acid yields a major diagnostic ion at m/z 95, the C_8-acid an ion at m/z 109 and normal acyl ions for C_2 (acetyl, m/z 43) to C_6 (methylpentanoyl, m/z 99) are observed.

Although the parent ion for TMS derivatives of SE is generally not observed,

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

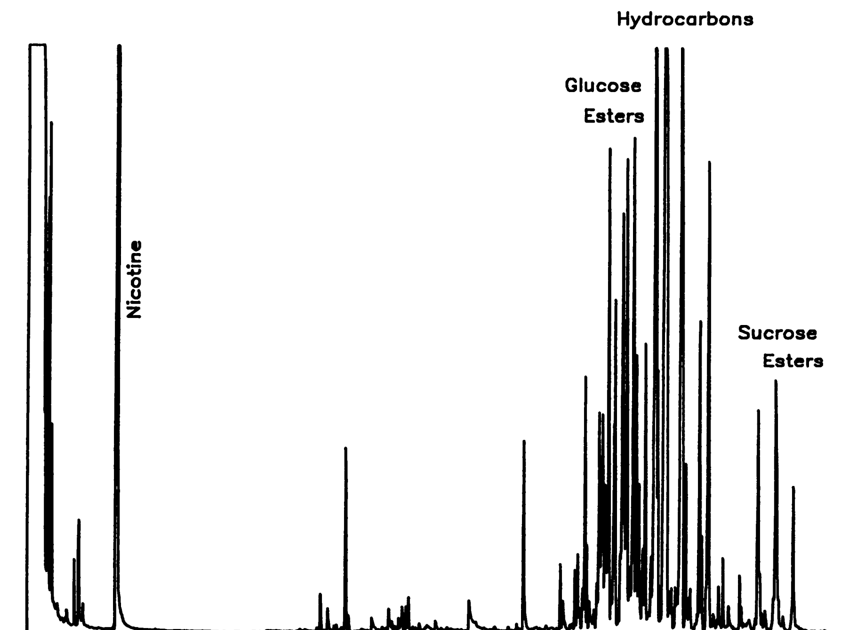


Figure 2. Capillary Gas Chromatogram of the Cuticular Extract of *N. gossei.*

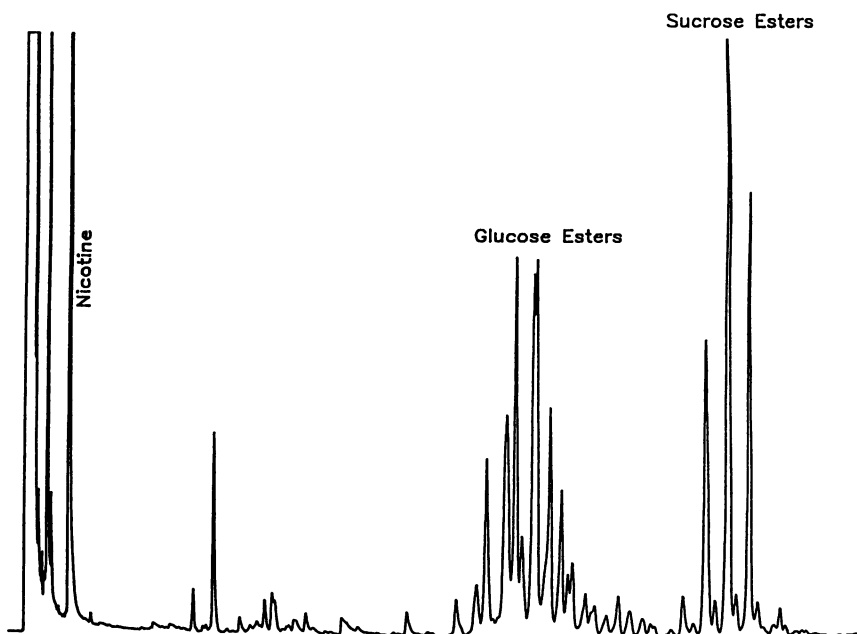


Figure 3. Capillary Gas Chromatogram of the Acetonitrile Solubles of *N. gossei.*

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

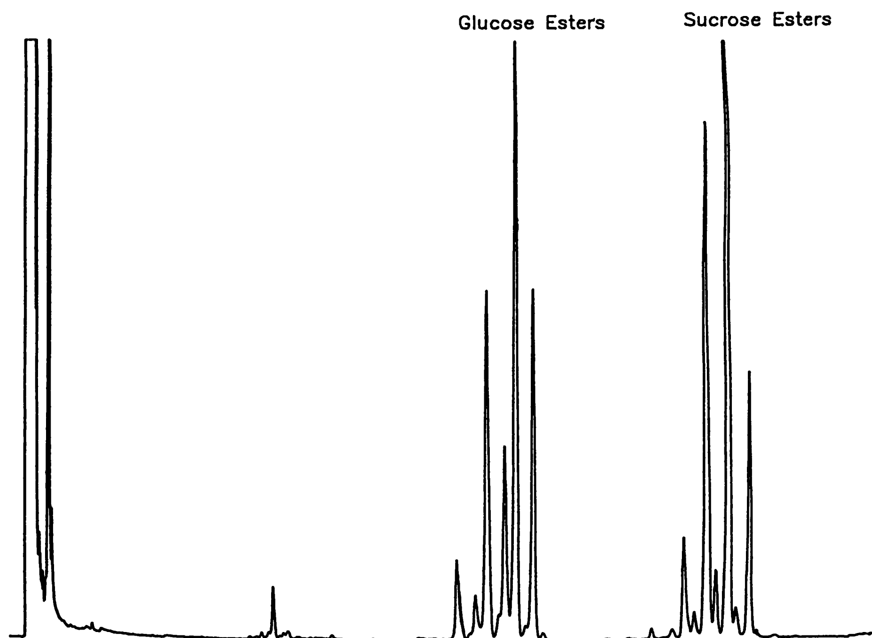


Figure 4. Capillary Gas Chromatogram of the Alkaloid-Free Sugar Ester of *N. gossei.*

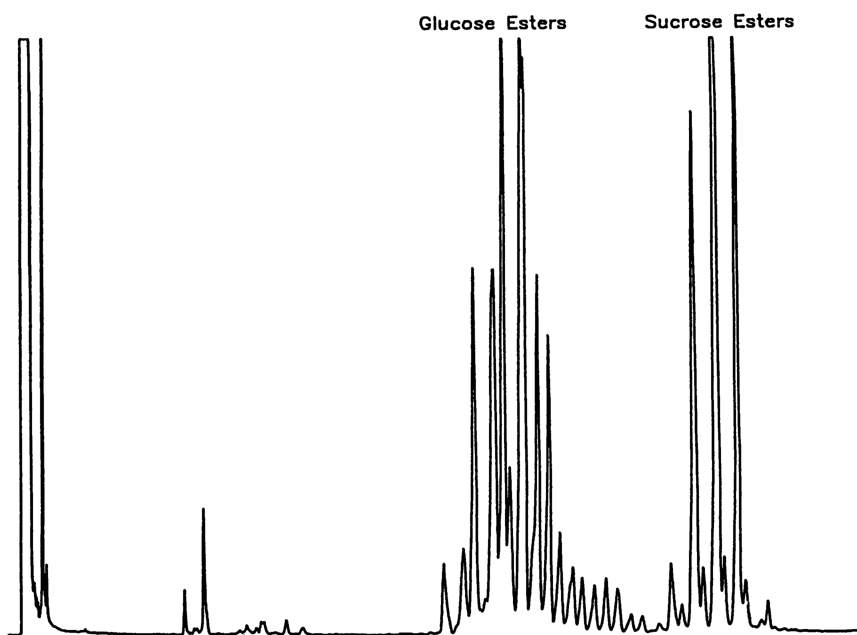


Figure 5. Capillary Gas Chromatogram of the *N. gossei* Biorational.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

Table I. Relative % Distribution of Acids in the Sugar Ester of *N. gossei*

Acid Moiety	Total SE & GE Acids
Acetic	100.0
Propionic	1.0
Isobutyric	8.4
2-Methylbutyric	3.0
3-Methylbutyric	0.9
Valeric	3.1
3-Methylvaleric	2.1
4-Methylvaleric	14.9
5-Methylhexanoic	30.6
4-Methylhexanoic	6.3
6-Methylheptanoic	10.4
5-Methylheptanoic	81.8
7-Methyloctanoic	1.1

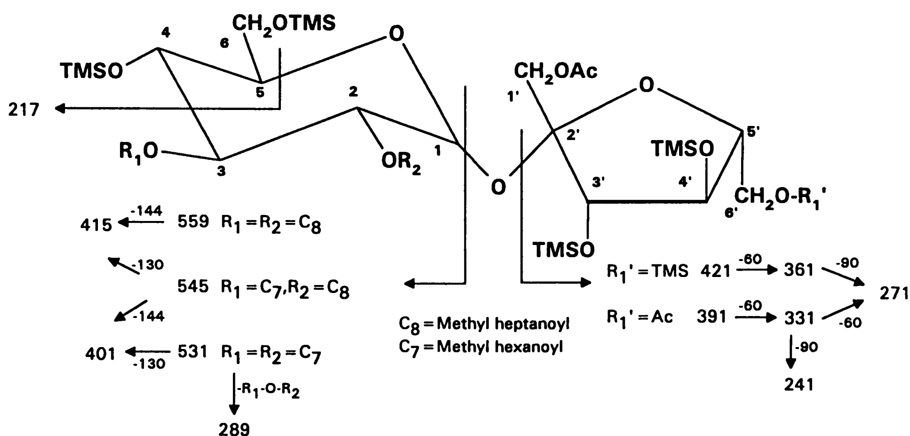


Figure 6. MS Fragmentation of Sucrose Ester Fractions SEI and SEII from *N. gossei*.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

fragmentation produces glucose and fructose ions, which permit general characterization of the molecule. When a SE has a fructose moiety with four free hydroxyls, the tetraTMS fructose ion has an m/z of 451, a fructose with an acetyl and three TMS moieties produces a m/z 421 fragment and diacetyl-di TMS fructose yields a m/z 391 ion. A TMS group on carbon 1' will rearrange to carbon 2' resulting in the loss of CH_2O to yield an ion 14 amu less than the parent fructose ion (*16,26*). Each sugar ester type produces a series of ions differing one from the other by 14 amu, which defines acyl moieties on glucose.

In determining structures from MS data, one searches not only for specific fructose ions, but also for glucose ions that define the numbers and types of C_3-C_8 acids attached to glucose. Thus, for example, in past work with *N. tabacum* SE, the presence of a 499 ion indicated that there were three C_6-acids attached to the hydroxyls of the 2, 3, 4 carbons of glucose and that the 6-carbon hydroxyl carried an acetate group. If there were no acetate group, the free hydroxyl would become silylated and the 529 fragment would be observed. For the isolated *N. gossei* sugar esters, we found only two C_3-C_8 aliphatic acids were attached to glucose positions. Thus, two C_8-acids on glucose leave 2 free hydroxyls for silylation to produce a m/z 559 glucose fragment for the ester isomer containing two C_8 acyls. As shown in Figure 6, the 217 ion (TMSO-CH=CH-CH-OTMS) and the 289 ion (M+ - R_1-O-R_2) indicated a 2,3-O-acylglucose. Thus, a fructose fragment at 421 (no 407) and a glucose fragment at 559 prove that the original molecule had an acetate group on the 1' carbon of fructose and two C_8-acids are esterified to glucose, suggesting the structure 2,3-di-O-(5-methylheptanoyl)-1'-O-acetylsucrose. For the other SE, a glucose fragment at 559 was obtained in the same spectrum as a fructose ion at 391. As there was no fructose rearrangement ion at 377, it is apparent that there were two acetate groups on fructose, with one at the 1' carbon position. These data led to the structure for SEI of 2,3-di-O-(5-methylheptanoyl)-1',6'-di-O-acetylsucrose.

Analyses of the mass spectral data of the GEII fraction (Figure 7), indicated the presence of the α- and ß-isomers of 2,3-di-O-acylglucose, and indicated the structure of GEI to be 1-O-acetyl-2,3-di-O-acylglucose (Figure 8). In this manner, we identified the four following ester types as the constituents of the *N. gossei* sugar esters: 2,3-di-O-acylglucose, 1-O-acetyl-2,3-di-O-acylglucose, 2,3-di-O-acyl-1'-O-acetylsucrose, and 2,3-di-O-acyl-1',6'-di-O-acetylsucrose, where the acyl groups are predominantly 5-methylhexanoyl and 5-methylheptanoyl groups (Table I).

The results of the tests of these biorationals against nymphs of the greenhouse whitefly in the greenhouse showed that the NGB was essentially as active as its SE mixture (SEI & SEII) and resulted in 95% or more mortality, even down to 0.1% levels (Table II). The NGB was consistently as effective as the Safer Soap, which was tested at much higher concentrations and was much more active than the Neem product.

In the field, on cotton, the NGB was evaluated against two commercial products. The results (Table III) showed the greater effectiveness of the *N.*

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

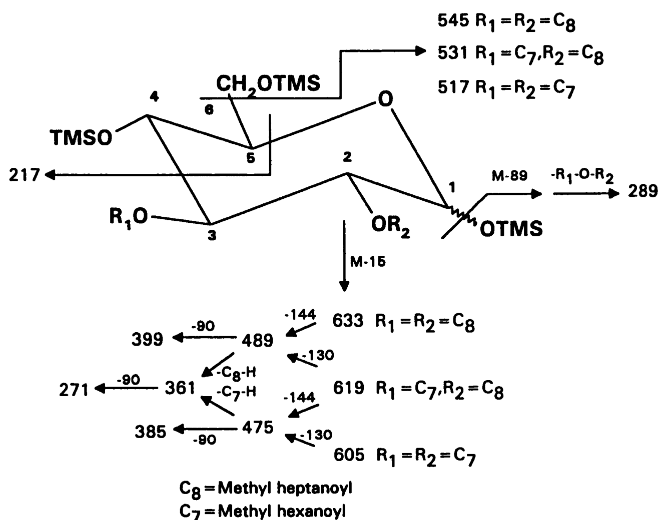


Figure 7. MS Fragmentation of Glucose Ester Fraction GEII from *N. gossei*.

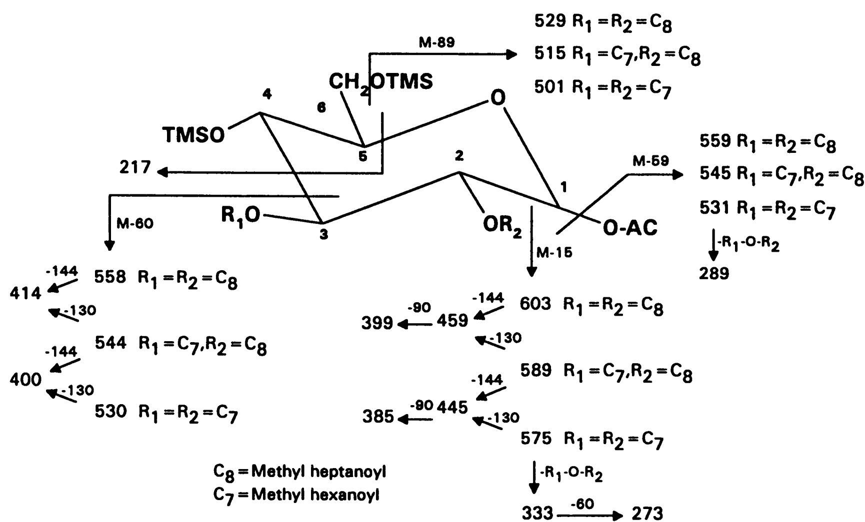


Figure 8. MS Fragmentation of Glucose Ester Fraction GEI from *N. gossei*.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

Table II. Evaluation of the *N. gossei* Sugar Esters Biorational Against Nymphs of the Greenhouse Whitefly

Spray Material	Spraying Rate Percent	% Mortality
N. gossei SE (SEI & SEII)	0.10	99
N. gossei Biorational	0.20	90
N. gossei Biorational	0.15	96
N. gossei Biorational	0.10	95
N. gossei Biorational	0.05	89
M-Pede[a]	2.00	100
M-Pede[a]	1.00	99
Neem[b]	2.50	37
Neem[b]	5.00	24

[a]Potassium salts of naturally derived fatty acids, Mycogen Corporation, San Diego, CA.
[b]Clarified neem oil, 90% conc., W. R. Grace & Company, Lexington, MA.

Table III. Comparison of Effectiveness of the *N. gossei* Biorational, M-Pede and Sun Spray Ultrafine Oil Against Nymphs of the SweetPotato Whitefly on Cotton[a]

Spray Material	Rate	% Mortality[b]
N. gossei Biorational	0.1%	78
M-Pede[c]	2.0%	58
Sun Spray Ultrafine Oil[d]	2.0%	65

[a]Conducted at the Western Cotton Research Laboratory, Phoenix, AZ
[b]Relative to untreated control
[c]Potassium salts of naturally derived fatty acids, Mycogen Corporation, San Diego, CA.
[d]Petroleum oil base, Safer, Newton, MA.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

gossei material. Akey also conducted a small field test against the SPWF on cotton at the Maricopa Agriculture Center, Maricopa, AZ. Applying the NGB at a rate of 0.51 lb ai/ac, 94% reduction of SPWF immatures relative to the untreated control was achieved. Topical treatment of tobacco aphids with NGB resulted in 100 % mortality after 48 hours. Survival for the control was 23/30 for untreated aphids and 18/30 for those treated with the solvent blank.

This report has demonstrated the feasibility of growing *N. gossei* to obtain sufficient amounts of cuticular extract for isolation of the sugar ester fraction for field evaluation. Methodology to isolate the esters for bioassay was developed which permits the recovery of solvents and the reactivation and use of the LH-20 chromatographic system. The *N. gossei* sugar esters are a very active biorational against the SPWF, GHWF and tobacco aphids. Further tests, including dose-response tests, tests against other insects, and mechanism of action are needed.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

LITERATURE CITED

1. Severson, R. F.; Arrendale, R. F.; Chortyk, O. T.; Johnson, A. W.; Jackson, D. M.; Gwynn, G. R.; Chaplin, J. F.; Stephenson, M. G. *J. Agric. Food Chem.* **1984**, *32*,556-570.

2. Severson, R. F.; Johnson, A. W.; Jackson, D. M. *Recent Advances in Tobacco Science* **1985**, *11*,105-174.

3. Severson, R. F.; Eckel, R. V. W.; Jackson, D. M.; Sisson, V. A.; Stephenson, M. G. In *Natural and Engineered Pest Management Agents*; Hedin, P, Ed; ACS Symposium Series 551. American Chemical Society: Washington, DC, 1991, pp 172-179.

4. Jackson, D. M.; Severson, R. F.; Johnson, A. W. J *Chem Ecol.* **1986,** *133*,1359.

5. Jackson, D. M; Severson, R. F; Johnson, A. W.; *Recent Advances in Tobacco Science* **1989**, *15*,26-116.

6. Jackson, D. M.; Severson, R. F.; Sisson, V. A.; Stephenson, M. G. *J. Chem. Ecology.* **1991,** *17*(2),2489-2515.

7. Severson, R. F.; Jackson, D. M.; Johnson, A. W.; Sisson, V. A.; Stephenson, M. G. In *Naturally Occurring Pest Bioregulators*; Hedin, P., Ed.; ACS Symposium Series 449; American Chemical Society: Washington, DC, 1991, Vol. 17,pp 264-277.

8. Severson, R. F.; Arrendale, R. F.; Chortyk, O. T.; Green, C. R.; Thome, F. A.; Stewart, J. L.; Johnson, A. W. *J. Agric. Food Chem.* **1985**, *33*,870-875.

9. Cutler, H. G.; Severson, R. F.; Cole, P. D.; Sisson, V. A.; Jackson, D. M.; Stephenson, M. G. *PGRSA Quarterly*, **1992**, *20*(1),18-28.

10. Matsuzaki, T.; Shinozaki, Y.; Hagimori, M.; Tobita, T.; Shigematsu, H.; Koiwai, A. *Biosci. Biotech. Biochem*, **1992,** *56*,1565-1569.

11. Matsuzaki, T.; Shinozaki, Y.; Suhara, S.; Shigematsu, H,; Koiwai, A. *Agric. Biol. Chem.*, **1989.** *53*,3343.

12. Matsuzaki, T.; Shinozaki, Y.; Suhara, S.; Ninomiya, M.; Shigematsu, H.; Koiwai, A. *Agric. Biol. Chem*, **1989**, *53*,3079-3082.
13. Buta, J. G.; Lusby, W. R.; Neal, J. W. Jr.; Waters, R. M.; Pittarelli, G. W. *Phytochemistry*, **1993**, *32*,859-864.
14. Shinozaki, Y.; Matsuzaki, T.; Suhara, S.; Tobita, T.; Shigematsu, H.; Koiwai, A. *Agric. Biol. Chem.*, **1991**, *55*,751-756.
15. Shinozaki, Y.; Matsuzaki, T.; Suzuki, H.; Shigematsu, H.; Koiwai, A. *Biosci. Biotech. Biochem.*, **1992**, *56*,1482-1483.
16. Arrendale, R. F.; Severson, R. F.; Sisson, V. A.; Costello, C. E.; Leary, J. E.; Himmelsbach, D. S.; van Halbeeck, H. *J. Agric. Food Chem.*, **1990**, *38*,75-85.
17. Matsuzaki, T.; Koseki, K.; Koiwai, A. *Agric. Bio.. Chem.*, **1988**, *52*,1889-1897.
18. Matsuzaki, T.; Shinozaki, Y.; Suhara, S.; Tobita, T.; Shigematsu, H.; Koiwai, A. *Agric. Biol. Chem.*, **1991,** *55*,1417.
19. Cutler, H. G.; Severson, R. F.; Montemurro, N.; Cole, P. D.; Sisson, V. A.; Stephenson, M. G. *PGRSA Quarterly*, **1991**, *19*,69.
20. Neal, J. W., Jr.; Pittarelli, G. W.; Gott, K. M. *Tob. Sci.*, **1987**, *31*,61.
21. Pittarelli, G. W.; Buta, J. G.; Neal, J. W. Jr.; Lusby, W. R.; Waters, R. M. Biological Pesticide Derived from Nicotiana Plants, U. S. Patent # 5,260,281.
22. Severson, R. F.; Sisson, V. A.; Smith, L. B., King, B. G., Chortyk, O. T. **1988**, *Abstracts of Papers*, Tob. Chem. Res. Conf. *42*,38.
23. Akey, D. H.; Neal, J. W. Jr.; Severson, R. F.; Stephenson, M. G.; Pittarelli, G. W.; Buta, J. G. In *First Annual Review of the 5-year National Research and Action Plan*, ARS-112, USDA-ARS, Tempe, AZ. 1993, p54.
24. Severson, R.F.; Stephenson, M. G.; Johnson, A. W.; Jackson, D. M.; Chortyk, O. T. *Tob. Sci.*, **1988**, *32*,99-103.
25. Severson, R. F.; Chortyk, O. T.; Stephenson, M. G.; Pittarelli, G. W.; Neal, J. W. Jr.; Buta, J. G.; Jackson, D. M.; Sisson, V. A. *Abstracts of Papers,* Tob. Chem. Res. Conf. 1992, *46*,39-40.
26. Binkley, W.W.; Dougherty, R. C.; Horton, D.; Wanter, J. D. *Carbohydr. Res.*,**1971**, *17*,127-144.

RECEIVED March 11, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch009

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

Chapter 10

New Flavone C-Glycosides from Corn (*Zea mays* L.) for the Control of the Corn Earworm (*Helicoverpa zea*)

Maurice E. Snook[1], Neil W. Widstrom[2], Billy R. Wiseman[2], Richard C. Gueldner[1], Richard L. Wilson[3], David S. Himmelsbach[4], John S. Harwood[5], and Catherine E. Costello[6]

[1]Phytochemical Research Unit, Richard B. Russell Research Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 5677, Athens, GA 30613
[2]Insect Biology and Population Management Research Laboratory, P.O. Box 748, Tifton, GA 31793
[3]Agricultural Research Service, U.S. Department of Agriculture, University of Iowa, Ames, IA 50011
[4]Plant Structure & Composition Research Unit, Richard B. Russell Research Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 5677, Athens, GA 30613
[5]Department of Chemistry, University of Georgia, Athens, GA 30602
[6]Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139

Zapalote Chico cornsilks are resistant to the corn earworm, *Helicoverpa zea* (Boddie), because of the presence in the silks of one major luteolin-C-glycoside called maysin. A recent HPLC screening of large numbers of corn germplasm for maysin content has resulted in the discovery of several other inbreds and populations with high levels of maysin. This screening led to the discovery of several lines with relatively high levels of flavone-C-glycosides, other than maysin. Tx501 contained high levels of maysin, 3'-methoxymaysin and their dihydro-derivatives. NC7 and SC353 had high levels of the apigenin-analogue of maysin (apimaysin). T218, T315 and PI340853 silks contained large levels of 6-C-galactosyl-luteolin. Laboratory bioassays were used to determine the antibiosis activity of these new flavones as compared to maysin. 6-C-galactosyl-luteolin was almost as active as maysin, while apimaysin and 3'-methoxymaysin were about half as active in reducing corn earworm growth. Incorporation of these new flavones into corn silks should yield additional corn earworm resistance.

0097–6156/94/0557–0122$08.00/0
© 1994 American Chemical Society

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

The natural resistance of Zapalote Chico (ZC) corn silks to the corn earworm (*Helicoverpa zea*) has been attributed to the presence in the silks of a single flavone called maysin [2"-*O*-α-L-rhamnosyl-6-C-(6-deoxy-xylo-hexos-4-ulosyl)-luteolin] (Figure 1.) (*1-3*). Waiss et al. (*1*) and Wiseman (*4-5*) demonstrated that the activity of maysin and of Zapalote Chico cornsilks was true antibiosis. Maysin can thus be considered a natural insecticide. Wiseman showed that maysin was also active against the fall armyworm *Spodoptera frugiperda* (*6*). Elliger et al. (*3*) tested a number of flavonoids for growth inhibition against the corn earworm and demonstrated that the presence of a vicinal dihydroxy group was essential for activity, a structure that maysin possesses.

We recently developed a high performance liquid chromatographic (HPLC) method for the determination of maysin in corn silks (*7*) and showed that HPLC provided a more accurate quantitation of maysin levels (*8*) than the previous spectrophotometric method (*1*). Further, HPLC allowed a more complete profile of the flavone contents of the silks than was previously possible. Using HPLC, we recently identified several germplasm sources, other than ZC, that had maysin levels in their silks approaching that of ZC (*6-7*). To date, we have surveyed the maysin content of the silks from over 600 corn inbreds, populations, Plant Introductions (PI), and various unassigned collections. In addition to discovering many new sources of corn with high silk maysin, several lines were identified that contained high levels of related flavones. This paper reports preliminary identifications of these new flavones and their biological activity towards the corn earworm in a laboratory bioassay.

Materials and Methods

Plant Material. The majority of the plants were grown in 1989 and 1991 at the Coastal Plain Experiment Station, Tifton, GA under standard cultural practices of fertilizer and weed control. Silks were covered to prevent pollination and were sampled when 3-5 days old.

HPLC Analysis. Sufficient numbers of plants were sampled to give approximately 30 g of silk/sample. The silks were weighed, immediately placed in 8 oz jars (Teflon-lined cap) and the jars were filled with 100% MeOH (approx. 180 mL). Samples were stored at 0° until analysis. Samples were warmed to room temperature and 6 mL of chrysin, dissolved in methanol (1.6 mg/mL, recrystallized from amyl alcohol), was added as internal standard. After ultrasonication for 20 min, aliquots of the solution were analyzed by reversed-phase HPLC, as described before (*7*), using a H_2O/MeOH linear gradient from 20% to 90% MeOH in 35 min, a flow rate of 1 mL/min, and detection at 340 nm. Each solvent contained 0.1% H_3PO_4. Most analyses were performed with an Altex Ultrasphere C18, 5 micron (4.6 X 250 mm) (Beckman Instruments, Norcross, GA) column. Additional analyses were made with a Hypersil Phenyl, 5 micron (4.6 X 250 mm, Alltech Associates, Deerfield, IL) column.

Isolation of flavone glycosides. All solvents were analytical reagent grade. Fast atom bombardment mass spectroscopic (FAB-MS) data were obtained in a glycerol

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

matrix, as described before (*8*). ^1H and ^{13}C NMR data were obtained in CD_3OD or DMSO-d_6 at either room temperature or 60° with Bruker AM250 or AC300 spectrometers.

Typical isolation procedures for silk flavone glycosides were as follows.

(1) Extraction. Silks were slurried with 100% MeOH, filtered, and the MeOH/H_2O solution was concentrated with a rotary evaporator until only an aqueous solution remained. The resulting water/sample mixture was extracted with CH_2Cl_2 followed by extraction with n-butanol. HPLC analysis of the aqueous layer showed that a near quantitative extraction of the flavone glycosides was obtained into the n-butanol extract. The n-butanol was evaporated to dryness (a small amount of water, added at the end of the evaporation, facilitated the removal of the last traces of n-butanol). The residue was dissolved in 40% MeOH/H_2O and submitted to preparative reversed-phase column chromatography.

(2) Isolation. Isolation was mainly by preparative reversed-phase column chromatography. The packing material from a Waters PrepPAK 500 C18 cartridge (Millipore Corp., Milford, MA) was repacked into a smaller glass Cheminert LC column (54 X 2.54 cm, Valco Instruments Co., Inc., Houston, TX), washed with MeOH and recycled to H_2O. The n-butanol residue (dissolved in water) was chromatographed by applying the sample to the column with a loop injection valve and eluted with water (to remove sugars) and then 50% MeOH/H_2O to elute the flavones.

The flavonoid fraction was evaporated to dryness and submitted to silicic acid (SA- Mallinckrodt, 100 mesh, washed with methanol and activated at 155° for 1 hr) column chromatography. The column was packed in CH_2Cl_2 and after applying the sample to the top of the column (as a SA/sample deposited mixture), eluted with CH_2Cl_2 followed by ethyl acetate or acetone/ethyl acetate mixtures. Most of the flavones of interest were found in the ethyl acetate eluant. After evaporation to dryness, the SA separated flavones were dissolved in 40% MeOH/H_2O and submitted again to reversed-phase chromatography using the following linear solvent program: 40-60% MeOH/H_2O in 400 min; 8 mL fractions were collected; column effluent monitored at 340 nm.

(3) Identification. Isolated flavones were identified by UV, NMR (^1H and ^{13}C), and FAB/MS spectrometric methods.

Bioassay Procedures

(1) Silk extract bioassay. Silk/methanol extracts were bioassayed by the method of Wiseman, et al. (*6*). Silks were excised at the ear tip and immediately weighed; 30 g were placed in 200 mL MeOH and frozen. The silk/solutions were then warmed to room temperature, ultrasonicated for 20 min, and a 4 mL aliquot removed for maysin analysis. The remaining solution was filtered into a 1 L roundbottom flask, 5 g of celufil (U.S. Biochemical, Cleveland, OH) was added and the solvent was evaporated to deposit the extract onto the celufil. The dried

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

celufil/extract mixture was then added to 50 g of diluted pinto bean diet (3 mL diet:2 mL water), 10 mL were dispensed into plastic diet cups and one neonate corn earworm added. After 8 days the weights of the worms were recorded. Appropriate MeOH/celufil blanks were used. The experiment was arranged in a randomized complete block design with 15 replications.

(2) Model compound bioassay. Isolated flavones or commercially available compounds (rutin, luteolin, and chlorogenic acid) were deposited onto celufil as above. Concentrations for each compound were 240, 120, 60, and 30 mg/2 g celufil. Each compound/celufil mixture was added to 25 g of diluted pinto bean diet. Detached, disposable plastic pipette bulbs were filled with 2 g of the diet/celufil mixture, allowed to solidify, and one neonate corn earworm was placed on the diet. The bulbs were placed in diet cups and worm weights measured after 8 days. There were ten replications for each compound concentration.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

Results and Discussions

I. New sources of high maysin germplasm

Previously, Waiss (*9*) showed that a level of maysin of 0.15% (wgt/wgt of diet) in laboratory bioassays reduced corn earworm larval weights by 50%. Wiseman (*6*) subsequently showed, in a more detailed study, using a variety of different corn lines and crosses, that a highly significant negative relationship existed between maysin concentration in fresh silks and weights of corn earworm larvae fed diets containing methanol-silk extracts. Their study indicated that silk maysin concentration of 0.2% (fresh wgt) reduced larval weights to about 50% and that higher maysin levels (>0.4%) reduced larval weights to <70% of controls. From these data, 0.2% has been selected as the minimum level needed for resistance, with levels >0.3% most desirable, because of possible yearly variation.

Since the first report of the resistance of Zapalote Chico to the corn earworm, effort has been made to cross ZC with other corn lines in order to develop new, stable corn inbreds with a sufficient level of maysin for resistance but, with more desirable agronomic characteristics than ZC. The development of a high-performance liquid chromatograpic (HPLC) method, which gave a more accurate measure of maysin concentration in corn silks, stimulated this research and has led to searches for other corn lines with high maysin and more favorable characteristics. Flavone analyses were performed on the methanol extracts of the silks of 370 inbreds and 160 populations of corn, selected as representing a broad genetic base (*10*). In addition, 81 Plant Introductions (PI's) and 36 unassigned germplasm sources of corn from the North Central Regional PI Station, Ames, Iowa, were also analyzed. The results of these analyses showed that there is a wide range in silk maysin levels, from <0.01% to >0.5% fresh weight. Table I gives a partial listing of inbreds and populations with levels of maysin >0.3%.

