Allelopathy

Organisms, Processes, and Applications

Inderjit, K. M. M. Dakshini,
and Frank A. Einhellig

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Allelopathy

Organisms, Processes, and Applications

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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

Preface

ALMOST EVERYONE HAS AN AWARENESS that certain plants can irritate the skin or trigger our sense of smell, but it is less well recognized that plants often release chemicals that influence the growth and distribution of other plants. In spite of the fact that observations of this type of phenomenon occurred more than 2000 years ago, the community of scientists still struggles with elucidating these chemical interactions and placing them in working perspectives in ecology.

In this book, we will hold with a definition of allelopathy that includes all types of chemical interactions among plants, and in so doing utilize the broad, historical definition of plants that encompasses algae, fungi, and bacteria, as well as higher plants. We also recognize that too strict a definition for allelopathy sets artificial limits on the action of chemicals produced by plants. This book is not a comprehensive treatise. Its aim, however, is to provide some of the most recent insights into the range of allelopathic functions. The contributed papers aptly illustrate allelopathy at work in agroecosystems, and they suggest a vision of challenges and potential rewards in this field of science.

The sequence in this book starts with an overview followed by several chapters on allelopathic interactions involving specific organisms and some of the chemicals isolated in those situations. The progression leads to discussion in Chapters 7–12 of some of the mechanisms of action of chemicals involved in allelopathy, both their physiological activity and some of the processes involved in soil transfers. Studies in agroecosystems are the focus of the next six chapters, and from Chapter 19 onward, ideas of biological control and/or direct conversion of compounds for use in agriculture are presented.

During the past three decades, the breadth and depth of research on chemical interactions among plants has established this field as a mature science that involves the contributions of biologists, botanists, microbiologists, agronomists, crop scientists, chemists, biochemists, ecologists, soil scientists, and others. Contributions have come from academia and industry alike. Many affects that plant chemicals have are not well understood because they are interwoven with stresses of the environment, and this situation renders attempts to investigate these complexities a formidable task. However, any rational evaluation of the literature suggests that chemical interactions among all types of organisms are a pervasive aspect of all ecology, including both natural communities and agricultural

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systems. We cannot ignore this reality, and we are challenged to capitalize on the opportunities that these chemical interactions present.

Most nations of the world face serious environmental problems, and a portion of those evolve from a heavy reliance on herbicides and pesticides for agricultural production. Efforts toward a more sustainable agriculture must inevitably take into account the adaptive mechanisms that have allowed the success of certain plants, and these include the allelochemicals they produce. The insights in this book illustrate that a better understanding of the allelopathic phenomenon can help in progress toward a more sustainable agriculture worldwide. To this end, this book is timely, and we hope it will stimulate the thinking of young scientists to take up some of these challenges.

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We gratefully acknowledge authors who have contributed their data and perspectives. The authorship of chapters is drawn from scientists around the world, and they truly provide a global perspective on the potential impacts and action of plant chemicals in a diversity of environments.

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Chapter 1

Allelopathy: Current Status and Future Goals

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The phenomenon of allelopathy encompasses all types of chemical interactions among plants and microorganisms. Several hundred different organic compounds (allelochemicals) released from plants and microbes are known to affect the growth or aspects of function of the receiving species. Many new allelochemicals have been identified in recent years and it has become clear that the actions of allelochemicals are important features characterizing the interrelationships among organisms. These compounds influence patterns in vegetational communities, plant succession, seed preservation, germination of fungal spores, the nitrogen cycle, mutualistic associations, crop productivity, and plant defense. Allelopathy is tightly coupled with competition for resources and stress from disease, temperature extremes, moisture deficit, and herbicides. Such stresses often increase allelochemical production and accentuate their action. Allelopathic inhibition typically results from a combination of allelochemicals which interfere with several physiological processes in the receiving plant or microorganism. Other than the autecological study of specific species, there are persistent challenges in allelopathy to determine the mechanism of action of compounds, isolate new compounds, evaluate environmental interactions, and understand activity in the soil. New frontiers will focus on ways to capitalize on allelopathy to enhance crop production and develop a more sustainable agriculture, including weed and pest control through crop rotations, residue management, and a variety of approaches in biocontrol. Other goals are to adapt allelochemicals as herbicides, pesticides, and growth stimulants, modify crop genomes to manipulate allelochemical production, and better elucidate chemical communications that generate associations between microorganisms and higher plants.

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The writings of some natural philosophers that date back more than two millenniums show that they recognized chemical influences in nature (*1*). However, it is the evidence accumulated over the last several decades which has established that external roles for biochemicals, meaning roles that do not directly affect the basic physiology of the producing organism, are pervasive themes characterizing the interrelationships among organisms. This realization does not diminish the respective importance that competition for resources has on the relative success of an organism or a species. Instead, chemical ecology extends our dimensions of understanding and provides new insights into the intricacies of interchanges that occur in an ecosystem, community, or population.

Allelopathy, a subset within the broader scope of chemical ecology, is concerned with effects that chemicals of plant or microbial origin have on growth, development, and distribution of other plants and microorganisms in natural communities or agricultural systems. The aims of this overview chapter are to provide a synthesis of the scope of allelopathy, suggest general principles, illustrate some complications in this field, and project future opportunities for study and application.

Scope of Allelopathy

Background. The definition of allelopathy has not been static and the term continues to be applied in slightly different ways. It was first used by Molish (*2*) to indicate all effects that are either directly or indirectly the result of chemicals transferred from one plant to another plant. At that time more than half a century ago, the accepted parameters of the plant kingdom included algae, fungi, and the various microorganisms as well as higher plants. It also is clear that Molish intended allelopathy to encompass both inhibitory and stimulatory activity. When Rice (*3*) wrote the first comprehensive treatise on allelopathy, he limited the term to inhibitory effects in keeping with most of the available information. However, Rice (*4*) revised his view and the second edition of *Allelopathy* embraced the original definition of the term which included stimulatory as well as inhibitory effects (*1*).

Both Waller (*5*) and Rizvi and Rizvi (*6*) included the plant-insect and plant-higher animal interactions in the terms allelopathy and allelochemicals as used in the books they edited. Lovett (*7,8*) has been articulate in pointing out that many of the same plant-produced chemicals that affect associated plants also influence other organisms, and he has called for expanding the context of allelopathy. He has focused on allelopathy as the complex of subtle communications between plants and also between plants and other organisms. In referring to the scope of biological activities that arise because plant-produced chemical messengers are added to the environment, Lovett (*8*) has adopted a perspective that broadens the parameters of allelopathy to include some aspects of plant defense. There has been no other umbrella terminology to encompass chemical defenses of plants, so Lovett's usage may eventually become established in the literature.

Most of the investigators in the field of allelopathy have adopted the original definition of Molish (*2*). However, terms do not set the limits on the actions of plant allelochemicals and it is useful to recognize that some of the same compounds active in plant-plant interactions also impact insects and other animals.

Interference. Theories in classical ecology assumed that competition for space and resources such as water, nutrients, and light was responsible for the mythical "balance of nature" and the relative success of an individual or species in plant communities. Allelopathic effects can not be considered a part of competition since they do not rely on removal of resources. To avoid confusion and recognize that both competition and allelopathy are often involved, Muller (*9*) designated the collective effects of one plant on another as interference. Interference is a term now widely used in the literature, especially in the literature of weed biology where the perspective has generally focused on the deleterious effects of a weedy species on a crop.

Communications. There has been considerable recent work on chemical communications or signals between plants, including those involving the plant-microbe interface. Many bacteria and fungi in the soil form mutualistic or symbiotic associations with plant roots, and evidence exists for roots excreting specific chemical signals which influence microbial activity (*10-14*). Initiation of *Rizobium*-legume symbiosis involves a complex series of steps wherein the bacteria and plant each influence each other. *Rhizobium* are chemotactic toward plant roots and root colonization, growth, and nodulation by rhizobia inoculants are stimulated by seed and root exudates which include certain flavonoids (*11,15,16*). Recent studies show that both plant growth-promoting rhizobacteria and vesicular-arbuscular mycorrhizal fungi produce chemical signals that mediate root growth (*14,17,18*).

Volatile flavor compounds from fungal spores, seeds, bacteria, and plant tissue have been implicated in a number of bioregulatory actions (*19*). Stem rust of wheat urediniospores (*Puccinia graminis* var. *tritici,* causal agent of wheat stem rust) are stimulated to germinate by nonanal at levels as low as 0.01 ppm (*20*). These volatile compounds are not found alone and an array of compounds may be active stimulators of fungal spores. French and Leather (*21*) found nonanal and several related compounds stimulated germination in some weed seeds. Exposure to octyl thiocyanate as low as 1 ppm/v stimulated *Rumex crispus* germination.

Likewise, host signals are required for stimulating germination and several stages of development in witchweed (*Striga asiatica*) parasitism of *Sorghum bicolor* and several other crops (*22,23*). Another type of communication is seen from the evidence that insect feeding on one plant may result in biochemical changes in neighboring plants as the result of an airborne cue originating in the damaged tissue (*24*). Although the picture is not clear, recent work suggests that when insect or mechanical damage occurs, volatile methyl jasmonate released from one plant may signal defense responses in another plant (*25,26*). The

biology of these communications fits within the parameters of the original definition of allelopathy.

Allelochemical Release and Transfers

A key concept in allelopathy is that chemicals transfer through the environment from one organism to another. As illustrated in Figure 1, Grummer (*27*) proposed specific designations for the agents of allelopathy based on the type of producing plant and the type of plant affected. However, with the exception of antibiotic, these designations have not received wide usage. Most investigators have opted to use the general terminology of allelopathic chemicals, or allelochemicals. I feel there are several reasons for the lack of promulgation of the more specific terms. Often the immediate source of a compound involved in allelopathy is obscure. For example, compounds released from higher plants may be altered by microorganisms before the altered substance is contacted by another higher plant. Similarly, it is very difficult to establish the source when a compound of any origin is contacted through the soil medium. A further complication is that the same compound is likely to have multiple roles, affecting different kinds of recipient plants. Alternative usage of these terms has added to the confusion, with antibiotic sometimes designated to encompass any allelopathic chemical (*28*).

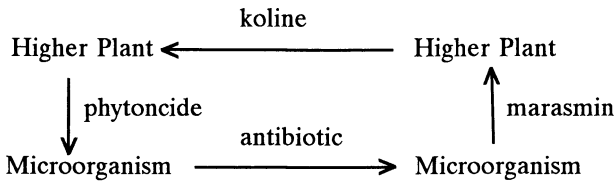

Figure 1. Terms for chemical agents that indicate the type of donor and receiver plants, as shown by the arrows.

While investigators in allelopathy refer to the organic agents that affect the receiving plant as allelochemicals, this term is applied throughout chemical ecology. The active agents in the interorganismic chemical effects involving insects are routinely referenced as allelochemicals. As stated by Reese (*29*), the context of allelochemicals includes the array of nonnutritional chemicals produced by living organisms that affect the growth, health, behavior, or population biology of other species.

Modes of Release. Higher plants regularly release organic compounds by volatilization from their surfaces and through leaf leachates and root exudates. Since the pioneer work on leaf leachates and root exudates (*30-35*), an array of literature confirms that plants loose many metabolites as well as allelochemicals. There is compelling evidence that a wide range of allelochemicals present in

seeds contribute to the prevention of seed decay (*1,36*), and a few experiments illustrate that inhibitors may leach from seeds (*37-41*). Likewise, even during their limited life span, the various microorganisms do not retain all that they produce. Toxic releases are the mechanisms of action of many fungal pathogens, antibiotic zones improve the success of certain bacteria, and microbial signals lead to some associations.

Eventually, the chemical constituents of all organisms are released to the environment through the processes of decomposition. These decomposition products are often added to the soil matrix. Some are volatile compounds that permeate the air environment of the soil as well as having some solubility in the aqueous phase. Allelopathic effects of *Amaranthus palmeri* residue result from an array of volatile methyl ketones and alcohols (*42-46*). There is evidence that volatile seed germination inhibitors that include C_2 - C_{10} hydrocarbons, alcohols, aldehydes, ketones, esters, and monoterpenes arise from a variety of weed and crop plants (*47,48*). The most inhibitory volatile to seed germination tested by Bradow and Connick (*48*) was (*E*)-2-hexenal, an emission from purple nutsedge (*Cyperus rotundus*) residue.

In addition to allelochemicals, organic releases include many chemicals which either have no negative or positive impacts on an associated plant or these possibilities have not been investigated. For example, methanol appears to be a volatile routinely released from plant leaves and it probably is a carbon source for methylobacteria that colonize leaf surfaces (*49,50*), yet we have no information about its effects on adjacent plants. In contrast, volatile phytotoxic monoterpenes such as cineole, piene, and camphor have long been reported as allelopathic inhibitors (*51-54*).

Very little is known about the cellular mechanisms involved in the release of allelochemicals from living tissue, including any modes of regulation or environmental influences on these processes. This area should eventually be a focus of investigation. It will be particularly important to determine the role of environmental factors on the type and quantity of compounds released. Sterling et al. (*55*) showed that the toxicity of exudates from velvetleaf (*Abutilon theophrasti)* glandular trichomes was twice as high in temperature-stressed plants compared to plants grown under more moderate temperatures, yet the volume of exudate production remained fairly constant. Alsaadawi et al. (*56*) reported that gamma irradiation of grain sorghum increased the allelopathic activity of future root exudates. A variety of factors appear to affect the amount and chemical content of root exudates (*57,58*), but generalizations as to how various stresses affect losses of allelopathic compounds into the rhizosphere are premature. Tang and Young (*59*) developed a trapping system for root exudates that allows collection and subsequent identification of small quantities of novel compounds in the exudate. Coupling techniques like this with a manipulation of plant conditions should provide a better understanding of the interplay between environmental stress and the release of allelochemicals.

In most cases, it is an open question whether escaping allelochemicals are actively exuded or simply passive leakage. Even in chemically influenced mutualism or parasitic associations, little is known about regulation. Parasitism of crop plants by members of the genus Scrophulariaceae has been shown to be

facilitated by small amounts of germination stimulants in root exudates, yet whether these exudate compounds are regulated releases is unknown (*60*). Another challenge is that substances are released from plants which cannot be isolated from the tissue, implicating a transforming role of membrane enzymes. Inhibitory *p*-benzoquinones, known as sorgoleone, are abundant in *Sorghum* root exudates but have not been found in the root tissue (*61*).

Distance of Transfer. Allelochemical transfers from one higher plant to another in a terrestrial community can be either through volatiles, aqueous leachates, or various exudates. Volatiles may move through the atmosphere from a donor plant to a receiving species; alternatively, these compounds are adsorbed on soil particles and solubilized in the soil solution. Water-soluble allelochemicals leach from shoot tissue into the soil matrix and exudates from roots are a regular occurrence. Hence, spacial movements of allelochemical can be of some distance and they often infer that the soil acts as an allelochemical pool (*62*). Roots of a receiving plant take up allelochemicals from the soil solution or lipid-soluble compounds adsorbed on soil particles can partition directly into root tissue. As pointed out by Patrick (*63*), plant residues decomposing in the soil will result in localized regions of higher allelochemical concentrations and the impact of allelochemicals in the soil on a receiving plant often depends on the chance encounters of the root system with such regions.