As expected, the majority of lines tested (82%) contained levels of maysin below that considered necessary for activity. Fully 24% of the lines were completely

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Table I. High Silk Maysin Corn Inbreds and Populations

Inbreds[a] (% Fresh Weight)							
GE37	0.90	Ab616	0.49	CI64	0.36[b]	Akd24	0.31[b]
GE80	0.85	CoeG12	0.47[b]	CoeG21	0.36	NC64	0.31[b]
L329	0.65[b]	Ab602	0.46[b]	0-835	0.33	CI83A	0.31[b]
9-96A	0.53	Ab16	0.44[b]	0-1836	0.32[b]	SC249	0.30[b]
GE58	0.52	894OC	0.42	E2629P	0.32[b]	T315	0.30[b]
91201Y	0.52	0-909	0.39	B14(T)	0.32[b]	SC102	0.30[b]
WF-038B	0.52	SC114	0.38	CI317B	0.31[b]	GE74	0.30
T226	0.52	1-1566	0.38	SC245A	0.31[b]	GE84	0.30
GT169a	0.51	NC45	0.36[b]	A102	0.31[b]	F98	0.30

Populations[a] (% Fresh Weight)			
Oax Comp Grp.	0.56	1299X 1T#	0.37[b]
RFC-RI (C9)	0.56	MAS:gk	0.36
998x 1T#	0.45[b]	PR70B602-604	0.36
2280x 1T#	0.41[b]	Z. Chico (2451)#	0.35[c]
Catito Limon	0.39	Tabloncillo X's	0.34

[a]Single year determination.
[b]2-Year average.
[c]3-Year average.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

devoid of maysin or only possessed trace amounts of maysin. In this study, we found a number of corn inbreds and populations with high silk maysin levels above the 0.2% fresh weight threshold, considered significant for corn earworm antibiosis. Approximately 1/5 of both the inbreds and populations were found to have maysin levels >0.2%. Fully 1/3 of these high maysin lines contain silk-maysin, that is far greater than ZC, based on the amount of maysin per quantity of silk. Of the PI's evaluated, the silks of PI340856 averaged 0.743% maysin, over 3 years. This PI is a popcorn from the Eldredge collection. These lines form an important, new genetic base for breeding studies to produce agronomically acceptable corn earworm resistant germplasms.

II. Isolation and Identification of New Corn Silk Flavones

In addition to identifying corn germplasm with high maysin contents, the survey resulted in the discovery of several inbreds, populations and PI's with very high levels of flavone glycosides related to maysin. These compounds were isolated by a combination of solvent partitioning and silicic acid, followed by preparative reverse-phase column chromatography. HPLC profiles of two of these unique inbreds (NC7 and Tx501) are given in Figure 2. They are compared to the HPLC profile of Zapalote Chico. Zapalote Chico contained low levels of two flavone glycosides (peaks A & B), eluting after maysin. The inbred, NC7, was found to contain high levels of Compound A, while Tx501, contained high levels of Compound B [subsequently identified as the apigenin-analogue of maysin (A- called apimaysin) and 3'-methoxymaysin (B) (Figure 1]. These two varieties were convenient sources for the isolation of these two compounds.

Ultraviolet spectra, fast-atom bombardment mass spectroscopy (FAB-MS), and NMR, especially ^{13}C-NMR, proved particularly helpful in identifying these unknown flavones. The UV spectra indicated Compound A was an apigenin-derivative, while Compound B was a luteolin-derivative. Usually acid hydrolysis is the method of choice for identification of flavonoids with ether-linked sugars, yielding the aglycone and both individual types of sugars in their appropiate ratios (by GC of their silyl-derivatives). Maysin will yield rhamnose under acid hydrolysis but, being a C-glycoside, will then rearrange and decompose with no liberation of the aglycone. This Wessely-Moser rearrangement is well known for C-glycosides (*11*). Only FAB-MS will give a molecular weight (MW+1 in positive ion mode) for these types of compounds plus additional information of the nature of ether-linked sugars (MW+1 minus 146 = rhamnose; MW+1 minus 162 = hexose, i.e. galactose or glucose). FAB-MS of Compound A showed a MW of 560 and a loss of 146 amu, indicating an ether-linked rhamnose. Compound B had a MW of 590 and also a loss of rhamnose.

The ^{13}C-NMR of maysin, Compound A, and Compound B are given in Figure 3 and illustrate how a comparison of the three can provide important structural features for the unknown compounds. Absorptions in all three compounds at about 204 ppm indicates that all still retain the carbonyl on the deoxy-xylo-hexos-4-ulosyl (the ketorhamnosyl) portion of the molecule. The wide spacing of the two rhamnosyl methyl absorptions at 17 and 14 ppm of A & B indicate these methyls are in the same environment as in maysin. This is further confirmed by the nearly identical sugar absorptions between 68 and 80, and at 100 ppm. The ^{13}C-NMR prove that A & B both retain the 2"-*O*-α-L-rhamnosyl-6-C-(6-deoxy-xylo-hexos-4-ulosyl) sugar structure present in maysin. Absorptions between 90 and 182 ppm indicate the type of aglycone present. The typical luteolin pattern of maysin (confirmed by comparison to standards (*12*) is present in Compound B, while the pattern of apigenin is present in Compound A. Further inspection shows that Compound B has a methoxyl absorption at 56 ppm. Analysis of all the data indicates that Compound A is 2"-*O*-α-L-rhamnosyl-6-C-(6-deoxy-xylo-hexos-4-ulosyl)-apigenin (which we call apimaysin) and Compound B is 2"-*O*-α-L-rhamnosyl-6-C-(6-deoxy-xylo-hexos-4-ulosyl)-3'-methoxyluteolin (3'-methoxymaysin) (Figure 1).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

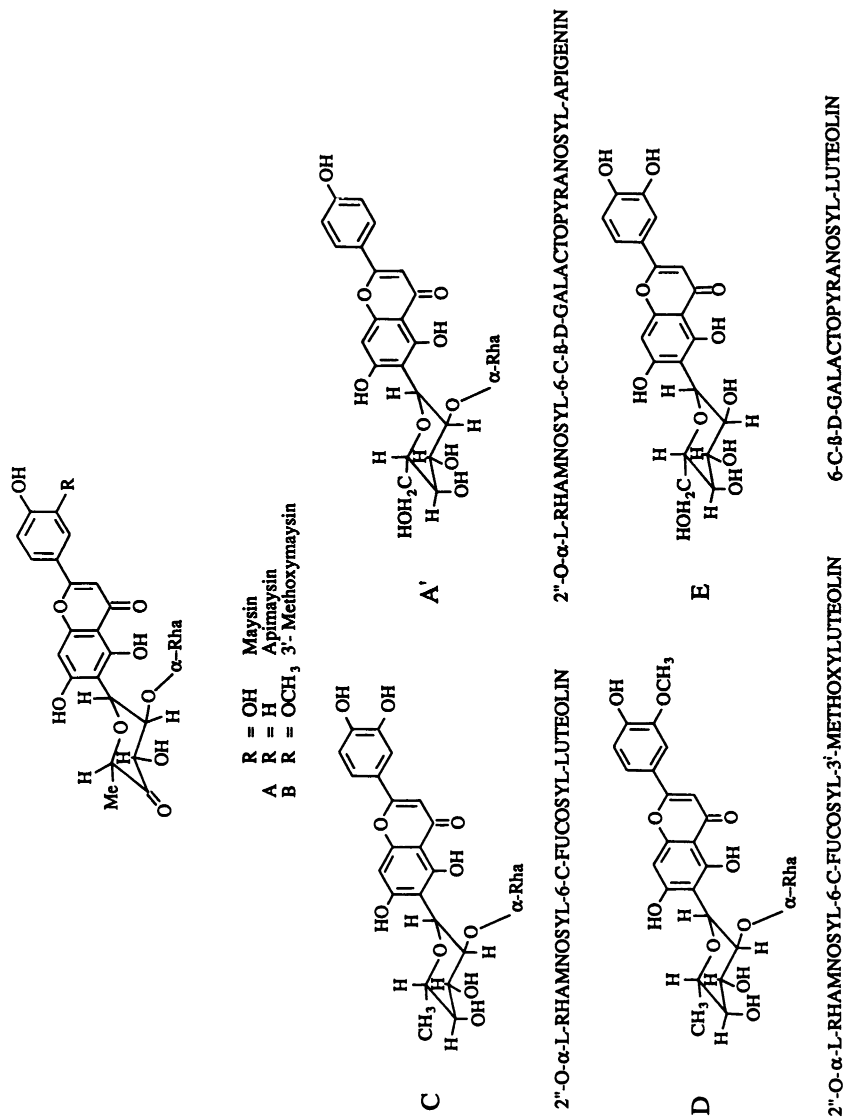


Figure 1. Structures of maysin and maysin-analogues isolated from corn silks.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

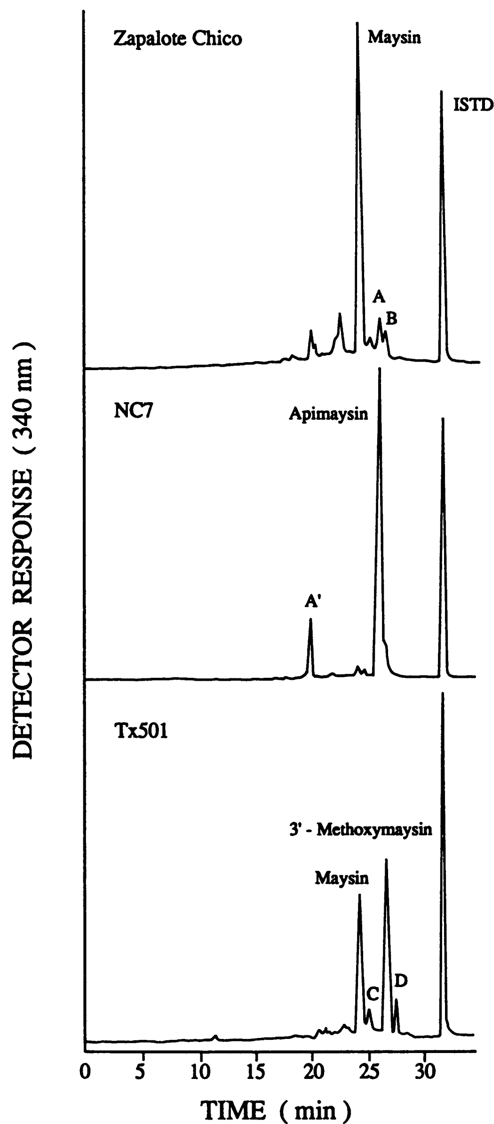


Figure 2. HPLC profiles of Zapalote Chico, NC7, and Tx501 silks.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

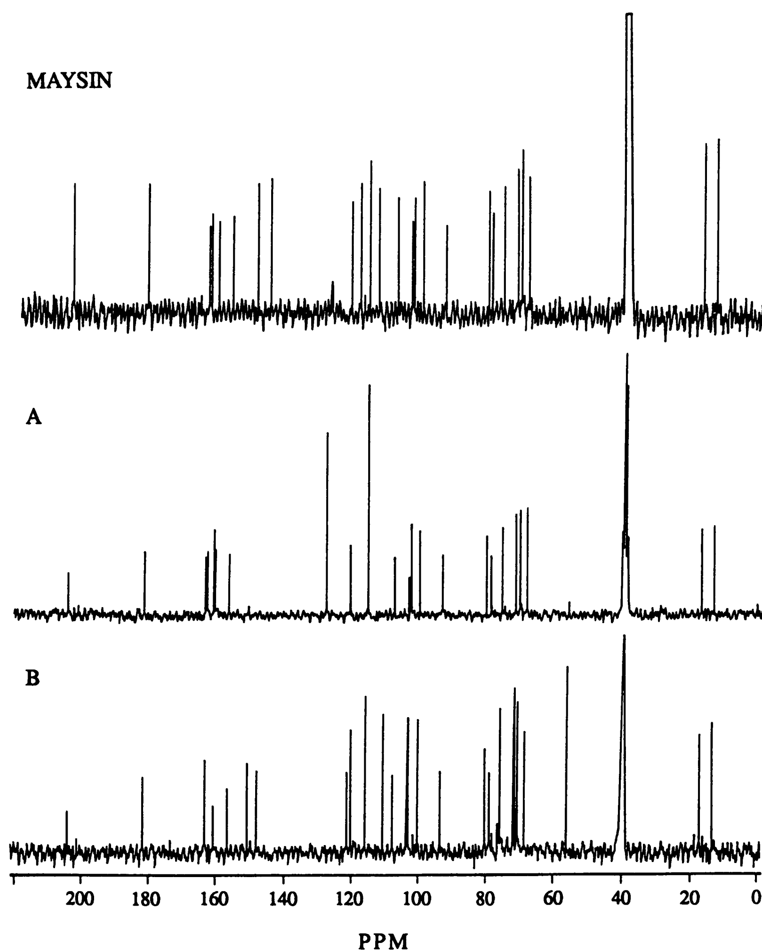


Figure 3. ^{13}C-NMR spectra of maysin, Compound A (apimaysin), and Compound B (3'-methoxymaysin).

Although most corn lines with high maysin levels also contained small amounts of either apimaysin, 3'-methoxymaysin, or both compounds, very few lines contained large amounts of these flavones. Elliger et al. (*2*) previously isolated apimaysin and 3'-methoxymaysin from Zapalote Chico, in which they occur in minor amounts. Our analysis of Zapalote Chico showed apimaysin and 3'-methoxymaysin to be present in only 0.019 and 0.045% fresh weight, while maysin was at the 0.35% level (averaged over 4 years). Only one line, the inbred NC7, was found to contain appreciable levels of apimaysin. It is unique in the quantity of this flavone it produces (0.614% fresh weight), along with only a minor amount of maysin. SC353 is another good source of maysin and apimaysin (0.40% and 0.22% respectively). Only one population line was found to contain high apimaysin (3146 1T#). Good sources of 3'-methoxymaysin are inbreds 9-201, SC144, CI64, and Tx501.

Inbred line Tx501, contained two other flavones, Compounds C and D (Figure 2), eluting immediately after maysin and 3'-methoxymaysin, respectively. Both compounds possessed luteolin UV spectra. FAB-MS indicated C had a MW of 2 more than maysin and facile loss of rhamnose, while D had MW of 2 more than methoxymaysin and also loss of rhamnose. The ^{13}C-NMR showed that, in both compounds, the carbonyls on the keto-rhamnoses have been reduced and both rhamnose methyls are almost equivalent. A more detailed analysis of the ^{1}H-NMR data indicates the new hydroxyls at C-4" are axial. The conversion of the keto group to a hydroxyl explains the now almost equivalent methyls. The postulated structures of C and D are 2"-*O*-α-L-rhamnosyl-6-C-fucosyl-luteolin (dihydromaysin) and its 3'-methoxy-derivative respectively (dihydro-3'-methoxymaysin) (Figure 1).

In our HPLC survey of the inbreds and populations, one line was unusual in that it was found to contain all of the compounds identified above. Population 2-043 (Figure 4) contained, in addition to maysin, apimaysin (Compound A), 3'-methoxymaysin (Compound B), and the dihydro-derivaties of maysin and 3'-methoxymaysin (Compounds C and D).

Inbred NC7 contained another apigenin flavone (Figure 2, peak A') in addition to apimaysin. Its ^{13}C-NMR exhibited no carbonyl absorption for C-4" and only one rhamnose methyl. This, together with a MW of 578, loss of rhamnose from the molecular ion, and an apigenin UV, indicated the methyl at C-6" had been oxidized to a CH_2OH group. Assuming an axial hydroxyl at C-4, the compound has been tentatively identified as 2"-*O*-α-L-rhamnosyl-6-C-β-D-galactopyranosyl-apigenin (Figure 1).

The inbred, T218 (Figure 5), contained relatively large levels of a luteolin-flavone (UV spectra), that eluted several minutes earlier than maysin from the HPLC (Peak E). After isolation, the compound was submitted to spectroscopic analysis. FAB-MS gave a MW of 448 indicating the ether-linked rhamnose was not present. The ^{13}C-NMR confirmed that no rhamnose methyls were present proving the O-linked rhamnose was absent, that the methyl at C-6" was now a CH_2OH and that the ketone at C-4" was reduced. Further analysis of the ^{1}H-NMR indicated the hydroxyl group at C-4 was in the axial configuration. The structure of Compound E has been tentatively assigned 6-C-galactopyranosyl-luteolin (Figure 1).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

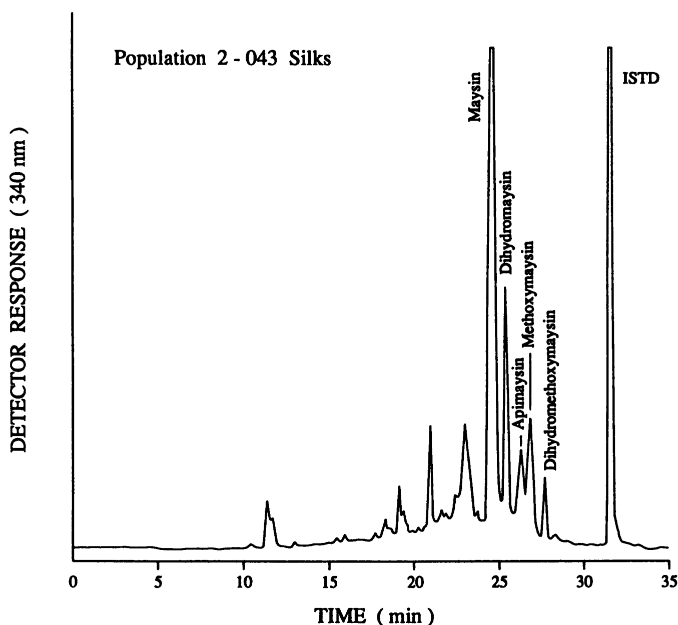


Figure 4. HPLC of silk extract of population 2-043 containing various maysin-analogues.

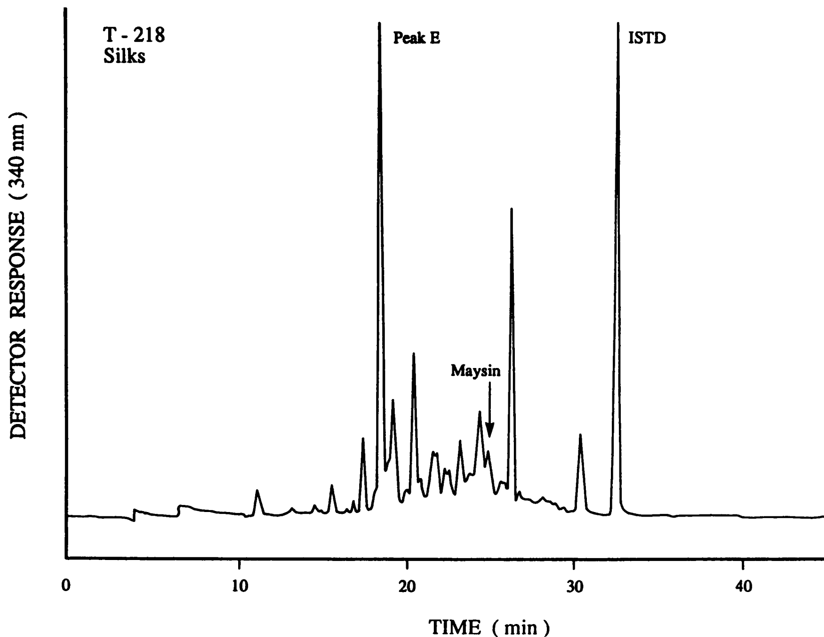


Figure 5. HPLC profile of T218 silks showing presence of early eluting maysin analogue (Peak E, 6-C-galactosyl-luteolin).

III. Biological Activity of Maysin and Maysin-analogues

Silks from various corn inbreds and their crosses with Zapalote Chico and a high silk maysin popcorn line- PI340856, along with Zapalote Chico, PI340856, and Stowell's Evergreen controls were extracted with methanol and the extracts were deposited onto celufil and incorporated into pinto bean diets (*10*). Corn earworms were allowed to feed on the diets and their weights were recorded after eight days. A plot of maysin concentration of the silks versus corn earworm weights is given in Figure 6. Maysin levels varied from 0 to 1.085% fresh weight. A highly significant ($p < 0.001$) negative relationship was observed for the weights of the corn earworm and concentration of maysin in the diet-extract mixtures ($r = -0.81$, n=54). A concentration of >0.2% maysin reduced larval growth to >50% of that of the control, while higher levels of maysin (up to 1%) reduced weights of the larvae to >80%.

Corn earworm laboratory bioassays were performed on the following polyphenols: maysin, luteolin, rutin, and chlorogenic acid. As shown in Figure 7, maysin reduced the weight of corn earworms by about 84% of controls (at 12.6 mM conc.). Rutin, (quercetin-3-rutinoside) possessing the requisite ortho-dihydroxy structure, was found to be just as active as maysin. In fact, the presence of the sugar moiety is not even needed, since the aglycone- luteolin was found to be just as active as maysin (diglycosyl-luteolin). Chlorogenic acid also has an ortho-dihydroxyphenyl structure and is found in small amounts in corn silk. It was found to be active against the corn earworm, resulting in an 80% reduction of growth at 20.5 mM concentration.

The isolated corn flavones-maysin, apimaysin, 3'-methoxymaysin, and galactoluteolin were tested in the microbioassay method (Figure 8). In this test,

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

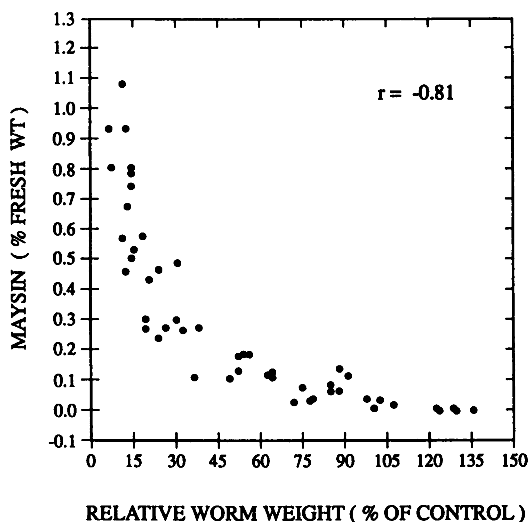


Figure 6. Levels of silk maysin versus corn earworm weights obtained in the laboratory bioassay.

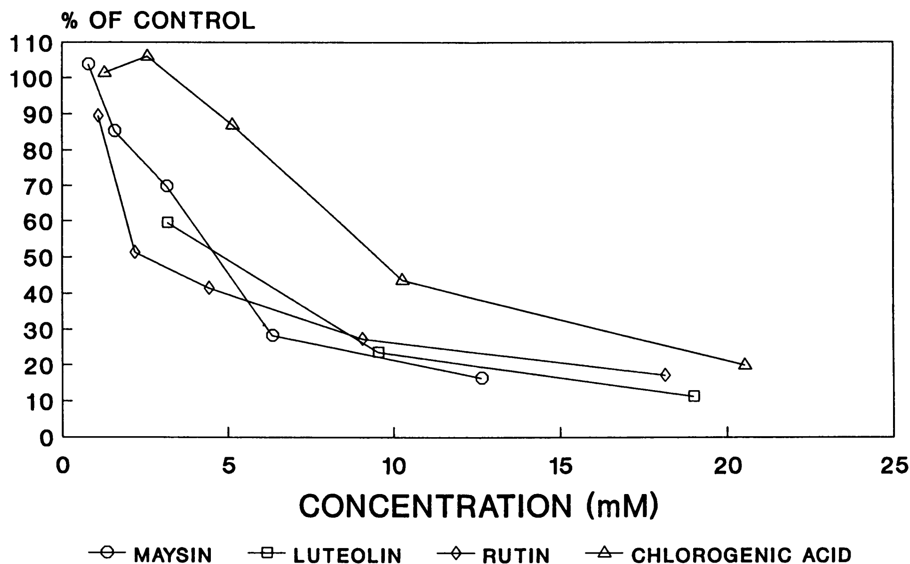


Figure 7. Growth of the corn earworm versus concentration of polyphenols in laboratory pinto bean diets.

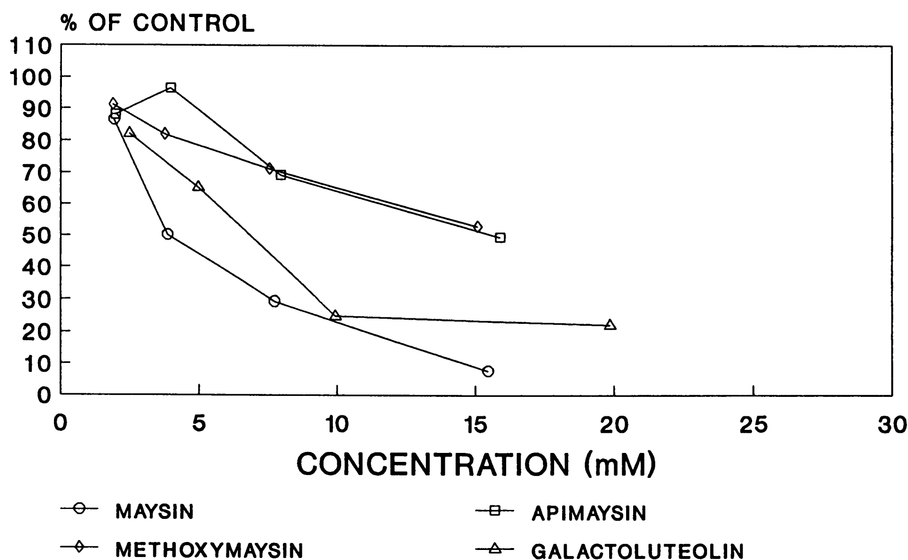


Figure 8. Growth of corn earworm versus concentration of corn flavones in pinto bean diets.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

maysin reduced the weights of the corn earworm by 92% (15.4 mM) while galactoluteolin gave worm weights about 76% of controls at the highest level tested (19.85 mM). Apimaysin and 3'-methoxymaysin both gave about 50% inhibition of growth at the maximum concentrations tested (15.9 and 15.1 mM respectively). Elliger (*3*) reported 3'-methoxymaysin as about half as active as maysin based on ED_{50} concentrations (mM/kg to retard growth to 50% of control).

The bioassay data show that maysin, galactoluteolin, and chlorogenic acid are comparable in activity against the corn earworm. Recently, one line has been identified with relatively high levels (>0.3% fresh wt) of maysin and galactoluteolin while another line was found with high levels of chlorogenic acid. Breeding experiments are currently underway to incorporate all three active compounds into one line that hopefully, will possess extremely high antibiosis activity against the corn earworm and be useful for production of naturally resistant hybrids.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch010

Literature Cited

1. Waiss, Jr., A. C.; Chan, B. G.; Elliger, C. A.; Wiseman, B. R.; McMillian, W. W.; Widstrom, N. W.; Zuber, M. S.; Keaster, A. J. *J. Econ. Entomol.* **1979**, *72*, 256-258.
2. Elliger, C. A.; Chan, B. G.; Waiss, Jr., A. C.; Lundin, R. E.; Haddon, W. F. *Phytochemistry* **1980**, *19*, 293-297.
3. Elliger, C. A.; Chan, B. C.; Waiss, Jr., A. C. *Naturwissenschaften* **1980**, *67*, 358-360.
4. Wiseman, B. R.; Widstrom, N. W.; McMillian, W. W. *Florida Entom.* **1981**, *64*, 395-399.
5. Wiseman, B. R.; Widstrom, N. W.; Waiss, Jr., A. C. *J. Agric. Entomol.* **1984**, *1*, 53-57.
6. Wiseman, B. R.; Snook, M. E.; Isenhour, D. J.; Mihm, J. A.; Widstrom, N. W. *J. Econ. Entomol.* **1992**, *85*, 2473-2477.
7. Snook, M. E.; Widstrom, N. W.; Gueldner, R. C. *J. Chromatogr.* **1989**, *477*, 439-447.
8. Widstrom, N. W.; Snook, M. E.; McMillian, W. W.; Waiss, Jr., A. C.; Elliger, C. A. *J. Agric. Food Chem.* **1991**, *39*, 182-184.
9. Waiss, Jr., A. C.; Chan, B. G.; Elliger, C. A.; Dreyer, D. L.; Binder, R. G.; Gueldner, R. C. Insect growth inhibitors in crop plants. *Bull. Entomol. Soc. Am.* **1981**, *27*, 217-221.
10. Snook, M. E.; Gueldner, R. C.; Widstrom, N. W.; Wiseman, B. R.; Himmelsbach, D. S.; Harwood, J. S.; Costello, C. E. *J. Agric. Food Chem.* **1993** (in press).
11. Chopin, J.; Bouillant, M. L. In *The Flavonoids*, Harborne, J. B.; Mabry, T. J.; Mabry, H., Eds. Academic Press, New York, NY 1975.
12. Markham, K. R.; Chari, V. M., Eds. *The Flavonoids: Advances in Research*, Chapman & Hall, New York, NY 1982.

RECEIVED February 10, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch011

Chapter 11

Inhibition of Phytotoxin (Viridiol) Biosynthesis in the Biocontrol Agent *Gliocladium virens*

R. D. Stipanovic and C. R. Howell

Southern Crops Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, College Station, TX 77845

Triazole fungicides that inhibit cytochrome P-450 enzymatic 14-α-demethylation of sterols also inhibit the biosynthesis of the phytotoxin viridiol produced by the biocontrol agent *Gliocladium virens*. Concentrations (0.5 - 1 ppm) of fungicides required to inhibit viridiol biosynthesis do not inhibit growth of the biocontrol agent.

The plant pathogens *Pythium ultimum* and *Rhizoctonia solani* cause significant losses to cotton and other economically important agricultural crops. These pathogens generally attack the plant during the early stages of growth and are thus termed "seedling diseases." Until recently, various agrochemicals applied as a seed treatment were the only methods available to protect plants from these pathogens. The current emphasis on reducing the use of agrochemicals has led to a search for alternative means of controlling the plant pathogens that cause seedling diseases, and the exploitation of beneficial microbes as biocontrol agents has received increased scrutiny.

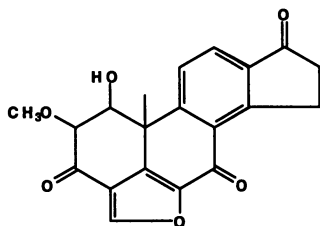
Gliocladium virens as a Biocontrol Agent

The fungus, *Gliocladium virens*, appears to offer a viable alternative or enhancement to agrochemicals in controlling seedling diseases (1). The biocontrol efficacy of *G. virens* has been attributed to both mycoparasitism and antibiosis (2,3) but the former appears to play only a minor role as a control mechanism (4). Rather, the production of antibiotics appears to be the principal method by which *G. virens* controls diseases incited by *R. Solani* or *P. ultimum*.

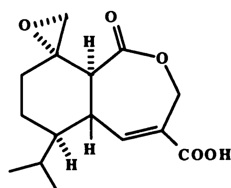
Antibiotics. Four antibiotics isolated from *G. virens* have been identified: gliotoxin (5,6), gliovirin (7), viridin (8), and heptelidic acid (9-11). Of these, gliotoxin and gliovirin are the most active against *R. solani* and *P. ultimum*, respectively (Table I) (12).

This chapter not subject to U.S. copyright
Published 1994 American Chemical Society

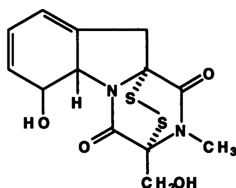
In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.



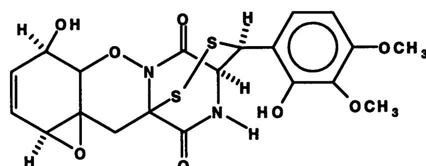
Viridin



Heptelidic Acid



Gliotoxin



Gliovirin

Table I. Minimum Inhibitory Concentration (μg/ml) of antibiotics from *Gliocladium virens*

Antibiotic	*Rhizoctonia solani*		*Pythium ultimum*	
	Conidia	Mycelia	Oospores	Mycelia
Gliotoxin	0.5	5	1	30
Viridin	50	>30	25	>30
Gliovirin	50	>30	0.05	0.5
Heptelidic Acid	25	>30	10	20

Gliotoxin. Gliotoxin is classified as an epidithiodiketopiperazine. It shows a broad spectrum of activity against fungi, bacteria, and viruses (1) and inhibits farnesyl-protein transferase (13). A bridged disulfide appears to be essential for antimicrobial activity in gliotoxin (14) and other epidithiodiketopiperazines (15). Gliotoxin is highly toxic to *R. solani* but shows less activity against *P. ultimum*.

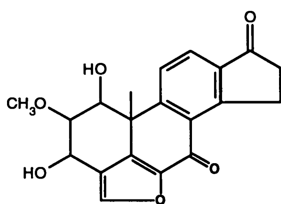
Gliovirin. Gliovirin also contains a diketopiperazine ring and a disulfide group (8). However, unlike gliotoxin and related compounds, the disulfide group does not bridge directly across the diketopiperazine ring. Its activity appears to be more specific than gliotoxin and is active only against the Oomycetes, including *P. ultimum* (16).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch011

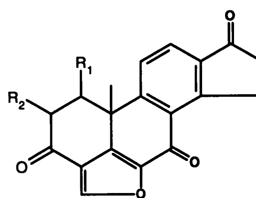
G. virens Strain Differences. Strains of *G. virens* show significant differences in their activity against *P. ultimum* and *R. solani.* Weindling (17) divided *G. virens* into two groups (i.e. P- and Q- strains) based on their pigment production on specific media. We find P-strains control *P. ultimum* more effectively than Q-strains, and Q-strains control *R. solani* more effectively than P-strains (Howell, C. R., unpublished data). HPLC analysis of extracts from P-strains show that gliovirin, but not gliotoxin, is produced by these strains, whereas Q-strains produce gliotoxin but not gliovirin. Both strains produce viridin and the related steroid, viridiol. Thus, gliovirin in the P-strains and gliotoxin in the Q-strains appear to play pivotal roles in the biocontrol efficacy of these respective biocontrol agents.

Phytotoxicity. The use of *G. virens* as a biocontrol agent has been plagued by the phytotoxicity of some of its metabolites. Both viridin and gliotoxin have been shown to inhibit seedling root growth of wheat, white mustard and red clover (18). More importantly, however, when cotton seed was coated with high concentrations of *G. virens*/rice preparations, they did not emerge, and the radical tips were necrotic (12). This indicated a more serious phytotoxic activity.

In addition to the steroid viridin, the related compound viridiol has also been isolated from *G. virens* (19). GV-P strains of *G. virens,* when grown on rice, produce significant quantities of viridiol but only small quantities when grown on potato-dextrose agar (PDA). Seed treated with extracts from the latter produced healthy seedlings, while those from the former produced necrotic radicals (20). Indeed, *G. virens* grown on rice was found to be an effective pigweed herbicide. When viridiol was purified, it was found to be phytotoxic (20).