Our perspective also must be one that recognizes the importance of microclimates and microdistances involved in many interfaces between organisms. This is the case in the rhizosphere where antibiotic effects occur between bacteria that interact for space in colonizing root tissue (*10,64,65*). Although allelopathy is not a common term in the literature of plant pathology, the actions of disease organisms are frequently mediated by toxins they release that cause chlorosis, neucrosis, wilting, or modification of growth (*66*). The distance of chemical transfer is obviously minute for the numerous pathogenic fungi whose deleterious action comes by release of phytotoxic substances.

Allelochemicals of Allelopathy

Origin and Diversity. With a few exceptions, the allelopathic agents reported from higher plants are secondary compounds that arise from either the acetate or shikimate pathway, or their chemical skeletons come from a combination of these two origins (Figure 2). This is not to suggest that the details of biosynthesis are always known. These compounds do not appear to have central metabolic functions and the more novel ones have limited occurrence throughout the plant world, being absent from the majority of species. Hence, the genetic capacity for their biosynthesis is important to explaining their roles in a plant community.

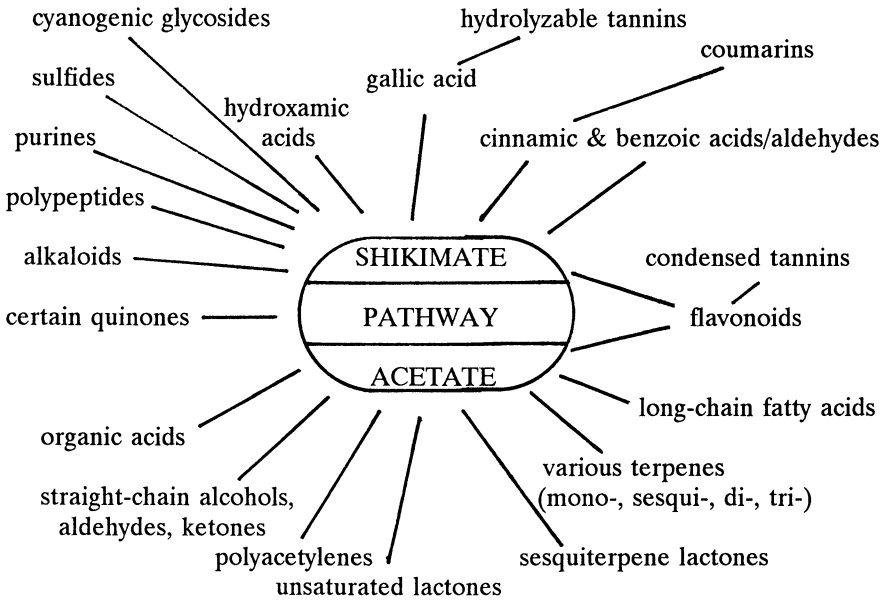

Figure 2. Some of the diversity of allelochemicals implicated in allelopathy. The sketch does not list all classes of allelochemicals and it is not intended to show amino acid intermediates or other pathway details.

Whittaker and Feeny (*67*) classified allelochemicals into five groups: phenylpropanes, acetogenins, terpenoids, steroids, and alkaloids. Based on chemical similarity, Rice (*1*) designated 14 categories of allelopathic compounds, plus a miscellaneous group. Although some commonality in the primary pathways of their biosynthesis is evident, there is an extensive diversity of structures among the several hundred known allelopathic chemicals and a review of their chemistry is beyond the scope of this overview.

Activity of Compounds. The range for biological activity of different allelochemicals reported in the literature covers several orders of magnitude. Activity of compounds within a particular chemical class can be quite different and the sensitivity among species and in the numerous bioassay systems varies a great deal. Many coumarins, cinnamic and benozic acids, flavonoids, monoterpenes, and sesquiterpene lactones affect growth of whole-plant seedlings at thresholds of inhibition between 100 to 1000 μM, but certain ones from these groups have lower inhibition thresholds (*68,69*). Sorgoleone, a *p*-benzoquinone, is more toxic than most of these phenolics and terpenes, inhibiting seedling growth at 10 μM (*70*). When tested at the chloroplast level, exposure to 0.1 μM sorgoleone inhibited chloroplast photosynthesis (*71*). Polyacetylenes also are highly toxic, reducing root elongation of barnyard grass (*Echinochloa crus-galli*) and several other seedlings at 10 ppm or less (*72*). Relatively few structure-

function studies within chemical classes have been done (*69,73*), and none have established what mechanisms cause differences in toxicity among compounds in a chemical class. Interestingly, often an inhibitory compound will stimulate growth when its concentration is relatively low.

Allelochemicals Within Plants. Other than their activity in allelopathy, certain allelopathic compounds also have structural or physiological functions within the producing plant. Cinnamic acid, ferulic acid, *p*-coumaric acid, and other phenylpropanoids commonly identified in the phenomenon of allelopathy are intermediates of lignification. Salicylic acid, in my experience the most growth inhibitory of the benzoic acid allelochemicals (*74*), appears to be an endogenous signal in systemic activation of plant defenses after a localized exposure to certain viral and fungal pathogens (*75-77*). It does not appear to be a signal compound in response to ultraviolet-C radiation (*78*). An array of chemicals implicated in allelopathy have protective functions against various disease organisms, insect predation, and other herbivores (*79-81*), and we should not be quick to dismiss them as of no value to the producing plant.

Mode of Action

Bioassays. The primary tool used to establish allelopathic activity for plant residue, plant extracts, substances in plant releases, or compounds that have been isolated from these sources has been a bioassay. This is a critical step for investigations because answers obtained in a study are intimately tied to the sensitivity of the test system employed. The range of bioassays include effects on seed germination, radicle elongation, whole-plant growth, microbial numbers, or some functional process (*82-84*). In spite of advances in analytical chemistry, these techniques cannot determine biological responses and bioassays will continue at the heart of studies in allelopathy.

Bioassay results are altered by solubility of the allelochemical, the relative amount of the chemical to bioassay tissue, and numerous environmental conditions (*85-88*). Investigators frequently use several different assays in a screening procedure. We have employed a whole-plant *Lemna* bioassay because it is applicable with a small quantity of putative allelochemical and has sensitivity to effects on a range of physiology process (*74,89*).

Physiological Effects. A major future challenge is to determine the mechanisms of action of allelochemicals. At present, we know that the coumarins and phenolic compounds derived from cinnamic and benzoic acids interfere to some degree with many vital plant processes, including cell division, mineral uptake, stomatal function, water balance, respiration, photosynthesis, protein and chlorophyll synthesis, and phytohormone activity (*68*, Einhellig, this book). No clear separation of primary from secondary effects has been possible, but membrane perturbations may be a starting point for the multiple actions of these compounds. Information on the physiological effects and possible mode of action for other groups of allelochemicals on higher plants is even more embryonic.

One of the greatest deficits in our knowledge about allelochemical activity is an explanation for differences in species sensitivity to these compounds. A large seed size or seedling biomass may explain why some species are more tolerant than species with smaller seeds or biomass. However, physiological mechanisms that can explain differences in sensitivity have seldom been investigated.

Activity of allelochemicals against plants is often indirect through inhibition of the growth of microbial symbionts. Suppression of fungal-root colonization hinders water and nutrient absorption, slowing growth and perhaps contributing to delays in reforestation or decline problems in perennial crops (*90-92*). The sensitivity of *Rhizobium* spp. to allelochemicals leads to poor nodulation and subsequent reduction in nitrogen available to legumes growing in association with allelopathic plants or residue (*93,94*). There is even a more indirect affect on higher plants as allelochemicals inhibit free-living nitrogen-fixing bacteria and blue-green algae, thus disrupting the nitrogen cycle and having ramification for mineral nutrition. Allelochemicals from higher plants have been reported to alter microbial respiration (*92*), but for the most part their mode of action is unknown. On the other hand, mechanisms for certain antibiotics are well characterized.

The challenge to explain how allelochemicals act is complicated by the many chemical classes and array of different structures identified as agents in allelopathy. There is no generic allelochemical, and certainly we should anticipate different mechanisms of action among allelopathic chemicals. In many instances, the lack of a sufficient quantity of a substance for study is a hindrance to elucidating its mechanism or mode of action. Further advancements in isolation and purification procedures and synthesis of natural products are critical to pushing the frontiers on compound uptake, transport, and function. Likewise, information on how they alter growth is central to explaining differences in species sensitivity to allelopathy. These mode of action questions are among several keys to exploring the use of allelochemicals or avoiding their detrimental effects in agroecosystems.

Stress Combinations

It is important to recognize that the allelopathic phenomenon is not independent of other stresses. Allelochemical and environmental stress act in concert to affect plant growth. More work is needed on stress interactions, but there is sufficient data to conclude that both the production and impacts of allelochemicals are suspectable to other conditions in the environment (*95,96*). In this paradigm, stresses such as moisture and temperature conditions not only directly affect plant growth, they may enhance allelochemical production which subsequently impacts the growth of associated plants. A further coupling of allelopathy with environmental stress is apparent as effects of allelochemicals are greater when a plant is also stressed by other environmental conditions.

Quantity of Allelochemicals. Some of the pioneer investigations of effects of abiotic stress on allelochemicals demonstrated that coumarins, such as scopoletin

and scopolin, in tobacco and sunflower increased in response to herbicide, nutrient, temperature, and radiation stresses (*97*). Similarly, Hanson et al. (*98*) found barley (*Hordeum vulgare*) alkaloids increased when plants were grown under high temperature. Moisture stress caused an increase of allelopathic monoterpenes in *Pinus taeda* and cyclic hydroxamic acids in corn (*Zea mays*) (*99,100*). On the other hand, aspen tissue culture plantlets grown under water stress had lower levels of catechol, salicortin, and salicin (*101*). Hall et al. (*102*) found nutrient deficiency enhanced the allelopathic activity of sunflower (*Helianthus annuus*) debris and this was attributed to modification in total phenolic compounds. Nutrient and water stresses appear to favor a general increase in secondary plant metabolites (*103*). There are many reports on the increased production of secondary metabolites, particularly a variety of phenolics, in plants damaged by insect or disease (*26,79*). While it appears that both abiotic and biotic stresses often stimulate production of certain allelochemicals, research is needed to determine what plants and which secondary compounds exhibit this type of plasticity, the conditions that will induce an accumulation of allelochemicals, and any quantitative or qualitative changes in these compounds found in leachates and exudates due to stress.

Allelochemical Plus Nonallelochemical Stress. Plants are regularly subjected to environmental conditions that are less than optimal, and these collective stresses may interact in additive, synergistic, or antagonistic ways (*96*). Temperature, moisture, nutrient, herbicide, and disease stresses are common. Einhellig and Echrich (*88*) found grain sorghum (*Sorghum bicolor*) and soybean (*Glycine max*) were more susceptible to damage from ferulic acid when they were grown at temperatures at the higher end of the plant's range of tolerance. Soybean seedlings grown with a day temperature of 23° C were not affected by 100 μM ferulic acid in the culture medium, but plants grown at 34° C were significantly inhibited even though the temperature difference by itself had no effect on growth. Similarly, under laboratory conditions almost any degree of water stress seems to lower the ferulic acid-inhibition threshold on germination or seedling growth (*96*). In some cases the pathogenesis of disease organisms, such as root rot fungi, is increased by allelopathic effects from decomposing plant residue (*104-106*).

Data from my laboratory indicated that herbicide stress from atrazine and trifluralin works in concert with allelopathy to impair seedling growth (*95,96*). This type of interaction is a very important area for future investigation in view of weed control strategies designed to reduce herbicide inputs.

When allelochemicals enter the soil, microbial transformations often occur or their biological activity may be altered as they are adsorbed on soil particles. Yet the impacts of these phenomena and other interactions in the soil on allelopathy are not well understood. Recently, Blum et al. (*107*) reported that the amount of nitrates and other organic carbon sources in the soil modified the allelopathic action of *p*-coumaric acid. Higher levels of nitrate increased the amount of *p*-coumaric acid required to reduce growth of morning-glory (*Ipomoea hederacca*), but elevated glucose or methionine in the soil reduced the concentration for growth inhibition. This interaction with soil carbon shows a

new dimension in allelopathy-environmental interrelationships and it illustrates the need for data that will help predict the influence of the soil matrix on allelopathy.

Combinations of Allelochemicals

It my opinion almost all cases of allelopathic inhibition in a plant community result from the combined effect of a several compounds. Perhaps the action of juglone in black walnut (*Juglans nigra*) allelopathy may be one of a few rare exceptions (*108*), but even then I suspect other allelochemicals make a contribution.

Einhellig (*96*) reported the joint action of 50 μM each of ten benzoic acid allelochemicals was generally as inhibitory to the growth of velvetleaf (*Abutilon theophrasti*) as 500 μM of a single compound. As is a common reality when comparing molecular derivatives of a particular chemical class, certain of these phenolic acids were more toxic than others. A review of investigations on phenolic acids, alkaloids, sesquiterpene lactones, monoterpenes, and volatile fatty acids, volatile methyl ketones and alcohols indicates that when several compounds are present together their inhibitory action will either be additive or sometimes synergistic, depending on the relative concentrations of the allelochemicals (*96,109*).

In the 1980s, the challenge went out to develop more specific proofs of allelopathy by adhering to a specific protocol like Koch's postulates for demonstrating pathogenicity (*106,110,111*). A cardinal point was to isolate and identify the chemical(s) responsible. Unfortunately, critics of the science of allelopathy have often formulated their questions in terms of the amount, presence, and biological activity of a specific allelochemical. Such questions do not fit with the reality that case histories repeatedly show several compounds implicated as the agents in the allelopathic action of a particular situation, and they do not recognize interactive effects with other stresses. The bias that carried over from Koch's rules also has frustrated work in allelopathy by not enough attention being given to disease and abiotic stresses that complement allelopathy.

Impacts on Ecosystems

Allelopathy influences vegetational associations and patterns, succession, invasion of exotic plant species, nitrogen fixation, seed preservation, the extent of disease and other dynamics of natural plant communities. Although terrestrial ecosystems have been the focus of most of the investigations, allelopathy occurs in aquatic ecosystems as well. Occasionally the role of allelochemicals dominates, but more often it is a subtle, difficult to measure component of community relationships. The case studies chosen to illustrate the capacity of plant chemicals to influence community relationships not only show the direct action of allelochemicals on higher plants, they also illustrate effects on microorganisms that subsequently impact the vegetational community.

Vegetational Patterns. The most visual evidences of allelopathy in natural communities are instances of bare-looking areas, a "halo" zone, around a plant or stand of one type of vegetation. This effect has been intensely scrutinized in the chaparral of southern California and in the sand pine scrub community of Florida's central ridge and costal dunes.

The classic studies on the zonation of vegetation in the chaparral showed *Saliva leucophylla*, *Artemisia californica*, and other aromatic shrubs release a variety of volatile terpenes that solubilize into the leaf cuticle of associated species or adsorb on soil particles from which they may later transfer into root tissue of seedling that will try to establish in proximity to the shrub (*53,112*). Water-soluble phenolic acids are leached from leaves of these and other shrubs, adding to the complex of inhibitory allochemicals that will be encountered by the associated herb and grassland vegetation. Annual grasses are sensitive to these allelochemicals and the net result is a border zone of sparse vegetation near the shrubs that may wax and wane to some degree with seasonal precipitation. As pointed out by Halligan (*113*), the situation is complicated by moisture conditions, mammals, and other factors interacting with the chemical environment to create the vegetational pattern.