Viridiol



Demethoxyviridin R_1=OH, R_2=H

Dehydroxydemethoxyviridin R_1, R_2=H

Viridiol Biosynthesis. A number of publications indicate that viridiol is a byproduct of the biosynthetic conversion of lanosterol to ergosterol (Figure 1). Hanson, et. al. (21) have shown that *G. deliquescens* converts both demethoxyviridin and dehydroxydemethoxyviridin into viridiol. Jones and Hancock (22) found viridin was irreversibly reduced to viridiol and that viridin, but not viridiol, was rapidly taken up by *G. virens* mycelium from liquid culture.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch011

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

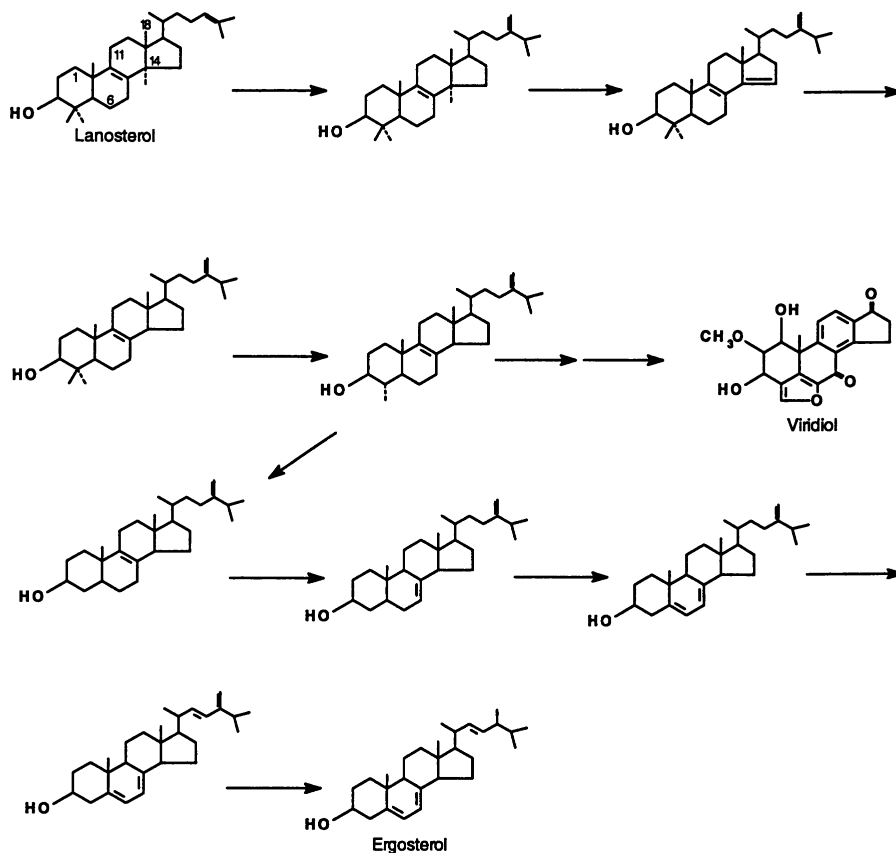
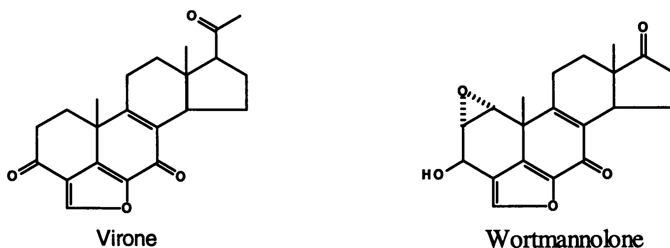


Figure 1. Steroid biosynthesis in fungi: lanosterol to ergosterol.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch011



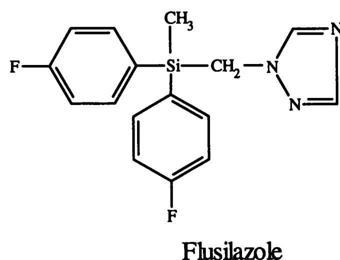
Biosynthetic studies with ^{14}C-mevalonate (23) and ^{13}C-acetate have shown the steroidal origin of viridin, demethoxyviridin (24), and wortmannin (25). The conversion of lanosterol to viridin (26) and squalene and lanosterol to viridiol (21) have been reported. Based on the various metabolites identified from the viridiol series, Hanson, et. al. (21) proposed that oxidative modification of ring A probably occurs in the later stages of biosynthesis. Based on the isolation of various precursors including virone and wortmannolone, they propose that earlier stages involve the formation of the furan ring, removal of the side chain, loss of the C18 methyl and aromatization of ring C. These observations lead one to conclude that viridiol is the end product in a series of steroid modifying steps which branch off from the main biosynthetic pathway of lanosterol to ergosterol.

A teleologist would recognize that reduction of the ketone in viridin to an alcohol in viridiol would increase the water solubility of this compound and allow its diffusion into the surrounding soil. Its ability to act as a phytotoxin could aid survival of the microorganism by destroying root tissue and thus providing nutrients for the organism.

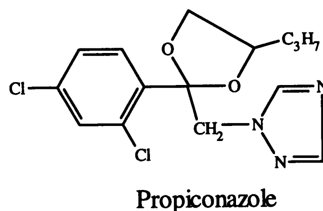
Viridin exhibits some antibiotic activity (Table I), and thus probably increases the biocontrol efficacy of *G. virens*. It would, therefore, appear advantageous to retain this metabolite but eliminate viridiol. One may also conclude that the conversion of viridin to viridiol may be accomplished by a nonspecific alcohol dehydrogenase. Thus, attempts to selectively inhibit this conversion may be futile. Therefore, the usefulness of *G. virens* as a biocontrol agent could probably be improved by interrupting the viridiol pathway at an earlier stage, even though this would result in the loss of viridin production by the fungus.

Inhibition of Viridiol Biosynthesis. There are a number of commercially available fungicides that operate by interrupting the conversion of lanosterol to ergosterol in fungi. These compounds have been shown to inhibit the cytochrome P-450 responsible for the 14α-demethylation of sterols. We found compounds such as propiconazole and flusilazole to effectively inhibit the production of viridin and viridiol at very low concentrations.

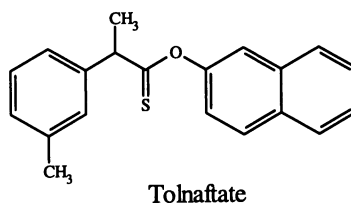
August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch011



Flusilazole



Propiconazole



Tolnaftate

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch011

Treatment of *G. virens* cultures with O.5 to 1 ppm of these fungicides inhibited viridin and viridiol biosynthesis (Table II) without adverse effects on

Table II. Effects of Fungicides on the Production of Viridiol and Other Metabolites of *Gliocladium virens* in culture[a]

Strain (Treatment)[b]	Viridiol	Viridin	Gliotoxin	Gliovirin
P strains				
G-4 (NT)	15.1	11.0	-	26.8
G-8 (NT)	12.5	7.0	-	16.8
G-4 (PC)	-	4.0	-	25.6
G-8 (PC)	-	5.5	-	28.3
Q strains				
G-6 (NT)	24.1	3.0	7.7	-
G-11 (NT)	31.0	4.0	9.8	-
G-6 (FL)	-	-	6.3	-
G-11 (FL)	-	-	7.3	-

[a]Concentration of metabolite expressed in µg/100ml of culture medium.
[b]NT = No treatment; PC = Propiconazole; FL = Flusilazole.

the growth of the organism or the production of the nonsteroid antibiotics gliotoxin and gliovirin (Howell, C. R. and Stipanovic, R. D., unpublished data). Cottonseed treated with *G. virens* and a sterol inhibitor produced healthy and normal radicals, while those treated with *G. virens* alone produced radicals that were stunted and apical meristems that were necrotic.

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

The site of inhibition of these fungicides in the viridiol pathway is not known. As indicated above, the conversion of lanosterol to viridin (26) and viridiol (21) has been documented. Thus, viridin is a side product of the main lanosterol to ergosterol pathway (Figure 1). Fungicides that inhibit 14α-demethylase effectively block viridiol biosynthesis, but the fungicide tolnaftate which inhibits squalene synthase does not effectively inhibit viridiol production. Blockage of the 14α-demethylase reaction is unlikely since the organism appears to grow normally. Rather, these fungicides appear to be acting on another enzyme which is exquisitely sensitive to these compounds. Hanson's (21) proposed stages in the biosynthesis of viridiol led us to conclude that a likely site of action may be the cytochrome P-450 enzyme responsible for C-18 demethylation (i.e. the C-ring aromatase enzyme). This enzyme apparently operates after formation of the furan ring and removal of the side chain. Experiments to probe the validity of this hypothesis are presently under investigation.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch011

Literature Cited

1. Jones, R. W.; Hancock, J. G. *J. Gen. Microbiol.* 1988, 134, 2067.
2. Tu, J. C.; Vaartaja, O. *Can. J. Bot.* 1980, 59, 22.
3. Howell, C. R.; *Phytopathology* 1982, 72, 496.
4. Howell, C. R.; Phytopathology 1987, 77, 992.
5. Weindling, R.; Emerson, O. H. *Phytopathology* 1936, 26, 1068.
6. Weindling, R.; Phytopathology 1941, 31, 991.
7. Stipanovic, R. D.; Howell, C. R. *J. Antibiotics* 1982, 35, 1326.
8. Grove, J. F.; McCloskey, P.; Moffatt, J. S. *J. Chem. Soc.* (C) 1966, 1966, 743.
9. Arigoni, D. *Pure App. Chem.* 1975, 41, 219.
10. Itoh, Y.; Takahshi, S.; Haneishi, T.; Arai, M. *J. Antibiotics* 1980, 33, 468.
11. Stipanovic, R. D.; Howell, C. R. *Tetrahedron* 1983, 39, 1103.
12. Howell, C. R. *Phytopathology* 1991, 81, 738.
13. Van der Pyl, D.; Inokoshi, J.; Shiomi, K.; Yang, H.; Takeshima, H.; Omura, S. *J. Antibiotics* 1992, 45, 1802.
14. Cavillito, J. C.; Bailey, J. H.; Warner, W. F. *J. Amer. Chem. Soc.* 1946, 68, 715.
15. Michel, K. H.; Chaney, M. O.; Jones, N. D.; Hoehn, M. M.; Nagarajan, R. *J. Antibiotics* 1974, 27, 57.
16. Howell, C. R.; Stipanovic, R. D. *Can. J. Microbiol.* 1983, 29, 321.
17. Weindling, R.; *Phytopathology* 1934, 24, 1153.
18. Wright, J. M. Ann. Bot. 1951, 60, 493.
19. Moffatt, J. S.; Bu'Lock, J. D.; Tse Hing Yuen, *T.L.S. J. Chem. Soc.* (D), 1969, 1969, 839.
20. Howell, C. R.; Stipanovic, R. D. *Phytopathology* 1984, 74, 1346.
21. Hanson, J. R.; O'Leary, M. A.; Wadsworth, H. J.; Yeoh, B. L. *Phytochemistry* 1988, 27, 387.
22. Jones, R.W.; Hancock, J. G. *Can. J. Microbiol.* 1987, 33, 963.

23. Grove, J. F.; *J. Chem. Soc.* (C) 1969, 1969, 549.
24. Hanson, J. R.; O'Leary, M. A.; Wadsworth, H. J. *J. Chem. Soc. Perkin Trans. I* 1983, 1983, 867.
25. Simpson, T. J.; Lunnon, M. W.; MacMillan, J. J. Chem. Soc. Perkin Trans. I. 1979, 1979, 931.
26. Golden, W. S.; Watson, T. R. *J. Chem. Soc. Perkin Trans. I* 1980, 1980, 422.

RECEIVED December 6, 1993

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch011

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch012

Chapter 12

After Discovery: The Issue of Supply Strategies in the Development of Natural Products

James D. McChesney

Research Institute of Pharmaceutical Sciences, School of Pharmacy, University of Mississippi, University, MS 38677

Natural products, especially those derived from higher plants, have historically played a pivotal role in the discovery of new pharmaceuticals and agrochemicals. However, in the recent past less emphasis has been placed on natural products as new leads, especially for agricultural applications. An important, if not predominant, consideration fostering this lack of interest in natural product-derived leads has to do with the issue of sustainable and economic supplies of the natural product to meet the needs of the development process and ultimately the clinical use of the drug or the agricultural application of the substance. A brief overview of alternative strategies to resolve supply issues and their relative strengths and weaknesses is presented.

Chemicals derived from higher plants have played a central role in the history of humankind. The Age of Discovery was fostered by explorations to find more economic trade routes to the East to bring back plant-derived spices and other products. Indeed, the discovery by Europeans of the "New World", whose 500th anniversary we recently noted, was a direct consequence of that effort. The prototype agent for a majority of our classes of pharmaceuticals was a natural product of plant or microbial origin. Numerous examples could be cited. However, with the increasing sophistication of synthetic organic chemistry, interest in natural products as prototypes for pharmaceuticals and agrochemicals waned greatly during the decades of the 60's, 70's and 80's. Today a re-interest in the potential of substances of natural origin to provide prototypes for new pharmaceuticals, agrochemicals and consumer products is being evidenced. Until recently, efforts to realize the potential of plant-derived natural products have been very modest and largely restricted to discovery programs centered in academic settings. The evaluation of fermentation products by the pharmaceutical industry was largely restricted to efforts to identify antibiotic and antitumor agents. A number of convergent factors are bringing about a renaissance in natural products research and development. Some of those factors are: advances in bioassay technology; advances in separation and

0097–6156/94/0557–0144$08.00/0
© 1994 American Chemical Society

structure elucidation technology; advances in our understanding of biochemical and physiological pathways; the biotechnology revolution; historical success of the approach; loss of practitioners of traditional medicine; loss of biological diversity; loss of chemical diversity, and world-wide competition. However, an important consideration or nagging concern which is still responsible for the significant reluctance to initiate a natural products research and development program, especially on the part of the private sector, is the issue of natural product chemical supply.

We often refer to the "pipeline" when discussing discovery and development. Portrayed in Figure 1 is this pipeline concept. Three basic stages are evident; discovery, development and marketing. Each of those periods is, in turn, subdivided according to recognized descreet activities with their specific attendant costs and time for accomplishment. In Tables 1 and 2 are summarized usual and mean times for accomplishment of the various discovery and development activities. From these numbers it is clear that pharmaceutical and agrochemical companies must take a long view of product development, in most instances a much longer view than almost any other industry. Table 3 outlines the specific costs associated with each of these discovery and development activities. Each new pharmaceutical or agrochemical represents a major cash investment on the part of the company to get it to market. What is not immediately obvious from these tables are the hidden costs of discovery and development. What does it cost to identify that first lead compound? How many of the candidates that enter the development phase fall out before making it to market? How much "lost opportunity" is represented by the necessary investments in discovery and development? For example, it is estimated that more than 10,000 chemical substances are ordinarily evaluated before a lead is identified. What does it cost to generate or produce all those substances? A majority of candidates prove to be unsuitable as drugs or agrochemicals for one reason or another - too toxic, too costly, not sufficient benefit beyond present agents, etc. And unfortunately, on occasion, drugs or agrochemicals are removed from use even after making it to the market due to unacceptable side effects, or other problems. In summary, the successes must pay for all the failures so that on average, the cost of each successful agent is approximately 70 million for new agrochemicals and 225 million dollars for new drugs.[1] Because of these very high development costs, most companies now limit their discovery and development efforts to agents which hold promise of 100 to 200 million dollars per year markets. Consequently, anything that can make the process more efficient and cost effective is very desirable. Evaluation of new natural products holds significant promise of lowering present day drug and agrochemical discovery and development costs.

Table 1 - Typical Periods of Time Required to Develop New Drugs

Stage of Development	Usual Range of Time Required (Years)	Approximate Mean Time Required (Years)
1. Project Formation to IND[1] Filing	1.5 to 3.5	2.5
2. Phase I Clinical Studies	0.5 to 1.5	1.0
3. Phase II Clinical Studies	1.0 to 5.0	3.0
4. Phase III Clinical Studies and Preparation of NDA[2]	1.0 to 5.0	3.0
5. FDA[3] Review of NDA	1.0 to 5.0	2.5
	4.0 to 19.0	12.0

[1]IND = Investigational New Drug Application, [2]NDA = New Drug Application, [3]FDA = U.S. Food and Drug Administration

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch012

Table 2 - Typical Periods of Time Required to Develop New Agrochemicals

Stage of Development	Usual Range of Time Required (Years)	Approximate Mean Time Required (Years)
1. Project Formation to Experimental Use Permit	1.5 to 3.5	2.5
2. Greenhouse and Field Trials	2.0 to 5.0	3.0
3. EPA[1] Registration Wait	1.0 to 3.0	1.5
	4.5 to11.5	7.0

[1]EPA = U.S. Environmental Protection Agency

Table 3 - Direct Costs at Various Stages of Development

Drug Stage	Drug Cost(10^6)	Agrochemical Stage	Agrochemical Cost(10^6)
Pre-IND	2.4	Pre-Experimental Use	2.4
Phase I	2.6	Greenhouse and Field Trials	6.0
Phase II	11.4	Registration Wait	1.1
NDA Wait	2.2		
Total	31.0	Total	9.5

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch012

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch012

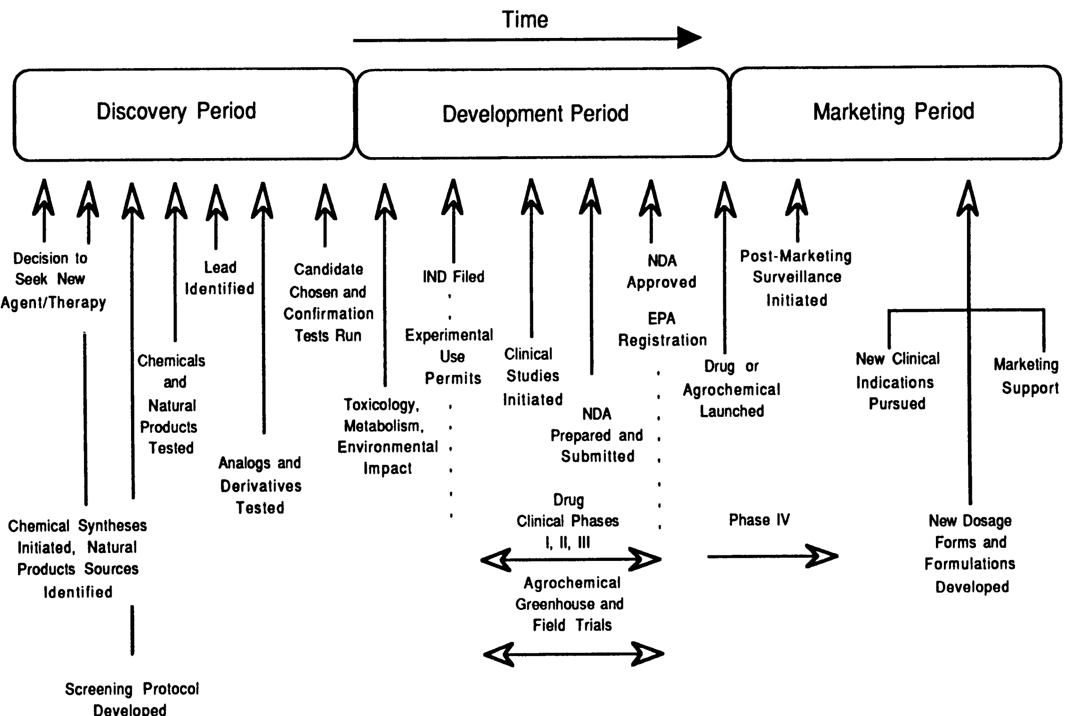


Figure 1. Pipeline concept of development.

With the advances in bioassay, separation and structure elucidation technologies mentioned above, we are now able to discover and identify substances with potential utility from natural sources quickly and with very modest amounts of material. The sensitivity of the bioassays routinely in use today and the desired potency for new prototypes make it easily possible to etect substances which are present at very low concentrations in fermentation broths or extracts of biomass.

Ordinarily 50 milligrams (mg) or so of pure chemical substance is sufficient to determine its complete structure including stereochemistry. In order to obtain 50 mg of a chemical which is present at 0.001% of the dry weight of the plant biomass, one needs at least 5 kilograms (kg) of biomass. Practically, one actually needs as much as 10 kg of biomass since no isolation procedure is 100% efficient.

Herbaceous plants are roughly 75-80% water when fresh so to obtain 5 to 10 kilgrams of dry plant materials one must collect 20 to 50 kilgrams of fresh plant material. For woody plants, the situation may be somewhat more complex since we usually process a specific part of the plant - bark, leaves, fruits, roots, wood. For the leaves and fruits the situation is like that for herbaceous plants. In the case of wood, bark or roots, the dry weight yield from fresh weight is often much higher, sometimes as high as 75%. However, digging roots or peeling bark is more difficult than collecting leaves or fruits. So for woody plant parts one may need 20 to 50 kilgrams of leaves of fruits; 10 to 25 kilgrams of roots or bark and 8-15 kilgrams of wood in order to get sufficient substance to accomplish isolation and chemical characterization of the active principle.

In the case of fermentation products, an original observation of activity is often made with active principals at a concentration of a few micrograms per liter of fermentation medium. In order to prepare the requisite 50 mg of pure substance for chemical characterization, either enhanced production of the natural products must be stimulated or 100's of liters of medium may need to be prepared and processed.

Having isolated a bioactive natural product and determined its structure, we arrive at a decision point - is the structure of the active principal novel? Does the substance represent a potential new prototype? In order to assess the full potential of the substance so that a valid decision can be made whether to proceed further with development, quantities are needed for an array of evaluations: secondary and confirmatory bioassays; preliminary toxicology and initial in vivo evaluation. Ordinarily at least 400-500 mg of material are needed for this next phase of evaluation. This represents roughly a 10-fold increase needed over the original quantities required for detection of the lead and its chemical characterization. To isolate and purify the required quantities of material may require 100 - 500 kg of biomass or 1000's of liters of fermentation medium to be processed. It is already at this point that concerns about supplies of the natural product are beginning to surface. If one must invest in bulk collection of plant material or large scale fermentation and subsequent processing to obtain quantities for these next evaluations, what are you going to do for the larger quantities of the active principal, perhaps as much as two kilograms (kg), required to take the drug candidate into preclinical evaluation and subsequent clinical trials and ultimately to provide for actual marketing and clinical use of the new drug, maybe 10's or 100's of kilograms per year? For agrochemical candidates, quantities required for greenhouse and field testing may reach 10's of kilograms and final use applications will require thousands of kilograms per year.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch012

Two more or less extreme examples illustrate the range of quantities of active drug substance that would be required to provide for clinical use of a plant-derived drug. Let us assume that the condition to be treated is an acute condition, that the agent is relatively potent, i.e. 2 grams are required for therapy and that only a modest patient population is expected - 10,000 patients per year. To provide for this market roughly 20 kilograms of drug substance would be required. If again we assume our worst case of 0.001% of active substance isolated from our biomass then 2,000,000 kilograms of biomass will be necessary to provide the quantity of drug needed to meet market demand. This may seem like a great deal, but 2,000,000 kilograms of biomass is the same as 75,000 bushels of wheat, a quantity of wheat produced by even a modest-sized family farm in America. If we assume the plant-derived drug would be used to treat a chronic condition in a much larger population would that make a great difference? For a chronic condition a moderately potent agent might require administration of 50 mg per day per patient which is equivalent to 20g per year per patient. If 100,000 patients could benefit from this drug then 2,000 kilograms of active drug substance would be needed to meet market demand. This is 100 times our example above or in other words 200,000,000 kilgrams of biomass would need to be processed to provide the required material. Looked at another way, that is equivalent to 7,500,000 bushels of wheat, a quantity easily produced in an average wheat producing county of a wheat producing state such as Kansas. Clearly these are quantities of biomass which can be readily produced and processed if thought and planning for a system for drug plant production is initiated.

With our current capabilities in biochemical engineering and experience with manipulation of product formation in microorganisms, the production within the pharmaceutical industry of 10's or 100's of kilograms of bulk active by fermentation is routine. To expand this capability to produce 1000's of kilograms for agrochemical applications can be expected to be accomplished if modest additional investment were to be made. With our current understanding of natural product production, development of an economic production system for plant-derived natural products is most needed.

Are there any actual examples which might illustrate the point we are making? First let me comment on two plant derived drugs, marijuana and cocaine which are problems for society. The best estimates on marijuana production worldwide in 1990 placed the figure at 30,000 tons.[2] The U.S. Drug Enforcement Agency estimated that U.S. production was at least 5,000 tons, more than 4,500,000 kilograms. At an average price of $1,200 to $2,000 per pound, there is apparently incentive enough for growers to risk even imprisonment to provide for the market demand. Cocaine, a purified drug from the leaves of the coca plant, was produced at a rate of at least 1,000 tons worldwide in 1990.[2] With prices of $6,000 to $18,000 per pound wholesale this was enough incentive to cause illicit procucers to grow and process enough drug to meet market demand. It is to be emphasized that these levels of natural product drug production are in place even though governments are making a concerted effort costing billions of dollars to stop or eradicate the growing of these plants.

Any system for production of a plant-derived natural product must meet certain criteria. It must be economic, sustainable, reliable and non-environmentally impacting. These over-arching criteria can be met if a careful and systematic evaluation of each step of the production system is made. One must discover and develop: a superior source of the natural product which contains a consistently high yield of agent, an effective production and harvest system, appropriate technology for processing and storage of biomass, and an

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch012

economic and efficient extraction and purification system which minimizes waste product generation.

Wild populations of plants may not be a reliable source of drug or agrochemical entity and their harvest may be counter-productive to the development of a reliable, cost-effective, long-term production system. Several unpredictable and uncontrollable phenomena preclude establishment of a stable cost of production of the natural product if it is produced by harvest from wild populations: Forest fires, annual climatic variations; natural variation and presence of chemotypes of wild populations; increasing societal pressures to protect and regulate harvesting of wild plants, high cost of collection of scattered plants often in rough or inaccessible terrain and transportation to processing facilities and lack of assured accessibility of populations which occur on public and privately held lands. Reliance on harvest of wild plants for production may lead to uncontrollable interruption of supply.

Even more critical is the fact that wild harvest risks the destruction of germplasm essential for the future cultivation of the plant for natural product production. This includes genes for disease and pest resistance, hardiness, and tolerance to full sunlight, drought and flooding as well as genes for high growth rates and high chemical production. The preservation of these wild genes can be critical to development of long-term, cost-effective supplies whether produced by cultivated plants, tissue culture, or genetically modified microorganisms. Because of the critical role wild germplasm will serve in future production strategies, the preservation of wild populations should be considered an essential component of the development strategy for natural product production.

Lack of a stable and reliable supply of plant-derived natural product at a predictable cost will even more significantly impede commercial utilization of the agent. Procurement of biomass by harvest of limited wild populations may not be an appropriate strategy to provide the agent once utility is established. Development of a sustainable, economic and reliable source is imperative.

The appropriate alternative may be production from a cultivated plant source An advantage is the known genetic origin and uniformity of cultivated plants. Additionally, cultivation will provide high plant densities in defined locations which will significantly reduce collection and transportation costs. Two strategies may be taken to accomplish this: (1) to bring into cultivation the currently recognized source of the substance; (2) if the plant source of the natural product is already in cultivation, to evaluate and select currently cultivated varieties for substance or precursor content. Strategy One is fraught with many problems and uncertainties associated with the introduction of a new plant into cultivation, a process which has been successful in bringing into cultivation approximately 3,000 species of the estimated 300,000 plant species thought to occur in the world. We will not detail all of those uncertainties. However, as our understanding of agronomics and plant biology has increased, we can have ever-increasing confidence in our ability to bring into cultivation the plant source of a natural product. Even with success, a period of several years may be required for this strategy to provide a reliable source for production. Additionally, the necessity of recovering the investments made during the development period will impact the economics of this strategy to provide a source for the natural product. In contrast, Strategy Two, selection of currently cultivated varieties for chemical production, presents the advantage that a proven cultural system is in place which may be rapidly expanded to provide biomass for production of the desired natural product chemical.

As noted above, any system for production of a plant-derived natural product must meet certain criteria: it must be economic, sustainable, reliable and have minimal environmental impact. A plant source of the agent must be

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch012

discovered which contains a high and consistent yield of either the natural product itself or a closely related natural product or precursor which may be converted economically to the desired agent. We must learn how to optimize chemical content of the plant through appropriate agronomic practices. A system for harvesting or collection of the biomass must be developed which is responsive to time of harvest for highest chemical content, correct handling to retain chemical content in freshly harvested biomass and also provides for mechanization of the process, if possible. Harvested biomass will ordinarily require drying and storage which must be accomplished so as to retain its chemical content. Development of storage conditions which assure stability of the biomass is especially critical as this will allow subsequent processing of the biomass and purification of the natural product throughout the calendar year. And finally, an efficient and high-yielding extracting and purification process must be developed which is safe and economic and which minimizes production of hazardous wastes.

The potential for developing new sources of valuable plant chemicals is largely unexplored and the benefits from doing so unexploited. Plants are known sources of medicines, insecticides, herbicides, and other useful substances; developing new industries and crops based upon plant extracts and extraction residues provides opportunities for agricultural and industrial expansion that will benefit farmers, consumers and industry, both in the developing as well as the developed nations.

New crop and plant product development will:

- provide consumers with new products, including new drugs to treat diseases,
- provide less environmentally hazardous pesticides,
- diversity and increase the efficiency of agricultural production,
- improve land resource use,
- offer increased economic stability to farmers,
- create new and improve existing agriculturally related industries,
- increase employment opportunities.

In conclusion, careful and thoughtful analysis of the issue of supply of sufficient quantities of natural products to meet development and commercial needs brings one to the realization that this is **not** an issue which should cause us to preclude the carrying forward of such agents through the development process to commercial use. Indeed, it is clear that adequate supplies of most naturally-derived substances can be assured with only modest additional research and investment.

Literature Cited

1. Pharmaceutical Manufacturers Association, 1994, "Drug Prices and Profits, 1993"; Washington, D.C.

2. The National Narcotics Intelligence Consumers Committee Report, 1990. "The Supply of Illicit Drugs to the United States", Drug Enforcement Administration, Office of Intelligence, Washington, D.C.

RECEIVED March 2, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch012

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

Chapter 13

Development of Economical Population Control of Codling Moth (*Cydia pomonella*) by Disrupting Communication with Semiochemicals

Leslie M. McDonough and Harry G. Davis

Agricultural Research Service, U.S. Department of Agriculture, 3706 West Nob Hill Boulevard, Yakima, WA 98902

The feasibility of controlling codling moth populations by disrupting communication with semiochemicals was established about 20 years ago. Nevertheless, an economical and effective control procedure based on communication disruption has not yet been developed in the USA. To develop economical control, several critical issues must be addressed. First, when the communication disruptant semiochemical is the sex pheromone, it is believed that an exact replica of the natural pheromone (same components and same component ratios) would be the most efficacious disruptant. Sex pheromones of insects are almost invariably multicomponent, and the codling moth sex pheromone had been reported to consist of three components, but subsequently two components were shown to be inactive. It is not known whether there are other active components remaining to be discovered. Second, the potential value of nonpheromone semiochemicals for communication disruption of moths is unknown. For the codling moth, certain nonpheromone semiochemicals appear to have efficacy superior to the single pheromone component. Third, to effect population control of codling moth in the Yakima valley, the disrupting semiochemical must be continuously disseminated throughout the orchard canopy for a period of five months. At present no known controlled release device can fulfill this requirement as efficiently as desired. A major problem with controlled release of the codling moth sex pheromone is the ease of chemical decomposition.

The well recognized problems from the use of insecticides including high mammalian toxicity, environmental contamination, destruction of beneficial arthropods, and insect resistance have stimulated research on alternate insect control methods. One of these methods is the use

This chapter not subject to U.S. copyright
Published 1994 American Chemical Society

of sex attractant pheromones or other semiochemicals to prevent mate finding. A pheromone is a substance secreted by an organism to the outside that causes a specific reaction (such as mate seeking) in a receiving organism of the same species and semiochemicals are chemicals that mediate interactions between organisms (1,2). Since the late 1980s, control with sex attractant pheromones has appeared highly promising. In this paper we review our research of the last few years that has addressed some of the problems of controlling codling moth with semiochemicals.

Historical Perspective

Knowledge of the existence of insect sex pheromones predates the twentieth century, but chemical identification of the minute quantities produced by insects only became feasible after the development of the various chromatographic devices and procedures in the 1950s.

Following the identification of the silkworm sex pheromone in 1959 (3), intense interest in identifying sex pheromones of economically important insect pests developed. One of the projected applications of sex pheromones was mass trapping, i.e., traps baited with a sex pheromone could effectively remove the male insects from the insect's habitat, and thereby cause a collapse of the population levels of the pest. Another potential application was continuous permeation of the air of the insect's habitat with the synthetic sex pheromone. With the odor of the sex pheromone everywhere, the ability of the males to locate the females would be disrupted and again the population levels would collapse.

Besides the use of pheromones for direct control, it was also anticipated that pheromones would be used to detect new insect introductions, to determine insect distribution, and to monitor population changes as a guide for determining timing and measuring efficacy of other control measures such as application of insecticides or biological control agents. Since the early 1970s, these uses of pheromones have become firmly established.

After the identification of the sex pheromones of several important economic pests in the late 1960s, mass trapping was evaluated and was first successfully demonstrated for the redbanded leafroller (4). The requirements for successful implementation were so stringent that development of economical applications did not seem feasible, and as a result there has been limited interest in developing this technique for moths. This technique appears to offer more potential for other insect types, such as beetles because for many species both sexes are attracted to the pheromone (5).

In the early 1970s, tests with the communication disruption method were encouraging, but the dispenser technology then available (sprayable microcapsules) only provided very short periods of communication disruption (6).