In Florida, sharp ecotones often marked by persistent bare zones exist between the sandhill and pine scrub communities, and there is little ground cover under the scrubs. Both allelopathy and fire cycles appear to contribute to vegetational patterns associated with the scrubs (*114-117*). Grasses are excluded from the immediate vicinity of *Polygonella myriophylla* (*118*). *Ceratiola ericoides* and several of the other early scrub colonizers also inhibit grasses. A variety of allelochemicals have been implicated in this activity. For *C. eriocoides*, these include mono-, di-, and triterpenes and several flavonoids (*115-117*). Ceratiolin, a novel flavonoid, may be among the more important compounds since it degrades by photochemical action to produce the very toxic hydrocinnamic acid. It appears that allelopathy interacts with other environmental stresses to generate some discreetness to the distribution of the vegetation in the pine scrub community.

Succession. Rice and coworkers at the University of Oklahoma provided the most comprehensive documentation of allelopathy in succession (*1*). Their studies focused on the sequence of natural succession after fields of low fertility in Central Oklahoma had been abandoned. These "old fields" revegetated with a 2 - 3 year period of robust, annual weeds, followed by an extended period of perhaps a dozen years wherein an annual grass, triple awn grass (*Aristida oligantha*), dominated. This second stage of succession eventually gave way to an abundance of the prairie bunchgrass, little bluestem (*Schizachyrium scoparius*). If left undisturbed long enough, succession reached a stable mixture of the true prairie grasses.

Investigations in the 1960s and 1970s established that the short tenure of the weed stage was a case of autotoxicity. These weeds, *Helianthus annuus, Ambrosia psilostachya, Sorghum halepense, Digitaria sanguinalis* and others, released chemicals which reduced their ability to survive, while the second-stage triple awn grass was more tolerant to this chemical environment (*119-122*).

Scopoletin, chlorogenic acid, tannic acid, and many of the phenolic derivatives of cinnamic and benzoic acid were identified as causative agents. Improvement in nitrogen availability was slowed during the early successional stages because allelochemicals from the first stage weeds and triple awn grass suppressed nitrogen fixation by blue-green algae and both free-living and nodulating bacteria (*93,123*). As a low nitrogen-requiring species, triple awn grass was favored. While activity of nitrifying bacteria was somewhat affected by allelochemicals early in succession, further work suggested that plants of the climax community were more allelopathic to nitrification and caused retention of a higher percentage of available nitrogen in the ammonium form (*124-126*). The kind of plant material added to soil markedly affected the kinds and numbers of microorganisms; soil invertase, amylase and cellulase activity also decreased with progression of old-field succession (*127*).

The studies that have been cited on succession and patterning of vegetation pioneered valuable techniques in allelopathy. These include (a) extensive use of bioassays for detection of allelochemical activity, (b) use of bioassay species relevant to the field situation, (c) isolation and identification of putative allelochemicals, and (d) a variety of experimental designs to isolate allelopathic from competitive interference. There continues to be considerable allelopathy work on the roles of certain species in community dynamics.

Seed Preservation. The mechanisms of how seeds can remain viable many years in the soil without destruction by decay organisms are still not well understood. However, there is abundant evidence showing allelochemicals in the seed coat and other seed tissue are a deterrent to microbial action and in some cases these compounds help regulate when germination can occur (*1,36*). Investigations are needed to evaluate the extend to which allelochemicals exit and form a localized sphere of influence around seeds.

Agricultural Ecosystems

Productivity of agricultural fields, including pasture land and agroforestry environments, is routinely influenced by allelopathy. The source of allelochemicals may be either the crop, weeds, or microorganisms of the decomposition processes (*62,128*). Alternately, any of these groups could be the affected species and allelochemical transformations in the soil always complicate our insights. Even though a variety of scenarios are possible, it is the net effect on crop yield that has been a primary concern. As shown in this book and other literature (*6*), agricultural allelopathy issues have drawn the attention of scientists from many regions of the world. A few specific examples will be used to illustrate the range of allelopathic impact on the agricultural economy.

Weed Interference. Since weeds are a major cause of yield losses, the aggressive growth habits of some of the most tenacious species have come in for scrutiny. Putnam and Weston (*129*) listed 90 species that show allelopathic potential and others have been reported since then. The data implicate some of the world's worst weeds in allelopathy, including ragweed parthenium (*Parthenium*

hysterophorus) (*130*), quackgrass [*Elytrigia repens* (L.) Nevski (*Agropyron repens*)](*129,131*), Johnsongrass (*Sorghum halepense* (*42,119*), Canada thistle (*Cirsium arvense*)(*132*) and giant foxtail (*Setaria faberi*)(*133*).

The most complete investigations on weeds have tied field-based evidence with a search for allelochemicals. *Parthenium hysterophorus*, a tropical weed endemic to America, has done great damage since arriving as an exotic to the India landscape and other places. Numerous reports of the last two decades document the phytotoxicity of its living and decomposing tissue, leachates, and root exudates (*134-137*). Effects on the receiving crop plants and other weeds include reductions in chlorophyll, water uptake, nutrient uptake, and legume nodulation. Several sesquiterpene lactones, phenolic acids, and organic acids have been identified as the responsible agents.

Quackgrass is a second example where multiple investigations spread over many years have elucidated toxicity problems. Decomposing residues and foliage and rhizomes of living plants all reduce crop growth. Putnam and Weston (*129*) found quackgrass residues left on the surface in no-till systems reduced the biomass of eight crop species tested by at least 50%, with alfalfa (*Medicago sativa*) and carrot (*Daucus carota*) reduced more than 90%. Quackgrass inhibited legume nodulation, crops associated with living quackgrass exhibited symptoms of mineral deficiency, and added fertilizer did not solve the problem. These examples show there is a need for awareness of allelochemical toxicity in residue management practices.

Allelopathy of Crops. Some of the major agronomic crops produce allelochemicals which can affect weed growth, result in autotoxicity, or influence growth of the next crop (*128*). Sunflower (*Helianthus annuus*), *Sorghum* crops, and rye (*Secale cereale*) are perhaps the better documented examples of both living biomass and residue allelopathy, albeit a number of other crops could be cited. Although allelochemical production and activity is apparent in crop plants, it has been postulated that through the processes of domestication and plant breeding for yield parameters the current crop varieties are less capable of producing allelochemicals than ancestral germplasm. This hypothesis has important implications for more sustainable agricultural practices that are being demanded, yet it needs more rigorous testing. Supporting data was obtained on cucumber (*Cucumis sativus*) and oat (*Avena sativa*)(*138,139*). Some accessions of oats produced three times as much scopoletin as a standard cultivar and the former provided better weed control. However, varietal differences in allelopathic potential were not shown when comparing a number of sunflower cultivars (*140,141*).

Crops Affect Weeds. It is more than a tantalizing idea that crop plants may provide their own herbicides---some do, but modern agriculture has seldom acted on this reality. Leather (*141*) reported that sunflower grown without a herbicide had no more weed problems than when a herbicide was employed. Our three years of field data from eastern Nebraska demonstrated that grain sorghum greatly reduced weediness in the next growing season (*142*). When grain sorghum was strip cropped with corn and soybean, weed biomass where *Sorghum*

had previously grown was reduced by more than 50% compared to the other two crops. Since that data was reported, I have had personal communication from several agronomist indicating they have observed the weed suppressing action of grain sorghum. I project the mechanism of *Sorghum* action is a combination of phenolic allelochemicals, cyanogenic glycosides, and sorgoleone (Einhellig, this book).

Crop Autotoxicity. Grain sorghum and sunflower yields show a marked decline when these crops are replanted year after year, and to a lesser extent this is seen with wheat (*Triticum aestivum*), rice (*Oryza sativa*), corn, and several others (*128*). Proper crop rotations can avoid this pattern and sometimes stimulatory effects are obtained from the rotation. Production of alfalfa and other perennial legumes diminishes due to autotoxicity, and immediate replanting in the same field is problematic. The alfalfa replant problem has been controversial and certainly unique factors may be involved according to local conditions, but my experience (*143,144*) fits with the allelopathy scenario proposed by Miller (*145*).

Agroforestry. Lichens, a variety of understory vegetation, and many weeds contribute to regeneration problems in managed forestry systems. Either direct or indirect allelopathic effects are often part of the complexities in regeneration failure (*146,147*). Indirect effects on mycorrhizal fungi and organisms of the nitrogen cycle appear to be particularly important (*147,148*). Economic realities of allelopathy in black walnut (*Juglans nigra*) plantations show a different dimension. When a nurse crop of black alder (*Alnus glutinosa*), a tree species forming a microbial nitrogen-fixing association, was interplanted with walnut to improve fertility, the alder eventually died due to the black walnut toxicity (*108,149*).

Applications of Allelopathy

Modern agriculture is challenged to reduce environmental damage and health hazards from chemical inputs, minimize soil erosion, and yet maintain a high level of production (*150*). Strategies capitalizing on allelopathy can help in efforts toward this ideal of a more sustainable agriculture (*128,151*). The actions of allelochemicals should be a consideration in crop rotations, residue management, tillage practices, and implementation of biological control. Opportunities also exist for alteration of crop genomes to enhance production of herbicide and pesticide constituents, and direct commercial uses of allelochemicals may be feasible.

Field Strategies. After several decades of limited advocacy, crop rotations are again being promoted and these decisions need to recognize allelopathic activity. Although specific effects must be worked out for local regions, the weed suppressing action of *Sorghum* spp. (*142*), sunflower (*141*), and other crops can reduce the use of herbicides both in the crop year and subsequent year. Similarly, cover crops and residues of rye, oats, barley, wheat, grain sorghum,

and sudangrass (*Sorghum arundinaceum*) are effective in limiting weed growth (*151-157*). Scenarios for using these crops for weed control include (a) their direct suppression of weed populations when they are the harvested crop, (b) their use as a ground cover in orchards with subsequent desiccation from freezing or a herbicide, (c) double cropping into the stubble and surface residue left from such a crop, and (d) a crop rotation with no-till planting in the year following an allelopathic crop.

Another approach is to coplant a weed control crop simultaneously with a production crop. However, we found when rye and soybean were seeded together the living rye depressed soybean yield (*158*). The risk of interference from the weed control crop must be evaluated in any management strategy. Interplanting of two or more harvestable crops is another way to take advantage of natural product-mediated weed and pest control (*159*). Gliessmann (*160*) reported that some combinations of interplanted crops, as well as allowing "good weeds" to persist, can enhance yield and reduce the reliance on chemical control measures. Peach growers in southeastern United States have found a reduction in nematode problems when they interplant wheat in their orchards (*161*).

If allelopathy only provides a partial control of weed populations, some combination of allelopathy management along with herbicides may be desired. The complementary action of natural phytotoxins and herbicides has received very little attention, but there is some indication that their additive action would justify a reduced level of herbicide input (*95,96*). Since control of weeds and pests is the key issue to any of the management options mentioned, the strategies can be employed without a complete understanding of the interference mechanisms.

Market Products. A few allelochemicals or products that have a functional basis in allelopathy have been marketed and a number of companies are actively pursuing programs focused on these opportunities. These include screening for and isolation of biological active allelochemicals, modification and development of natural products as herbicides, efforts to formulate and market growth-enhancing microorganisms, and ways for expanded use of biological control.

Protectants and Stimulants. DeFreitas and Germida (*65,162*) found applications of certain pseudomonads promoted winter wheat growth and they indicated antibiosis towards phytopathogens may be part of the mechanism. Chemical signals also may be involved in the initial colonization. Agrochemical companies have recognized the value of seed-applied inoculants and plant growth-promoting rhizobacteria which colonize roots and enhance aspects of plant growth. Quantum 4000 and Kodiak, marketed by Gustafson, Inc., are seed-treatment biological fungicides which contain *Bacillus subtilis*. This beneficial bacteria takes root space from pathogens and it has been suggested that antibiotic production may be involved (*64,163*). These products have been used on cotton (*Gossypium hirsutum*), peanuts (*Archis hypogaea*), soybean, and several other beans. Stanley (*161*) reported ISK Biotech Corporation is developing a strain of *B. subtilis* for protection against brown rot fungus (*Monilinia frucitcola*) on peaches and grapes.

In Allelopathy; Dakshini, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Biological Herbicides. The success of two mycoherbicides marketed several years ago, Collego and DeVine (*164,165*), fostered an expansion of efforts in this direction. These mycoherbicides control northern jointvetch (*Aeschynomene virginica*) and stranglevine (*Morrenia odorata*). DeVine contains a strain of the fungus *Phytophthora palmivora* that kills stranglevine, although it is not known for certain that toxic substances are involved.

A range of viral, bacterial, and fungal pathogens are being tested as biological control agents against specific target weeds. I have no doubt that research will eventually establish that the action of some of these biological herbicides is mediated by chemicals they produce. The use of higher plants, including noncrop plants, to control weedy species has received much less attention, but this avenue needs exploration. Leafy spurge (*Euphorbia esula*), the worst rangeland weed of the United States northern plains, does not encroach into areas with small everlasting (*Antennaria microphylla*), perhaps due to the sensitivity of leafy spurge to hydroquione and other substances produced by small everlasting (*166,167*).

Natural Product Herbicides. The thousands of secondary compounds produced by plants and microorganisms provide an amazing diversity in chemical structures which offer opportunities for new herbicides, pesticides, growth stimulants, or growth regulators (*168*). Isolating and testing compounds for biological activities is an ongoing endeavor of several agrochemical companies. Important insecticides have their basis in natural products, but gaining herbicides from plant and microbial sources has been more difficult. On balance, microbial compounds appear to have greater potential as herbicides than do many of the allelochemicals from higher plants (*169-174*). Microbial compounds are often more selective and have higher phytotoxicity. Of the many natural-product compounds with herbicidal activity, only two discovered from nonpathogen bacteria are currently marketed; bialaphos, a tripeptide, and glufosinate, a phosphonate amino acid analog.

The chemistry of natural product isolation and identification is complicated, screening bioassays to detect active compounds are problematic, and many barriers challenge the synthesis and production of the more complicated structures. Nevertheless, it is likely that allelochemicals will provide some useful products to aid crop production. The hope is that these compounds will be less toxic to nontarget organisms and, have a shorter residence time in the environment.

Future Directions and Challenges

Certain aspects for the future of allelopathy are a replay of the past; that is (a) in-depth autecological studies on species, (b) evaluation of allelopathy in plant associations and crop production (c) isolation, identification and characterization of alleochemicals, (d) efforts to determine mechanisms of action, (e) learning more about soil transformations and residence times, and (f) continuation of work on practical applications for allelochemicals and the allelopathic phenomenon. However, I anticipate a much stronger focus on the

last point, including all the chemistry, biotechnological manipulations, and field management strategies this will entail. More scrutiny also will be given to allelochemical activity in disease and defense roles, including chemical communications from one plant to another. The role of chemicals as control factors in plant-microbe and host plant-parasite association will be more fully explored. Finally, advances in biological control programs will evolve in concert with a better understanding of the role of allelopathy in these interactions.

In summary, the pervasive involvement of plant-produced chemicals in plant-plant and plant-microorganism interactions provides many challenging frontiers. This science has the potential to contribute greatly to agricultural production and stability. It should be no surprise that many of the chapters in this book are focused on agroecosystem problems and ways to capitalize on allelopathy. These include some specifics on weed interference, tillage practices, rotations, studies on specific agronomy systems, parasitic plant-host plant communications, and a number of perspectives on problems and approaches to biological control. Topics in this book also address findings on a few specific chemicals, how allelochemicals function, stress interactions, and other issues that have only been touched on in this overview chapter. Collectively, they provide valuable insights into allelopathy and the functions of allelochemicals.

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Chapter 2

Lichen Allelopathy: A Review

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Secondary metabolites produced by lichen-forming fungi have attracted attention of investigators for over 100 years. Approximately 500 compounds have been reported from lichens, of which about 350 appear to be unique. Most of these compounds are weak phenolic acids which are produced by the fungal partner and accumulate on the outer walls of fungal hyphae. Concentrations vary considerably within and among species; however, values of 1-2% air dry wt are commonly observed and values as high as 20% have been reported in some. Ecologists have long assumed an antibiotic role for these compounds, and recent evidence suggests that they play an important adaptive role defending lichen thalli from herbivores and pathogens; they also suppress neighboring lichen and moss competitors, and there is some evidence that they can inhibit higher plants. As such, they are allelochemicals in the broadest sense of the term.

The distinctive phenolic compounds produced by lichens began to attract attention of investigators well over 100 years ago, and information about their structure, biogenic origin and phylogenetic significance has accumulated steadily ever since. At the present time, our understanding of their biological roles is meager, although a number of hypotheses have been proposed in recent reviews (*1-4*). Among the adaptive roles assumed for these compounds is allelopathy, and the evidence pertaining to this role will be discussed in this review.

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Lichens and Lichen Substances

Lichens are stable, symbiotic associations between fungi (called mycobionts) and photosynthetic chlorophytes or cyanophytes (called photobionts). Found in all terrestrial habitats, lichens represent one of the most successful groups of symbiotic organisms, and are frequently studied as models of symbiosis. The group is polyphyletic and includes approximately 18,000 named species (the name is assigned to the fungal partner), representing nearly 20% of all named fungi. Although they are difficult to maintain under laboratory conditions, some have been successfully cultured and maintained *in vitro* (*5*).

Lichens are known to produce numerous extracellular secondary metabolites (*6*), some of which are not seen outside the lichen symbiosis. Approximately 500 compounds have been described from over 6000 taxa of lichens and their chemotypes; of these, approximately 350 are considered to be unique to lichens (Table I). Most of these metabolites are fungal in origin, and although it was once thought that lichenization was required for production of these compounds, recent evidence demonstrates that isolated fungi can be induced to form characteristic compounds in culture (*7*).

The chemistry, biogenic origin and methods used to study these compounds have been discussed at length in a recent review (*6*). In general, however, the most characteristic lichen products are aromatic compounds that are formed from simple phenolic units via the acetyl-polymalonyl pathway, a pathway that can also generate numerous aliphatic acids and esters. Numerous terpenoids and steroids are formed via the mevalonic acid pathway and some pulvinic acid derivatives are formed via the shikimic acid pathway. Some compounds frequently used in the study of lichen taxonomy are of unknown structure, but have been given common names and are recognizable in the most frequently used microchemical tests used for the identification of these compounds (*6*).

Crystalline deposits of many of the compounds can be visualized in scanning electron micrographs of lichens (Figure 1). They appear to be formed on the outer surfaces of fungal hyphae, and there is some localization of the compounds to reproductive and various vegetative portions of the lichen thallus (a term indicating a lichenized body with morphological features unique to the species). For example, some compounds are apparently more concentrated in the outer layers of the stroma (cortex) while others are found only in the central hyphae (medulla). These patterns are generally consistent across taxonomic boundaries, indicating biogenetic and possibly functional differences for compounds from localized regions of the lichen thallus.

Biological Roles of Lichen Substances: Defense and Light-Screening

Numerous adaptive roles have been proposed for lichen secondary metabolites, but until recently little evidence existed to support them. The most commonly-held view early in this century, based largely on anecdotal evidence (*8*), was

Table I. Major Categories of Secondary Metabolites in Lichens, Arranged by Biogenetic Pathway[a]

Product Category	Pathway	Number[b]
Secondary aliphatic acids, esters and related compounds	Acetate-polymalonate	42
Mononuclear phenolic compounds[c]	Acetate-polymalonate	17
para-Depsides, tridepsides, benzyl esters and *meta*-depsides[c,d]	Acetate-polymalonate	133
Depsidones, depsones and related diphenyl ethers[c,d]	Acetate-polymalonate	88
Dibenzofurans, usnic acids and related compounds[c,d]	Acetate-polymalonate	17
Chromones[c,d]	Acetate-polymalonate	13
Naphthoquinones[c,d]	Acetate-polymalonate	4
Xanthones[c,d]	Acetate-polymalonate	26
Anthraquinones and related xanthones[c,d]	Acetate-polymalonate	54
Di-, sester- and triterpenoids	Mevalonic acid	62
Steroids	Mevalonic acid	20
Terphenylquinones	Shikimic acid	2
Pulvinic acid derivatives	Shikimic acid	12

[a]SOURCE: Reproduced with permission from reference 6. Copyright 1989 Academic Press.

[b]Number of known compounds.

[c]Polyketide-derived aromatic compounds.

[d]Di- and triaryl derivatives of simple phenolic units.

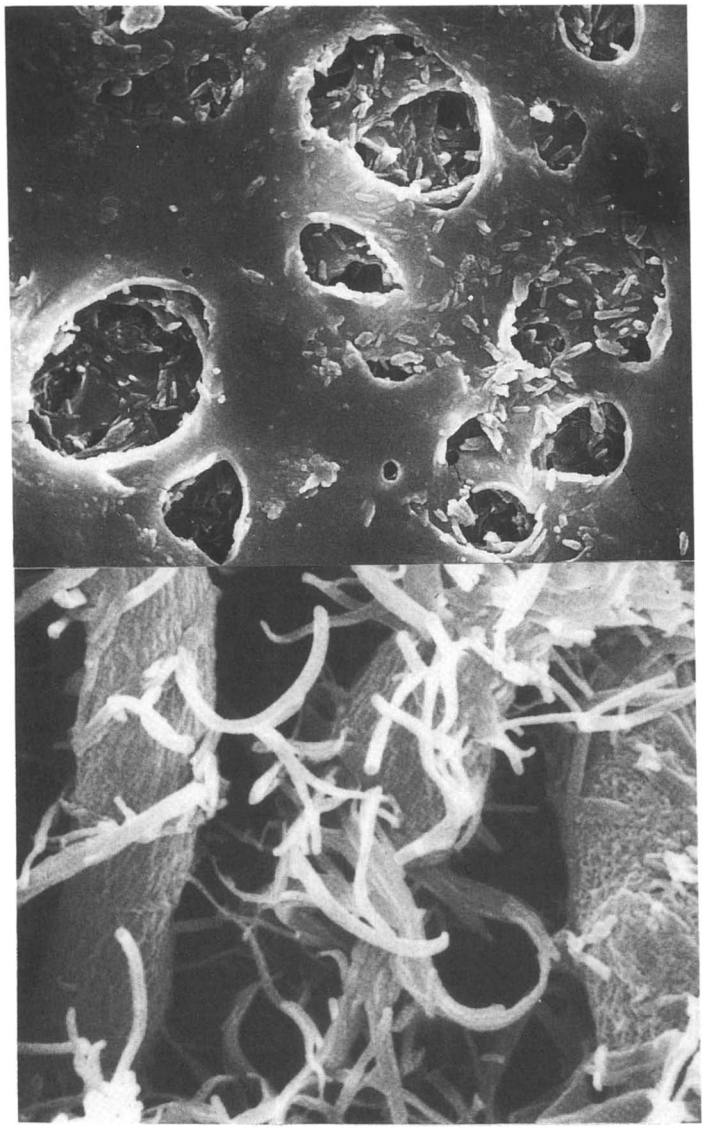

Figure 1. Top: Crystals of atranorin embedded in the pored epicortex of *Parmelina enormis* (Hale) Hale from Africa (x2000). Bottom: Crystals of lecanoric acid on medullary hyphae of *Pseudevernia consocians* (Vain.) Hale & Culb (x5000).

that they protected slow-growing lichen thalli from attack by microorganisms and herbivorous animals. However, there seemed to be little interest in establishing this idea experimentally. Most of the research on the biological activity of these compounds began after 1940, when the antibiotic nature of many of these compounds was recognized (*9*), and was aimed exclusively at clinical uses. Interest in clinical applications of lichen compounds has continued to the present, and a number of antitumor, antifungal and antibacterial properties of lichen compounds have been discovered.

The ecological role of these compounds was largely ignored during this period, however, and was not taken up again until the 1960's and 1970's, when investigators began testing some of the ideas from the early literature and developing some new theories. At this time, three hypotheses emerged concerning these compounds: (1) they were light-screening agents; (2) they were antibiotic defense compounds; and (3) they were allelopathic. The light-screening idea emerged from an early study (*10*) demonstrating that lichen compounds, especially those produced in cortical regions, rendered tissues relatively opaque; this implied that they protected sensitive algal cells from high light intensities. The idea was given further support by studies like that of Rundel (*11*) showing that concentrations of usnic acid in the cortex of *Cladina subtenuis* increased with increasing light intensity of the habitat. However, a number of recent studies, notably that of Culberson et al. (*12*), indicate that light may have less effect on compound production than other factors, especially those having to do with tissue age and genotype.

The defensive role of lichen compounds has been established by a number of investigators (*1-4*). Many lichen compounds are now known to deter feeding by generalist herbivores, some of which are known to feed regularly on lichens in nature. This suggests an important antiherbivore role for these compounds. For example, Lawrey (*13*) showed that the feeding ecology of the lichenivorous slug *Pallifera varia* was regulated to a large extent by the chemistry of the available lichen species in its habitat. Lichen species preferred by the slug were generally those that produce the lowest diversity of secondary metabolites; the slugs avoided lichens that produce a wide range of compounds. Furthermore, extracts of these avoided lichens inhibited feeding by slugs in laboratory experiments; other factors, such as essential element content and thallus texture, seemed to be relatively unimportant.

In addition to an antiherbivore role, lichen compounds definitely have antifungal and antibacterial properties as well, which may explain why lichens are so infrequently attacked by microorganisms in nature. Based on the clinical research done in the 1950's, lichen compounds seem to be most effective against gram-positive bacteria and fungi (*9*). This would indicate a general defensive role for these compounds. Lawrey (*14*) recently determined the antimicrobial function of several lichens that differed markedly in palatability to the lichenivore *Pallifera varia*. Several, rather generalized gram-positive and gram-negative bacteria, were assayed for their sensitivity to extracts of lichens known to be either well-defended or poorly-defended against *P. varia*. Results of these assays showed a distinct correspondence between the antiherbivore and antimicrobial potential of the test lichens, indicating that lichens can defend against both types of consumers but their

secondary compounds are not designed to defend specifically against either group.

Despite the fact that lichens are seldom attacked by generalized microorganisms, there are rather specialized fungal parasites known to attack lichens. These are members of a relatively small group (approximately 1000 taxa) of lichenicolous fungi which form a wide variety of interactions with lichens ranging from commensalism to extremely virulent parasitism (*15*). However, even the parasitic groups, which are highly adapted to lichen hosts, can be inhibited by lichen compounds (*16*). Taken together, therefore, the available evidence indicates that of all the ecological roles proposed for lichen compounds, a defensive one is the most generally applicable.

Lichen Allelopathy: Inhibition of Vascular Plants

At about the same time lichen compounds were being investigated as light-screening and defensive agents, there was interest in the potential allelopathic role they played in ecosystems. The early investigations of allelopathy in lichens are reviewed briefly by Kershaw (*17*) and Lawrey (*2*). These established that aqueous extracts of various lichens were capable of inhibiting seed germination and growth of numerous vascular plants (*18-26*). The ecological importance of this is not clear in most studies since the plants used as bioassays do not normally grow with lichens, although some investigators have demonstrated an allelopathic effect of trees in lichen-dominated systems (*27-30*). As Kershaw (*17*) points out, however, results of these studies are difficult to interpret since factors other than allelopathy (such as nutrient availability, soil moisture retention and gas exchange) are likely involved in the vascular plant responses observed.

Cowles (*30*) attempted to isolate the various factors beside allelopathy that are involved in growth of black spruce trees in Canadian boreal habitats where the soil surface is frequently covered by mats of the terricolous lichen *Cladina stellaris*. Using a number of combined treatments that included removal of the lichen mat, fertilization, mulching with plastic and straw (to moderate soil temperatures), and treatment with lichen extracts, Cowles was able to establish that exudates from *C. stellaris* were quite inhibitory to spruce growth; however, destruction of the lichen mat had a far more deleterious effect on spruce growth (by worsening soil temperature and moisture conditions), suggesting that the negative allelopathic effect in this case was more than counterbalanced by the positive moderation by the lichen mat of the soil conditions favorable to spruce.

Inhibition of Soil, Decay and Mycorrhizal Microorganisms

When Burkholder and colleagues (*31,32*) first established that lichens produced antibiotic substances effective against numerous gram-positive bacteria, this stimulated research into the biochemistry and clinical application of lichen compounds and the ecological significance of this also began to be investigated. For example, several studies showed reductions in the frequency

of soil fungi and bacteria below ground-dwelling lichens (*33-37*). These were attributed to the antibiotic effect of lichen compounds.

Other studies demonstrated that water extracts of lichens inhibited activity of various decay fungi (*38-39*), suggesting lichen metabolites may be involved in various soil processes, especially biogeochemical ones, mediated by microorganisms. This aspect of lichen allelopathy has been studied very little, and would seem to have important implications in terrestrial ecosystems.

Lichen compounds have also been shown to inhibit mycorrhizal fungi, the consequences of which are important not only to the fungus but to the associated vascular plant. Indeed, as Rundel (*1*) has pointed out, the inhibitory effect of lichen compounds on vascular plants may have less to do with direct toxicity than with the indirect disturbance of their mycorrhizal associations. There is some evidence to support this idea (*27,38,40*). However, it is difficult to interpret such information ecologically since inhibition of vascular plants via their mycorrhizal fungi would seldom benefit lichens in most habitats. Nevertheless, the evidence suggests that lichens may play a prominent role as regulators of vascular plant colonization and community succession in ecosystems where they dominate the flora.

Inhibition of Bryophytes by Lichen Compounds

If lichen compounds confer competitive advantages to the lichens that produce them, they must be able to inhibit organisms that frequently share lichen substrates, and these are generally other lichens and bryophytes. There are numerous accounts, most of them quite old now, of encounters between lichens and bryophytes that involve competition for light and substrate (*41*). In many of these early accounts, there is speculation that chemical inhibition of mosses by lichens could influence the outcome of these interactions. More recently, Heilman and Sharp (*42*) suggested an allelopathic cause for the striking patterns of occurrence of slow-growing lichens amongst dense patches of bryophytes in the southern Appalachian Mountains. However, this idea was not tested experimentally; indeed, the importance of allelopathy in competitive interactions between lichens and bryophytes was not established experimentally until relatively recently.