A second wave of interest in communication disruption developed in the middle-late 1970s because of pheromone dispensers which were effective for much longer periods: sprayable hollow fibers (7), and plastic laminates (8). Nevertheless, and despite some successes (9,10,11), by the early 1980s there was widespread belief that the use of sex pheromones for communication disruption would never be a

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

significant part of accepted control methods. Compared to insecticide applications, communication disruption was perceived to be less reliable, to involve more difficult application techniques, and to be more expensive (12).

By the late 1980s, the third wave of interest in communication disruption had developed. The interest was stimulated by the success with controlling the oriental fruit moth in Australia, Canada, and California (13-14). Here communication disruption offered control equal to or superior to insecticides at about the same cost. Two of the factors important to success were chemical identification of all of the sex pheromone components (15-18) and development of an effective controlled release dispenser (13-14).

Codling Moth Control

Except in Japan, the codling moth (Cydia pomonella) is a world wide pest of apples, pears and walnuts. Since about 1960 the codling moth has been controlled with Guthion (azinphos-methyl), an organophosphate insecticide. Because of environmental concerns and evidence of growing resistance in codling moth (19), control with Guthion may be limited or canceled in the future. The most promising alternate control methods are semiochemicals such as the sex pheromone or the sterile male technique.

The codling moth has one to three generations per year depending on geographic location. To effect control with its sex pheromone, the habitat of the insect must be continuously permeated with pheromone vapor at a high enough concentration to prevent mate finding for the entire season. This requirement is obviously more demanding of pheromone use and of controlled release dispensers in locations having two or three generations per year than in locations having one generation per year.

Studies in the early 1970s at Yakima indicated communication disruption had potential for codling moth control, but the first used dispensers, microcapsules, only disrupted communication for about one week (20). Subsequently, hollow fiber dispensers were shown to provide about 2 months of communication disruption (21). More recently, in extensive tests using plastic laminates and rubber tube filled dispensers, Charmillot (22) demonstrated control of codling moth in commercial apple orchards in Switzerland, where the pest has one generation per year.

The impediment to developing a control program in the USA where the pest has two or three generations per year is economics. Therefore, our studies were directed to issues that affect the economics of communication disruption. Two of the principle issues which must be addressed to optimize communication disruption are an effective controlled release dispenser and the most efficacious communication disruptant. There are other semiochemicals besides the sex pheromone of the codling moth which can disrupt sexual communication, e.g., the acetate derivative of (E,E)-8,10-dodecadien-1-ol (23-26).

It has been proposed that an exact replicate of the natural pheromone will be a more efficacious communication disruptant than any single pheromone component or a mixture of pheromone components with

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

a ratio different from the natural (27-28). For communication disruptants which are not pheromone components, no theory of efficacy relative to the pheromone has been advanced. In practice, nonpheromone communication disruptants have not been superior to sex pheromones (26, 29).

Codling Moth Sex Pheromone

In 1971, Roelofs and coworkers reported identification of the codling moth sex pheromone as (E,E)-8,10-dodecadien-1-ol, "codlemone" (30). Later Bartell and coworkers (1981) presented evidence from a wing flutter bioassay, that female sex pheromone gland extract induced more male activity than codlemone alone (31). Subsequently, Einhorn et al. (32) and Arn et al. (33) identified several other compounds in female sex pheromone gland extract. Additionally, Arn et al. identified the components in the effluvium of calling females (33). These compounds and their ratios are shown in Table I. Both Einhorn's and Arn's groups

Table I. Compounds and their amounts relative to codlemone = 100 identified in the effluvium of calling females by Arn et al. (33). The compounds are listed in a condensed nomenclature: the OH after the colon indicates a primary alcohol; the number between the colon and the dash is the number of carbon atoms in the straight chain; the numbers and letters before the dash indicate positions and configurations of double bonds that may be present.

E8,E10-12:OH, 100	10:OH, 2.8
Z8,E10-12:OH, trace	12:OH, 64.8
E8,Z10-12:OH, 5.6	14:OH, 9.3
E9-12:OH, 14.8	16:OH, 18.5
	18:OH, 22.2

reported behavioral activity of 12:OH in combination with E8,E10-12:OH (codlemone) in flight tunnel tests. Using their wing flutter bioassay, Bartell and coworkers (1988) found no increased activity when 12:OH was added to codlemone, but when both 12:OH and 14:OH were added, activity was increased to the level obtained with sex pheromone gland extract (34).

We undertook flight tunnel studies of components of the effluvium of calling female codling moths in order to more completely define the sex pheromone, which we hoped would produce a more efficacious mating disruptant (35). First we compared codlemone with a combination of codlemone, 12:OH and 14:OH. The ratios in the 3-component lure were chosen to reproduce the vapor ratios reported by Arn et al. (33). Surprisingly, our results did not support the previously mentioned reports ascribing activity to 12:OH and 14:OH. Figure 1 shows percent response versus logarithm of dose obtained for dosages varying from 0.1 to 100,000 ug for codlemone alone, and for codlemone in combination with 12:OH and 14:OH. Response was defined as upwind flight

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

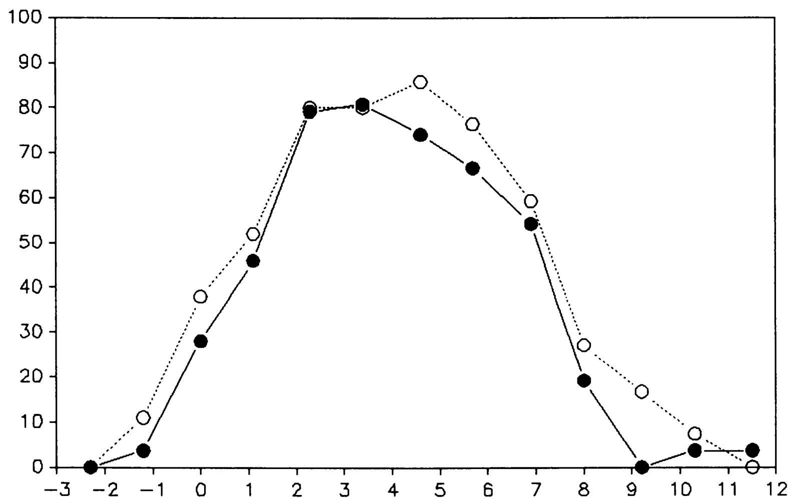


Figure 1. Percent of male codling moths making oriented upwind flights culminating in contacting the source (ordinate) versus natural logarithm of dose in μg of codlemone (abscissa). Dotted line, codlemone; solid line, codlemone plus 12:OH and 14:OH.
(Reprinted from reference 35. Copyright 1993)

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

culminating in contacting the source of the lure. As can be seen, there is no significant difference between the curves for these two compositions. Also, because 12:OH and 14:OH did not have any effect on the response to codlemone, one would not expect these compounds to improve the efficacy of communication disruption of codlemone.

We also tested components of the effluvium of calling females for which data had not been reported. Figure 2 shows a comparison of codlemone with a 5-component composition consisting of codlemone, E9-12:OH, 10:OH, 12:OH, and 14:OH. For the 5-component composition, the range of maximum response extended from ln dose = 2.3 to 6.9 (10 -1,000 ug), which was slightly larger than codlemone alone (10-300 μg). Nevertheless, these values are well within the standard deviations; the 5-component composition also did not produce a response superior to that of codlemone alone. The codling moth could be one of the few examples of a moth that possesses a single component pheromone, but this is not certain yet. Further studies in a flight tunnel comparing codlemone to sex pheromone gland extract or to effluvium of calling females are needed to fully resolve this question.

Comparison of Efficacy of Communication Disruptants

In 1991 and 1992 field tests were conducted to evaluate both pheromone and nonpheromone compositions for efficacy of communication disruption (36). Figure 3 shows the section of the orchard that was used for the

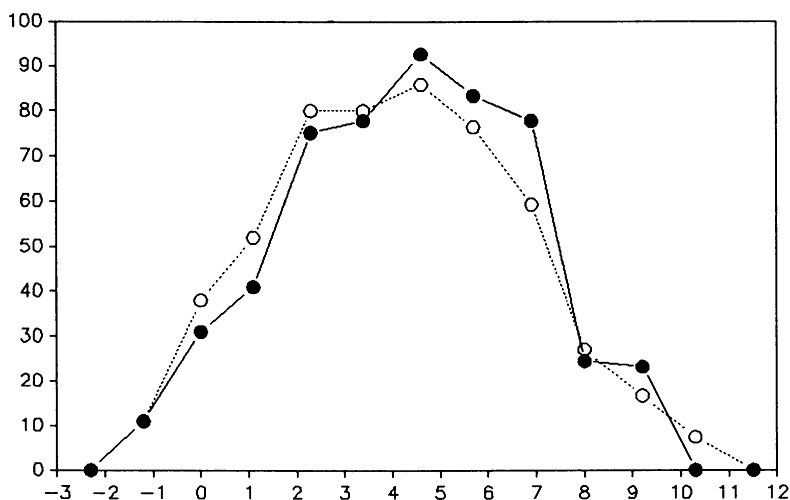


Figure 2. Percent of male codling moths making oriented upwind flights culminating in contacting the source (ordinate) versus natural logarithm of dose in μg of codlemone (abscissa). Dotted line, codlemone; solid line, codlemone plus 10:OH, 12:OH, 14:OH, and E9-12:OH. (Reprinted from reference 35. Copyright 1993)

tests. The test area was downwind of the control area. Traps baited with 10 females each were placed in the designated trees. Surrounding each trap were six gray, elastomer septa dispensers, which were blank in the control area and loaded with the test composition in the test area. Dyed male moths were released at the designated trees in each area (30/tree, twice a week). For each treatment, total captures in test and control areas were compared. Because any estimation of percent communication disruption presupposes equal numbers of males in test and control areas, different colored moths were released in the test and control areas so that any disparity in migration between these areas could be taken into account when calculating the percent communication disruption. For this situation the following formula (36) was derived to calculate the percent communication disruption, D:

$$D = 100 - \frac{100\,(N_{tt} - N_{tc})}{(N_{cc} - N_{ct})}$$

In this equation, N_{tt} is the number of males caught in the test area that were released in the test area; N_{tc} is the number of males caught in the test area that were released in the control area. N_{cc} and N_{ct} were similarly defined.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

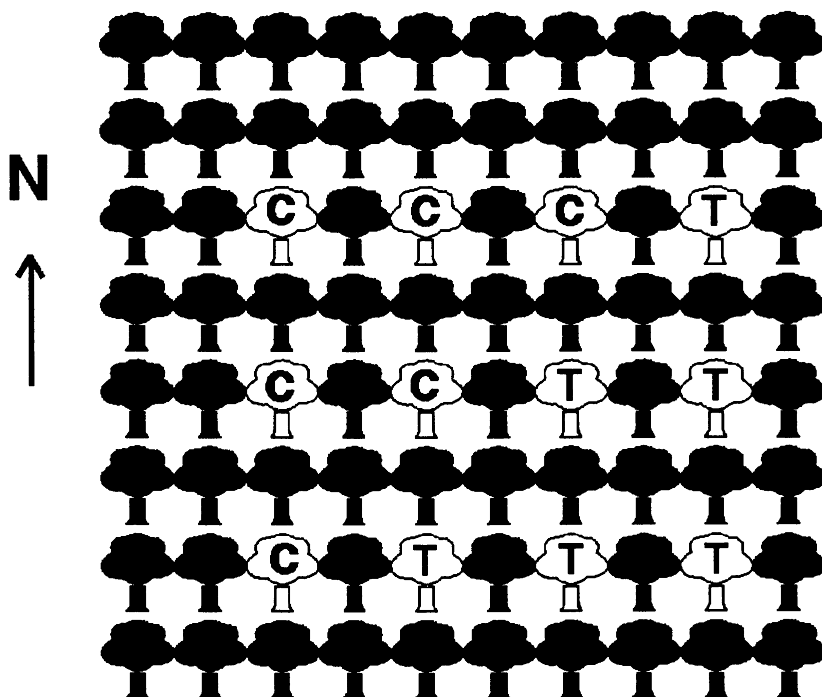


Figure 3. Map of southeast corner of apple orchard used in communication disruption tests. The traps were placed in the white trees: C = control trees and T = test trees. During codling moth flights (late afternoon and early evening) prevailing winds were from the northwest. (Reprinted from reference 36.)

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

In 1991, three compositions were evaluated: 1, codlemone; 2, codlemone + 12:OH + 14:OH; and 3, an equilibrium mixture of isomers of codlemone. Data were analyzed according to the regression of percent communication disruption, D, versus natural logarithm of dose. The data and regression lines are shown in Figure 4. All three compositions produced good regression lines with r^2 values greater than 0.9. Based on the 95% confidence limit, the slopes and the intercepts were equivalent for codlemone and the three component mixture and different from the equilibrium mixture of isomers of codlemone. Therefore, these results are consistent with the flight tunnel studies, which indicated that there was no difference in male response to codlemone and the three component composition. In contrast, the equilibrium mixture of isomers was a superior communication disruptant at all doses. In field tests, the isomers of codlemone are known to decrease catch of males in traps baited with

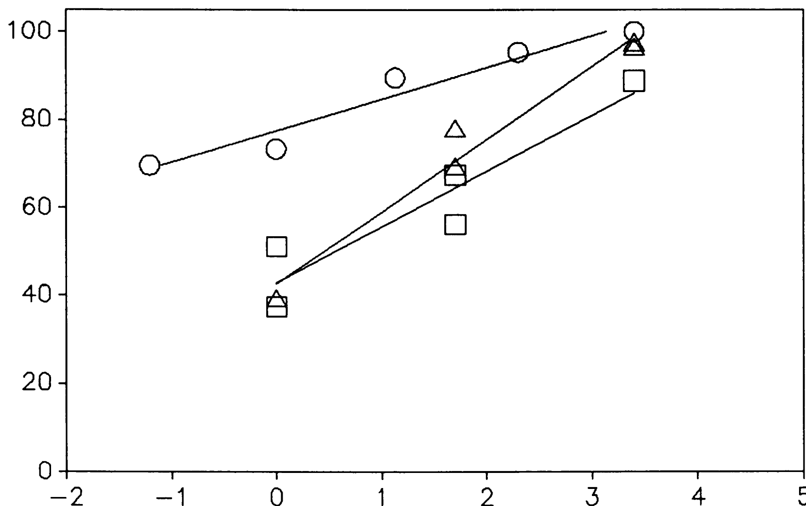


Figure 4. Regression curves for percent communication disruption (ordinate) versus natural logarithm of dose (mg/dispenser) of 8,10-12:OH (abscissa) for 3 compositions: □, codlemone (E8,E10-12:OH); ▲, E8,E10-12:OH + 12:OH + 14:OH; ○, equilibrium mixture of isomers of 8,10-12:OH (61% EE, 20% EZ, 14% ZE, and 5% ZZ). (Reprinted from reference 36.)

codlemone (37), and in flight tunnel tests, the isomers also decreased response (35). As a result, most researchers assumed the isomers would decrease the effectiveness of codlemone as a communication disruptant, and isomerization of codlemone in controlled release dispensers used for communication disruption would be detrimental to efficacy. Therefore, these results were surprising, and raised the question of whether the disruption was due to the mixture or whether the individual isomers were disruptants.

To further determine the activity of the isomers, E8,Z10-12:OH and Z8,E10-12:OH were individually synthesized and tested (36). In 1992, five treatments were compared at one dose: E8,Z10-12:OH, Z8,E10-12:OH, equilibrium mixture of isomers of 8,10-12:OH, E8,E10-12:OH (codlemone), and blanks. Here, the catches of the native population were also tabulated. The orchard had been heavily infested for several years and 100% of the apples were wormy every year. Therefore, the native population might be fairly evenly distributed. The percent communication disruption for the native population was calculated from the following formula (36):

$$D = \frac{100\,(N_c - N_t)}{N_c}$$

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

Where N_c and N_t are the numbers caught in the control and test areas, respectively.

The data for both released and native males are given in Table II. At the 95% confidence limit in either the tabulation of

Table II. Percent communication disruption of released and native male codling moths for different treatments. Numbers followed by different letters are different at the 95% confidence limit.

Treatments	Calculated % Communication Disruption	
	Released Males	Native Males
E8,Z10-12:OH	80.9a	91.9a
Z8,E10-12:OH	80.8a	96.4a
8,10-12:OH Isomers	63.1a	89.2a
E8,E10-12:OH (codlemone)	42.4b	55.2b
None	18.9c	17.3c

native or laboratory reared males, the EZ and ZE isomers were equivalent to each other and superior to codlemone and the blanks. The equilibrium mixture of isomers produced numerically less disruption than the EZ and ZE isomers, but was not different at the 95% confidence limit. The mixture of isomers was superior to codlemone which was superior to the blanks.

Therefore, the 1992 results confirm the 1991 results that the 8,10-12:OH isomers produced more disruption than codlemone and establish that both the EZ and ZE isomers individually disrupt sexual communication of codling moths to a greater degree than codlemone in these tests. Further tests are needed to determine the practical value of these results. The principal question is whether the EZ or ZE isomer will be superior to codlemone under practical conditions of commercial communication disruption. At the least, we think these findings indicate that when codlemone is used for mating disruption, it does not need to be of high isomeric purity, and that controlled release dispensers that allow isomerization are acceptable and perhaps desirable.

Controlled Release Dispenser of Codling Moth Sex Pheromone

Because the Pacific Biocontrol dispenser had been so successful in controlling oriental fruit moth infestations (13-14), its formulation of the codling moth pheromone was evaluated (38). The Biocontrol dispenser is a hollow polyethylene tube filled with pheromone and sealed at the ends. An aluminum wire is imbedded in the wall of the dispenser so it can be conveniently tied to a branch of foliage. The dispensers were formulated with codlemone + 12:OH + 14:OH.

The Biocontrol dispenser was expected to release pheromone by a first order mechanism (39). The integral form of the equation of a first order evaporative loss is:

$$\ln P = -kt + \ln P_o \quad \text{Equation 1}$$

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

P is the amount of pheromone remaining at any specified time, t; P_0 is the amount of pheromone at the start and k is the rate constant. Because the evaporation rate, E = kP, the equation can be rewritten:

$$\ln E = -kt + \ln E_o \qquad \text{Equation 2}$$

Equations 1 and 2 show that when the evaporative loss is first order, that a plot of lnP versus time and a plot of lnE versus time will give parallel straight lines with slopes equal to -k.

These plots for the total of all three components are shown in Figure 5. The plot for the amount of pheromone remaining versus time

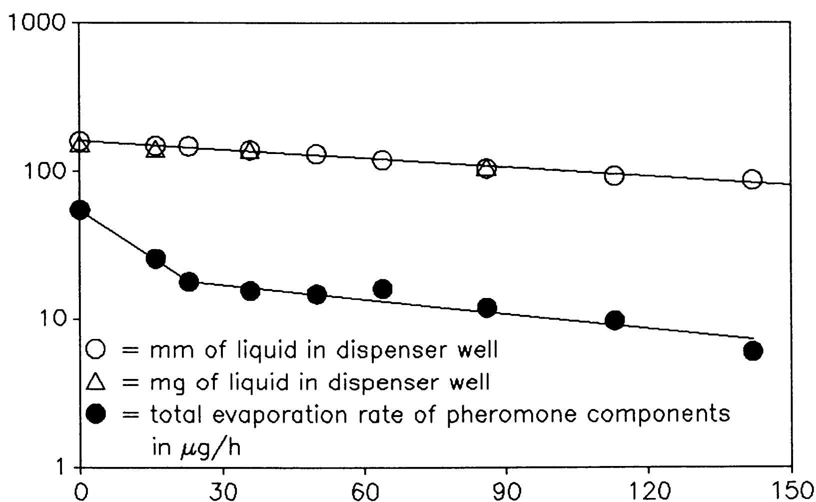


Figure 5. Loss of dispenser well contents as measured by well length and weight of well components vs. time, and loss of evaporation rate, E (total of all components) vs. time during 1991. Ordinate numbers are on logarithmic scale of titer in millimeters and milligrams, and E in μg/h. Abscissa numbers are days. (Reprinted from reference 38. Copyright 1992)

gave an excellent straight line, but the plot for evaporation rate versus time showed a much faster decrease during the first three weeks, and after the third week, the second leg of the line is steeper than the line for the amount of pheromone remaining.

Figure 6 shows a plot of lnE versus time for each individual component. The fast loss during the first three weeks occurred for each individual component. The second leg of the codlemone line is steeper

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

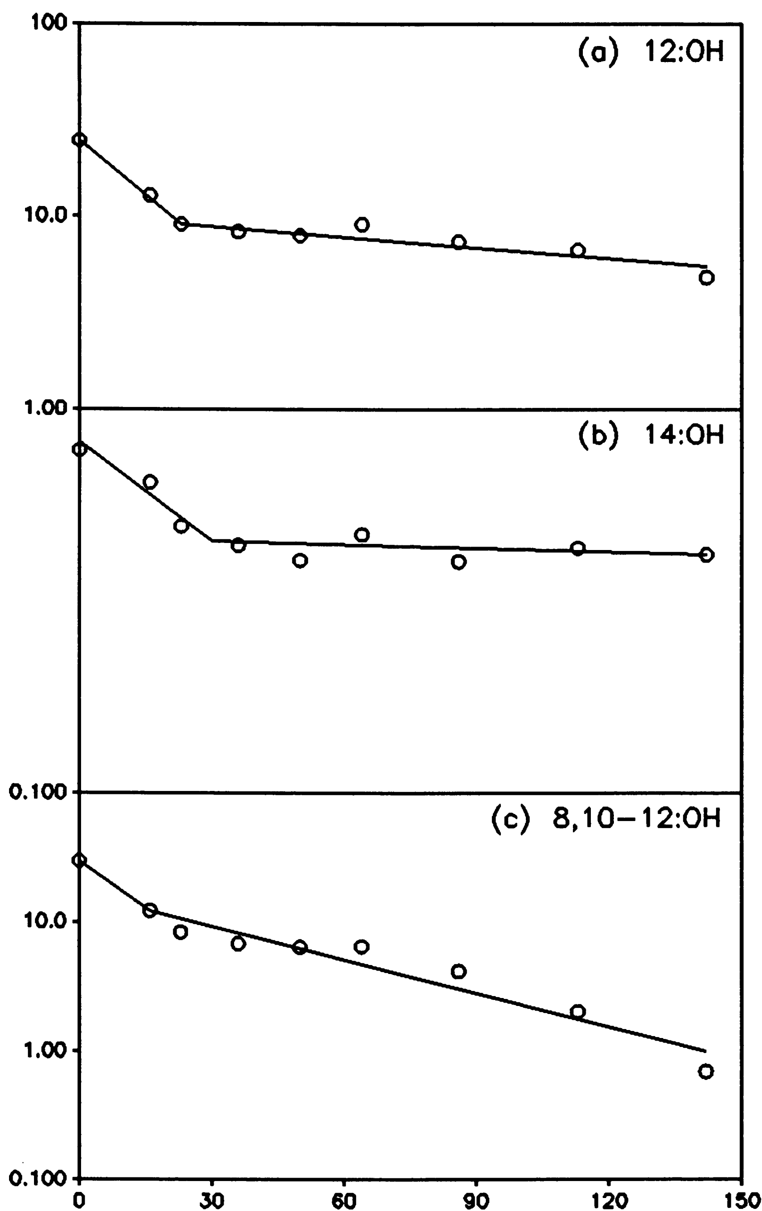


Figure 6. Evaporation rate, E (μg/hr), ordinate, versus time (days), abscissa, for 1991 dispensers for each pheromone component: (a) 12:OH; (b) 14:OH; (c) 8,10-12:OH.
(Reprinted from reference 38. Copyright 1992)

than the equivalent line for 12:OH. Since codlemone was expected to evaporate more slowly, this result suggested that concurrent chemical degradation of codlemone was occurring.

In order to understand the reason for the fast loss during the first 3 weeks and to determine the degree of chemical decomposition of codlemone, the basic processes involved in evaporative loss from the dispenser were analyzed. Two steps are involved in evaporative loss: first the contents of the dispenser well must dissolve in the dispenser wall, and second, the wall contents must evaporate into the atmosphere.

For a first order evaporative loss, the differential equation is:

$$E = -dP/dt = kP \qquad \text{Equation 3}$$

Note that the rate constant, k, is the ratio of the evaporation rate, E, to the amount of pheromone in the dispenser well, P. Two new constants were then defined: k_1 is the ratio of the amount of pheromone in the dispenser wall to that in the well; k_2 is the ratio of the evaporation rate to the amount of pheromone in the wall. If no chemical decomposition occurs, it follows that $k = k_1k_2$. If there is a first order chemical decomposition, then $k = k_1k_2 + k_d$, where k_d is the rate constant for chemical decomposition.

There are two methods of determining k. It can be evaluated from the slope of a plot of lnE versus time (Equation 2), or it can be determined by separately determining k_1 and k_2 at time intervals by measuring evaporation rates, wall contents, and well contents. If these two methods of estimating k agreed, then chemical decomposition did not occur. If the k values did not agree, then the difference between the values would be equal to k_d. Over the 86 day test period, k_2 was constant for each component and was $1.27*10^{-3}$ h^{-1} for codlemone, $1.96*10^{-3}$ h^{-1} for 12:OH, and $0.33*10^{-3}$ h^{-1} for 14:OH. These data showed that the evaporation rate of each pheromone component was proportional to the concentration of that component in the polyethylene wall, and that 12:OH evaporated faster than codlemone as expected from data on rubber septa. For all three components, k_1 values were initially 0.25 and decreased to 0.1 during the first three weeks and then remained constant thereafter. Therefore, the change in k_1 accounted for the rapid change in evaporation rates of the components during the first three weeks. It was speculated that the change in k_1 values was a result of weather induced changes in the polyethylene, perhaps crosslinking (38).

When k values were determined from Equation 2 (ignoring the data for the first three weeks), the value for 12:OH was $1.94*10^{-4}$ h^{-1} which was in excellent agreement with $k_1k_2 = 1.96*10^{-4}$ h^{-1}. For codlemone, the value of k from Equation 2 was $8.23*10^{-4}$ h^{-1} which was much greater than the value obtained from $k_1k_2 = 1.27*10^{-4}$ h^{-1}. The difference between these values gave $k_d = 6.96*10^{-4}$ h^{-1}.

These rate constants were then used to evaluate the performance of the dispensers during use in the apple orchard in 1991. After 150 days, 95% of the codlemone was lost from the dispensers; 61% was lost by chemical decomposition and 39% was lost by evaporation. Because of the large loss by chemical decomposition, one might think that this dispenser would be inadequate. However, during 4 years of testing (1988-1991), the dispenser was usually successful. During

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

1992, there were more reports of failure than in previous years, perhaps because 1992 was cooler than usual.

The minimum evaporation rate of codlemone needed for control of codling moth is not adequately established. The needed rate may vary with moth population, size of trees, and other environmental factors. Charmillot estimated the value at 10-40 mg ha^{-1} h^{-1} (22); Hathaway et al. estimated the value at 15 mg ha^{-1} h^{-1} (26); and Carde et al. estimated the value at 2 mg ha^{-1} h^{-1} (21). The values of Charmillot are based on the most extensive data. A needed rate of 10 mg ha^{-1} h^{-1} is a good working hypothesis for most situations.

Table III shows the change in evaporation rates at 20°C of codlemone in the Biocontrol dispensers during the 1991 season

Table III. Evaporation rate, E, of codlemone from Biocontrol dispensers (1,000 dispensers ha^{-1}) at 20°C at 3 week intervals

Time, days	E, mg ha^{-1} h^{-1}
0	29
21	11
42	7.2
63	4.7
84	3.1
105	2.1
126	1.4
147	0.9

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

(1,000 dispensers ha^{-1}). During the first generation of codling moths (0-42 days), evaporation rates were generally above the desired value of 10 mg ha^{-1} h^{-1}; but the rates were below this value during the second generation (84-126 days). It may be that good results were obtained with these dispensers because good control of the first generation produced low second generation populations which were relatively easy to control.

Codling moths can mate at temperatures as low as 12°C. At that temperature, evaporation rates would only be about 40% of those listed (40). Therefore, the lower temperatures during the first codling moth flight in 1992 might account for the lower success rates in that year.

Conclusions

Charmillot (22) showed that communication disruption with codlemone can be used to control codling moth. The main impediment to broad acceptance of this technique is economics. If the pheromone is used as the disruptant, then efficacy of control will be improved when an exact replicate of the natural pheromone is used. Only one of the three proposed components of the pheromone was found to be active, and if other active components exist, they remain to be discovered. The discovery of nonpheromonal semiochemicals (E8,Z10-12:OH and Z8,E10-12:OH) which are superior disruptants appears to offer

significant potential for improvement of the economics, but this discovery needs to be verified with tests under conditions of commercial use. Commercial controlled release dispensers, which prevent decomposition of codlemone, would also substantially improve the economics of communication disruption, and are perhaps the most important need if this technology is to be widely accepted.

Acknowledgment

We thank the Washington State Tree Fruit Commission for grants that partially supported this research.

Literature Cited

1. Karlson, P. and Luscher, M. Nature. 1959, 183, 55-56.
2. Law, J. H. and Regnier, F. E. Annual Review of Biochemistry. 1971, 40, 533-548.
3. Butenandt, A.; Beckman, R.; Stamm, D.; Hecker, E. Z. Naturforsch. 1959, 14b, 283-284.
4. Roelofs, W. L.; Glass, E. H.; Tette, J.; Comeau, A. J. Econ. Entomol. 1970, 63, 1162-1167.
5. Bedard, W. D.; Wood, D. L. In Management of Insect Pests with Semiochemicals: Concepts and Practice; Mitchell, E. R., Ed.; Plenum Press, New York, 1981; Chapter 11.
6. McDonough, L. M. U. S. Dept. of Agriculture/Science and Education Administration/Agricultural Research Results 1978, ARR-W-1/May.
7. Ashare, E.; Brooks, T. W.; Swenson, D. W. Insect Suppression with Controlled Release Pheromone Systems, Vol. 2; Kydonieus, A. F.; Beroza, M., Eds.; CRC Press Inc., Boca Raton, Florida, 1982; Chap. 9.
8. Quisumbing, A. R.; Kydonieus, A. F. Insect Suppression with Controlled Release Pheromone Systems, Vol. 2; Kydonieus, A. F.; Beroza, M., Eds.; CRC Press Inc., Boca Raton, Florida, 1982; Chap. 8.
9. Mitchell, E. R., ed.; Management of Insect Pests with Semiochemicals. Plenum Press, New York and London, 1981.
10. Ritter, F. J., ed., Chemical Ecology: Odour Communication in Animals. Elsevier, North-Holland Amsterdam, 1979.
11. Shorey, H. H. and McKelvey, J. J., eds., Chemical Control of Insect Behavior, John Wiley and Sons, New York, 1977.
12. Silverstein, R. M. In Behavior Modifying Chemicals for Insect Management; Ridgway, R. L.; Silverstein, R. M.; Inscoe, M. N., Eds.; Marcel Dekker, Inc. New York, 1990; Chapter 1.
13. Vickers, R. A. In Behavior Modifying Chemicals for Insect Management; Ridgway, R. L.; Silverstein, R. M.; Inscoe, M. N., Eds.; Marcel Dekker, Inc. New York, 1990; Chapter 12.
14. Rice, R. E.; Kirsch, P. In Behavior Modifying Chemicals for Insect Management; Ridgway, R. L.; Silverstein, R. M.; Inscoe, M. N., Eds.; Marcel Dekker, Inc. New York, 1990; Chapter 13.
15. Roelofs, W. L.; Comeau, A.; Selle, R. Nature 1969, 224, 723.
16. Roelofs, W. L.; Carde, R. T.; Tette, J. P. Environ. Entomol. 1973, 2, 252-254.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

17. Beroza, M.; Gentry, C. R.; Blythe, J. L.; Muschik, G. M.; J. Econ. Entomol. 1973, 66, 1307-1311.
18. Carde, A. M.; Baker, T. C.; Carde, R. T. J. Chem. Ecol. 1979, 5, 423-427.
19. Varela, L. G.; Welter, S. C.; Jones, V. P.; Brunner, J. F.; Riedl, H. J. Econ. Entomol. 1993, 86, 1-10.
20. Butt, B. A.; Beroza, M. 1973, Unpublished personal communication.
21. Carde, R. T.; Baker, T. C.; Castrovillo, P. J. Entomol. Exp. Appl. 1978, 22, 280-288.
22. Charmillot, P. J. In Behavior Modifying Chemicals for Insect Management; Ridgway, R. L.; Silverstein, R. M.; Inscoe, M. N., Eds.; Marcel Dekker, Inc. New York, 1990; Chapter 11.
23. Hathaway, D. O.; McGovern, T. P.; Beroza, M.; Moffitt, H. R.; McDonough, L. M.; Butt, B. A. Environ. Entomol. 1974, 3, 522-524.
24. Arn, A.; Schwartz, C.; Limacher, H.; Mani, E. Experentia 1974, 30, 1142-1144.
25. George, D. A.; McDonough, L. M.; Hathaway, D. O.; Moffitt, H. R. Environ. Entomol. 1975, 4, 606-608.
26. Hathaway, D. O.; McDonough, L. M.; George, D. A.; Moffitt, H. R. Environ. Entomol. 1979, 8, 318-321.
27. Minks, A. K.; Carde, R. T. Entomol. Exp. Appl. 1988, 49, 25-36.
28. Roelofs, W. L. J. Chem. Ecol. 1978, 4, 685-699.
29. McLaughlin, J. R.; Mitchell, E. R.; Chambers, D. L.; Tumlinson, J. H. Environ. Entomol. 1974, 3, 677-680.
30. Roelofs, W. L.; Comeau, A.; Hill, A.; Milicevic, G. Science 1971, 174, 297-299.
31. Bartell, R. J.; Bellas, T. E. J. Aust. Entomol. Soc. 1981, 20, 197-199.
32. Einhorn, J.; Beauvis, F.; Gallois, M.; Descoins, C.; Causse, R. C. R. Acad. Sci. Paris 1984, 299, 773-778.
33. Arn, H.; Guerin, P. M.; Buser, H. R.; Rauscher, S.; Mani, E. Experientia 1985, 41, 1482-1484.
34. Bartell, R. J.; Bellas, T. E.; Whittle, C. P. J. Aust. Entomol. Soc. 1988, 27, 11-12.
35. McDonough, L. M.; Davis, H. G.; Chapman, P. S.; Smithhisler, C. L. J. Chem. Ecol. 1993, 19, 1737-1748.
36. McDonough, L. M.; Davis, H. G.; Chapman, P. S.; Smithhisler, C. L. J. Chem. Ecol., in press.
37. Roelofs, W. L.; Bartell, R. J.; Hill, A. S.; Carde, R. T.; Waters, L. A. J. Econ. Entomol. 1972, 65, 1276-1277.
38. McDonough, L. M.; Aller, W. C.; Knight, A. L. J. Chem. Ecol. 1992, 18, 2177-2189.
39. Weatherston, I. In Behavior Modifying Chemicals for Insect Management; Ridgway, R. L.; Silverstein, R. M.; Inscoe, M. N., Eds.; Marcel Dekker, Inc. New York, 1990; Chapter 6.
40. Brown, D. F.; Knight, A. L.; Howell, J. F.; Sell, C. R.; Krysan, J. L.; Weiss, M. J. Econ. Entomol. 1992, 85, 910-917.

RECEIVED February 16, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch013

Chapter 14

Metabolites of *Pseudomonas corrugata* That Elicit Plant Defense Reactions

D. L. Gustine[1], R. T. Sherwood[1], F. L. Lukezic[2], B. G. Moyer[1], and W. S. Devlin[3]

[1]Pasture Systems & Watershed Management Research, U.S. Regional Pasture Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, University Park, PA 16802
Departments of [2]Plant Pathology and [3]Agronomy, Pennsylvania State University, University Park, PA 16802

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

Pseudomonas corrugata, a bacterial pathogen of tomato, and its metabolites elicited components of the hypersensitive reaction (HR) in two nonhost plants, Ladino white clover and Havana 44 tobacco. Live bacterial cells (8 x 10^8 colony-forming units/mL) elicited 1) biosynthesis of the phytoalexin medicarpin in white clover leaflets, 2) medicarpin production and transient formation of the active oxygen (AO) species, hydrogen peroxide and superoxide, in white clover callus suspension cells, and 3) potassium ion efflux and hypersensitive necrosis in tobacco leaf tissue. The AO burst was not required for induced phytoalexin synthesis or for elicitation of hypersensitive necrosis. Elicitors of the responses were isolated from *P. corrugata* and characterized. A fraction consisting of unidentified carbohydrate and phosphate synergistically elicited the phytoalexin response in combination with either of two chromophoric peptides isolated from *P. corrugata* culture fluids. The two peptides, HR1 and HR2, elicited hypersensitive necrosis.

Breeders must continually develop crop cultivars having superior resistance to microbes and insects if farmers are to maintain high yields and quality in the face of evolving pests. Understanding the biochemical and physiological bases of the many mechanisms of plant resistance will yield information that can then be used to genetically engineer crops. The explosion of reported work on molecular biology of plant growth and regulation and of genetic engineering of crops is already resulting in improved crops. Examples include insertion of antisense messages against pectinase to delay softening of the fruit and thus yield better tasting tomatoes, against caffeic acid O-methyltransferase to reduce lignin content for increased digestibility of forages and against pterocarpan synthase to directly determine the role of the phytoalexins in disease resistance (*1*). Other examples are insertion of viral coat protein genes into plant genomes to effectively increase

0097–6156/94/0557–0169$08.00/0
© 1994 American Chemical Society

resistance to more than 20 viruses (*2*) and overexpression of rate-limiting enzymes to provide herbicide resistance (*3,4*). These intellectually and scientifically satisfying approaches were possible because of the recently gained ability to apply the tools of molecular biology to cultured plant tissues and to regenerate genetically stable engineered plants. Thus, genetic engineering has greatly increased our knowledge of the biochemistry, physiology, cellular structure, and biology of plants.

The research reported here focuses on the biochemical and physiological interactions between the tomato pathogen *Pseudomonas corrugata* and the nonhost plants, white clover (*Trifolium repens* L.) and tobacco (*Nicotiana tabacum* L.). We have partially characterized bioregulators produced by *P. corrugata* that may be useful in investigating basic mechanisms of induced plant resistance.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

The Hypersensitive Reaction is a Mixed Bag of Defense Responses

When host plant cells and microbes interact in an incompatible fashion (resistance expression), host processes are set in motion that lead to autotoxicity and visible signs of resistance. Hypersensitive necrosis spreads until all host cells within and at the periphery of the infection court collapse and die. In this way, spread of the pathogen is checked (*5*) with minimal sacrifice of tissue. This localized, rather than systemic response, is thus advantageous to the plant. In field-grown plants, such resistance is observed as necrotic flecks on leaf or stem tissue. This response is called the hypersensitive reaction, or HR (*6*).

At the cellular level, leaf tissue and cell cultures produce a transient burst of active oxygen (AO) within minutes of exposure to pathogenic bacteria (*7,8*), fungi (*9*), fungal cell wall components (*10,11*) and abiotic elicitors (*7,12*). At least three AO species are generated: superoxide (O_2^-), hydroxyl radical ($^{\cdot}OH$) and H_2O_2. These AO species are thought to initiate a chain reaction that leads to lipid peroxidation in the plasma membrane of the host (*13-16*). The peroxidation of lipid then leads to membrane leakage and influx of signal molecules into the cell (*10,12,16*). Membrane leakage is detected as increased rate of K^+/H^+ exchange during an incompatible interaction (*17-19*).

Other induced plant defense responses that have been described may occur along with, or independently of, hypersensitive necrosis. These include synthesis and accumulation of antimicrobial metabolites called phytoalexins; strengthening of the cell wall through synthesis and deposition of hydroxy-rich glycoproteins (HRGP), callose, and lignin; synthesis of lytic enzymes such as chitinases and endo β-glucanases (*1,20*); and synthesis of fungal-specific ribosome inactivating proteins (RIP) (*21*). All or some of the responses described above may be detected during the HR. It follows that observation of any one of them indicates that HR has occurred.

Elicitors Initiate Signalling of Defense Responses. An elicitor as defined by Keen and Holliday (*22*) is a microbially produced factor that induces the localized synthesis and accumulation of phytoalexins to concentrations that are toxic to the invading microbe. An elicitor as defined by us is a factor(s) of a pathogen that is directly responsible for eliciting plant signals in a nonhost that transduce one or

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

more of the defense responses described above. For bacteria, the factor that induces HR (detected as hypersensitive necrosis) in a nonhost, appears to be required for pathogenicity in its host (*5,6,23*). Bacterial elicitors of HR in nonhosts are determined by *hrp* (hypersensitive response and pathogenicity) genes (*24*). The *avr* (avirulence) genes determine host specificity.

P. corrugata **Produces Elicitors of HR and the Phytoalexin Response.** We observed that live cells or culture fluid of *P. corrugata* elicited the synthesis and accumulation of the phytoalexin medicarpin in Ladino white clover callus (*25*) and hypersensitive necrosis in infiltrated Havana 44 tobacco leaf panels (*26,27*). Various authors have proposed that hypersensitive necrosis as well as phytoalexin synthesis is mediated by active oxygen, but it is still not clear whether the AO burst is involved in mediating the phytoalexin response (*7*). The objectives of our research are to 1) compare defense responses for tobacco and white clover to *P. corrugata* and to biotic and abiotic elicitors, 2) determine the role of the oxidative burst in elicitation of the phytoalexin response in white clover tissue culture, 3) isolate elicitors of HR and phytoalexin synthesis from *P. corrugata*, and 4) chemically characterize the elicitors.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

Indications of Different Mechanisms for Individual HR Responses

The pterocarpan phytoalexin medicarpin is found in white clover, red clover, alfalfa, and jackbean. It can be elicited by bacteria, fungi, sulfhydryl reagents, or pectic fragments in plant tissues, callus tissue, or suspension cultures (7,25,28-32). Medicarpin is derived from the phenylpropanoid pathway (*33*), which provides precursors for lignin and many induced plant defense compounds. Pterocarpan phytoalexins also occur in soybean (glyceollins) and French bean (phaseollin) (*34*).

The scheme in Figure 1 depicts components of signalling, regulation, and molecular biology of expression of plant defense genes involved in phytoalexin synthesis as the result of fungus/plant cell interaction. Fungal pectinases and cellulases begin to digest accessible parts of the host cell wall and release oligosaccharide fragments, some of which are specifically bound by elicitor receptors in the host plasma membrane. The binding process causes production of signal molecules through unknown mechanisms, perhaps involving cAMP, phosphoinositol, or phosphorylation/dephosphorylation of certain proteins. These uncharacterized signals rapidly propagate through the nucleus and into the nucleolus where they act directly in derepressing or repressing promoters which activate expression of genes for the enzymes in the medicarpin biosynthetic pathway. Rapid synthesis of mRNAs coding for the five enzymes shown in Figure 1 and *de novo* synthesis of those enzymes is well established (*35*). Other pathogenesis-related proteins such as chitinases and glucanases are also synthesized as a result of defense gene activation, and are thought to be important in degrading the cell wall of any "potential pathogen" (*20*). Lipid peroxidation, increased permeability of the plasma membrane, loss of K^+, production of AO species, and hypersensitive necrosis may occur concurrently in the host.

Responses of White Clover and Tobacco to *P. corrugata* and $HgCl_2$. The synthesis and accumulation of medicarpin in Ladino white clover callus treated with optimum concentrations of *P. corrugata* cells or the abiotic elicitor $HgCl_2$ was rapid (Figure 2). Medicarpin concentrations reached maximum levels in a matter of hours in callus and suspension cultures, but levels were 5- to 10-fold lower in detached leaflets (*25*). Suspension cells treated with *P. corrugata* cells or high concentrations of $HgCl_2$ (150 μM or greater) produced a transient AO within minutes (*7*), with peak AO production occurring about 10 to 20 min after elicitation (Figure 3).

Hypersensitive necrosis was produced in tobacco leaf panels. Tissue of tobacco leaf panels infiltrated with either suspended *P. corrugata* cells or $HgCl_2$ (300 μM or greater) recovered from water soaking within minutes; for 6 to 15 h treated tissue was indistinguishable from control tissue infiltrated with water. Faint chlorosis developed after 6 to 15 h and was followed closely by cell collapse. The tissue was desiccated within 12 to 36 h after infiltration. This response was typical of HR, and was indistinguishable from that produced by *P. syringae* pv. *syringae*, an incompatible pathogen on tobacco (*36*).

Rapid loss of K^+ from tobacco leaf disks was detected. Tobacco leaf disks infiltrated with *P. corrugata* cells and floated on 0.5 mM Tris/Mes buffer and 0.5 mM $CaCl_2$, (pH 6.0), rapidly lost K^+ within 2 h, and reached a maximum external K^+ level by 10 h (Figure 4), indicating membrane deterioration. This was accompanied by uptake of H^+, which was detected as a pH increase in the medium. These changes in K^+ and H^+ concentrations were comparable to those reported for *P. syringae* in both tobacco leaf disks and tobacco suspension cultures (*36*).

Active Oxygen is Not Required for HR. Devlin (*37*) proposed the membrane deterioration model (Figure 5) to account for the elicitation of HR by bacteria in an incompatible interaction with host cells. Since the AO species O_2^- and H_2O_2 are produced at the interface, removal of these species would prevent the HR if they are part of the signalling mechanism or the cause of HR. Addition of superoxide dismutase (SOD) would prevent formation of the highly reactive hydroxyl radical $^{\cdot}OH$ and convert O_2^- to H_2O_2, and addition of catalase would remove H_2O_2. This can be tested in the presence of luminol, which reacts with H_2O_2 to form an unstable product that during decay produces measurable chemiluminescence in proportion to the amount of H_2O_2. Devlin and Gustine (*7*) found that addition of SOD to white clover suspension cells two minutes prior to adding *P. corrugata* cells slightly reduced the AO burst, while addition of catalase greatly reduced it. They concluded that H_2O_2 was the main component of the AO burst and that no O_2^- was produced. Addition of catalase or both SOD and catalase completely eliminated the transient production of AO. They then tested whether elimination of the AO burst would also prevent elicited medicarpin synthesis in white clover suspension cells and/or HR in infiltrated tobacco leaf panels. In the presence of SOD and catalase, *P. corrugata* at 0.4 x 10^8 cfu still elicited medicarpin synthesis, suggesting no involvement of AO in the phytoalexin defense response. Further indication of this was demonstrated by the fact that concentrations of 25 or 42 μM $HgCl_2$, which elicited synthesis and accumulation of medicarpin, did not elicit an

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

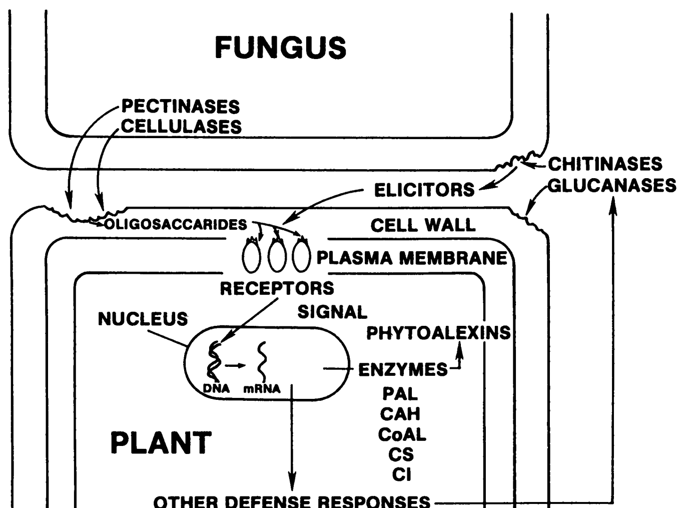


Figure 1. Receptor model for generation of defense signalling and defense responses during host plant/fungus interaction. In this model, host plasma membrane receptors combine with fungal elicitors and initiate activation of defense genes necessary for phytoalexin synthesis and generation of hydrolytic enzymes that attack the fungal cell wall. **PAL**, phenylalanine ammonia lyase; **CAH**, cinnamic acid hydroxylase; **CoAL**, CoA ligase; **CS**, chalcone synthase; **CI**, chalcone isomerase. Adapted from ref. *38*.

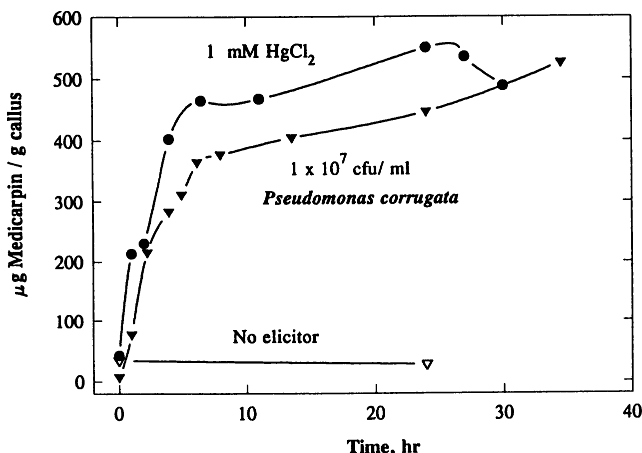


Figure 2. Elicitation of medicarpin in Ladino white clover callus by biotic and abiotic elicitors. Callus (1 to 2 g) in 1 mL of culture medium was incubated with live cells of *Pseudomonas corrugata* and with $HgCl_2$ at 24 C for the indicated times. The phytoalexin medicarpin was determined in callus extracts for each time point by analytical HPLC. The preparation of callus and experimental conditions were as described by Gustine (*28*). **cfu**, colony forming units.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

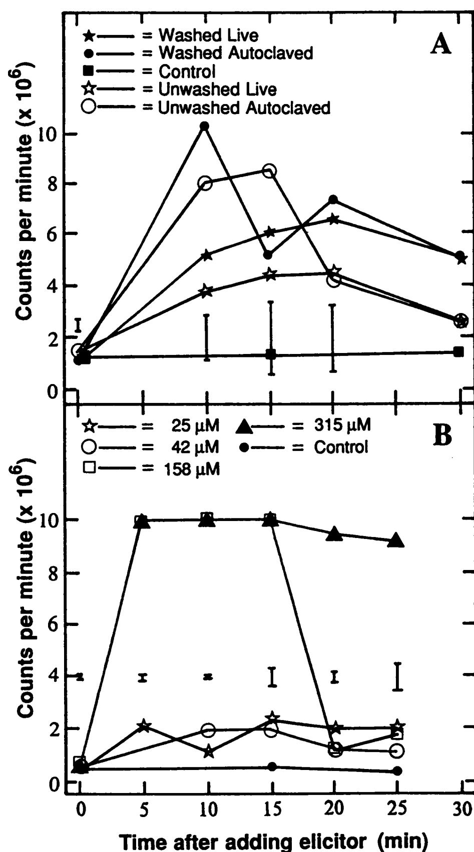


Figure 3. Short-term elicitation of the oxidative burst. Following addition of elicitors (A, *Pseudomonas corrugata*, 0.4 x 10^8 colony forming units/mL; or B, $HgCl_2$) to Ladino white clover suspension cultures, luminol-coupled chemiluminescence was measured during a 30-min period. These cultures were examined after 24 hours of culture, and had increased levels of the phytoalexin medicarpin. Reproduced with permission from ref. 7. Copyright 1992 American Society of Plant Physiologists.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

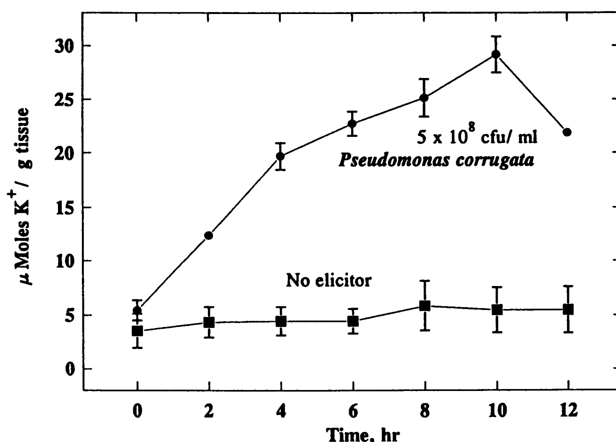


Figure 4. Efflux of K^+ ion from tobacco leaf disks exposed to *Pseudomonas corrugata*. Tobacco leaf disks were infiltrated with live cells of *P. corrugata* and potassium levels monitored over 12 hours. Experiment conducted as described (*18*); potassium in buffer was determined by atomic absorption spectroscopy.

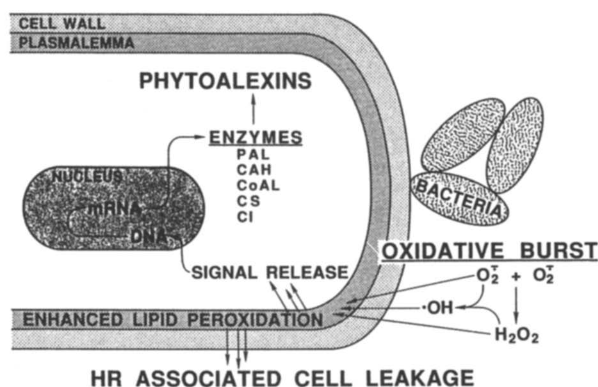


Figure 5. Membrane deterioration model for generation of oxidative burst, defense signalling, and defense responses during host plant/bacteria interaction. Bacteria at the host cell surface activate rapid autolysis initiated by oxidative reactions in the plasma lemma. This host regulated response leads to lipid peroxidation and subsequent electrolyte leakage and generation of signals that activate defense genes. **PAL**, phenylalanine ammonia lyase; **CAH**, cinnamic acid hydroxylase; **CoAL**, CoA ligase; **CS**, chalcone synthase; **CI**, chalcone isomerase. Reproduced with permission from ref. *37*. Copyright, 1991, Winifred S. Devlin.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

AO burst. Further, autoclaved *P. corrugata* elicited the AO burst, but did not elicit medicarpin accumulation. Taken together, these results provide compelling evidence that transient production of AO species neither signals nor directly causes the expression of the phytoalexin response in Ladino white clover suspension cultures.

The role of AO production in rapid necrosis in tobacco leaf tissue was less clear. The AO burst was not tested in tobacco leaves, although it does occur in tobacco suspension cultures (*19*). $HgCl_2$ at 315 μM elicited hypersensitive necrosis in tobacco leaf panels, but the lower concentrations which elicited AO production in clover suspension cells failed to elicit rapid necrosis (*7*). Both live *P. corrugata* (8 x 10^8 cfu) and autoclaved *P. corrugata* elicited the AO burst in clover suspension cells, but only live bacteria elicited the rapid necrosis typical of HR in tobacco leaf panels. These results again indicated lack of AO involvement. Infiltration of tobacco leaf panels with live *P. corrugata* and catalase and SOD, resulted in a typical HR that was indistinguishable from *P. corrugata* cells alone (*37*). While SOD and catalase prevented the AO burst within the first 20 minutes, this may not have been true at 24 hours, since the enzyme activities were not established at the time of HR evaluation (*7,37*). Even so, it appears that the AO burst was not the cause of hypersensitive necrosis.

P. corrugata Elaborates Elicitors

Liquid cultures (2 liters) of *P. corrugata* grown on an orbital shaker for 24 h at 25 C in modified King's B medium provided elicitor-active cells and culture fluid, from which a dialysate fraction containing elicitor-active metabolites >3.5 kD (Figure 6) was prepared (*25*). Preparative reversed phase HPLC of the elicitor-active dialysate fraction yielded four active fractions (*38*) that seemed to be involved in elicitation of both phytoalexin and HR (Fraction 1, HR - and Med 197; Fraction 2, HR +++ and Med 24; Fraction 3, HR ++++ and Med 25; Fraction 4, HR - and Med 42. See Figure 6 legend for abbreviations and units.). While it was clear that Fractions 2 and 3 elicited HR in tobacco leaf panels, Fraction 1 elicited medicarpin synthesis in white clover callus at low levels and did not account for all of the activity prior to separation by HPLC. The activity was restored in a synergistic interaction when Fraction 1 was combined with Fraction 4 (*25*); however, mg quantities of Fraction 1, which contained mostly reducing carbohydrate and inorganic phosphate, were required. It is not known whether phytoalexin elicitor activity of Fraction 1 was due to osmotic/ionic stress, or whether it was due to a minor constituent of Fraction 1.

HR1 (Fraction 2) was purified by CM-Sephadex column chromatography (20 to 50 mM step gradient of sodium acetate, pH 5.0) and HR2 (Fraction 3) was purified by three cycles of partitioning from water to ethyl acetate and back to water. The purified preparations of HR1 always had 2 to 5% HR2, and vice versa. Microgram quantities of HR1 and HR2 accounted for the hypersensitive necrosis activity on tobacco leaves that was present in the original culture filtrate. A further indication of HR2 elicitor activity was the stimulation of K^+ efflux from tobacco leaf disks (Table I). Leaf disks were infiltrated under vacuum with HR2 or live bacteria in 0.05 mM Tris/Mes (pH 6.0)/0.5 mM $CaCl_2$ buffer. Levels of

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

K^+ in the buffer increased over 12 h as a result of exposure of tobacco leaf disks to HR2 or *P. syringae*.

Table I. Efflux of K^+ from Tobacco Leaf Disks Treated with HR2 and *P. Syringae* pv. *Syringae*[a]

Hours after Elicitation	*Buffer Control*	*HR2*	*P. Syringae pv. Syringae*
	μmoles K^+/g Tissue (+/-Standard Deviation, n=2)		
0	2.0 (0.4)	2.2 (0.1)	0.7 (0.1)
2	3.0 (0.4)	5.0 (0.5)	2.7 (0.6)
4	3.2 (0.4)	5.5 (0.5)	3.3 (0.6)
6	3.3 (0.4)	5.7 (0.5)	3.5 (0.9)
8	3.6 (0.4)	7.4 (0.7)	5.1 (1.3)
10	3.4 (0.3)	8.7 (0.6)	7.3 (1.5)
12	3.3 (0.3)	9.6 (0.3)	9.4 (1.7)

[a]Experimental conditions as in Figure 4.

The responses produced by *P. corrugata* in leaf disks (Figure 4) and HR2 (Table I) were similar to those by wild type *P. syringae* pv. *syringae* (*36*), an organism which elicits both hypersensitive necrosis and K^+ efflux in tobacco. These results suggested that HR2, *P. corrugata*, and *P. syringae* had essentially identical effects on the plasma membrane, namely the K^+/H^+ exchange. This early response to an elicitor is one of the defining indicators of HR.

HR1 and HR2 are Peptides

The fraction that elicited phytoalexin synthesis was not characterized, as it seemed unlikely that components needed in such large quantities to stimulate an HR response would have significance for *in vivo* plant/microbe interactions. However, HR1 and HR2 appear to be physiologically important because they were active at much lower concentrations. Hydrolysis of both elicitors in 6N HCl in sealed tubes and chromatographic separation of the products by TLC yielded ninhydrin positive constituents (Figure 7). Since the starting material did not react with ninhydrin, we concluded that the elicitors are peptides with blocked N-terminal amino groups. We also concluded that both elicitors contained a chromophore, since the compounds fluoresced under UV illumination on the TLC plate (Figure 7) and their UV spectra (absorbance maxima, nm: HR1 199, 242sh, 392; HR2 192, 250, 398) were not characteristic of a peptide. The free amino groups in hydrolysates were derivitized with o-pthalaldehyde and separated by analytical reversed phase HPLC

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

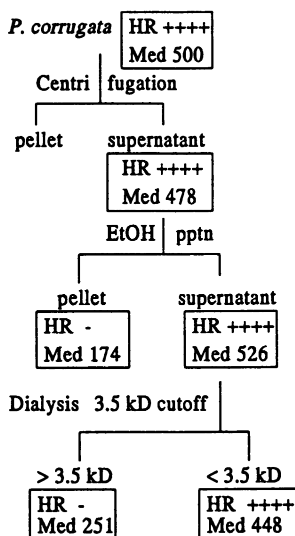


Figure 6. Summary of fractionation of phytoalexin and hypersensitive reaction (HR) activities from *Pseudomonas corrugata* culture fluids. Cultures were grown for 24 h at 25 C, and fractions prepared as described (*26*). Fractions were infiltrated into Havana 44 leaf panels and rated after 24 hours (*7, 26*). HR extent : ++++, full HR; +++, ++, and +, hypersensitive necrosis in 3/4, 1/2, and 1/4 of panel, respectively; - no HR. Phytoalexin activity: Med, μg medicarpin/g callus, fresh weight.

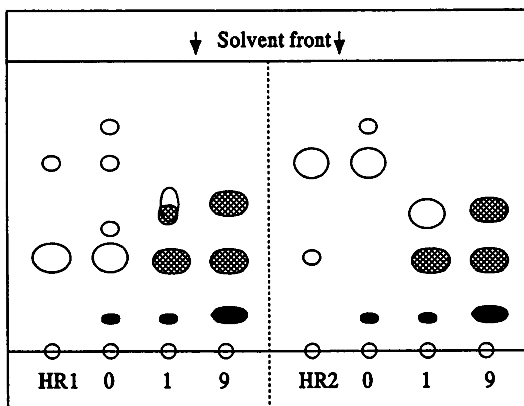


Figure 7. TLC separation of products from hydrolysis of HR1 and HR2 in 6N HCl. The elicitors were hydrolyzed in acid for 0, 1, and 9 hours at 110 C in sealed tubes. The hydrolysates were applied to a 0.25 μm silica gel (E.M. Merck) TLC plate and developed in 1-butanol-glacial acetic acid-water (4:1:1; v,v,v). The ovals indicate fluorescence under ultraviolet (UV) illumination; crosshatched ovals indicated fluorescence under UV and reaction with ninhydrin; dark ovals indicate reaction with ninhydrin only.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

as described (*39*). Amino acids were identified by elution profile and quantified by peak area integration: glutamic acid, aspartic acid, glycine, alanine or arginine, and leucine or isoleucine (10:3:1:1:1). HR1 and HR2 had similar amino acid compositions.

Conclusions

The discovery that *P. corrugata* produced metabolites which activate various defense responses associated with the HR, suggests that this bacterium may be a source of novel bioregulators. The properties of these metabolites might be useful in designing more effective bioregulators for increased crop resistance. The bioregulators could be delivered to crops through introduction of an engineered, non pathogenic form of *P. corrugata* or through application of purified bioactive compounds from fermentation cultures. The elicitors could be modified through chemical or genetic approaches to yield more active bioregulators, or to produce bioregulators affecting different plant functions. An important application of elicitor-active bacterial metabolites will be their use in examining the earliest events in signalling and regulating defense responses. These metabolites could be radiolabeled in culture and then used for locating the host plant sites of interaction. As more active modified forms of HR1 and HR2 are identified, other host components involved in resistance responses can be examined. Such studies will likely lead to identification of plant regulatory genes that control host resistance.

Acknowledgments

We thank Rick Pedersen for his excellent technical help and Scott Donahue for his help in data analysis.

Literature Cited

1. Lamb, C.J.; Ryals, J.A.; Ward, E.R.; Dixon, R.A. *Bio/Technology.* **1992**, *10*, 1436-1445.
2. Scholthof, K-B.G.; Scholthof, H.B.; Jackson, A.O. *Plant Physiol.* **1993**, *102*, 7-12.
3. Hartnett, M.E.; Chui, C.-F.;Mauvais, C.J.; McDivitt, R.E.; Knowlton, S.; Smith, J.K.; Falco, S.C.; Mazur, B.J. In *Fundamental and Practical Approaches to Combating Resistance*; LeBaron, H., Mowberg, W., Green, M., Ed.; Herbicide-resistant plants carrying mutated acetolactate synthase genes; ACS Books: Washington, D.C., 1989; pp 459-473.
4. Della-Cioppa, G.; Bauer, S.C.; Taylor, M.L.; Rochester, D.E.; Klein, B.K.; Shah, D.M.; Fraley, R.T.; Kishore, G.M. *Bio/Technology.* **1987**, *5*, 579-584.
5. Wei, Z-M; Laby, R.J.; Zumoff, C.H.; Bauer, D.W.; He, S.Y.; Collmer, A.; Beer, S.V. *Science.* **1992**, *257*, 85-88.
6. Klement, Z. In *Phytopathogenic Prokaryotes*; Mount, M.S., Lacy, G.H., Ed.; Hypersensitivity; Academic Press: New York, 1982; pp 149-177.
7. Devlin, W.S.; Gustine, D.L. *Plant Physiol.* **1992,** *100*, 1189-1195.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

8. Keppler, L.D.; Baker, C.J.; Atkinson, M.M. *Phytopathology*. **1989**, *79*, 974-978.
9. Doke, N. *Physiol. Plant Pathol.* **1985**, *27*, 311-322.
10. Apostol, I.; Heinstein, P.F.; Low, P.S. *Plant Physiol.* **1989**, *90*, 109-116.
11. Doke, N. *Physiol. Plant Pathol.* **1983**, *23*, 359-367.
12. Epperlein, M.M.; Noronha-Dutra, A.A.; Strange, R.N. *Physiol. Mol. Plant Pathol.* **1986**, *28*, 67-77.
13. Adam, A.; Farkas, T.; Somlyai, G.; Hevesi, M.; Kiraly, Z. *Physiol. Mol. Plant Pathol.* **1989**, *34*, 13-26.
14. Keppler, L.D.; Novacky, A. *Phytopathology*. **1986**, *76*, 104-108.
15. Keppler, L.D.; Novacky, A. *Physiol. Mol. Plant Pathol.* **1987**, *30*, 233-245.
16. Rogers, K.R.; Albert, A.; Anderson, A.J. *Plant Physiol.* **1988**, *86*, 547-553.
17. Hutcheson, S. W.; Collmer, A.; Baker, C.J. *Physiol. Plant.* **1989**, *76*, 155-163.
18. Atkinson, M. M.; Baker, C. J. *Phytopathology*. **1987**, *77*, 1273-1279.
19. Baker, C.J.; O'Neill, N.R.; Keppler, D.; Orlandi, E.W. *Phytopathology*. **1991**, *81*, 1504-1507.
20. Dixon, R.A.; Lamb, C.J. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1990**, *41*, 339-367.
21. Vigers, A.J.; Roberts, W.K.; Selitrennikoff, C.P. *Mol. Plant-Microbe Int.* **1991**, *4*, 315-323.
22. Keen, N.T.; Holiday, M.J. In *Phytopathogenic Prokaryotes*; Mount, M.S., Lacy, G.H., Ed.; Recognition of bacterial pathogens by plants; Academic Press: New York, 1982; pp 179-217.
23. Lindgreen, P.B.; Peet, R.C.; Panopoulos, N.J. *J. Bacteriol.* **1986**, *168*, 512-522.
24. Kamoun, S.; Kado, C.I. *J. Bacteriol.* **1990**, *172*, 5165-5172.
25. Gustine, D.L.; Sherwood, R.T.; Moyer, B.G.; Lukezic, F.L. *Phytopathology*. **1990**, *80*, 1427-1432.
26. Lukezic, F.L. *Phytopathology*. **1979**, *69*, 27-31.
27. Gustine, D.L.; Moyer, B.G.; Sherwood, R.T.; Lukezic, F.L. *Plant Physiol.* **1991**, *96* (Suppl.), 53.
28. Gustine, D.L. *Plant Physiol.* **1981**, *68*, 1323-1326.
29. Gustine, D.L. In *Natural Resistance of Plants to Pests: Roles of Allelochemicals*; Green, M.B., Hedin, P.H., Ed.; Regulation of accumulated phytoalexin levels in Ladino clover callus; ACS Books: Washington, D.C., 1986; pp 55-69.
30. Gustine, D.L.; Moyer, B.G. *Plant Cell Tiss. Org. Cult.* **1982**, *1*, 255-263.
31. Gustine, D.L.; Sherwood, R.T.; Vance, C.P. *Plant Physiol.* **1978**, *61*, 226-230.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

32. West, C.A.; Bruce, R.J.; Jin, D.F. In *Structure, Function, and Biosynthesis of Plant Cell Walls*; Dugger, W.M., Bartnicki-Garcia, S., Ed.; Pectic fragments of plant cell walls as mediators of stress responses; Waverly Press: Baltimore, 1984, pp 359-379.
33. Dalkin, K.; Edwards, R.; Edington, B.; Dixon, R.A. *Plant Physiol.* **1990**, *92*, 440-446.
34. Lamb, C.J.; Lawton, M.A.; Dron, M.; Dixon, R.A. *Cell.* **1989**, *56*, 215-224.
35. Hahlbrock, K.; Scheel, D. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 347-369.
36. Baker, C.J.; Atkinson, M.M.; Collmer, A. *Phytopathology.* **1987**, *77*, 1268-1272.
37. Devlin, W.S. *A Role for Oxidative Stress in the Elicitation of Plant Defense Responses*; Ph.D. Thesis, The Pennsylvania State University; Winifred Swope Devlin, State College, PA, 1991; pp 1-164.
38. Gustine, D.L.; Sherwood, R.T.; Lukezic, F.L.; Moyer, B.G. In *Mechanisms of Plant Perception and Responses to Environmental Stimuli*; Thomas, T.H.; Smith, A.R., Ed.; Phytoalexin biosynthesis is elicited by metabolites of the bacterium, *Pseudomonas corrugata*; British Society for Plant Growth Regulation Monograph No. 20; Parchments Ltd.: Oxford, UK, 1990; pp 173-183.
39. Gustine, D.L. *J. Chromatogr.* **1985**, *319*, 450-453.