The first experimental tests (*43,44*) verified the allelopathic nature of lichen compounds against bryophytes. These tests were done first with acetone extracts of various ground-dwelling species of *Cladonia*, using spores from three moss species that live in the same habitat as bioassays. Subsequent tests made use of pure lichen compounds. In all cases, lichen compounds were found to be quite inhibitory to the germination and growth of moss spores. A later study (*45*) demonstrated the inhibitory effect of numerous pure lichen compounds on spore germination and sporeling growth of the moss *Funaria hygrometrica* (Table II). These results indicated that lichen compounds were frequently quite toxic to mosses and that toxicity was both concentration and pH dependent. However, moss responses were variable and difficult to interpret ecologically, suggesting to the authors that the significance of

allelopathy in lichen-moss interactions had to be considered in relation to numerous other habitat factors.

Inhibition of Lichens by Lichen Compounds

Given the antibacterial and antifungal nature of lichen compounds reported in numerous clinical studies (*9*), one is easily persuaded that lichens prevent establishment and growth of lichen neighbors by inhibiting them chemically. This idea appears commonly in the literature, but usually with little direct evidence to support it. For example, Barkman (*41*) reports numerous cases of lichen death thought to be caused by chemical exudates from neighboring lichens; he also mentions that certain lichen species never co-occur despite having similar habitat requirements, suggesting a possible chemical interference between them.

Nonrandom spatial patterns of lichens are also sometimes used to indicate an allelopathic effect among lichens. For example, Culberson *et al.* (*46*) discovered that specimens of *Neofuscelia verruculifera* collected in eastern Europe frequently had an epiphytic *Lepraria* species attached to them while specimens of *N. loxodes* collected at the same time and in the same localities were almost entirely free of the epiphyte (Table III). This interesting nonrandom spatial pattern was thought to be the consequence of an allelopathic effect since the more chemically-diverse lichen (*N. loxodes*) was the one rarely colonized.

The first attempts to establish the allelopathic potential of lichen compounds in lichen-lichen interactions made use of lichen ascospore germination as an indicator of this potential (*47-49*). These studies demonstrated that lichen compounds are quite effective inhibitors of lichen spore germination, in some cases inhibiting germination completely. This was found to be true especially of vulpinic and evernic acids, which are known to be especially effective antibacterial and antifungal compounds. For example, Whiton and Lawrey (*49*) observed complete spore inhibition by these compounds for the crustose lichens *Graphis scripta* and *Caloplaca citrina*, although spores of the fruticose *Cladonia cristatella* were not as severely inhibited (Figure 2). The negative effect on spore germination was considered most damaging to crustose lichens since these lichens have no alternative method of propagation.

The degree to which these chemical interactions influence lichens in their natural habitats is yet to be established unequivocally. However, there is little doubt that lichen compounds have the potential to inhibit lichen fungi, especially in the sensitive ascospore stage, which would seem to strengthen the argument that these compounds are allelopathic.

Role of Lichen Allelochemicals in Lichen Communities

The importance of allelopathy in lichen communities is difficult to gauge with so little information to work with. However, if it is true that lichen compounds are allelopathic agents in lichen communities, then the secondary chemistry

Table II. Effect of pH on the Relative Toxicity of Several Lichen Secondary Metabolites on the Spore Germination of *Funaria hyrometrica*. Data are Percentages Based on Spore Germination Under Control (no compounds) Conditions. Sample Size is 6. Lichen Compound Concentration in All Cases is 2.7×10^{-3} M

	Percent Spore Germination			
Lichen Compound	pH 5	pH 6	pH 7	pH 8
Vulpinic acid	0	0	0	0
Psoromic acid	11	0	20	0
Fumarprotocetraric acid	41	97	97	97
Evernic acid	75	41	1	44
Lecanoric acid	94	86	90	96
Atranorin	97	98	97	99
Stictic acid	100	102	101	105
Usnic acid	102	98	25	1

SOURCE: Some data are from reference 45.

Table III. Distribution of a *Lepraria* Epiphyte on *Neofuscelia loxodes* and *N. verruculifera* Collected in Czechoslovakia

	Without epiphyte		With epiphyte	
Parameter	*N. lox.*	*N. verr.*	*N. lox.*	*N. verr.*
Number of specimens	26	20	4	55
Percentage by species	87	27	13	73
Weight (g)	5.96	1.00	0.23	4.52
Cover (cm^2)	129.0	39.4	6.0	139.0

SOURCE: Some data are from reference 46.

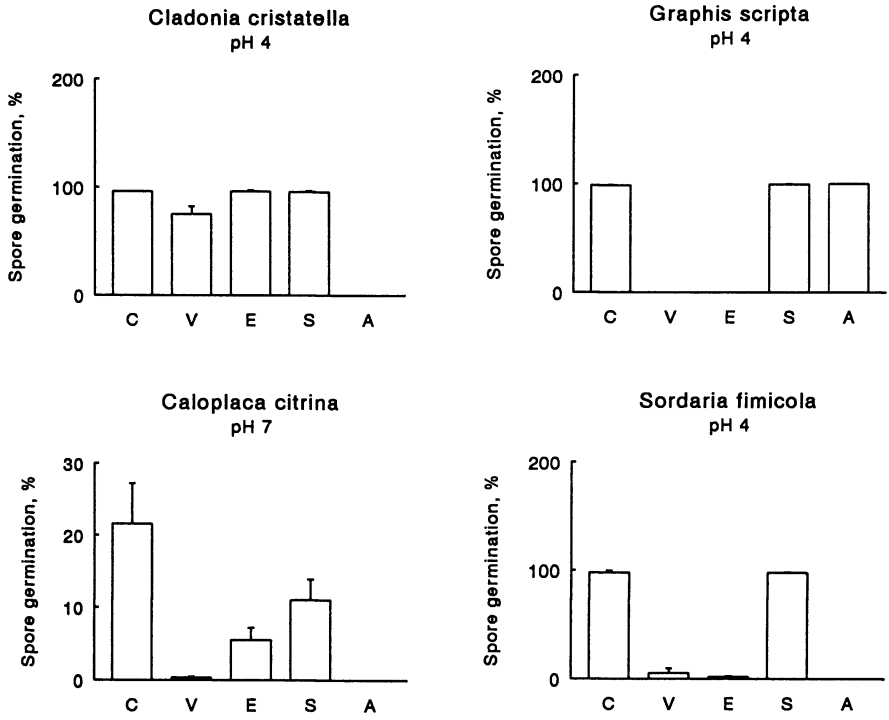

Figure 2. Effect of vulpinic (V), evernic (E) and stictic (S) acids and the cortical compound atranorin (A) on ascospore germination of three lichen-forming fungi (*Cladonia cristatella*, *Graphis scripta* and *Caloplaca citrina*) and the non-lichenized *Sordaria fimicola* at media pH eliciting highest germination responses in controls (C) to which no compounds were added. (Adapted from references 48 and 49.)

of a lichen's neighbors would appear to be an important determinant of its success. For example, certain pairs of species might not be expected to co-occur in communities because of chemical interference. It is also possible that certain species will replace others as a result of chemical inhibition (or the lack of chemical defense against herbivore consumers and pathogens).

There are a number of detailed descriptive studies of lichen successions in the literature (*50*), but little evidence that chemistry influences species replacements during lichen successions. One of the successional trends frequently predicted by theorists is that chemically well-defended species will replace poorly-defended species over successional time. This is because poorly-defended species are more susceptible to attack by herbivores and pathogens and intense competition by neighbors. Lawrey (*50*) considered this prediction to have little support in lichen communities, not because there is no ecologically meaningful variation in chemical complexity, but because chemistry seems not to cause species replacements in lichen successions. Indeed, there is little evidence for species replacements in lichen communities at all.

However, this is not to say that chemistry is not involved in the successional status of lichens during community development. There is some indirect evidence indicating that chemistry is correlated with competitive ability in lichens, and it is likely that competitive ability is a strong determinant of successional status. This idea was discussed by Rogers (51), who considered chemistry an important attribute of a lichen's ecological strategy. He hypothesized that good competitors will tend to produce allelopathic (offensive) compounds capable of inhibiting neighbors while stress-tolerators will produce antibiotic (defensive) compounds that function as protective agents (it is unclear from his discussion how specific lichen compounds differ in this regard). A review of the chemistry of (*34*) lichens revealed that chemical complexity (whatever the function) is positively associated with competitive ability and negatively associated with ruderality. Since early stages of succession tend to favor ruderal strategies and later stages competitive strategies (*52*), one should expect to see increased chemical complexity during lichen community development. This is probably an oversimplification, however, since chemical inhibition of competitors, herbivores or pathogens is not likely the principal regulator of lichen successional patterns (50); it is an hypothesis that certainly merits consideration by investigators, however.

Summary and Directions for Future Research

Research done to date has established a number of important facts about lichen compounds as allelopathic agents:

1) Lichen compounds are not simply waste products, but seem to have a number of functions, including allelopathy.

2) The effectiveness of compounds as allelopathic agents against vascular and nonvascular plants, algae, and fungi (including other lichens) varies, but seems to be related in many cases to the diversity of compounds produced; in addition, certain compounds seem to be more effective than others.

3) There is still little convincing information about the mode of action of these compounds. Indeed, this is the single unexplored area of research that would seem to merit the greatest attention by interested investigators.

Lichens represent one of the best-known groups of organisms in terms of secondary metabolite chemistry, and researchers will undoubtedly continue to find them attractive subjects for the study of allelopathy. The study of lichen compounds as antibiotic and antiviral agents will also contribute much to our understanding of lichen allelopathy. Most of what we presently understand of the phenomenon has resulted from the study of interactions of lichens with competitors and predators in their natural environments, and it is hoped that ecological investigations of this sort will continue. In addition, laboratory screening studies of lichen allelochemicals (*53*) have provided, and likely will continue to provide, evidence of chemicals that merit further study.

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Chapter 3

Overcoming Allelopathic Growth Inhibition by Micorrhizal Inoculation

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Failure of conifer regeneration in *Kalmia*-dominated, nutrient poor sites of Newfoundland is attributable at least partly to *Kalmia* allelopathy. The aggressive regeneration behaviour of *Kalmia* after forest disturbance and its resistance to the commonly used herbicides makes it difficult to control the shrub in order to achieve silvicultural success. An alternative approach was taken to test the growth potential of black spruce seedlings preinoculated with mycorrhizal fungi in the presence of *Kalmia*. Aqueous extracts of fresh leaves and humus of *Kalmia* were found to be inhibitory to primary root growth of black spruce. Further analysis of the leaf extract of *Kalmia* by TLC and HPLC isolated and identified eight phenolic acids. These were *m*-coumaric, *p*-coumaric, ferulic, gentisic, *p*-hydroxybenzoic, *o*-hydroxyphenylacetic, syringic and vanillic acid. All the compounds were inhibitory to the root growth of black spruce, with *o*-hydroxyphenylacetic acid being the most toxic and *m*-coumaric acid the least toxic. Toxicity of the compounds was greater with increasing concentration and decreasing pH. Tolerance of 51 isolates of ectomycorrhizal fungi was tested in laboratory where 19 isolates were able to grow in presence of *Kalmia* leaf extracts. Mycelial growth of four ectomycorrhizal isolates, NF4, GB45, GB23 and GB12 was stimulated in presence of *Kalmia* leaf extract. Black spruce seedlings were inoculated with these four fungal isolates for subsequent experiments. The inoculated seedlings were grown in greenhouse pots in presence of *Kalmia* plants. The seedlings inoculated with three of the four isolates had better growth compared to the noninoculated control. All these three fungal isolates, NF4, GB45 and GB24 have potential in overcoming *Kalmia* growth inhibition in black spruce. Particularly important is isolate NF4, that caused 2-3 fold increase in seedling biomass compared to the control. A field trial is recommended to test the growth response of preinoculated black spruce seedlings in *Kalmia*-dominated sites in central Newfoundland.

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The widely observed conifer regeneration failure in medium to poor quality site types of Newfoundland forests was attributed at least partly to allelopathic property of *Kalmia angustifolia* L. var *angustifolia* (hereafter referred to as *Kalmia*) (*1-3*). This ericaceous understorey shrub grows vigorously following forest disturbance such as clear cutting and fire (*4-8*). Rapid sprouting from stem bases and rhizomes is the principal mode of regeneration (*9*); seedling regeneration is very slow. The plant, however, maintains a large soil seed bank (*10*) as other ericaceous plants do, for example *Calluna vulgaris* L. Hull, *Erica cinerea* L. and *E. teralix* L. (*11,12*). Irrespective of habitat conditions and disturbance regimes, *Kalmia* gains dominance within 5-8 years after forest clearing (*13*). Rapid vegetative growth and slow litter decomposition of *Kalmia*, in cool and moist oceanic climate, cause build up of a thick duff layer under *Kalmia* (*14*). It has been reported that *Kalmia* occupancy in a site can cause irreversible soil degradation as a result of thick duff accumulation, high acidity, poor nutrient availability and iron pan formation (*15*). Meades (*16,17*) suggested that long term occupancy of *Kalmia* in a site can bring about a permanent vegetation shift from forest to heathland thereby precluding any tree regeneration. Other ericaceous plants, such as *Calluna vulgaris* and *Gaultheria shallon* L., have been known to cause a vegetation shift from forest to heathland following disturbance (*13*).

In addition to the unfavourable soil pH and nutrient conditions for tree seedling growth in *Kalmia* sites it has been reported that leaf litter and soil of *Kalmia* contain allelopathic substances that affect the primary root growth of black spruce (*1,3,18*). Similar organic accumulation and soil acidity increases have been reported for other ericaceous plants of cool, moist, temperate climate, particularly *Calluna vulgaris* and *Erica cinerea* (*19,20*). These and other heath forming species in western Europe have been reported to produce allelopathic substances in soil (*21-25*). Handley (*26*) reported *Calluna*-induced "growth check" in Sitka spruce (*Picea sitchensis* (Bong.) Carriere). Robinson (*27,28*) demonstrated that root exudates of *Calluna vulgaris* contained allelopathic compounds that were inhibitory to some mycorrhizal fungi of conifer seedlings. Jalal et al. (*29*) and Jalal and Read (*23,24*) isolated and identified a number of phenolic compounds from *Calluna* plant and soil material. Some of these compounds were highly phytotoxic and were believed to contribute to the exclusion of other plants including trees from ericaceous heath (*30*).

In the case of *Kalmia*-black spruce forests of Newfoundland, silvicultural success following forest harvesting is predicated upon the normal growth of black spruce. This could be achieved either by controlling *Kalmia* growth or by overcoming the *Kalmia*-induced growth inhibition in black spruce. Since the traditional methods of vegetation control by commonly used herbicides, NPK fertilization, cutting and burning were not successful in controlling *Kalmia* (*31,32*), an alternative approach was taken to address the problem. The basic question was that instead of disturbing *Kalmia* sites, which often results in enhancement of vegetative growth of *Kalmia*, can we manipulate black spruce seedlings to make them more efficient to grow in sites where *Kalmia* is a problem? We hypothesized that allelopathy and soil acidity were the major problems for black spruce regeneration

and that inoculation of black spruce seedlings with appropriate mycorrhizal fungi capable of withstanding habitat acidity and allelopathy would help improve black spruce growth. We have conducted several experiments to test these hypotheses. The present chapter summarizes our findings.