RECEIVED February 9, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch014

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

Chapter 15

Mechanisms of *Heliothis virescens* Resistance to Exogenous Ecdysteroids

Minli Zhang[1] and Isao Kubo[2]

Division of Entomology, Plant and Soil Microbiology, College of Natural Resources, University of California, Berkeley, CA 94720

Tobacco budworm, *Heliothis virescens* can tolerate high doses of ecdysteroids introduced by feeding. We studied the absorption, distribution and metabolic fate of ecdysone in this insect in order to understand the mechanisms involved in this resistance. Ecdysone hydroxylation at 20- and 26-C followed by oxidation to 20-hydroxyecdysonoic acid was the major metabolic pathway when ecdysone was injected into the larval hemocoel. However, transformation to ecdysteroids-22-acylesters was dominant when ecdysone was introduced by feeding. Ecdysteroid-22-*O*-acyltransferase in the gut was found to be responsible for this unique metabolic pathway. This enzyme located on the gut epithelial cell membrane and may serve as a detoxifying enzyme. In both treatments, most of metabolites were excreted into feces a short time after ecdysone was applied. Furthermore, gut epithelial membrane serves as additional protection since the rate of ecdysteroid intake was low and no evidence for carrier mediated absorption was observed. These studies suggest that the low permeability, ecdysteroid acylester formation in the gut, and high rate of metabolite excretion were the major factors that reduced the bioactivity of the ingested ecdysteroids.

[1]Current address: Department of Entomology, Comstock Hall, Cornell University, Ithaca, NY 14853
[2]Corresponding author

0097–6156/94/0557–0182$08.00/0
© 1994 American Chemical Society

Ecdysteroids, together with other hormones such as juvenile hormone and neuropeptide hormones play an important role in regulating insect growth and development from larval to adult stage. Insects require these hormones to initiate metamorphosis which results in the growth in size as well as transformation. The titer of ecdysteroid hormones is strictly controlled by the kinetics of biosynthesis and metabolism (*1, 2*). Insects obtain steroids from food sources and transform the steroids into their molting hormones (*3*). After the ecdysteroid action in target tissues, these ecdysteroids are converted into inactive forms and excreted (*2*). When these processes are disrupted, for example, by inhibiting ecdysteroid biosynthesis, metabolism or by introducing additional ecdysteroids into the insects, normal growth and molting will be disturbed. These perturbations may result in insect mortality.

Since the first isolation of ecdysone, many ecdysteroids have been studied (*4, 5*) and characterized in many plants (*6*). Compared to the zooecdysteroids, phytoecdysteroids occur in much larger quantity and diversity. Some phytoecdysteroids can account for up to 2.9% of dry weight of the plant (*7*).

The discovery and rich resources of ecdysteroids in plants enhanced other fields of ecdysteroid study, such as the possible function, the evolutionary relationships of phytoecdysteroids with insects, and the potential applications of these compounds (*8*). Phytoecdysteroids, especially 20-hydroxyecdysone, have now been successfully used in the silk industry to control silkworm pupation (*9, 10*). Phytoecdysteroids have also been studied extensively as natural occurring insect control agents (*8, 11*). The normal molting and development could be disrupted by the ingested phytoecdysteroids in some insects, such as the house fly, *Musca domestica* (*12, 13*), the silkworm, *Bombyx mori* (*14-16*), the pink bollworm, *Pectinophora gossypiella* and the fall armyworm, *Spodoptera frugiperda* (*16*).

Theoretically, using ecdysteroid hormones to control insect pests has many advantages. For example, they are active at very low concentrations in the target sites. They are very specific and affect mainly insects or related arthropods. The development and reproduction of helminths can also be affected by ecdysteroids (*17*). On the other hand, the acute toxicity to mammals is extremely low and no sub-acute toxicity in long-term feeding experiments was observed (*18*), although, some pharmacological properties such as analgesic affects (*19*), have been observed in mammals.

Many difficulties occurred, however, when attempts were made to use ecdysteroids in insect pest control. It is still very costly to isolate them in large

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

quantities from plant resources. Large scale synthesis of ecdysteroids is difficult because of the complexity of the structures. Another major problem for the use of ecdysteroids as pest control agents concerns the low activity of these steroid compounds against several important insect pests, such as the *Heliothis* species. These insects have a wide host range (*20*), and most importantly, they have already developed resistance to many insecticides (*21*). It has been shown that larvae of the tobacco budworm, *H. virescens*, are insensitive to ingested phytoecdysteroids (*22*). Some efficient processes must be responsible for this high resistance.

Many factors many contribute to the low activity of the exogenous ecdysteroids in insects. These include low permeability of the cell membrane, efficient metabolism, high rate of metabolite excretion, and species- or developmental stage-specific threshold differences in responding to the ecdysteroids. There may be other unknown mechanisms involved. In a particular insect, there may be some specific mechanisms that become the major factors in the inactivation of the foreign compounds including exogenous ecdysteroids.

In order to understand the major mechanisms leading to the low sensitivity of *H. virescens* to phytoecdysteroids, we have studied the metabolic fate of the exogenous ecdysone, tissue distribution and the excretion rate of ecdysone metabolites. The *in vitro* absorption of ecdysone in the larval gut tissue was also investigated using ^{3}H-ecdysone.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

Specific Ecdysteroid Metabolic Pathway in the Gut of Larval *H. virescens*

The metabolism of ecdysteroids has been studied in many insect species (*6, 23*) and polar ecdysteroid derivatives have been found as the major metabolites (*23-25*). However, a class of ecdysteroid acylesters has also been found in ticks and some insects (*26-32*), especially in the *Heliothis* larvae (*33, 34*). This class of metabolites may be produced in different physiological processes which serve different functions.

H. virescens larval gut plays an important role in detoxifying foreign compounds. When a steroid is introduced through feeding, it may also be subjected to the metabolism by the gut tissue and the metabolic pathway may be different from that of endogenous ecdysteroid hormones. This difference may be one of the major factors involved in the high resistance of the ingested ecdysteroids.

To understand the metabolism of ecdysone in these situations, we analyzed ecdysone metabolites from the

fifth instar larvae of *H. virescens*, each treated with 0.05 μCi of ^{3}H-ecdysone by oral or hemocoel injection. In the injection treatment, we assumed that the hemocoel injected ecdysone is metabolized as the metabolism of endogenous ecdysone. Two hours after ^{3}H-ecdysone oral or hemocoel administration, larvae were dissected and different tissues were extracted in methanol overnight. The ecdysone metabolites in the methanol extracts were analyzed using high performance liquid chromatography (HPLC) by comparing authentic ecdysteroids and their derivatives. We found that ecdysone introduced into larvae by oral injection faced completely different metabolic fate from that introduced by hemocoel injection (Figure 1). HPLC analyses of methanol extracts of feces and tissues from the oral treated larvae revealed three major apolar peaks together with some polar peaks (Figure 1a,c,g), however, hemolymph extract from orally treated larvae had only polar peaks were observed (Figure 1e). This was probably due to the aqueous environment of the hemolymph. By comparison with several synthetic ecdysone derivatives, these three major apolar peaks (in Figure 1a,c,g) were identified as ecdysone-22-linoleate, -22-palmitate/-22-oleate and -22-stearate, respectively. These peaks accounted for over 75% of radioactivity in the methanol extracts from the larvae. However, only minor amounts of ecdysone-22-acylesters were found in the tissue and fecal extracts of *H. virescens* larvae treated with ^{3}H-ecdysone by hemocoel injections (Figure 1b,d,f,h). Instead, over 88% of the injected ecdysone was metabolized into polar metabolites. These characteristics of ecdysone metabolism have also been found in other *Heliothis* species (*34*). In larvae treated with ^{3}H-ecdysone by oral injection, the major polar component in the feces was found as the unchanged ecdysone (Figure 2a); however, 20-hydroxyecdysonoic acid, together with several other hydroxylated ecdysteroids and conjugates, became dominant in the feces of the larvae treated by hemocoel injection (Figure 2b).

We also studied the rate of ecdysteroid excretion by measuring the distribution of radioactive ecdysteroids at different time intervals after ^{3}H-ecdysone treatment. Within two hours after administration by either oral or hemocoel injection, over 70% radioactivity could be recovered in the feces (Figure 3), indicating that the rate of ecdysone metabolite excretion was very high.

When the feces and different larval tissues were extracted, separated into polar and apolar fractions and analyzed by HPLC, the ratios of different ecdysteroid quantities were obtained (Table I, II). Two hours after both oral and hemocoel injection treatments, ecdysone itself became a minor component in the total ecdysteroid

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

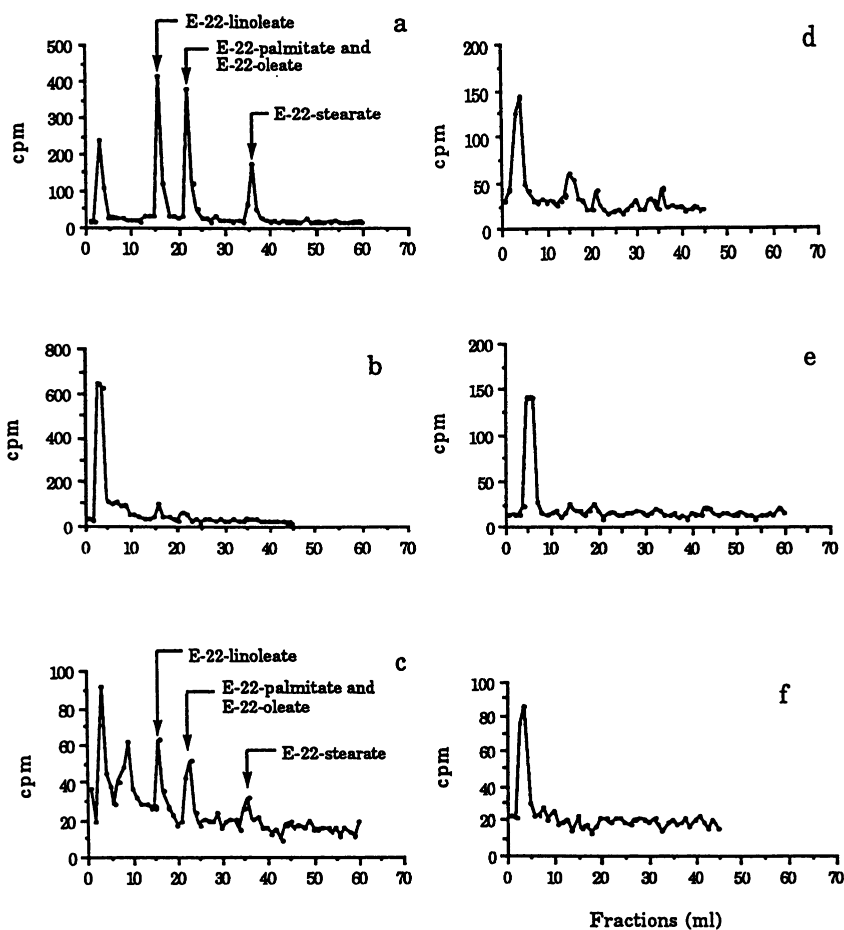


Figure 1. HPLC analysis of ecdysteroid metabolites in methanol extracts of feces and different *H. virescens* larval tissues, a: feces from the larvae treated by oral injection of ^{3}H-ecdysone; b: feces from the larvae treated by hemocoel injection; c: gut from the larvae treated by oral injection; d: gut from the larvae treated by hemocoel injection; e: hemolymph from the larvae treated by oral injection; f: hemolymph from the larvae treated by hemocoel injection. (Reproduced with permission from ref. 57. Copyright 1993 Pergamon Press Ltd).

Continued on next page.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

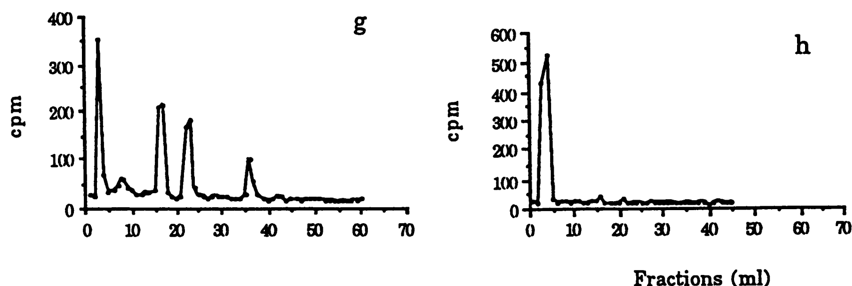


Figure 1. Continued. g: carcass from the larvae treated by oral injection; h: carcass from the larvae treated by hemocoel injection treatment. HPLC system: ZORBAX ODS column (4.6 mm x 25 cm), eluted with 93% MeOH containing 0.5% acetic acid at 1 ml/min (1 ml/fraction). E-22-linoleate: ecdysone-22-linoleate; E-22-palmitate: ecdysone-22-palmitate; E-22-oleate: ecdysone-22-oleate; E-22-stearate: ecdysone-22-stearate (Reproduced with permission from ref. 57. Copyright 1993 Pergamon Press Ltd).

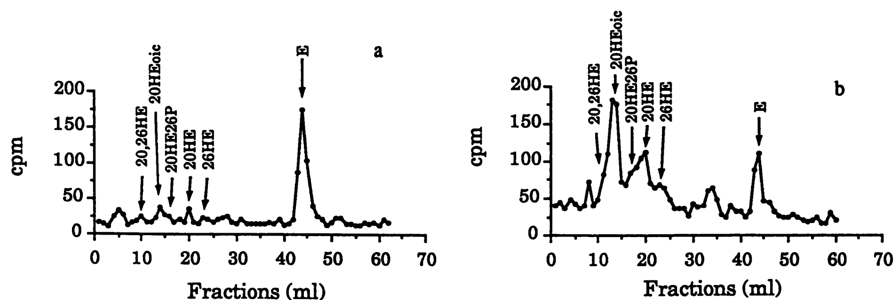


Figure 2. HPLC analysis of several minor polar ecdysteroid metabolites in *H. virescens* larval feces, a: feces from the larvae treated by oral injection of ^{3}H-ecdysone; b: feces from the larvae treated by hemocoel injection. HPLC system: ZORBAX ODS column (4.6 mm x 25 cm), eluted with 38% MeOH containing 0.5% acetic acid at 1 ml/min (1 ml/fraction). E: ecdysone, 20HE: 20-hydroxyecdysone, 26HE: 26-hydroxyecdysone, 20,26HE: 20,26-dihydroxyecdysone, 20HE26P: 20-hydroxyecdysone-26-phosphate, 20HEoic: 20-hydroxyecdysonoic acid (Reproduced with permission from ref. 57. Copyright 1993 Pergamon Press Ltd).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

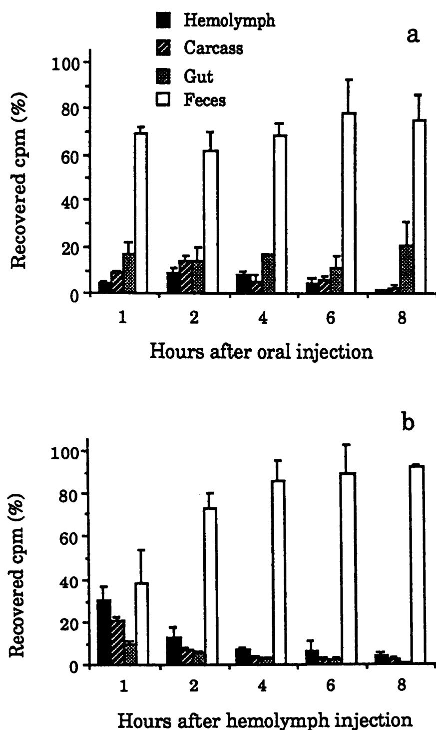


Figure 3. Radioactivity recovered in methanol extracts of feces and different tissues of larval *H. virescens*;, a: after oral injection of ^{3}H-ecdysone; b: after hemocoel injection of ^{3}H-ecdysone. Each result was the mean of three replicates (Adapted with permission from ref. 57. Copyright 1993 Pergamon Press Ltd).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

extracted. Ecdysone-22-acylesters became the major metabolites in tissues and feces of the larvae treated by oral injection of ^{3}H-ecdysone (Table I), while 20-hydroxyecdysonoic acid and other polar ecdysteroids and conjugates were the major metabolites in the hemocoel injected larvae (Table II).

Table I Approximate ratio of ecdysone metabolites from *H. virescens* larvae 2 hours after orally injected with ^{3}H-ecdysone

Metabolites	Amount in different extracts (%)				Total
	Feces	Gut	Hemolymph	Carcass	(%)
20,26-Dihydroxy-ecdysone	0.1	0.0	0.0	0.4	0.5
20-Hydroxy-ecdysone acid	1.0	0.4	0.7	1.4	9.8
20-Hydroxy-ecdysone	0.9	0.2	0.9	0.7	2.7
26-Hydroxy-ecdysone	0.3	0.2	0.4	0.1	1.0
Ecdysone	9.4	0.1	0.0	0.5	10.0
Ecdysone-22-linoleate	20.4	1.3	0.0	2.8	27.2
Ecdysone-22-palmitate/oleate	20.2	6.2	0.0	2.4	28.8
Ecdysone-22-stearate	9.2	2.1	0.0	0.9	12.2

Reproduced with permission from ref. *57*. Copyright 1993 Pergamon Press Ltd.

These studies indicate that, in *H. virescens*, the rate of ecdysone metabolism and metabolite excretion in both treatments was very high but the metabolic pathways were very different. Because the hemocoel injected ecdysone is not subjected to the processes of gut absorption, it may not be facing the detoxification processes that ingested compounds undergo. In this respect, the metabolic pathway may be similar to that of the endogenous ecdysteroids (Figure 4). In fact, ecdysone hydroxylation in 20- and 26-C followed by oxidation to its 26-oic acid has been shown as the major endogenous ecdysone metabolic pathway in several insect orders and it may be the common metabolic pathway in the insects (*24, 35*).

A very unusual ecdysone metabolic pathway was revealed when ^{3}H-ecdysone was orally injected into *H. virescens* (Figure 5). The apolar metabolites, ecdysone-22-acylesters, were the major products in this pathway.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

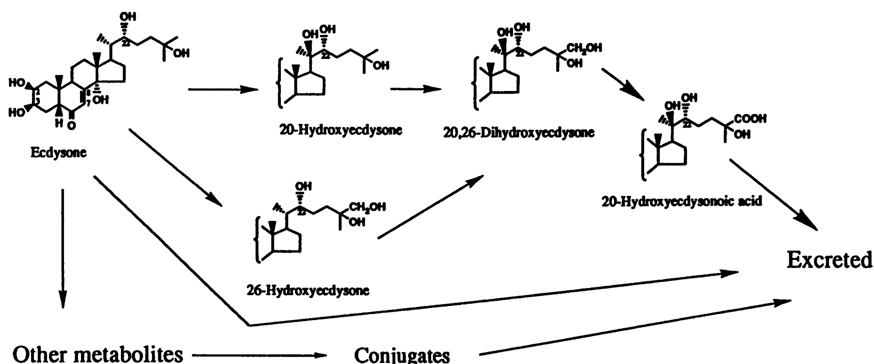


Figure 4. Major metabolic pathways of hemolymph injected ecdysone in *H. virescens* larvae.

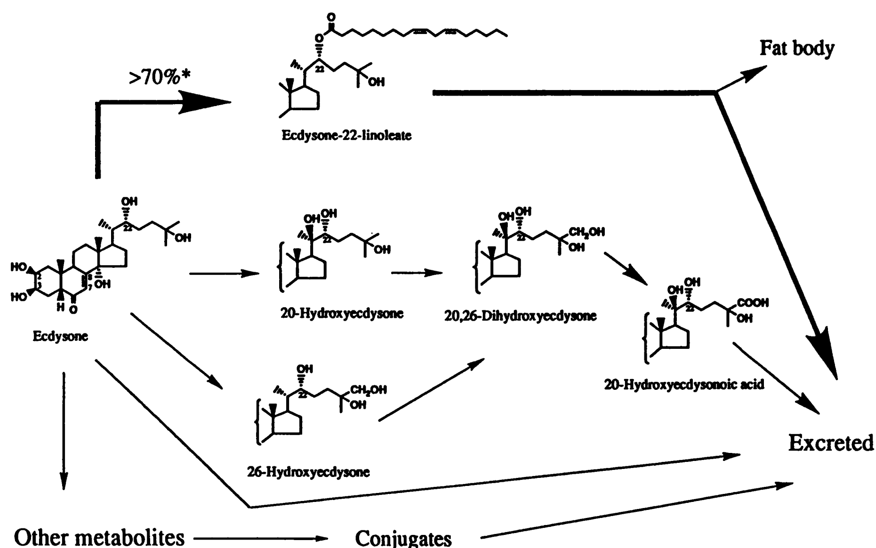


Figure 5. Major metabolic pathways of ingested ecdysone in *H. virescens* larvae. *Products include several other ecdysone-22-acylesters.

Table II Approximate ratio of ecdysone metabolites from *H. virescens* larvae 2 hours after ^{3}H-ecdysone was injected into hemocoel

Metabolites	Amount in different extracts (%)				Total
	Feces	Gut	Hemolymph	Carcass	(%)
20,26-Dihydroxy-ecdysone	2.0	0.1	0.0	0.1	2.2
20-Hydroxy-ecdysone acid	17.0	1.1	0.1	2.7	20.9
20-Hydroxy-ecdysone	5.8	0.2	0.1	0.7	6.8
26-Hydroxy-ecdysone	3.3	0.0	0.0	0.3	3.6
Ecdysone	7.4	0.3	0.0	0.0	7.7
Ecdysone-22-linoleate	4.2	1.0	0.0	0.2	5.4
Ecdysone-22-palmitate/oleate	5.9	0.3	0.0	0.0	6.2
Ecdysone-22-stearate	0.0	0.3	0.0	0.0	0.3

Reproduced with permission from ref. *57*. Copyright 1993 Pergamon Press Ltd.

It is suggested that this metabolic pathway is specifically located in the gut tissue of the *H. virescens* larvae, and may be important to the detoxification process (*36, 37*). In this situation, the exogenous ecdysone in the larval gut may not be recognized as the molting hormone. Instead, as a foreign toxin it is inactivated by transformation to esters. This is a unique pathway in that the end products are more lipophilic, whereas the normal metabolic pathway results in more polar products.

Ecdysteroid-22-*O*-acyltransferase in the Larvae of *H. virescens*

In the *H. virescens* larval gut tissue there is an unusual ecdysteroid metabolic pathway in which ecdysteroid-22-*O*-acyltransferase plays an important role. This enzyme converts ecdysteroids into their ecdysteroid-22-acylesters *in vitro* in the presence of fatty acly-CoA (*36*). It is different from ecdysteroid acyltransferase in the ovaries of *Periplaneta americana* (*36*).

We have studied some structural requirements for the substrates of ecdysteroid-22-*O*-acyltransferase.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

When different ecdysteroids were used in the enzyme assay to compete with ^{3}H-ecdysone as the substrate, those with the blocked 22-OH could not compete with ^{3}H-ecdysone (Table III). This indicates that the reaction requires a free 22-OH group (*36, 37*). Ecdysteroid-22-*O*-acyltransferase activity in *H. virescens* larvae was found highest in the midgut. To locate the enzyme activity in the specific part of gut tissue, a piece of larval midgut was dissected, cut open longitudinally and incubated with ^{3}H-ecdysone in Grace's medium for one hour. The gut was then extracted with methanol overnight. HPLC analysis of the methanol extracts showed that most of the ecdysone was metabolized into ecdysone-22-acylesters (Figure 6a). However, when a of piece intact midgut was ligated at both ends before incubation with ^{3}H-ecdysone, the quantities of ecdysone-22-acylesters formed were very low (Figure 6b). These results suggest that ecdysteroid-22-*O*-acyltransferase is located on the lumenal site of the gut tissue.

In a further experiment, larval midgut tissue was homogenized and separated into different fractions by centrifugation. The enzyme activity in these fractions was then analyzed. Since alkaline phosphatase has been shown to concentrate in the epithelial cell membrane (brush border membrane) in lepidopterous larval gut tissue and can be concentrated between the 3000g supernatant and 30,000g precipitate (*38*), it can be used as a marker enzyme of the brush border membrane. Our results showed that the distributions of ecdysteroid-22-*O*-acyltransferase and alkaline phosphatase activity are very similar in the centrifuge fractions (Figure 7), indicating that these two enzymes share a similar cellular localization.

The above evidence suggests that ecdysteroid-22-*O*-acyltransferase is located on the lumenal side of the gut epithelial cell membrane and is possibly concentrated in the brush border membrane where ingested foreign compounds are first contacted before being absorbed. This cellular localization may explain why the ingested ecdysone was immediately converted into its 22-acylester and excreted with the feces. This rapid biotransformation and excretion in *H. virescens* larval gut tissue can be one of the major mechanisms for the low sensitivity of the larvae to ingested ecdysteroids.

When ecdysteroid-22-*O*-acyltransferase activity in the fifth instar larvae was measured, we found that this enzyme activity was associated with the larval feeding. Ecdysteroid-22-*O*-acyltransferase activity is very high during the feeding stage, and it decreases when the larvae stop feeding and commit to pupation (Figure 8). This association further indicates that the level of the ecdysteroid-22-*O*-acyltransferase activity in *H. virescens* is related to the rate of the detoxification

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

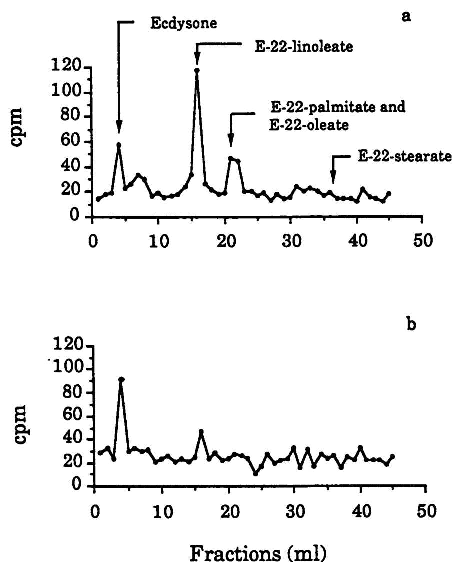


Figure 6. HPLC analysis of methanol extracts from the open (a) and the ligated (b) midgut after incubation with ^{3}H-ecdysone;. ZORBAX ODS column (4.6 mm x 25 cm); solvent system: 93% MeOH and 0.5% acetic acid at 1 ml/min (1 ml/fraction).

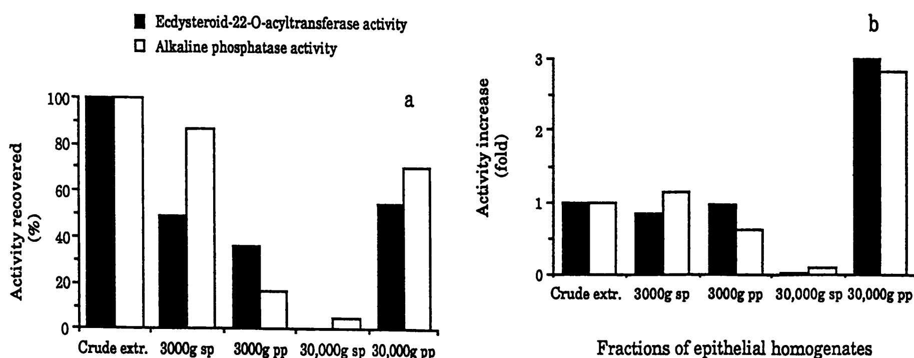


Figure 7. Ecdysteroid-22-*O*-acyltransferase recovery (a) and activity increase (b) in different *H. virescens* larval midgut epithelial homogenate centrifugal fractions;. Crude. extr.: crude gut homogenate; 3000g sp: 3000g supernatant; 3000g pp: 3000g precipitate; 30,000g sp: 30,000g supernatant; 30,000g pp: 30,000g precipitate. Each result was the mean of three replicates (Reproduced with permission from ref. 37. Copyright 1992 Plenum Publishing Corporation).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

Table III Enzymatic formation of ^{3}H-ecdysone-22-oleate from ^{3}H-ecdysone in the presence of other ecdysteroids

Ecdysteroids added to compete with ^{3}H-ecdysone	^{3}H-ecdysone-22-oleate formed (μM x 10^{-9}/min/mg ± SD)	^{3}H-ecdysone-22-oleate formed (%)
No competitors	2.13 ± 0.09	100
20-Hydroxyecdysone	0.74 ± 0.13	35
Ecdysone	0.62 ± 0.09	29
Abutasterone	0.89 ± 0.11	41
22-Acetyl-20-hydroxyecdysone-	2.12 ± 0.07	100
2-Acetly-20-hydroxyecdysone	0.51 ± 0.06	24
11α-Hydroxyecdysone	0.52 ± 0.10	24
Ajugasterone C	0.56 ± 0.10	26

of foreign compounds ingested with food. Since ecdysteroid-22-*O*-acylesters have been found in ovaries and eggs of some insects and were considered as the storage form of ecdysteroids (*30, 39-45*), ecdysteroid acyltransferase in these insects may serve a different function from that of ecdysteroid-22-*O*-acyltransferase in the gut of *H. virescens* larvae.

Studies have also been made on the induction of ecdysteroid-22-*O*-acyltransferase in *H. virescens* larvae. Because the major function of ecdysteroid-22-*O*-acyltransferase in *H. virescens* larvae is considered to be detoxifying noxious foreign compounds including ecdysteroids ingested with the food, these compounds (substrates) may be able to induce the enzyme activity in the larvae. However, when we fed the fifth instar larvae with 20-hydroxyecdysone for two days and measured the changes of ecdysteroid-22-*O*-acyltransferase activity, no activity induction was observed. Furthermore, no ecdysteroids have been found so far in the hosts of this insect, therefore, other compounds in these plants may serve as the original substrates for this enzyme and induce the activity. As ecdysteroid-22-*O*-acyltransferase is very specific to the 22-OH group in ecdysteroids, the potential substrate for this enzyme may share this structural similarity, but no other types of substrates for this enzyme have been studied except ecdysteroids. On the other hand, some plant steroid hormones such as the brassinosteroids share some of the structural characteristics with ecdysteroids (Figure 9) and may serve as a substrate for this enzyme. Brassinosteroids have been found as ecdysteroid agonists and can disrupt the growth of some insects (*46*). Studies of ecdysteroid-22-*O*-acyltransferase and its natural substrate spectra may help us understand why *H.*

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

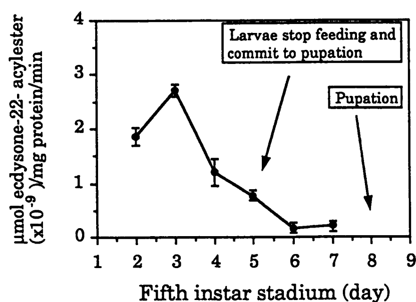
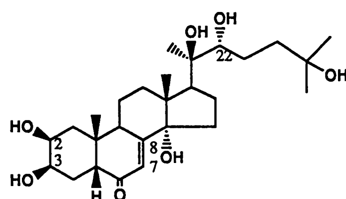
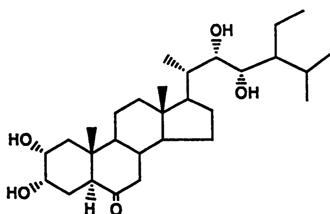


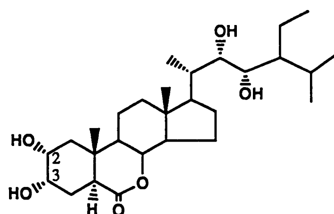
Figure 8. Ecdysteroid-22-O-acyltransferase activity in different day of fifth instar larvae of *H. virescens* (Adapted with permission from ref. 37. Copyright 1992 Plenum Publishing Corporation).



20-Hydroxyecdysone



22S,23S-Homocastasterone



22S,23S-Homobrassinolide

Figure 9. Structure of 20-hydroxyecdysone and brassinosteroids.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

virescens has broad host range and high level of resistance to many toxic substances.

Low Absorption Rate of Ecdysone in the Gut May Prevent the Accumulation in the Larvae of *H. virescens*

The insect cell membrane, especially gut epithelial cell membrane, plays an important role in allowing selected compounds entering into cells and preventing many toxic agents from entering target sites. Because many toxic compounds, especially natural products, are taken by feeding, the permeability of epithelial cell membrane becomes critical for the bioactivity of these compounds.

When a compound is absorbed into a cell, it is usually taken up by either passive diffusion or by a carrier-mediated process. A compound that can diffuse through a cell membrane is usually either a small molecule or a lipophilic compound. In general, steroids are highly lipophilic and many of them can readily pass through a cell membrane (*47, 48*). However, ecdysteroids are very unique among the steroid hormones because they are highly hydroxylated and much more polar than other steroid types. Thus, the permeability of cell membrane to ecdysteroids could be limited due to their high polarity. Low permeability of the gut epithelial cell membrane, therefore, may become another major factor that reduces their bioactivity.

Although studies of ecdysteroid uptake by insect tissues have been very limited and the exact mode of ecdysteroid absorption is unknown, some evidences have suggested the existence of ecdysteroid specific binding sites on the cell membrane of insect and other arthropod tissues (*49*) such as the integument (*50-53*), fat body (*54*), imaginal discs (*55*) and central nervous system (*56*). Many types of carrier mediated absorptions occur in insect gut, but, uptake of ecdysteroids in this tissue has not been well studied. To understand the possible modes of ecdysone absorption by the gut of larval *H. virescens*, we conducted *in vitro* studies by incubating under different conditions a section of midgut tissue in 0.5 ml Grace's medium, pH 6.8, containing 0.05 μCi (1.05 x 10-3 μM) ^{3}H-ecdysone. After incubation, the gut tissue was washed three times (1 min each) in 30 ml Grace's medium, at 4°C. The gut section was then extracted overnight with 1 ml methanol. An aliquot of the methanol extract was added to 4 ml liquid scintillation cocktail and the radioactivity was measured. The methanol extract for ecdysteroids was also analyzed by HPLC. Under these conditions, the kinetics of ecdysone absorption in midgut was obtained by measuring ^{3}H-ecdysone uptake in the gut during time intervals ranging from 30 seconds to two hours, by measuring the rate of absorption at different ecdysone

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

concentrations, ranging from 0.1 to 10 μM, and by measuring the rate of absorption at incubation temperatures ranging from 10°C to 37°C. The effects of a metabolic inhibitor, 2,4-dinitrophenol (DNP), was determined by measuring the change of ecdysone absorption rate in the presence of DNP.

These studies showed that accumulation of the radioactivity in *H. virescens* larval gut tissue could be saturated by increasing the incubation time or ^{3}H-ecdysone concentration (Figure 10a,b). The accumulation of radioactivity could also be affected by changing the temperature and by additions of a metabolic inhibitor such as the DNP (Figure 10c,d). All these results indicated the involvement of metabolic process in the accumulation of radioactivity in the gut tissue. However, HPLC analysis of the methanol extract of the gut tissues after incubation with ^{3}H-ecdysone indicated that the major radioactive compound in the gut is not from ^{3}H-ecdysone, but ^{3}H-ecdysone-22-acylesters (Figure 6a). As we have found that the major location of ecdysteroid-22-*O*-acyltransferase activity is on the lumenal side of gut epithelial cell membrane, the biotransformation of ecdysone to its 22-acylesters may occur on the surface of this membrane. Because of the ecdysteroid ester formation which possibly occurred before ecdysone entering into the epithelial cells, it is possible that the involvement of metabolic energy was not in the stage of absorption, but the stage of biotransformation when the fatty acid donor is activated to fatty acyl-CoA. These ecdysone acylesters are more lipophilic and more readily to pass through the membrane. Due to the increased membrane penetration ability of the lipophilic products, the esterification may become the rate limiting step affecting the accumulation of ecdysteroid in the gut. This may explain the kinetics of gut radioactivity accumulation which indicated the involvement of metabolic processes (Figure 10).

Even though the exact mode of ecdysone absorption in the gut of *H. virescens* larvae has not been identified, it appears that the rate of ecdysone accumulation in the gut tissue is very low and is possibly a passive process. In fact, our results showed that less than 6% of the total radioactivity was recovered in the gut after incubation with ^{3}H-ecdysone for 1 hour. Furthermore, the major radioactivity in the gut extract is not from ^{3}H-ecdysone itself but from the acyltransferase products, ^{3}H-ecdysone-22-acylesters. This low ecdysone absorption rate in the gut tissue, together with other metabolic processes, especially on the epithelial cell membrane, limit the access of active form of ecdysone to the larval target sites when introduced through feeding. Therefore, the adverse

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

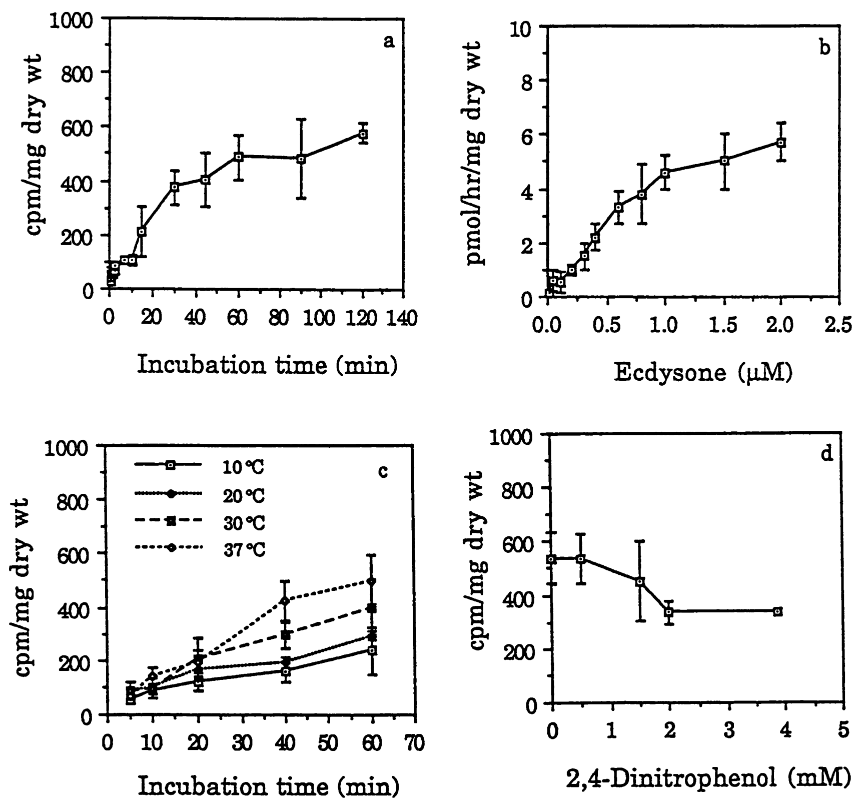


Figure 10. Kinetics of radioactivity accumulation in the midgut of *H. virescens* incubated with ^{3}H-ecdysone, a: in vitro accumulation of radioactivity in the midgut; b: dependence of uptake on substrate concentration; c: effect of temperature on radioactivity accumulation; d: effect of metabolic inhibitor on radioactivity accumulation (DNP, 2,4-dinitrophenol).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

effects of exogenous chemicals including ecdysteroid like compounds are decreased due to their limited absorption.

Conclusion

Many factors may contribute to the resistance of *H. virescens* to ingested ecdysteroids. Among the major factors are ecdysteroid-22-*O*-acyltransferase on the brush border membrane, the low permeability of this membrane to ecdysteroids, and the efficient metabolite excretion. Ecdysteroid-22-*O*-acyltransferase is a unique metabolizing enzyme in *H. virescens* larvae with regard to its location and metabolic activity. Over 80% of ingested ecdysone is metabolized by this enzyme in the larval gut, and before the absorption occurs, most of the enzyme products are excreted. Although some ecdysteroid acylesters enter the larval body, they may have a limited impact because they are not the active form of ecdysteroid. Furthermore, they are very lipophilic and can not be carried freely in the hemolymph to ecdysteroid receptor sites, instead, they are easily concentrated in the fat body where they can be further metabolized and excreted.

Acknowledgements

We thank Dr. Rene Lafont, Ecole Normale Superieure, Departement de Biologie, France, for providing samples of ecdysteroids derivatives, and Peter Korytko for editing the manuscript.

Literature Cited

1. Smith, S. L. In *Comp. Insect Physiol. Biochem. Pharm.;* Derkut, G. A. and Gilbert, L. I., Eds.; Pergamon Press: New York, 1985; Vol. 7; pp 295-341.
2. Koolman, J. and Karlson, P. In *Comp. Insect Physiol. Biochem. Pharm.;* Derkut, G. A. and Gilbert, L. I., Eds.; Pergamon Press: New York, 1985; Vol. 7; pp 343-362.
3. Rees, H. H. In *Comp. Insect Physiol. Biochem. Pharm.;* Derkut, G. A. and Gilbert, L. I., Eds.; Pergamon Press: New York, 1985; Vol. 7; pp 249-341.
4. Rees, H. H. In: *Ecdysone*; Koolman, J., ed.; Thieme: Stuttgart, 1989; pp. 28-38.
5. Hetru, C. and Horn, D. H. S. In: *Progress in Ecdysone Research*; Hoffmann, J. A., ed.; Elsevier/North-Holland: Amsterdam, 1980; pp. 13-28.
6. Lafont, R. and Horn, D. H. S. In: *Ecdysone*; Koolman, J., ed.; Thieme: Stuttgart, 1989; pp. 39-64.
7. Chou, W. -S. and Lu, M. -S. In: *Progress in Ecdysone Research;* Hoffmann, J. A., ed.; Elsevier/North-Holland: Amsterdam, 1980; pp. 218-297.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

8. Wilson, I. D. In: *CRC Handbook of Natural Pesticides;* Morgan, E. D. and Mandava, N. B., eds.; Insect Growth Regulators, Part A; CRC Press: Boca Raton, FL, 1987; Vol. 3; pp. 15-86.
9. Guo, F. In: *Ecdysone;* Koolman, J., ed.; Thieme: Stuttgart, 1989; pp. 442-446.
10. Nein, S. L.; Hsu, H. Y.; Ho, M. and Yo, Y. C. *Acta Chem. Sinica.* **1978;** *3,* 401.
11. Horn, D. H. S. In: *Naturally Occurring Insecticides;* Jacobson, N. and Crosby, D. G., eds.; Marcel Dekker: New York, 1971; pp. 333-459.
12. Robbins, W. E.; Kaplanis, J. N.; Thompson, M. J.; Shortino, T. J. and Joyner, S. C. *Steroids.* **1970,** *16,* 105.
13. Singh, P.; Russell, G. B. and Fredericksen, S. *Entomol. Exp. Appl.* **1982,** *32,* 7.
14. Hikino, H.; Ohizumi, Y. and Takemoto, T. *J. Insect Physiol.* **1975,** *21,* 1953.
15. Shigematsu, H.; Moriyama, H. and Agui, N. *J. Insect Physiol.* **1974,** *20,* 867.
16. Kubo, I.; Klocke, J. A. and Asano, S. *J. Insect Physiol.* **1983,** *29,* 307.
17. Barker, G. C.; Chitwood, D. J. and Rees, H. H. *Invert. Reprod. Devel.* **1990,** *18,* 1.
18. Simon, P. and Koolman, J. In: *Ecdysone, From Chemistry to Mode of Action;* Koolman, J., ed.; Thieme Medical Publisher, Inc.: New York, 1989, pp 245-259.
19. Hikino, H. and Takemoto, T. *Naturwiss.* **1972,** *59,* 91.
20. King E. G. and Coleman, R. J. *Ann. Rev. Entomol.* **1989,** *34,* 53.
21. Wolfenbarger, D. A.; Bodegas, P. R. and Flores, R. *Bull. Entomol. Soc. Am.* **1981,** *27,* 181.
22. Kubo, I.; Klocke, J. A. and Asano, S. *Agric. Biol. Chem.* **1981,** *45,* 1925.
23. Hoffmann, J. A. and Hetru, C. In: *Endocrinology of Insects;* Downer, R. G. H. and Laufer, H., eds.; Alan R. Liss: New York. 1983, Vol. 1; pp. 65-88.
24. Koolman, J. *Insect Biochem.* **1982,** *12,* 225.
25. Lafont, R. and Koolman, J. In: *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones;* Hoffmann, J. A. and Porchet, M. Eds.; Springer-Verlag: Berlin, 1984; pp. 196-226.
26. Diehl, P. A.; Connat, J. -L.; Girault, J. -P. and Lafont, R. *Int. J. Invert. Reprod. Dev.* **1985,** *8,* 1.
27. Hoffmann, K. H.; Bulenda, K.; Thiry, E. and Schmid, E. *Life Sci.* **1985,** *37,* 185.
28. Wigglesworth, K. P.; Lewis, D. and Rees, H. H. *Arch. Insect Biochem. Physiol.* **1985,** *2,* 39.
29. Bulenda, D.; Stecher, A.; Freunek, M. and Hoffmann, K. H. *Insect Biochem.* **1986,** *16,* 83.
30. Connat, J. -L.; Diehl, P. A. and Morici, M. *Gen. Comp. Endocrinol.* **1984,** *56,* 100.
31. Connat, J. -L. and Diehl, P. A. *Insect Biochem.* **1986,** *16,* 91.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

32. Crosby, T.; Evershed, R. P.; Lewis, D.; Wigglesworth, K. P. and Rees, H. H. *Biochem. J.* **1986**, *240*, 131.
33. Kubo, I.; Komatsu, S.; Asaka, Y. and De Boer, G. *J. Chem. Ecol.* **1987**, *13*, 785.
34. Robinson, P. D.; Morgan, E. D.; Wilson, I. D. and Lafont, R. *Physiol. Ent.* **1987**, *12*, 321.
35. Lafont, R.; Blais, C.; Beydon, P.; Modde, J. -F.; Enderle, U. and Koolman, J. *Arch. Insect Biochem. Physiol.* **1983**, *1*, 41.
36. Zhang, M. and Kubo, I. *Insect Biochem. Molec. Biol.* **1992**, *22*, 599.
37. Zhang, M. and Kubo, I. *J. Chem. Ecol.* **1992**, *18*, 1139.
38. Wolfersberger, M.; Luethy, P.; Maurer, A.; Parenti, P.; Sacchi, F. V.; Giordana, B. and Hanozet, G. M. *Comp. Biochem. Physiol.* **1986**, *86A*, 2301.
39. Hoffmann, J. A. *Insect Biochem.* **1986**, *16*, 1.
40. Slinger, A. J.; Dinan, L. N. and Isaac, R. E. *Insect Biochem.* **1986**, *16*, 115.
41. Slinger, A. J. and Isaac, R. E. *Gen. Comp. Endocrinol.* **1988**, *70*, 74.
42. Slinger, A. J. and Isaac, R. E. *Insect Biochem.* **1988**, *18*, 779.
43. Whiting, P. and Dinan, L. *Biochem. J.* **1988**, *252*, 95.
44. Whiting, P. and Dinan, L. *J. Insect Physiol.* **1988**, *34*, 625.
45. Whiting, P. and Dinan, L. *Insect Biochem.* **1989**, *19*, 759.
46. Koolman, J. *Zool. Science.* **1990**, *7*, 563.
47. Jensen E. V.; Suzuki, T.; Stumpf, W. E.; Jungblut, P. W. and Desombre, E. *Proc. Natn. Acad. Sci. U.S.A.* **1968**, *59*, 632.
48. Rao G. S. *Cell Endocrinol.* **1981**, *21*, 97.
49. De Loof A. *Insect Biochem.* **1986**, *16*, 169.
50. Spindler, K. D. and Spindler-Barth, M. In: *Ecdysone, From Chemistry to Mode of Action;* Koolman, J., ed. Thieme Medical Publisher: NY, 1989; pp 245-249.
51. Spindler, K. D. and Grossmann, D. *Molec. Cell. Endocrinol.* **1987**, *52*, 81.
52. Daig, K. and Spindler, K. D. *Molec. Cell. Endocrinol.* **1983**, *31*, 93.
53. Daig, K. and Spindler, K. D. *Molec. Cell. Endocrinol.* **1983**, *32*, 73.
54. Natori, S. and Ohtaki, T. *J. Insect Physiol.* **1976**, *22*, 227.
55. Yund, M. A. and Fristrom, J. W. *Dev. Biol.* **1975**, *43*, 287.
56. Bidmon, H. -J.; Stumpf, W. E. and Granger, N. A. *Cell Tissue Res.* **1991**, *263*, 183.
57. Zhang, M. and Kubo, I. *Insect Biochem. Molec. Biol.* **1993**, *23*, 831.

RECEIVED March 8, 1994

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch015

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016

Chapter 16

Biochemical Characterization of Auxin Transport Protein Using Phytotropins

S. Brunn, M. V. Subramanian, E. Walters, B. Patel, and J. D. Reagan

Sandoz Agro, Inc., 975 California Avenue, Palo Alto, CA 94304

Auxin transport inhibitors (phytotropins) are presumed to bind to the IAA-efflux protein and prevent auxin efflux. Membrane preparations from zucchini and *Arabidopsis* each showed only one specific binding site for the phytotropin, naphthylphthalamic acid (NPA) with a K_d = 7.12 nM, 23.8 nM and B_{max} = 6.70 pmol/mg, 2.55 pmol/mg, respectively. Several other phytotropins such as semicarbazone (SCB-1), pyrenoylbenzoic acid (PBA), 2,3,5 triiodobenzoic acid (TIBA) and quercetin completely displaced bound ^{3}H-NPA from zucchini membranes with I_{50} values of 16, 0.15, 12850 and 47560 nM, respectively. Hill plot analyses of NPA displacement by these phytotropins also indicated only one binding site on the membrane. Free indole-3-acetic acid (IAA), ethyl ester of IAA, as well as, amino acid and sugar conjugates of IAA, had no effect on ^{3}H-NPA bound to microsomal membranes (MM). Likewise, various auxin agonists like 2,4-dichlorophenoxy acetic acid (2,4-D), dicamba and α-naphthaleneacetic acid (NAA) also had no effect. Various cytokinins, calcium ions, calcium effectors such as chlorpromazine, flunarizine and verapamil as well as flavins, had no effect in the ^{3}H-NPA dissociation assay. Of the various phytotropins tested, only NPA, PBA, TIBA and SCB-1 showed significant IAA accumulation in the stem segment assay and loss of gravitropism in the tomato root bioassay. SCB-1 was very effective in the above bioassays. Quercetin, a weak phytotropin, and calcium effectors were not active in the bioassays. Solubilization of the NPA binding protein from zucchini MM was achieved with 0.4% CHAPS. The detergent solubilized protein exhibited similar ^{3}H-NPA saturation kinetics to that of the membrane bound protein (K_d = 2.0 nM, B_{max} = 0.2 pmol/mg).

Auxins are a group of weak organic acids that have dramatic and diverse effects on plant growth and development (1). Indole-3-acetic acid (IAA), the most common natural auxin is synthesized and transported unidirectionally away from growing

0097–6156/94/0557–0202$08.00/0
© 1994 American Chemical Society

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

apical meristems (2). This transmembrane polar transport has been reviewed extensively in the literature (2-5) and can be characterized by two stages: 1) simple diffusion across the membrane or *via* an influx carrier into the cell, and 2) basal efflux *via* a basally-located efflux carrier (2,6). This transport occurs primarily in parenchymatous cells associated with, but not of the vascular bundles themselves (2). Auxin transport is described by the chemiosmotic hypothesis (3) which outlines the factors driving polar auxin transport. Transport is dependent upon a pH gradient across the membrane which is maintained by a proton electrochemical gradient. The less polar nature of IAA at the prevailing apoplast pH of 5.5, allows it to cross the lipid bilayer passively following a concentration gradient.

Direct measurement of auxin uptake and transport into cells and vesicles is a difficult task. More readily measurable is the efflux process and inhibition of IAA efflux by auxin transport inhibitors (7). These inhibitors, referred to as phytotropins, are able to affect apical dominance, geotropic and phototropic responses (8).

The most extensively studied phytotropin is 1-N-naphthylphthalamic acid (NPA) and the binding protein is often referred to as the NPA binding protein. Competition studies using ^{3}H-NPA are useful in determining the relative affinities of other phytotropins for the NPA binding site (8,9). The structural requirements for binding have been defined to consist of an aromatic carboxylic acid (five- or six-membered ring system), which in turn is attached *via* the *ortho* position, by either a planar or conjugated bridge, to another aromatic ring. The minimum distance between the two rings has been proposed to be 7.3 Å (8).

Several naturally occurring flavonoid compounds have been shown to exhibit phytotropin effects (10) and some, such as quercetin, compete with NPA at the binding site. This has led researchers to consider the flavonoids as natural phytotropins for the control of auxin transport since they are ubiquitous and under stress, can approach high, physiologically effective concentrations. Their levels are also tightly regulated and known to be environmentally-responsive (11).

In the present paper, we have described several biochemical properties of the NPA binding protein and compared NPA with several other phytotropins and related natural ligands. The effects of phytotropins on IAA uptake by pea stem segments and tomato root gravitropism are also discussed.

MATERIALS AND METHODS

Binding Studies. The isolation of total membranes and plasmalemma from zucchini (*Curcubita pepo* L.) has been previously described (9). Solubilization of the NPA binding protein was achieved by incubating the membranes with 0.4% CHAPS in test buffer (0.25 M sucrose, 1 mM $MgCl_2$, 20 mM citrate, pH 5.3) for 2 hours on ice. An ultracentrifugation step for 1 hour at 100 000 · g separated the unsolubilized pellet from the solubilized proteins.

Quantitation of phytotropin binding (^{3}H-NPA) was achieved by either GF/B filter assay or by using the newly developed Millipore Multiscreen plate assay (12). Radiolabelled NPA, 2,3,4,5(n)-^{3}H naphthylphthalamic acid (48.9 Ci/mmol) was purchased from RPI Corp, Mt Prospect, Il.

For the GF/B assay, filters presoaked in 0.1% PEI (polyethylenimine) for 30 minutes were used. Different concentrations of inhibitors in test buffer in a volume of 5 µl were added to microcentrifuge tubes. To a membrane solution of 0.1 mg/ml total protein in test buffer (0.2 mg/ml for *Arabidopis*), was added ^{3}H-NPA to a final concentration of 5 nM. Aliquots of 195 µl of membrane-ligand mixture were added to the microfuge tubes for a final volume of 200 µl/assay. The samples were incubated at 4°C for 40 minutes on a gyratory shaker and then filtered over vacuum through the PEI treated membrane. Each tube was then rinsed with 1 ml test buffer and this was added to the filter. The filter was further washed with 7 ml test buffer to reduce background binding and left to dry on the vacuum for an additional 2

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

minutes. Filters were placed in 7 ml scintillation vials with 5 ml of Beckman ReadySafe cocktail and counted for 2 minutes in a Beckman LS 5000CE counter.

A novel Millipore multiscreen assay system has been developed (12) for ^{3}H-NPA dissociation analysis of large numbers of samples. The system consists of a 96 well plate fitted with a mixed cellulose ester filter at the base which is supported by a removable plastic sheet. The filters were pre-wetted with 200 µl distilled water and vacuum filtered using a Millipore vacuum manifold. Like the GF/B assay, inhibitor or buffer were added to the wells in a 5 µl volume followed by addition of 195 µl mixture of membranes and ^{3}H-NPA and incubated at 4° C. After 45 minutes the plate was vacuum aspirated and the filters washed with 200 µl test buffer. The plastic support was removed and the plate dried at 70° C for 20 minutes. The filters were punched out using either the Millipore Multiscreen Punch or a cork borer into 7 ml scintillation vials and soaked in 200 µl water prior to the addition of scintillation cocktail.

Physiological Studies. To demonstrate inhibition of auxin efflux and gravitropism inhibition caused by phytotropins, we have utilized the ^{14}C-IAA accumulation assay in pea (*Pisum sativum*) stem segments, and tomato root gravitropism bioassay response (*Lycopersicon esculentum* variety Celebrity) respectively.

The pea stem assay has been previously described (9). About 2 week old, light grown seedlings were the source of stem segments. Accumulation of ^{14}C-IAA in 5 mm stem segments at 1 µM (57 mCi/mmol, American Radiolabeled Chemicals Inc.), in the presence and absence of 1 and/or 10 µM phytotropins, was used to measure inhibition of the efflux process.

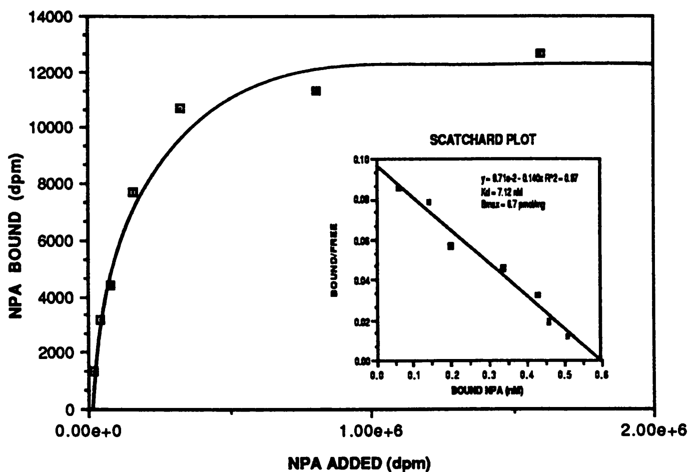
The tomato root assay was adapted from the descriptions of Brunn *et al.* (9). Pre-germinated seedlings were grown for 24 hours in a square petri dish oriented normal to gravity. The plate was then reoriented 90° with respect to gravity for another 24 hours. After a total of 48 hours the tomato roots were rated for root length and angle of curvature as a result of the reorientation to gravity. Various phytotropins were added as necessary to the growth medium to determine GI_{50} values.

RESULTS AND DISCUSSION

Binding Assays. Membrane vesicles isolated from etiolated zucchini hypocotyls can provide either total microsomal (MM) or purified plasma (PM) membranes. Either system is appropriate for ^{3}H-NPA binding studies; however, PM preparations yield higher specific activity, albeit with only about 10-12% recovery of total binding activity. The saturable binding of ^{3}H-NPA to zucchini MM shown in Figure 1a, gave a binding constant of 7.12 nM with a B_{max} of 6.7 pmol/mg. GF/B filtration assay gave very low nonspecific binding even at high concentration of the labelled ligand (15-20%). Scatchard analysis of the above data revealed only one population of binding site for NPA (Figure 1a, inset). Binding studies with *Arabidopsis* MM also revealed only one binding site (Figure 1b, K_d= 24 nM, B_{max}=2.6 pmol/mg). Multiscreen assay using zucchini PM provided further evidence for the presence of only one site (12). Extensive analysis of the binding data by double reciprocal, Hill, Scatchard plots and the program Ligand (13) have confirmed the presence of a single site (12). MM and PM from zucchini showed very similar results with respect to saturable NPA binding and kinetic constants (data not shown). In the literature, one NPA binding site has been proposed for pea and maize (14, 15, 16) and two distinct sites for sunflower (16) and zucchini (17).

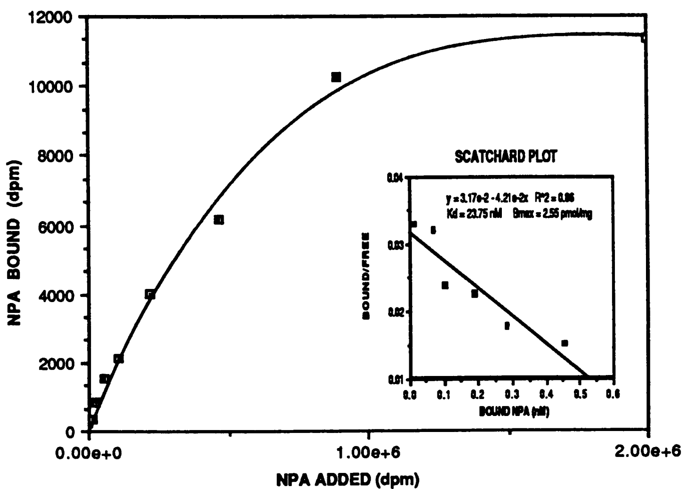
Other phytotropins (Figure 2) compete with NPA for binding as shown in Table I. The relative affinities of the inhibitors based on ^{3}H-NPA dissociation are related to their fulfilling of the parameters established by Katekar & Geissler (8).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016



a

Figure 1a. Saturation Curve of ^{3}H-NPA to Zucchini Membranes.



b

Figure 1b. Saturation Curve of ^{3}H-NPA to *Arabidopsis* Membranes.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

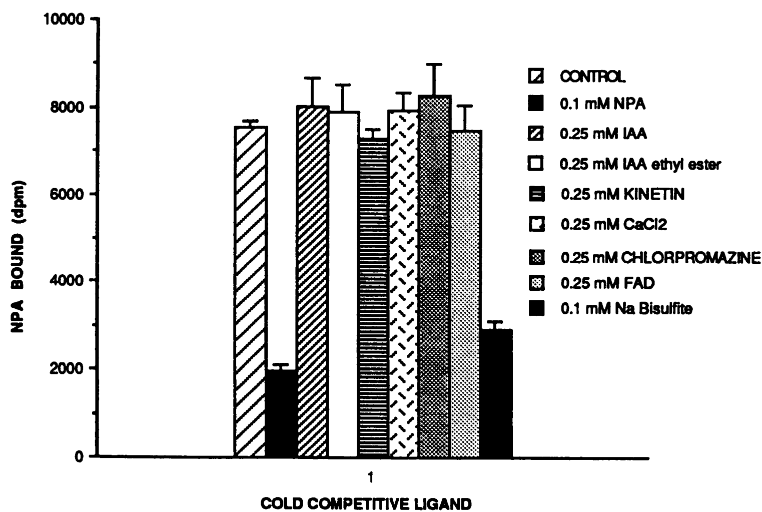


Figure 2. Dissociation of ^{3}H-NPA by Auxins, Cytokinins and Other Ligands.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Again, the ^{3}H-NPA dissociation constants for various phytotropins were very similar with MM or PM preparations (12). Hill plot analysis of the dissociation data of all phytotropins strongly supported the existence of one population of binding site previously established for NPA by saturation curves (Figure 1). The binding properties of these compounds and their physiological effects are discussed in the last section.

Table I. Characteristics of Several Phytotropins in the Dissociation, ^{14}C-IAA Uptake and Root Gravitropism Assays

Compound	*I_{50} (nM)* [a]	*^{14}C-IAA Uptake % of control* [b]	*GI_{50} (nM)* [c]
1. PBA	0.15	358 ± 60	44
2. SCB-1	16	424 ± 65	63
3. NPA	20	375 ± 68	118
4. TIBA	12850	360 ± 39	799
5. Quercetin	47560	130 ± 21	no effect

a Multiscreen binding assay system used to determine I_{50} (concentration required for dissociation of 50% bound ^{3}H-NPA) by Hill Plot.

b Uptake in stem segments treated with ^{14}C-IAA in the absence of phytotropins was rated as 100 ± 11%. Inhibitor concentration was 1μM.

c Concentration required to inhibit 50% of root angle of curvature compared to controls without phytotropins. The angle of curvature for controls were 84 ± 13°.

Quercetin, a flavonoid aglycone, is known to inhibit carrier-mediated auxin efflux (18) which was supported by an I_{50} value of 48 μM (Table I). Quercetin has much lower affinity compared to the synthetic phytotropins (Table I). It is not known if such high concentrations of flavonoids are present in plants under normal conditions. The proposal that flavonoids are the endogenous regulators of auxin transport is not well substantiated in literature.

It is not clear whether the NPA-binding protein and auxin efflux carrier are the same or two separate proteins (7). Hence, the biochemical relationship between NPA and various auxins was examined by means of dissociation analysis. Free IAA is present in very low concentrations in plants relative to various conjugated forms like IAA-glucose, IAA-myo-inositol, IAA-alanine and IAA-aspartate (19, 20). Although polar transport of IAA occurs in the free form, it has been proposed that conjugated forms of IAA are transported from maize seed to coleoptile (19, 20). However, free IAA and its ethyl ester did not dissociate ^{3}H-NPA bound to zucchini MM (Figure 2). Also, various conjugated forms of IAA mentioned above as well as auxin agonists like 2,4-D, dicamba and α-NAA had no effect on bound ^{3}H-NPA at 0.25 mM (data not shown). Binding analysis of different levels of ^{3}H-NPA at various concentrations of IAA, revealed no interaction between the two ligands. Sussman and Gardner (21) also observed lack of competition between IAA and ^{3}H-NPA for binding to corn coleoptile membranes. Recently, Zettl *et al.* (22) showed that labelling of a 23 kDa peptide by 5'-azido[3,6-$^3H_2$]-1-NPA in maize membrane vesicles was strongly reduced by pretreatment of membranes with IAA, NPA and

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016

2,3,5-TIBA. Although this implies competitive binding between IAA and NPA, no direct kinetic evidence has been presented. Zettl *et al.* (22) have concluded that a 23 kDa NPA binding protein in maize plasmalemma membranes has a binding site for IAA and that this protein and NPA-binding protein are related or identical (22, 23). It will be worthwhile to explore the relationship between auxins and phytotropins by direct kinetic analysis, using both NPA and IAA, as well as with both NPA-binding protein and various auxin binding proteins reported in the literature (24,25).

Cytokinins are naturally occurring compounds which have antagonistic effects to auxins. They are responsible for seed germination, lateral bud development, cell division induction as well as differentiation and retardation of senescence (26). The disruption of apical dominance by cytokinins is directly antagonistic with auxin action and prompted us to examine the effects of cytokinins on binding of ^{3}H-NPA. Kinetin (Figure 3) as well as zeatin and zeatin riboside showed no effect in displacing ^{3}H-NPA bound to zucchini MM at 0.25 mM.

Calcium has been implicated as a possible modulator of auxin transport and action (27). However, $CaCl_2$ at 1 mM and the calcium effector chlorpromazine at 0.25 mM showed no NPA dissociation activity (Figure 2). Although not shown in Figure 2, $CaSO_4$, flunarizine and verapamil were also inactive in the dissociation assay.

Thein and Michalke (28) have proposed that the NPA binding protein may be a flavoprotein, based on the inhibitory action of bisulfite on NPA binding. Indeed, bisulfite readily displaces ^{3}H-NPA (Figure 2) with an I_{50} of 9 µM. Bisulfite is also known to inhibit flavoprotein oxidases, although it is not a specific inhibitor of flavoproteins. The activity of certain flavoproteins can be enhanced by the addition of exogenous flavins to replace the dissociated cofactor during partial purification (29, 30). However, FAD (Figure 3) did not enhance ^{3}H-NPA binding or had any dissociation effect on the bound radioligand. The effect of FMN and riboflavin was likewise at 0.25 mM (not shown in Figure 3). The flavoprotein nature of NPA binding protein remains equivocal.