Allelopathic Effects of *Kalmia* Leaf and Soil Extracts

Aqueous extracts of leaves, roots, litter and soil of *Kalmia* are inhibitory to black spruce seedling growth (*1*). The extracts affected the growth and development of primary roots of the germinants with no significant effects on percent seed germination and stem growth of seedlings. The primary root growth of other conifers such as red pine (*Pinus resinosa* Ait.) and balsam fir (*Abies balsamea* (L.) Mill.) was also inhibited by the water extracts of *Kalmia* (*2,3,10*). In the present study *Kalmia* leaf extract was prepared by soaking 100g of fresh leaves in 1 litre distilled water for 24 hours. The filtrate of the extract was considered 100% (v/v) concentration. For soil extract, 2 litre distilled water was very slowly leached through 2 litre volume of *Kalmia* soil. The filtrate of the leachate was considered 100% (v/v) concentration. Other concentrations of the extracts were made by diluting the original extract (100%) with appropriate volume of distilled water (see *33* for detail). Root growth inhibition of black spruce was increased with increasing concentration of the aqueous extract of *Kalmia* (*33*). Significant reduction of primary root growth was occurred at or above 25% leaf extract and 10% humus extract of *Kalmia* compared to distilled water treated control (Table I). The shoot growth of treated seedlings was not significantly different from that of the control.

Allelopathy of *Kalmia* Extracts at Different pH

Soils of *Kalmia*-dominated sites in central and eastern Newfoundland were quite acidic with pH between 2.8 and 4.5 (*A.U. Mallik, unpublished data*). It was thought that acidity may have a detrimental effect on the primary root growth of black spruce. Thus a bioassay experiment was performed with 10, 25, and 50% water extracts of *Kalmia* leaves at pH 3, 4, and 5. For the control, black spruce germinants were treated with distilled water at pH 3, 4, and 5 without the extracts. In absence of extracts acidic pH alone caused a 50% reduction in root growth at pH 3 and pH 4 as compared to at pH 5 (Table II). In presence of water extracts of leaves, root growth inhibition was increased with decreasing pH and increasing extract concentration. The germinants were unable to grow in presence of 50% leaf extract at pH 3 and root growth was severely inhibited by 10% and 25% extracts at pH 3. Shoot growth was inhibited only at pH 3 by 25 and 50% of the extract (Table II). Although there was a strong interaction effect of pH and *Kalmia* leaf extract on black spruce root growth it was not caused by pH alone. The inhibition also did not appear to be caused by low osmotic potentials of the bioassay solution since the osmotic potentials were never lower than -2 bar. In most of the bioassays leaf extract concentrations used were lower than 50% and had osmotic potentials always higher

Table I. Effect of aqueous Extracts of *Kalmia* Leaves and Soil on the Growth of Black Spruce Seedlings

Treatment	Concentration (%)	Length (mm) of Root	Length (mm) of Shoot
Leaf water	0	16.0 ± 0.6a	19.5 ± 2.0
extract	10	13.9 ± 0.3ab	20.3 ± 0.7
	25	13.4 ± 0.8b	19.6 ± 1.2
	50	12.2 ± 1.2b	18.7 ± 0.5
	100	8.2 ± 0.9c	18.1 ± 1.0
Soil water	0	16.0 ± 0.6a	19.5 ± 2.0
extract	10	13.0 ± 1.2b	19.5 ± 1.0
	25	13.1 ± 0.8b	19.7 ± 0.7
	50	13.9 ± 1.0b	21.8 ± 1.0
	100	12.7 ± 1.2b	20.0 ± 1.2

NOTE: Data are means and standard deviations of 40 seedlings; mean values in a column within a treatment followed by the different letters are significantly different at $P \leq 0.01$ in Tukey's multiple-range test.

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Table II. Growth of Black Spruce Seedlings Exposed to Water Extract of Kalmia Leaves at Different pH Values

Concentration	Length (mm) of root			Length (mm) of shoot		
(%)	pH 5	pH 4	pH 3	pH 5	pH 4	pH 3
0	18.0 ± 1.3a	16.3 ± 0.9ab	10.8 ± 0.3b	20.0 ± 1.8	17.5 ± 2.1	17.5 ± 1.6
10	16.2 ± 1.5a	12.5 ± 1.0b	3.2 ± 0.5c	19.1 ± 1.8	19.9 ± 1.1	17.1 ± 1.7
25	13.9 ± 1.1a	9.4 ± 1.0b	2.0 ± 0.3c	18.3 ± 0.9a	16.8 ± 2.6a	2.1 ± 1.2b
50	9.5 ± 0.7a	6.5 ± 0.5b	0.5 ± 0.3c	18.7 ± 1.3a	18.2 ± 2.0a	1.0 ± 0.6b

NOTE: Data are means and standard deviations of 40 replicate seedlings; mean values within an extract concentration treatment followed by the different letters are significantly different at $P \leq 0.01$ in Tukey's multiple-range test.

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than -0.7 bar. Del Moral and Cates (*34*) determined the osmotic potentials of a larger number of plant extracts which were assayed for allelopathic activities and found that osmotic potentials even at -2 bar did not cause inhibitory effects. Therefore, inhibition of root growth of black spruce seedlings in the present study was most likely caused by water-soluble phytotoxic substances released from *Kalmia* leaves.

Isolation and Identification of Allelopathic Compounds

Allelopathic compounds were isolated from the water extracts of *Kalmia* leaves. Organic solvent extraction was done first with hexane and then acetic acid followed by two dimensional thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) (Figure 1). Details of the extraction method can be obtained from Zhu and Mallik (*33*). Black spruce germinants were used in conducting the bioassay to test the effects of various fractions of the extract. All the fractions exhibited a certain degree of inhibitory effects on primary root growth of the germinants. However, the strongest root growth inhibition was due to fractions 3 and 4. This indicates that the phytotoxic compounds in water extracts were effectively partitioned into the aqueous phase after hexane extraction and into the organic phase after ethyl acetate extraction.

Because of its high activity, ethyl acetate extract was used for further analyses by TLC and HPLC. Two dimensional TLC of fraction 4 revealed the presence of 18 bands and eight of them were identified as simple phenolic compounds by comparing with standards (Figure 2). The chemical nature of the other bands was not identified. It may be possible that compounds present in some of these bands have strong allelopathic effects. The identified phenolic compounds were *m*-coumaric, *p*-coumaric, ferulic, gentisic, *p*-hydroxybenzoic, *o*-hydroxyphenylacetic, syringic, and vanillic acid. HPLC analysis of ethyl acetate extract (fraction 4) detected at least 30 peaks (Figure 3). All the eight identified phenolic compounds were eluted between 0 and 22 min along with a number of unknown peaks. The 0-22 min fraction contained inhibitory activity and caused 53% inhibition in root growth of black spruce, while the 23-35 min fraction caused only 13% inhibition in root growth.

Allelopathic Activity of Identified Phenolic Compounds

Allelopathic activity of the identified phenolic compounds was assayed on black spruce at four concentrations. All compounds were inhibitory to growth of black spruce seedlings, particularly to root growth, and degree of inhibition varied depending on the compound and it's concentration (Table III). Among the eight phenolic acids, *o*-hydroxyphenylacetic acid was most toxic, causing 94% root growth inhibition at a concentration of 0.5 mM, while *m*-coumaric acid was least toxic, causing 75% inhibition at 5 mM. Toxicity of the other six compounds was between *o*-hydroxyphenylacetic and *m*-coumaric acid and caused 100% inhibition of root growth at 5 mM, 56-94% inhibition at 2 mM, 11-62% inhibition at 1 mM, and 0-19% inhibition at 0.5 mM. All of these phenolic compounds except *m*-coumaric acid

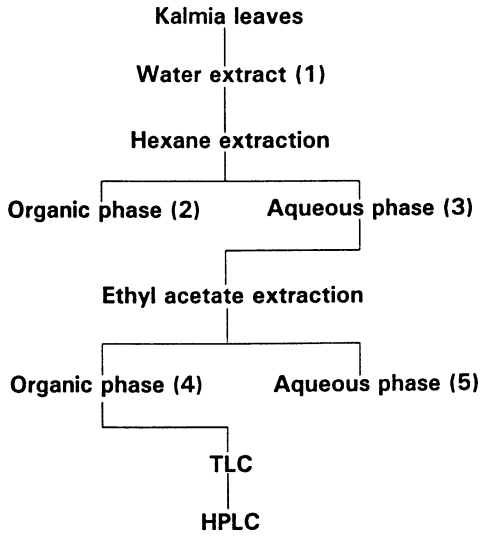

Figure 1. Flow diagram for the separation of compounds from water extract of *Kalmia* leaves. (Reproduced with permission from reference 33. Copyright 1994.)

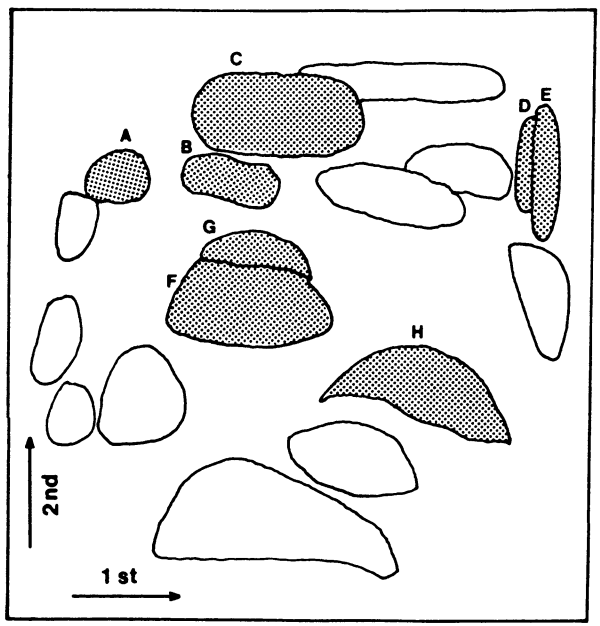

Figure 2. Two dimensional thin layer chromatogram of ethyl acetate of *Kalmia* leaf water leachate. A, gentisic acid; B, *p*-hydroxybenzoic acid; C, *o*-hydroxyphenylacetic acid; D, vanillic acid; E, syringic acid; F, *p*-coumaric acid; G, *m*-coumaric acid; and H, ferulic acid.

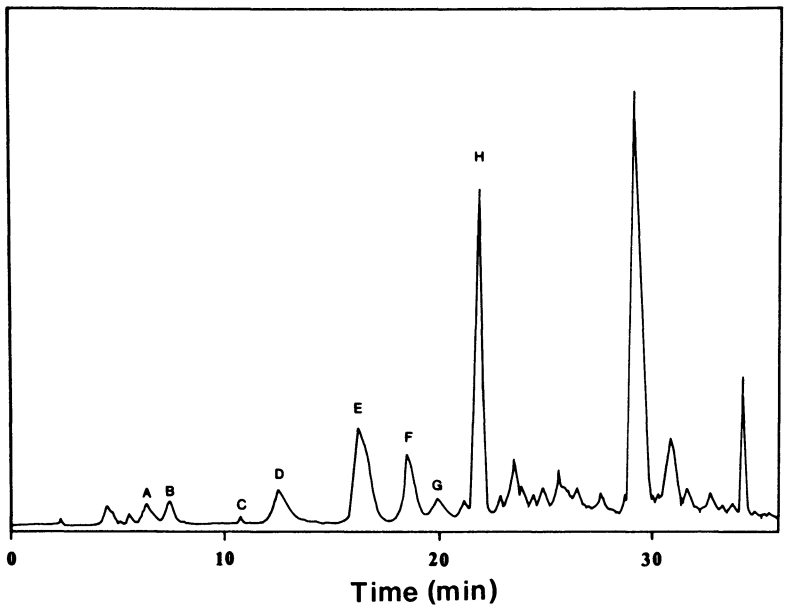

Figure 3. Separation of phenolic acids from ethyl acetate extract of *Kalmia* leaf water leachate by HPLC. A, gentisic acid; B, *p*-hydroxybenzoic acid; C, *o*-hydroxyphenylacetic acid; D. vanillic acid; E, syringic acid; F, *p*-coumaric acid; G, *m*-coumaric acid; and H, ferulic acid.

Table III. Effect of Phenolics Isolated from Water Extract of *Kalmia* Leaves on the Growth of Black Spruce Seedlings

Compound	Concen-tration (mM)	Root growth		Shoot growth	
		Length (mm)	% of control	Length (mm)	% of control
Control (H_2O)	0	16 ± 2.2a	100	19 ± 1.8a	100
Gentisic acid	0.5	15 ± 2.6a	94	22 ± 3.9a	100
	1	13 ± 3.7a	81	17 ± 3.9ab	89
	2	1 ± 0.3b	6	15 ± 2.7b	79
	5	0c	0	0c	0
p-Hydroxybenzoic acid	0.5	13 ± 3.7ab	81	19 ± 2.8a	100
	1	9 ± 2.9b	55	19 ± 3.0a	100
	2	1 ± 0.3c	6	15 ± 2.4b	79
	5	0d	0	0c	0

Table III. Continued.

Compound	Concen-tration (mM)	Root growth		Shoot growth	
		Length (mm)	% of control	Length (mm)	% of control
o-Hydroxyphenylacetic acid	0.5	1 ± 0.5b	6	16 ± 2.3ab	84
	1	0c	0	13 ± 1.6b	68
	2	0c	0	9 ± 2.1b	47
	5	0c	0	0c	0
Vanillic acid	0.5	14 ± 3.7a	87	16 ± 2.0ab	84
	1	6 ± 1.7b	38	15 ± 1.6b	79
	2	4 ± 1.3b	25	16 ± 2.0ab	84
	5	0c	0	13 ± 2.9b	68
Syringic acid	0.5	17 ± 2.7a	100	20 ± 2.5a	100
	1	9 ± 2.5b	57	18 ± 1.7a	95
	2	7 ± 2.1b	44	17 ± 3.2a	89
	5	0c	0	8 ± 2.0b	42
p-Coumaric acid	0.5	13 ± 2.6a	81	19 ± 3.3a	100
	1	9 ± 2.5a	56	15 ± 3.1ab	79
	2	3 ± 0.7b	19	13 ± 2.2b	68
	5	0c	0	13 ± 3.6b	68
m-Coumaric acid	0.5	16 ± 2.3a	100	19 ± 3.2a	100
	1	13 ± 1.9a	81	19 ± 3.1	100
	2	12 ± 3.2a	75	17 ± 3.4	89
	5	4 ± 1.5b	25	17 ± 3.5	89
Ferulic acid	0.5	15 ± 2.3a	94	17 ± 2.6a	90
	1	12 ± 1.9ab	89	18 ± 3.1a	95
	2	8 ± 2.2b	42	15 ± 3.0a	89
	5	0c	0	0c	0

NOTE: Data are means and standard deviations of 36 replicate seedlings; mean values in a column followed by the different letters are significantly different at $P \leq 0.01$ in Tukey's multiple-range test.

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have been reported previously in ericaceous plants. They are found in leaves of *Erica scoparia* (*21*) and *E. australis* (*22*) and in shoots and roots of *Calluna vulgaris* (*29*). Gentisic, vanillic, syringic and *p*-hydroxybenzoic acids are found in many angiosperm species, and the last three are also present widely in gymnosperms and ferns (*35*). Ferulic, *p*-coumaric and *m*-coumaric acids occur almost universally in higher plants (*36*). Some of these compounds, particularly *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids, have been found in heathland soils dominated by *Calluna vulgaris* (*23,24*) and other ericaceous plants (*22,37*). Recently, Oden et *al.* (*38*) identified a germination inhibitor called batatasin III (5-methoxy-3,3-dihydroxy-dihydrostilbene) from air dried leaves of *Empetrum hermaphorditum* Hagerup, a mat-forming understorey plant in northern Sweden. Phenolic compounds in soil could arise directly from residuals and living tissues of plants. There is also evidence that phenolic acids in soil originate in part from the decomposition of plant residues and from synthesis by soil microorganisms (*36*).