In order to characterize the NPA-binding protein, attempts were made to solubilize it from microsomal membranes. As stated earlier, PM was not used for solubilization studies due to the low recovery level in the phase separation step involved in PM preparation. Many different detergents were tried to solubilize the protein including Triton X-100, deoxycholate and taurodeoxycholate at various concentrations. The best recovery obtained was using 0.4% CHAPS, but it accounted for only 1-3% of the initial total binding activity. The majority of the binding activity was still retained in the MM. Repeated extraction did not solubilize any more binding activity. The solubilized protein exhibited similar ^{3}H-NPA saturation kinetics to that of the membrane bound protein with K_d = 2 nM (determined by Scatchard Plot) and B_{max}= 200 fmol/mg (Figure 4).

Physiological Studies. Pea stem segments is a good system to measure auxin accumulation in response to the blocking of the efflux carrier. The structures of phytotropins used in the present study are shown in Figure 3. Of the phytotropins tested (Table I), the semicarbazone, SCB-1, was most effective, increasing ^{14}C-IAA accumulation 4.2 fold over the control at 1 µM. NPA, TIBA and PBA were less active than SCB-1 at 1 µM, and quercetin had little effect on IAA accumulation at 1 µM (Table I) or 10 µM (120%). The calmodulin antagonist, chlorpromazine, was shown to increase IAA accumulation in hypocotyl segments of zucchini and had an additive effect with NPA at suboptimal concentrations (31). The calcium entry blocker, flunarizine has also been observed to increase IAA uptake in hypocotyl segments (31). Contrary to these reports, we were unable to demonstrate auxin accumulation significantly above control levels by using the calcium effectors chlorpromazine, flunarizine or verapamil at 10 µM. These calcium effectors also had no NPA dissociation activity (Figure 2).

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016

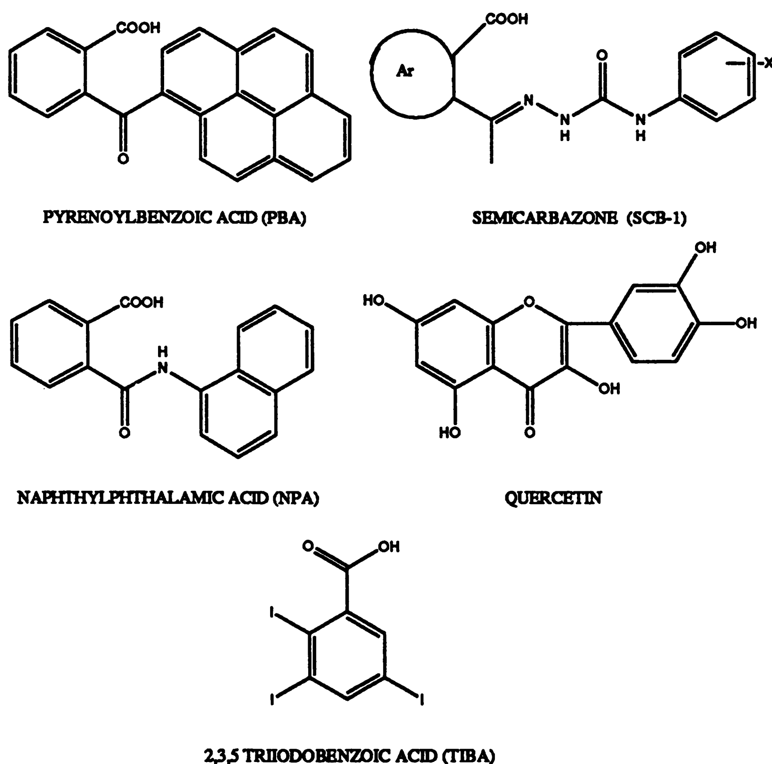


Figure 3. Structures of Several Synthetic and Natural Phytotropins.

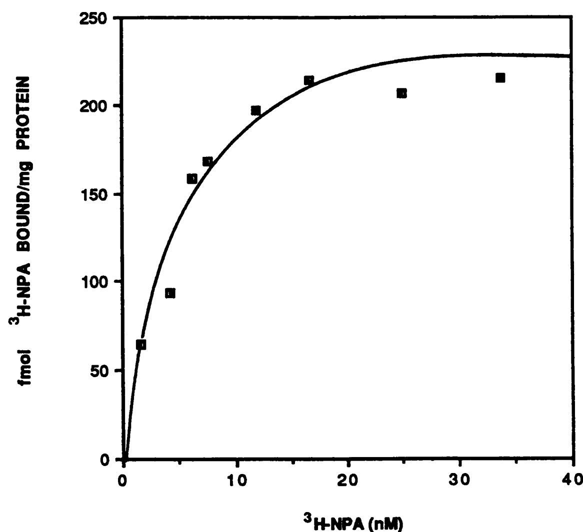


Figure 4. Saturation Curve of ^{3}H-NPA for Solubilized Protein Fraction from Zucchini Membranes.

The tomato root gravitropism assay is very specific for auxin transport inhibitors. Those compounds with phytotropin-like activity are able to inhibit the root response to gravity. In addition, affected roots are stunted with a proliferation of root hairs at high concentrations of the phytotropin. From dose response testing over a range of inhibitor concentrations, it is possible to calculate a GI_{50} value for various phytotropins (Table I). The semicarbazone and PBA were the most active compounds tested with GI_{50} values of 63 and 44 nM respectively. NPA was less than half as effective as PBA at inhibiting root gravitropism and TIBA was 18 fold less active than PBA. Quercetin had no effect on gravitropism even at concentrations as high as 1 mM. The calmodulin effector, chlorpromazine also showed no effect at 10 µM, on the angle of curvature or root length (data not shown).

A comparison of the structure of phytotropins (Figure 3), their relative NPA dissociation constants and their effects on ^{14}C-IAA uptake and root gravitropism (Table I), reveals some interesting features. The most potent compound based on the *in vitro* data is PBA with an I_{50} of 0.15 nM, which is about 100-fold less than SCB-1. However, this is not the case in the IAA uptake and root gravitropism assays. PBA is a highly hydrophobic molecule which might explain its relatively lower activity in the bioassays compared to the I_{50} value (Table 1). NPA, a less hydrophobic analog of PBA, has 100-fold higher dissociation constant, but is nearly as effective in the IAA uptake and only about 2.5 fold less active in the root gravitropism assay. Presumably, there is greater translocation of NPA into cells than PBA due to its higher water solubility. SCB-1 is effective in all the three assays (Table I). The dissociation constant of SCB-1 is 100 fold lower than PBA but it is more effective in the ^{14}C-IAA uptake assay and nearly as effective in the gravitropism assay (Table I). SCB-1 is more water soluble than PBA and NPA and hence, may be more readily translocated into the cells to achieve effective physiological concentrations. Quercetin and TIBA are rather weak in terms of their dissociation constants compared to synthetic phytotropins. Quercetin showed very little effect on the IAA uptake (at 10 µM) and no effect on the gravitropism assay. Quercetin may not readily partition into the cells under the bioassay conditions to exert a significant effect. Solubility problems precluded testing it at higher concentrations. TIBA, however displayed a stronger response relative to I_{50} in both root gravitropism and pea stem assays (Table I). It is possible that TIBA is blocking auxin efflux *via* another mechanism than *via* binding to the NPA-binding protein.

At the present time, very little is known about the auxin efflux carrier or the topography of the NPA binding site on the protein in PM, assuming they are indeed the same protein. All the information on the NPA binding site is based on the binding data of various ligands (8, 32). Judging from the high affinity of PBA, it can be inferred that this protein has a large hydrophobic pocket at the binding site. Also, it is clear that the binding site must accommodate an acidic function in the ligand. What is intriguing is, while all the phytotropins compete with NPA for the binding protein (presumably at the same site), it is not apparent where or how these structurally diverse molecules overlap and how they are functionally homologous to one another. Further understanding of the structure and function of this protein would depend on successful purification, characterization and/or isolation and sequencing of the gene for the NPA binding protein.

ACKNOWLEDGEMENTS We would like to thank Dr. J. Cohen for providing samples of IAA-glucose and IAA-myoinositol. We would also like to thank Laura Yamada for her excellent membrane preparations.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016

LITERATURE CITED

1. Guilfoyle, T.J. *CRC Crit. Rev. Plant Sci.* 1987, *4*, 247-276.
2. Rubery, P.H. In *Plant Hormones and Their Role in Plant Growth and Development;* Davies, P.J. Ed.; Auxin Transport; Martinus Nighhoff, Hingham, MA, 1987, pp 341-362.
3. Rubery, P.H.; Sheldrake, A.R. *Planta,* 1974, *118,* 101-121.
4. Sussman, M.R.; Goldsmith, M.H.M. *Planta,* 1981, *152, 1*3-18.
5. Lutzelscwab, M.; Asard, H.; Ingold, U.; Hertel, R. *Planta,* 1989, *177,* 304-311.
6. Jacobs, M.; Gilbert, S.K. *Science,* 1983, *220,* 1297-1300.
7. Jacobs, M.; Hertel, R. *Planta,* 1978, *142,* 1-10.
8. Katekar, G.F.; Geissler, A.E. *Aust. J. of Pl. Phys.,* 1977, *4,* 321-325.
9. Brunn, S.A.; Muday, G.K.; Haworth, P. *Plant Phys.,* 1992, *98,* 101-107.
10. Jacobs, M.; Rubery, P.H. *Science,* 1988, *241,* 346-349.
11. Rubery, P.H.; Jacobs, M. In *Plant Growth Substances;* Pharis, R.P., Rood, S.B. Eds.; Springer, Berlin, 1990, pp 428-440.
12. Muday, G.K., Brunn, S.A., Haworth, P., Subramanian, M. *Plant Phys.,* 1993, *103, in press.*
13. Munson, P.J.; Rodbard, D. *Anal. Biochem.*, 1980, *107,* 220-239.
14. Trillmich, F.; Michalke, W. *Planta,* 1979, *145,* 119-127.
15. Suttle, J. *Plant Phys.,* 1988, *88,* 795-799.
16. Suttle, J. *Plant Phys.,* 1991, *96,* 875-880.
17. Michalke, W.; Katekar, G.F.; Geissler, A.E. *Planta,* 1992,*187,* 254-260.
18. Faulkner, I.J.; Rubery, P.H. *Planta,* 1992, *186,* 618-625.
19. Bandurski, R.S. *Gior. Bot, Ital.,* 1989, *123,* 321-335.
20. Cohen, J.D.; Bandurski, R.S. *Ann. Rev. Plant Phys.*, 1982, *33,* 403-430.
21. Sussman, M.R.; Gardner, G. *Plant Phys.*, 1980, *66,* 1074-1078.
22. Zettl, R.; Feldwisch, J.; Boland, W.; Schell, J.; Palme, K. *Proc. Natl. Acad. Sci.,* 1992, *89,* 480-484.
23. Feldwisch, J.; Zettl, R.; Hesse, F.; Schell, J.; Palme, K. *Proc. Natl. Acad. Sci.,* 1992, *89,* 475-479.
24. Palme, K.; Hesse, T.; Campos, N.; Garbers, C.; Yanofsky, M.F.; Schell, J. *The Plant Cell,* 1992, *4,* 193-201.
25. Okada, K.; Ueda, J.; Komaki, M.K.; Bell, C.J.; Shimura, Y. *The Plant Cell,* 1991, *3,* 677-684.
26. Matsubara, S. In *CRC Crit. Rev. in Plant Sci.;* 1990, *9,* 17-56.
27. Hertel, R. *Z Pflanz.,* 1983, *112,* 53-67.
28. Thein, M.; Michalke, W. *Planta,* 1988, *176,* 343-350.
29. Subramanian, V.; Liu, T.N.; Yeh, W.K.; Narro, M.L.; Gibson, D.T. *J. Biol. Chem.,* 1981, *256,* 2723-2730.
30. Schloss, J.V. In *Flavins and Flavoproteins,* Walter de Gruyter & Co., Berlin, W. Germany, 1984, pp 737-740.
31. Astle, M.C.; Rubery, P.H. *Plant Sci.,* 1986, *43,* 165-172.
32. Katekar, G.F.; Venis, M.A.; Geissler, A.E. *Phytochemistry,* 1993, *32(3),* 527-531.

RECEIVED December 6, 1993

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ch016

Author Index

Akey, David H., 109
Aluja, M., 39
Bignami, Gary, 64
Brunn, S., 202
Chortyk, Orestes T., 109
Clarke, M. A., 26
Coats, Joel R., 92
Costello, Catherine E., 122
Davis, Harry G., 154
Devlin, W. S., 169
Godshall, M. A., 26
Greany, P. D., 39
Grothaus, Paul, 64
Gueldner, Richard C., 122
Gustine, D. L., 169
Harwood, John S., 122
Hedin, Paul A., 49
Himmelsbach, David S., 122
Howell, C. R., 136
Isman, M. B., 78
Jackson, D. Michael, 109
Kubo, Isao, 182
Legendre, B. L., 26
Lowery, D. T., 78
Lukezic, F. L., 169
Malavasi, A., 39
McCarty, Jack C., Jr., 49
McChesney, James D., 144
McDonald, R. E., 39
McDonough, Leslie M., 154
Moyer, B. G., 169
Neal, John W., Jr., 109
Nickell, Louis G., 1
Patel, B., 202
Pittarelli, George W., 109
Reagan, J. D., 202
Rice, Pamela J., 92
Schroeder, W. J., 39
Severson, Ray F., 109
Shaw, P. E., 39
Sherwood, R. T., 169
Sisson, Verne A., 109
Snook, Maurice E., 122
Stephenson, Michael G., 109
Stierle, Andrea, 64
Stierle, Donald, 64
Stipanovic, R. D., 136
Strobel, Gary, 64
Subramanian, M. V., 202
Walters, E., 202
Widstrom, Neil W., 122
Williams, M. W., 16
Wilson, Richard L., 122
Wiseman, Billy R., 122
Zhang, Minli, 182

Affiliation Index

Agricultural Research Service, U.S. Department of Agriculture, 16,26,39,49, 109,122,136,154,169
Hawaii Biotechnology Group Inc., 64
Insect Biology and Population Management Research Laboratory, 122
Instituto de Ecologia (Mexico), 39
Iowa State University, 92
Massachusetts Institute of Technology, 122
Montana College of Mineral Science and Technology, 64
Montana State University, 64
Nickell Research, Inc., 1
Pennsylvania State University, 169
Sandoz Agro, Inc., 202
University of British Columbia, 78
University of California—Berkeley, 182
University of Georgia, 122
University of Mississippi, 144
University of São Paulo, 39

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ix001

Subject Index

A

Abscisic acid, fruit and vegetable quality effect, 4
Abscission
  control by plant growth regulators, 6–8
  definition, 6
Acetylsalicylic acid, description, 11
Agricultural scientists, challenge, 49
Agriculture, role of plant growth regulators, 1–13
Agronomic traits of cotton, bioregulator-induced effects, 49–59
Alar, *See* Daminozide
Allelochemicals in cotton, bioregulator-induced effects, 49–59
Amidochlor, description, 10
Ancymidol, size control of plants and organs, 9
Anticancer agents, search, 64
Aphids, neem and azadirachtin effects, 78–87
Apples, chemical approaches for biennial bearing control, 16–24
Aspirin, description, 11
Auxin(s)
  fruit and vegetable quality effect, 4
  plant growth and development effect, 202–203
  transmembrane polar transport, 203
Auxin transport protein, biochemical characterization using phytotropins, 202–210
Azadirachtin
  aphid control
    antifeedant activity, 81
    field results, 80–81
    growth and development effect, 81–84
    laboratory results, 79–80
    natural enemy effect, 86–87
    reproduction effect, 84–86
  biological activities toward insects, 78–79
  insecticidal property studies, 78

B

BAS 105 and 109, use for insect control for cotton, 55–56
Bemisia tabaci (Gennadius), activity of *N. gossei*, 109–120
Benefits, gibberellic acid for fruit fly prophylaxis, 40–42
Biennial bearing, apples
  chemical examples, 16–17
  chemical thinning strategy, 18
  definition, 16
  disadvantages, 16
  economic impact, 17
  endothallic acid, 21–22
  new chemicals, need, 18–19
  pelargonic acid, 20–21,22*t*
  physiological basis, 17–18
  postbloom fruit thinning, 22–24
  sulfcarbamide, 19–22*t*
Biocontrol agent, *G. virens*, 136–142
Biodegradable pesticides, reasons for development, 93
Biological activity, maysin and analogs, 133–135
Bioregulator(s)
  advantages, 50
  history of use for insect control, 50–51
  plant growth and development process effect, 49
  responses at genetic level, 58–59
  sugar cane ripening agent use, 26–27
Bioregulator-induced effects
  allelochemicals and agronomic traits of cotton, 57–58
  insect effects, 51,54
  sugar cane composition effects
    dextran concentration, 31*t*,36
    experimental procedure, 27–29
    fiber, 30*t*,33–34
    inorganic ash, 31*t*,35
    invert sugar, 31*t*,34–35
    leucoanthocyanin, 32*t*,37
    purity, 30*t*,33
    starch concentration, 32*t*,36–37
    sucrose, 29,30*t*,33
    theoretical recoverable sugar per ton of cane, 30*t*,34
    total polysaccharide concentration, 31*t*,35–36
Botanicals, examples, 92

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ix002

In Bioregulators for Crop Protection and Pest Control; Hedin, P.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Brazil perspective, gibberellic acid for fruit fly prophylaxis, 41*t*,45–46
5-Bromosalicylic acid, description, 11
Buenos Aires, Argentina, recommendations from 1986 workshop, 1

C

Calcium, role in auxin transport and action, 208
Cancer, anticancer agent search, 64
Carbaryl
- abscission control, 7
- biennial bearing control use, 17
- postbloom thinner use, 22–24

Chemical approaches, biennial bearing control in apples, 16–24
Chemical(s) derived from higher plants, role in history of humankind, 144
Chemical Research Applied to World Needs II (CHEMRAWN II), recommendations, 1
Chlormequat chloride, insect effects, 51,54
(2-Chloroethyl)trimethylammonium chloride, use for insect control for cotton, 55–56
(2-Chloroethyl)tris(2-methoxyethoxy)silane, abscission control, 7
5-Chloro-3-methyl-4-nitro-1*H*-pyrazole, abscission control, 7
(4-Chlorophenoxy)acetic acid, role in fruit set and development, 6
5-Chlorosalicylic acid, description, 11
Chlorphonium chloride, size control of plants and organs, 9
Citrus fruit
- fruit fly susceptibility reduction using gibberellic acid, 39–47
- shelf life extension using gibberellic acid, 39

Codling moth
- communication disruption for control, 156–157
- control methods, 156
- sex pheromone
  - controlled-release dispenser, 162–166
  - efficacy of disruptants, 158–162
  - identification, 157–158,159*f*

Communication disruption, history, 155–156
Condensed tannin, insect effects, 54
Controlled-release dispenser, codling moth sex pheromone, 162–166
Corn earworm, natural resistance of Zapalote Chico corn silks, 123
Cotton, bioregulator-induced effects on allelochemicals and agronomic traits, 49–59
Cotton insect control, use of onium-type bioregulators, 54–56
Crop productivity, improvement approaches, 49–50
Cycloheximide, abscission control, 7
Cydia pomonella, See Codling moth
Cytokinins
- auxin inhibition, 208,209*f*
- fruit and vegetable quality effect, 4

D

Daminozide
- abscission control, 7
- flowering effect, 5
- fruit and vegetable quality effect, 4
- regulatory effects on development, 13
- size control of plants and organs, 9

Defense reactions of plants, elicitation by *P. corrugata* metabolites, 169–179
Defoliating cotton, use of plant growth regulators as harvest aids, 3
Development of natural products
- advantages, 151
- cost, 145
- material required, 148
- pipeline concept, 145–148
- supply concerns, 148–151

Dextran concentration, bioregulator-induced sugar cane composition effects, 31*t*,36
3,4-Dichloroisothiazole-5-carboxylic acid, insect effects, 51,54
(2,4-Dichlorophenoxy)acetic acid, use for insect control, 50–51
Diets, direct effects of plant regulators on insects, 51
Dikegulac sodium, size control of plants and organs, 9
1,1-Dimethylpiperidinium chloride, *See* Mepiquat chloride
Dinoseb, insect effects, 54

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ix002

Diquat, flowering effect, 5
Diuron, flowering effect, 5
Drawbacks, gibberellic acid for fruit fly prophylaxis, 46–47
Dropp, *See* Thidiazuron

E

Ecdysone, accumulation in *H. virescens* larval gut vs. absorption rate, 196–199
Ecdysteroid(s)
  accumulation in *H. virescens* larval gut vs. absorption rate, 196–199
  applications, 183
  insect growth and development effect, 183
  metabolic pathway in *H. virescens* larval gut, 184–191
  pest control, problems, 183–184
  pesticide activity, 184
Ecdysteroid-22-*O*-acyltransferase, role in *H. virescens* larvae, 191–196
Economic impact, biennial bearing of apples, 17
Elgetol, use for biennial bearing control, 16–17
Elicitor, initiation of defense responses, 170
Endophytic fungi of Pacific yew
  search
    pharmacophores, 75–76
    taxol, 68
  taxol
    confirmation of production, 69–75
    source, 66–67
Endothallic acid, use for control of biennial bearing of apples, 21–22
Ethephon
  abscission control, 7
  fruit and vegetable quality effect, 4
  fruit set and development effect, 6
Ethylene, role in flowering, 5

F

Fermentation procedure, confirmation of taxol production, 69
Fiber, bioregulator-induced sugar cane composition effects, 30*t*,33–34
Flavone *C*-glycosides from corn for corn earworm control
  biological activity, 133–135
  high-maysin germ plasm, sources, 125–126
  high-performance liquid chromatographic analytical procedure, 123
  isolation and identification, 127,130–132
  isolation procedures, 123–124
  model compound bioassay procedure, 125
  plant material, 123
  silk extract bioassay procedure, 124–125
  structures, 127,128*f*
Florida perspective, gibberellic acid for fruit fly prophylaxis, 41*t*,42–44
Flowering, control using plant growth regulators, 4–5
Flusilazole, viridiol biosynthesis inhibition, 140–142
Fruit fly control
  gibberellic acid use, 39–47
  new measures, need, 40
Fruit quality, role of plant growth regulators, 4
Fruit set and development, role of plant growth regulators, 5–6
Fungal taxol isolation protocol, confirmation of taxol production, 69–70
Fungicides, viridiol biosynthesis inhibition, 140–142
Fungus, pharmacophore source, 65

G

Genetic engineering of crops, examples, 169–170
Gibberellic acid
  fruit fly prophylaxis
    benefits, 40–42
    Brazil perspective, 41*t*,45–46
    drawbacks, 46–47
    Florida perspective, 41*t*,42–44
    induced pest problems, 46
    initial studies, 39–40
    long-term effects on tree, 46
    Mexico perspective, 41*t*,44–45
    phytotoxicity, 46
    retained green color, 46
    treatment cost, 47
  fruit set and development effect, 6

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ix002

Gibberellic acid—*Continued*
shelf life extension of citrus fruit, 39
Gibberellins
fruit and vegetable quality effect, 4
fruit set and development effect, 6
size control of plants and organs, 8–9
taxol, precedence for microbial source, 67
Gliocladium virens as biocontrol agent
antibiotics, 136–137
phytotoxicity, 138
strain differences, 138
viridiol biosynthesis, 138–140
viridiol biosynthesis inhibition, 140–142
Gliotoxin, biocontrol agent, 136–137
Gliovirin, biocontrol agent, 136–137
Glyphosate
insect effects, 54
sugar cane ripening agent, 26–27
Glyphosine, use as sugar cane ripener, 4
Gossypol
cotton insect control use, 55
insect effects, 54

H

Helicoverpa zea, natural resistance of Zapalote Chico corn silks, 123
Heliothis virescens
ecdysone accumulation in larval gut, 196–199
ecdysteroid-22-*O*-acyltransferase role in larvae, 191–196
ecdysteroid metabolic pathway in larval gut, 184–191
Heptelidic acid, biocontrol agent, 136–137
Herbicide programs, comparison to plant growth regulator programs, 12
History, plant growth regulation, 2–3
Horticulture, role of plant growth regulators, 1–13
Hydrazines, flowering effect, 4–5
Hypersensitive reaction
defense response, 170–171
elicitors, identification as peptides, 176*f*,178–179
individual responses, mechanisms, 171–176
oxygen effect, 172,175–176

I

Immunoassay techniques as screening tool, confirmation of taxol production, 70–71
Indirect competitive inhibition enzyme immunoassay, confirmation of taxol production, 71–73,74*f*
Indole-3-acetic acid, role in plant growth and development, 202–203
Inorganic ash, bioregulator-induced sugar cane composition effects, 31*t*,35
Insect control
bioregulators, history of use, 50–51
cotton, use of onium-type bioregulators, 54–56
Insecticides
alternate insect control methods, 154–155
disadvantages, 154
Invert sugar, bioregulator-induced sugar cane composition effects, 31*t*,34–35

K

Kinetin compounds and adducts, plant growth and development effect, 56–58

L

Leucoanthocyanin, bioregulator-induced sugar cane composition effects, 32*t*,37
Limit, description, 10

M

Maleic hydrazide
flowering effect, 5
size control of plants and organs, 9
Mass spectral analysis of fungal taxol, confirmation of taxol production, 70
Maysin
corn silks, HPLC determination, 123
Zapalote Chico corn silks, natural resistance to earworm, 123–135
Medicarpin, plant defense response effect, 171–176
Mepiquat chloride
description, 10
insect control use for cotton, 54–56
size control of plants and organs, 10

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ix002

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ix002

Metabolism of plants, regulation by plant growth regulators, 3
Methyl chlorflurenol, fruit set and development effect, 6
Mexico perspective, gibberellic acid for fruit fly prophylaxis, 41*t*,44–45
Microbial source for taxol, 66–67
Mode of action, monoterpenoids, 95–96
Monoclonal antibody development, confirmation of taxol production, 71
Monoterpenoids
  advantages as pesticides, 93
  mode of action, 95–96
  natural occurrence, 93–94
  toxicity, 94–95
Monuron, flowering effect, 5
Myzus nicotianae Blackman, activity of *N. gossei*, 109–120

N

Naphthaleneacetamide, abscission control, 7
Naphthaleneacetic acid
  abscission control, 7
  use as postbloom thinner, 22–24
2-Naphthoxyacetic acid
  fruit set and development, 6
  studies, 203
N-(1-Naphthyl)phthalamic acid binding protein
  binding assays, 204–209
  physiological studies, 208,210
  solubilization from microsomal membranes, 208,209*f*
  study procedure, 203–204
Natural pesticide from *Nicotiana gossei*
  acetonitrile-soluble components, 113,114*f*
  acid percent distribution in sugar ester, 113,116–117
  activity against whitefly, 117,119–120
  alkaloid-free sugar ester components, 113,115*f*
  birational bioassay procedure, 111,113
  biorational components, 113,115*f*
  biorational isolation procedure, 111,112*f*
  cuticular component extraction procedure, 111
  cuticular extract components, 113,114*f*
  experimental description, 110
Natural pesticide from *Nicotiana gossei*—*Continued*
  glucose ester fraction MS fragmentation, 117,118*f*
  plant production procedure, 11
  sucrose ester fraction MS fragmentation, 116*f*,117
  sugar ester characterization procedure, 111
Natural products
  factors affecting interest in research and development, 144
  supply concerns, 145–151
Naturally occurring bioregulators
  advantages, 50
  plant growth and development process effect, 49
Neem
  biological activities toward insects, 78–79
  insecticidal property studies, 78
Neem seed oil for aphid control
  antifeedant activity, 81
  factors affecting efficacy, 79
  field results, 80–81
  growth and development effect, 81–84
  laboratory results, 79–80
  natural enemy effect, 86–87
  reproduction effect, 84–86
Nicotiana gossei, natural pesticide characterization, 110–120
Nicotiana species, 110

O

Onium-type bioregulators, insect control use for cotton, 54–56
Organ size, control by plant growth regulators, 8–10

P

Paclobutrazol, description, 10
Pelargonic acid, use for control of biennial bearing of apples, 20–21,22*t*
Pesticide from *Nicotiana gossei*, natural, *See* Natural pesticide from *Nicotiana gossei*
Pharmacophores, search from *T. brevifolia*, 75–76

Phenylurea cytokinin, description, 10
Pheromone, definition, 155
N-Phosphonomethylglycine, *See* Glyphosate
Physiological basis, biennial bearing of apples, 17–18
Phytoecdysteroids, applications, 183
Phytotoxicity
- *G. virens*, 138
- gibberellic acid for fruit fly prophylaxis, 46

Phytotoxin biosynthesis, inhibition in *G. virens*, 136–142
Phytotropins
- binding assays, 204–209
- physiological studies, 208,210
- study procedure, 203–204

Pipeline concept of development, stages, 145–148
Pix, *See* Mepiquat chloride
Plant bioregulators, direct effects in diets of insects, 51
Plant defense reactions, elicitation by *P. corrugata* metabolites, 169–179
Plant growth regulators
- abscission control, 6–8
- advantages, 12–13
- examples developed in 1980s, 10–11
- flowering control, 4–5
- fruit set and development effect, 5–6
- future, 11–13
- history, 2–3
- metabolism regulation, 3–4
- plant and organ size control, 8–10
- programs, comparison to herbicide programs, 12
- regulatory issues, 13
- uses, 2

Plant metabolism, regulation by plant growth regulators, 3
Plant size, control by plant growth regulators, 8–10
Postbloom fruit thinning, improvements, 22–24
PP–333, description, 10
Productivity of crops, improvement approaches, 49–50
Propiconazole, viridiol biosynthesis inhibition, 140–142
Pseudomonas corrugata metabolites
- elicitor elaboration, 176–177,178*t*
- elicitor production, 171

Pseudomonas corrugata metabolites—*Continued*
- identification of elicitors as peptides, 176*f*,178–179
- mechanisms for individual defense responses, 171–176

Purity, bioregulator-induced sugar cane composition effects, 30*t*,33
Pythium ultimum, damage to crops, 136

Q

Quercetin, auxin inhibition, 207*t*

R

Radioisotopic labeling, confirmation of taxol production, 73
Regulatory issues, plant growth regulators, 12
2′′-*O*-α-L-Rhamnosyl-6-*C*-(6-deoxy-*xylo*-hexos-4-ulosyl)luteolin, *See* Maysin
Rhizobitoxine, fruit and vegetable quality effect, 4
Rhizoctonia solani, damage to crops, 136
Ripening of sugar cane, plant growth regulator use, 3–4
Rooting of cuttings, initiation and acceleration using plant growth regulators, 2–3

S

Secondary compounds, structural classes, 92
Seedling diseases, definition, 136
Semiochemicals for codling moth control
- controlled-release dispenser, 162–166
- economic issues, 156–157
- efficacy of disruptants, 158–162
- history of communication disruption, 155–156
- sex pheromone identification, 157–158,159*f*

Sex pheromone of codling moth, *See* Codling moth, sex pheromone
Size of plants and organs, control by plant growth regulators, 8–10

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ix002

Sodium 4,6-dinitro-*O*-cresylate, abscission control, 7
Sprouting of potatoes and onions, suppression using plant growth regulators, 3
Starch concentration, bioregulator-induced sugar cane composition effects, 32*t*,36–37
Structural requirements for monoterpenoid activity against insects
- acetates
  - vs. pivalates, 102
  - vs. propionates, 101–103,105*t*
  - vs. trichloroacetates and trifluoroacetates, 102
- alcohols vs. ketones, 101,103
- aromatic vs. acyclic, 101,103,104*t*
- derivatives vs. parent alcohol, 102–103
- experimental procedure, 96–98*f*
- fumigant toxicity evaluation procedure, 99
- monocyclic vs. bicyclic, 101
- ovicidal toxicity evaluation procedure, 99
- pivalates vs. trichloroacetates and trifluoroacetates, 102
- saturated vs. unsaturated, 101,103
- standard comparison, 101
- structural comparison procedure, 99–100
- topical toxicity evaluation procedure, 99
- trichloroacetates vs. trifluoroacetates, 102

Sucrose, bioregulator-induced sugar cane composition effects, 29,30*t*,33
Sugar cane
- bioregulator-induced composition effects, 27–37
- bioregulator use as ripening agents, 26
- high-yield cultivars, 26
- ripening, plant growth regulator use, 3–4

Sulfanilamide, description, 11
Sulfcarbamide, use for control of biennial bearing of apples, 19–22*t*
Supply of natural products
- cultivated plant source production, 150
- economic effects, 149
- production criteria, 149–151
- requirements, 148–149
- wild harvest risks, 150

Sweet potato whitefly, activity of *N. gossei*, 109–120
Synthetic bioregulators
- advantages, 50
- plant growth and development process effect, 49

Synthetic pesticides, problems, 93

T

Taxol
- applications, 65–66
- sources, 64–67
- structure, 65
- supply problem, 66

Taxol-producing microorganism
- confirmation, 69–75
- search, 68–69

Taxomyces andreanae, fungal source of taxol, 68–75
Taxus brevifolia, search for pharmacophores, 75–76
Terpenoids, natural occurrence, 93–94
Theoretical recoverable sugar per ton of cane, bioregulator-induced sugar cane composition effects, 30*t*,34
Thidiazuron, description, 10
Tobacco, response to *P. corrugata* and $HgCl_2$, 172–175*f*
Tobacco aphid, activity of *N. gossei*, 109–120
Tops of sugar cane, bioregulator-induced composition effects, 27–37
Total polysaccharide concentration, 31*t*,35–36
Toxicity, monoterpenoids, 94–95
Transmembrane polar transport, auxins, 203
Triazoles, size control of plants and organs, 9
Tributylphosphorotrithioite, defoliant use, 8
2,3,5-Triiodobenzoic acid, flowering effect, 5
Typey, definition, 6

V

Viridin, biocontrol agent, 136–137

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ix002

Viridiol
biosyntheses, 138–140
biosynthesis inhibition, 140–142

W

White clover, response to *P. corrugata* and $HgCl_2$, 172–175*f*

Z

Zapalote Chico corn silks, natural resistance to corn earworm, 123
Zea mays L., flavone *C*-glycosides for corn earworm control, 123–135

Production: Charlotte McNaughton
Indexing: Deborah H. Steiner
Acquisition: Anne Wilson
Cover design: Michele Telschow

Printed and bound by Maple Press, York, PA

August 18, 2012 | http://pubs.acs.org
Publication Date: May 27, 1994 | doi: 10.1021/bk-1994-0557.ix002