Most of the phenolic acids identified in the present study have been widely reported to have phytotoxic properties and to play an important role in allelopathic interactions (*36,37,39,40*). All the eight phenolic acids isolated in the present study inhibited root growth of black spruce seedlings significantly at concentration 1 mM or above. The presence of these phenolic compounds in water extract of *Kalmia* leaves may be of particular importance to allelopathic activity of *Kalmia* on black spruce. Although the estimated concentration of each phenolic acid in water extract was less than 0.1 mM, these compounds might interact in an additive or synergistic manner. It has been demonstrated in many studies that a combination of allelopathic compounds often exerts additive or synergistic effects on growth of plants (*36*). For example, Read (*30*) tested 11 acids identified from *Calluna* soil (including vanillic, ferulic, syringic and coumaric acids), singly and in combination, against *Agrostis tenuis* seedlings and found that a mixture almost completely inhibited root growth. The mechanisms of action of these phenolic acids on black spruce growth inhibition are not known.

Response of Mycorrhizal Fungi to *Kalmia* Allelopathy:

The objective of this research was to obtain some mycorrhizal fungi that are capable of growing in acidic soil with *Kalmia* allelopathy and which, at the same time, can form mycorrhizal association with black spruce. A total of 51 fungal isolates were collected from different fungal banks in Canada and some were isolated from Newfoundland soils. Nineteen isolates representing 11 species were selected on the basis of their mycelial growth in laboratory cultures and compatibility with black spruce (Appendix 1). The isolates obtained from the Botany Departments of the University of Guelph and the University of Alberta and from Newfoundland soils were coded as GB, AB, and NF, respectively. The isolates were grown in sterilized *Kalmia* extract-impregnated MMN agar medium. Since agar does not solidify at pH below 4, experiments to examine the water extract effect at more acidic pH were conducted using liquid medium. Mycelium radial growth of the fungi was tested in

presence of 0, 10, 25 and 50% *Kalmia* leaf extracts. The effect of *Kalmia* leaf extracts on mycelial dry weight of the fungi was tested at different pH. This experiment was conducted in liquid culture to obtain the fungal biomass. The nineteen fungal isolates were also tested for their tolerance to eight phenolic acids that were identified from *Kalmia* leaves. This fungal bioassay was conducted in MMN agar containing pure substances of ethyl, acetic, ferulic, vanillic, syringic, *o*-hydroxyphenylacetic, *m*-coumaric, *p*-hydrobenzoic, *p*-coumaric and gentisic acids (*H. Zhu and A.U. Mallik, unpublished data*).

The effect of *Kalmia* leaf extracts on mycelium radial growth varied depending on the fungal isolate and concentration of the water extract. The mycelial growth of GB6, NF4 and GB41 was stimulated; that of GB40, GB24, NF1, GB45, GB50, GB12 and GB8 remained unaffected, while in the rest growth was severely inhibited at all the three concentrations. Similar growth stimulation and inhibition of mycorrhizal fungi in presence of plant residues, leaf leachates and soil extracts have been reported by others (*41,42,43,44*).

A combination of high acidity and *Kalmia* leaf extracts had a strong growth inhibitory effect on most of the fungal isolates except GB12. At pH 4, the mycelial growth of 6 isolates, NF4, GB40, GB50, GB45, GB12 and GB23, were either stimulated or unaffected by the water extracts of *Kalmia* leaves. The growth of the other 13 isolates was inhibited by 10 - 70% due to the extracts. At pH 5 growth of 9 isolates was not inhibited by the water extracts. The mycelial growth was reduced in 10 other isolates at pH 5. Influence of acidity on the growth of fungi is well known. In general, mycorrhizal fungi have a growth optimum between pH 4.5 and 6.5.

When the ethyl acetate extract of *Kalmia* leaves was tested against the mycelium growth of the 19 isolates, four of them, NF4, GB12, GB23 and GB45, showed less growth inhibition compared to the rest of the isolates. Sensitivity of the isolates was also dependant on the type of phenolic acid and their concentrations. For example, growth of NF4 was inhibited by ferulic acid and stimulated by *p*-hydroxybenzoic acid at concentrations of 0.1 mM (*H. Zhu and A.U. Mallik, unpublished data*).

Mycorrhization of Black Spruce in Presence of *Kalmia* Leaf Extract

Black spruce germinants were inoculated with NF4, GB12, GB23 and GB24 strains of mycorrhizal fungi. These isolates showed maximum tolerance to *Kalmia* leaf extracts, as determined by the previous experiment. The black spruce seedlings were grown aseptically in plastic plates filled with autoclaved vermiculite mixed with MMN liquid medium. First the plastic plates were inoculated with discs of the fungal isolates and then 25 and 50% *Kalmia* leaf extracts were added. The plates holding the seedlings were incubated in a growth chamber at 18°C with 60-80% humidity and 16h photoperiod.

Mycorrhizal formation on the seedlings varied among the 19 isolates (Table IV). Four isolates, namely GB23, GB12, GB45 and NF4, formed mycorrhizae on 60-

Table IV. Effect of Water Extracts of *Kalmia* Leaves and Soil on Mycorrhizal Formation on Black Spruce in Pure Culture

Fungus		Ectomycorrhizal formation: Control	Ectomycorrhizal formation: Leaf
Hebeloma crustuliniforme	AB2	++	++
Hebeloma cylindrosporum	GB6	+	+
Paxillus involutus	NF4	+++	+++
Paxillus involutus	GB24	++	+
Paxillus involutus	GB40	++	++
Paxillus involutus	GB41	++	+
Leccinum scabrum	NF1	+	-
E-Strain	GB45	+++	+++
Thelephora terrestris	GB50	-	-
Pisolithus tinctorius	GB25	-	-
Pisolithus tinctorius	GB4	++	+
Pisolithus tinctorius	GB14	++	+
Cenococcum geophilum	AB1	+	-
Cenococcum geophilum	GB12	++	++
Laccaria bicolor	GB8	+	+
Laccaria laccata	AB5	++	+
Laccaria laccata	GB20	+++	-
Laccaria laccata	GB23	+++	+++
Lycoperdon perlatum	GB56	-	-

NOTE: Mycorrhizal formation was arbitrarily classified into four groups: -, no mycorrhizal formation; +, 5-20% mycorrhizae of total short roots; ++, 21-60% mycorrhizae; +++, 61-100% mycorrhizae (45).

80% of the total short roots. The isolates GB56, GB50, GB25, GB20, AB1 and NF1 were unable to form mycorrhizae in presence of leaf extracts.

The effect of *Kalmia* extracts on mycelial growth and mycorrhizal formation in black spruce has important silvicultural implications. Failure and reduction in mycorrhizal formation by some isolates may be due to the toxicity of the extracts affecting mycorrhizal colonization by restricting host-fungus contact. This could be at least partly responsible for low mycorrhizal inoculum potential for *Kalmia* dominated sites of central Newfoundland (*H. Zhu and A.U. Mallik, unpublished data*). It has been noted that allelopathy of ericaceous shrubs on ectomycorrhizal fungi may produce dramatic effects on conifer regeneration. For example, 'growth check' of Sitka spruce (*Picea sitchensis* L.) plantation in Scotland was attributed to the inhibition of spruce mycorrhizae by substances leaching from roots of *Calluna vulgaris* (*28*) and/or raw humus (*23,24*). Brown and Mikola (*45*) reported unidentified substances leached from Reindeer lichen (*Cladina* spp.) inhibited ectomycorrhizal formation on tree species. Other studies reported that allelopathy altered the composition of ectomycorrhizal fungi rather than ectomycorrhizal formation (*42,46*). Thus, allelopathic substances may affect black spruce root growth directly due to their toxicity and indirectly by affecting the growth of mycorrhizal fungi associated with black spruce. Other possibilities such as low pH and low available nutrient status of soil may have significant adverse effects on black spruce in *Kalmia*-dominated sites.

Response of Inoculated Black Spruce Seedlings to *Kalmia* Allelopathy

Two separate greenhouse experiments were conducted with four-month-old black spruce seedlings inoculated with GB12, GB23, GB45, and NF4 isolates. The seedlings were inoculated in tree nursery multipots four months prior to the experiments. In one experiment the inoculated seedlings were examined for mycorrhizal formation and growth by planting them in potted *Kalmia* soil which also received 300 ml *Kalmia* leaf extract at pH 4 and 5. At the end of this experiment, four months after commencement, the number of mycorrhizal and nonmycorrhizal short roots, height and diameter of seedlings, and the oven dry biomass of root and shoot were determined.

In the second experiment the four-month-old black spruce seedlings preinoculated with the selected mycorrhizal fungi, GB12, GB23, GB45 and NF4 were grown with live *Kalmia*. The *Kalmia* plants were transplanted in plastic pots with their own soil. No additional leaf extract of *Kalmia* was added to these pots. The experiment was conducted in greenhouse for four months at the end of which growth response of black spruce was evaluated as in the previous experiment.

Ectomycorrhizae were abundant on seedlings inoculated with NF4, GB23 and GB45 isolates. Over 90% of the mycorrhizae were attributable to the inoculated fungi although indigenous mycorrhizae were also found in the seedlings. Seedlings inoculated with GB12 formed an average 72% mycorrhizae. Noninoculated (control) seedlings formed about 45% ectomycorrhizae with unidentified fungi. The addition

of *Kalmia* leaf extracts enhanced mycorrhizal formation in GB12 and GB23 whereas in NF4 and GB45 it had no effect. The mycorrhization was 15% lower at pH 4 compared to pH 5 with the highest inhibition occurring in GB23 isolate. Mycorrhizal colonization with indigenous fungi was greatly reduced in noninoculated seedlings at pH 4. Among all the fungal treatments, seedlings inoculated with NF4 had the greatest shoot height, shoot dry weight, short and lateral root numbers. In general, black spruce seedlings inoculated with NF4, GB23 and GB45 grew better than the control and GB12 inoculated seedlings. Significant interaction effect was obtained on shoot height, shoot dry weight and short root number due to inoculation treatments. Seedlings receiving leaf extract produced lower shoot height and dry weight compared to seedlings receiving no leaf extract in all the inoculated seedlings. However, significant inhibition was found within fungal treatments GB45, and GB12.

Persistence of inoculated mycorrhizae was not affected by living *Kalmia* plants. Over 80% of mycorrhizae on seedlings inoculated with NF4, GB23 and GB45, and 53% on seedlings with GB12 were attributable to the inoculated fungi. Indigenous mycorrhizae were found on all the noninoculated seedlings and on most of the inoculated seedlings. The seedlings inoculated with NF4, GB23 and GB45 responded well with higher shoot and root growth compared to the noninoculated and GB12 inoculated ones. The NF4 treated seedlings had the highest shoot and root growth of all the treatments (Table V).

Our studies demonstrated that NF4 isolate, a fungus collected from a *Kalmia*-black spruce forest in central Newfoundland is the most effective fungus to inoculate black spruce seedlings in order to overcome the allelopathic growth inhibition of *Kalmia*. It also suggests that inclusion of local mycorrhizal fungi is important in the screening program for selecting the potential candidate for seedling inoculation. Trappe (*47*) pointed out the need for selection of mycorrhizal strains suitable for a particular host-soil-climate combination.

When inoculated black spruce seedlings were grown in presence of living *Kalmia* the seedling biomass was increased in the order of control, GB12, GB45, GB23 and NF4 treatments (Figure 4). The shoot and root biomass was 2-3 times higher in NF4 inoculated seedlings compared to the control. The remarkable increase in biomass of NF4 inoculated seedlings is likely due to the direct effect of increasing mycorrhizal formation which may result in reducing *Kalmia* allelopathy and increasing competitive ability of black spruce seedlings. Results presented in the first part of this chapter provided some evidence that several mycorrhizal fungi, including NF4, GB23, GB45 and GB12 are able to withstand *Kalmia* allelopathy. Abundant mycorrhizal short roots in seedlings inoculated with these fungi in the presence of living *Kalmia* or *Kalmia* leaf extract is also indicative of their ability to reduce *Kalmia* allelopathic effect. Increase in competitive ability of host plants infected with mycorrhizal fungi has been well documented in the literature (*48*). The difference in biomass accumulation of seedlings inoculated with the four fungi may be explained by differences in mycorrhization of the seedlings and their ability to detoxify the *Kalmia* allelopathic compounds.

Table V. Growth and Mycorrhizal Characteristics of Black Spruce Seedlings Grown in the Presence of *Kalmia* Plants

	Fungal treatment				
Measurement	Control	NF4	GB23	GB45	GB12
Shoot height (cm)	10.4bc	14.2a	12.2b	11.8bc	10.4c
	±2.2	±2.0	±1.8	±1.9	±1.9
Shoot dry weight (mg)	154c	356a	256b	250b	171c
	±42	±37	±64	±53	±51
Root dry weight (mg)	43c	124a	83b	74bc	54c
	±11	±28	±26	±24	±21
Short root tip	4.3c	6.2a	4.8bc	5.2b	3.3d
number/cm	±1.0	±0.7	±1.1	±1.0	±0.7
1st order lateral root	7.1b	11.2a	7.8b	5.8c	4.4c
Number/plant	±1.5	±1.8	±1.9	±1.16	±0.7
Percent mycorrhizal root tips	_	81b	80b	88a	53c
attributable to inoculation		±6	±8	±4	±9
Unknown	60a	13c	11cd	4d	22b
	±12	±6	±8	±2	±10

NOTE: Fifteen replicates per treatments. Means within a given parameter followed by the same letter are not significantly different at p=0.05 as determined by one-way ANOVA and Tukey's multiple range test(46).

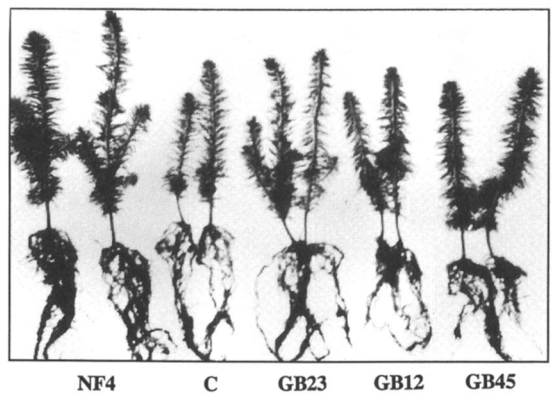

Figure 4. Root and shoot growth of black spruce seedlings preinoculated with NF4, GB45, GB23 and GB12 isolates of ectomycorrhizal fungi. The seedlings were grown in the presence of *Kalmia* for four months.

Conclusions

Regeneration failure of conifers in the harvested and burnt over forests of central Newfoundland with *Kalmia* understorey has been a serious problem. The aggressive regeneration strategies of *Kalmia* after disturbance (*13*) combined with its long-lasting allelopathic effects on conifer seedlings (*1,2,3,33*) make the traditional silvicultural treatments unsuccessful in promoting black spruce growth in *Kalmia* sites. An alternate approach was taken to overcome the *Kalmia* induced growth inhibition of black spruce by inoculating black spruce with mycorrhizal fungi. Growth of seedlings preinoculated with any of the three ectomycorrhizal fungi, isolates NF4, GB45 and GB23 was increased significantly in presence of *Kalmia*. The NF4 isolate obtained from central Newfoundland forest soil showed the best results. When inoculated with this isolate black spruce seedlings' shoot and root biomass attained a 2-3 fold increase compared to the noninoculated seedlings. This greenhouse experiment showed potential for developing a practical method to stimulate black spruce seedling growth in *Kalmia* sites. A field trial is recommended in *Kalmia* dominated sites of central Newfoundland by planting black spruce seedlings preinoculated with the mycorrhizal fungi.

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Appendix 1. Growth of 51 Mycorrhizal Isolates Obtained from the Botany Departments of the University of Guelph, (GB) and University of Alberta, (AB), and Kalmia-black Spruce Forest Soil of Newfoundland (NF).

Mycorrhizal isolates	Code	Growth on MMN
Pisolithus tinctorius	GB4	+++
Hebeloma cylindrosporum	GB6	+
Cenococcum geophilum	GB7	+
Laccaria bicolor	GB8	++
Tricholoma populinum	GB9	+
Piloderma bicolor	GB10	+
Paxillus involutus	GB11	++
Cenococcum geophilum	GB12	++
Pisolithus tinctorius	GB14	+++
Paxillus involutus	GB15	++
Fuscobletinus acruginascens	GB16	+
Laccaria laccata	GB20	++
Laccaria laccata (L. bicolor)	GB21	++
Laccaria laccata	GB23	++
Paxillus aframentarius(unverified)	GB24	+
Pisolithus tinctorius	GB25	++
Suillus spp.	GB28	+
Laccaria proxima	GB29	++
Sphaerosporella brunnea	GB32	++++
Sphaerosporella brunnea	GB34	++++
Paxillus involutus	GB37	+
Paxillus involutus	GB38	++
Paxillus involutus	GB39	+
Paxillus involutus	GB40	++
Paxillus involutus	GB41	++
Paxillus involutus	GB42	+

Appendix 1. Continued.

Mycorrhizal isolates	Code	Growth on MMN
Laccaria ochropurporea	GB43	+
E-Strain	GB45	+++
Thelophora terrestris	GB50	++
Lactarius deliciosus	GB54	+
Suillus brevipes (unverified)	GB55	++
Lycoperdon perlatum(unverified)	GB56	++
Suillus spp. (unverified)	GB57	++
Suillus spp. (unverified)	GB60	++
Suillus americanus	GB59	++
Cenococcum geophilum	AB1	++
Hebeloma crustuliniforme	AB2	++
Hebeloma crustuliniforme	AB3	+
Hebeloma crustuliniforme	AB4	+
Laccaria laccata	AB5	++
Suillus tomentosus	AB6	++
Suillus tomentosus	AB7	++
Leccinum scabrum	NF1	++
Suillus cavipes	NF2	+++
Lactarius spp. (unverified)	NF3	+
Paxillus involutus	NF4	++
Paxillus involutus	NF5	++
Coetnarius spp. (unverified)	NF6	++
Suillus cavipes	NF7	++
Suillus cavipes	NF8	++
Paxillus involutus	NF9	++

NOTE: Mycelial growth on MMN was classified as +, slow growth; ++, active growth; and +++, fast growth.

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Chapter 4

Allelopathic, Herbaceous, Vascular Hydrophytes

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A review of the literature since 1970 shows 67 genera and 97 species of herbaceous vascular hydrophytes reported to be allelopathic. The most frequently cited genus is *Eleocharis* with 11 included species. Lettuce is the most frequently used target plant. Comparison of lettuce and *Lemna minor* as bioassay target plants shows *L. minor* to be the more selective plant assay. *Nuphar lutea* and *Nymphaea odorata* are highly inhibitory toward both lettuce seedling radicle growth and *L. minor* frond production. Alkaloids from *Nuphar lutea* are being re-isolated to examine their allelopathic potential. Allelochemicals present in *Nymphaea odorata* are extracted by 95% ethanol, and are ethyl acetate soluble.

It has been over twenty years since McClure (*1*) reiterated the postulation that "Aquatic angiosperms are considered to be descendants of terrestrial plants which have reverted to the aquatic habits of their remote ancestors." He suggested that the initial change from an aquatic to an emergent habitat appears to involve a shift from an essentially anaerobic to an aerobic type of metabolism, and would surely be expected to influence the qualitative and quantitative production of secondary constituents (*1*). He then provided a synopsis of the secondary constituents of aquatic angiosperms, organized into alkaloids, terpenoids, simple phenolics, flavonoids and others.

Some 40 years ago Oborn *et al.* (*2*) suggested that aquatic macrophytes could serve as important sources of natural herbicides. They gave no experimental details or references, but stated that "laboratory evidence over a two-year period indicated that either or both of these plants (dwarf arrowhead, *Sagittaria subulata*, or needle spikerush, *Eleocharis acicularis*) growing in association with the taller more obnoxious pond weed *Potamogeton*, would, over a period of time, crowd out the pond weed growth." In spite of Oborn *et al.*'s suggestion that aquatic macrophytes

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could be involved in allelopathy and McClure's prediction that these plants are likely to produce unique secondary constituents, there is a dearth of information on the allelopathic potential of hydrophytes.

Allelopathic, Herbaceous, Vascular Hydrophytes

This chapter provides a partial listing of the work to date on allelopathic herbaceous vascular hydrophytes. This review of the literature excludes woody (trees and shrubs) and non-vascular (algae, liverworts, mosses, and fungi) hydrophytes. Species are included as hydrophytes based on the information provided in the papers or the habitat descriptions given in regional taxonomic manuals. Where classification was difficult, as in the case of plants which grow in various habitats including moist soil, the species were generally considered hydrophytes. Citations are included in the list only if the authors stated that their results indicated that allelopathy was involved. Hence, some reported results could be due to allelopathic activity but were interpreted by the authors as indicative of competition. The reference list is not exhaustive; a representative publication of information on a given species from authors is included rather than all relevant papers by those same authors. As shown in Table I, some 67 genera and 97 species are included. The genus appearing most frequently is *Eleocharis* with 11 included species. A total of 302 allelopathic plant-target plant interactions are listed. The most frequently used target plant is lettuce, listed 52 times.

The allelopathic potential of dwarf spikerush, *Eleocharis coloradoensis*, has been examined more extensively than that of any other hydrophyte (Table I). Frank and Dechoretz (*3*) planted *Potamogeton nodosus* and *P. pectinatus* in *E. coloradoensis* sod and also in aquaria to which were daily added 500 mL of leachate from *E. coloradoensis* sod. Numbers of new shoots and biomass of *Potamogeton* were significantly reduced in each case. *Potamogeton pectinatus* was more sensitive to the influence of *E. coloradoensis* than was *P. nodosus*. Yeo (*4*) reported his observations of *E. coloradoensis* in several water systems in California over a 12-year period. He found that *P. pectinatus*, *P. nodosus*, *P. pusillus*, and *Najas guadalupensis* were displaced by *E. coloradoensis* within two years. Two species of *Elodea (canadensis* and *nuttallii*) were displaced, but required longer than two years. In addition to these field observations, Yeo and Thurston (*5*) conducted outdoor competitive experiments. Planting schemes included seven individually grown species of aquatic weeds, each grown co-planted with *E. coloradoensis*, and *E. coloradoensis* grown alone. Dry masses of all seven of the aquatic weeds were reduced when the plants were grown with *E. coloradoensis*. For six of the seven, dry mass was less than 35% of the dry masses of the aquatic weeds in monoculture. Ashton *et al.* (*6*) examined the allelopathic potential of organic compounds leached from axenically cultured *E. coloradoensis*. They separated the leached organics into several fractions and separately bioassayed them using the aquatic plants *Hydrilla verticillata* and *P. pectinatus*, as well as tomato cell cultures and lettuce seedling roots, as the bioassay target species. Some fractions were found to be inhibitory to all of these target species.

Table I. Allelopathic Herbaceous Vascular Hydrophytes

Allelopathic plant	Affected plant(s)	Reference
Acorus gramineus	green and blue green algae (7 strains)	*18*
Agrostis stolonifera	grasses, clover	*19*
Aldrovanda vesiculosa	*Carex* sp. *Stratiotes aloides* *Hydrocharis morus-ranae*	*20*
Alternanthera philoxeroides	*Brassica campestris* *Oryza sativa*	*21*
Ambrosia trifida	lettuce, radish, tomato, cucumber	*22*
Anagallis arvensis	lettuce, radish	*23*
Andropogon nodosum	lettuce	*24*
Aster nova-angliae	*Acer saccaharum*	*25*
Azolla caroliniana	*Lemna paucicostata*	*26*
Bidens laevis	lettuce, radish, tomato, cucumber	*22*
Brachiaria mutica	rye, lettuce	*27*
Brasenia schreberi	lettuce, 9 bacteria	*28*
	lettuce *Lemna minor*	*8*
Cabomba caroliniana	lettuce *Lemna minor*	*8*
	Echinochloa crus-galli *Lactuca sativa* *Triticum aestivum*	*29*
	Hydrilla verticillata	*30*
Carex hudsonii	*Phragmites communis*	*31*

Table I. Continued.

Allelopathic plant	Affected plant(s)	Reference
Ceratophyllum demersum	lettuce	*8*
	Hydrilla verticillata	*30*
	Myriophyllum spicatum	*32*
	Lepidium sativum	*33*
Christella dentata	fern gametophytes	*34*
Cicuta virosa	photoreduction of NADP+	*35*
Cyperus brevifolius	lettuce, oats	*36*
Cyperus esculentus	*Glycine max* *Zea mays*	*37*
	oat coleoptile *Beta vulgaris* *Lactuca sativa* *Lolium perenne* *Lotus corniculatus* *Lycopersicum esculentum* *Pisum sativum* *Trifolium repens*	*38*
Cyperus kyllingia	lettuce, oats	*36*
Cyperus rotundus	*Gossypium hirsutum*	*39*
	lettuce, oats	*40*
	Digitaria sanguinalis *Rumex* white clover	*41*
Cyperus serotinus	*Cyperus serotinus* lettuce, rice	*42*
Echinocloa crus-galli	lettuce, mung bean	*43*
Eichhornia crassipes	algae	*44*
	Mimosa pudica turnip, beans	*45*
Eleocharis acicularis	lettuce *Lemna minor*	*8*
	Potamogeton	*2*

Continued on next page

Table I. Continued.

Allelopathic plant	Affected plant(s)	Reference
	Elodea canadensis *Potamogeton crispus* *Potamogeton pectinatus*	46
Eleocharis cellulosa	*Hydrilla verticillata*	47
	Lemna paucicostata	26
Eleocharis coloradoensis	*Hydrilla verticillata* *Potamogeton pectinatus* tomato cell culture lettuce seedling roots	6
	Potamogeton nodosus *Potamogeton pectinatus*	3
	radish *Nasturtium officinale*	48
	Elodea canadensis *Elodea nuttallii* *Najas guadalupensis* *Potamogeton foliosus* *Potamogeton nodosus* *Potamogeton pectinatus* *Potamogeton pusillus*	4
	Elodea canadensis *Elodea nuttallii* *Hydrilla verticillata* *Myriophyllum spicatum* *Potamogeton nodosus* *Potamogeton pectinatus* *Zannichellia palustris*	5
Eleocharis equisetoides	lettuce	49
Eleocharis flavescens	lettuce	49
Eleocharis geniculata	*Hydrilla verticillata*	50
Eleocharis interstincta	*Lemna paucicostata*	26
	Hydrilla verticillata	47
Eleocharis montana	lettuce *Lemna minor*	49

Table I. Continued.

Allelopathic plant	Affected plant(s)	Reference
Eleocharis obtusa	lettuce *Lemna minor*	8
Eleocharis quadrangulata	lettuce	49
Eleocharis tuberculosa	lettuce *Lemna minor*	49
Elodea nuttallii	*Echinochloa crus-galli* *Lactuca sativa* *Triticum aestivum*	29
Equisetum fluviatile	*Phragmites australis*	51
Equisetum limosum	*Phragmites communis*	52
Equisetum palustris	*Phragmites australis* *Typha latifolia*	51
Eupatorium riparium	*Aspergillus flavus* *Galinsoga ciliata* *Galinsoga paviflora* *Trichoderma viride*	53
Galium aparine	crabgrass, alfalfa *Galium aparine*	54
Hemarthria altissima	lettuce	55
	Desmodium intortum	56
Heracleum laciniatum	lettuce, radish *Salix pentandra*	57
	lettuce, oats *Cladosporium cucumerinum*	58
Hydrilla verticillata	lettuce *Lemna minor*	8
	Ceratophyllum demersum *Ceratophyllum muricatum*	59
Hydrocotyle sibthorpioides	*Marchania polymorpha*	60
Ipomoea aquatica	*Pennisetum typhoideum*	61

Continued on next page

Table I. Continued.

Allelopathic plant	Affected plant(s)	Reference
Juncus repens	lettuce	*8*
Leersia hexandra	lettuce and rice	*62*
Lemna gibba	*Spirodela polyrrhiza* *Wolffia arrhiza*	*63*
Lemna minor	*Spirodela polyrrhiza* *Wolffia arrhiza*	*63*
Limnobium spongia	lettuce *Lemna minor*	*8*
Lippia adoensis	lettuce	*64*
Ludwigia adscendens	*Pennisetum typhoideum*	*61*
Myriophyllum sp.	*Echinochloa crus-galli* *Lactuca sativa* *Triticum aestivum*	*29*
Myriophyllum aquaticum	lettuce *Lemna minor*	*8*
	Hydrilla verticillata	*30*
Myriophyllum spicatum	*Najas marina*	*65*
	lettuce *Lemna minor*	*8*
	Hydrilla verticillata	*30*
Najas guadalupensis	lettuce *Lemna minor*	*8*
	Hydrilla verticillata	*30*
Nelumbo lutea	*Hydrilla verticillata*	*30*
Nuphar lutea	lettuce *Lemna minor*	*11*
Nymphaea odorata	lettuce *Lemna minor*	*8*
	Hydrilla verticillata	*30*
	Myriophyllum spicatum	*32*

Table I. Continued.

Allelopathic plant	Affected plant(s)	Reference
Nymphoides cordata	lettuce *Lemna minor*	8
Onoclea sensibilis	*Onoclea sensibilis*	66
Osmunda cinnamomea	*Dennstaedtia punctilobula* *Osmunda cinnamomea* *Osmunda claytoniana*	67
	Dryopteris intermedia	68
	Dryopteris goldiana	69
Osmunda claytoniana	*Quercus rubra*	70
	Dennstaedtia punctilobula *Osmunda cinnamomea* *Osmunda claytoniana*	67
Panicum repens	lettuce, rye	27
Peltandra virginica	lettuce, radish, tomato, cucumber	22
Phragmites australis	*Carex elata*	51
Pistia stratiotes	algae (17 strains)	71
Polygonum orientale	mustard	72
Polystichum munitum	*Bromus tectorum* *Hordeum vulgare*	73
Pontederia lanceolata	*Hydrilla verticillata*	30
Posidonia oceanica	*Staphylococcus aureus*	74
Potamogeton amplifolius	*Vallisneria americana*	75
Potamogeton foliosus	lettuce *Lemna minor*	8
Potamogeton illinoensis	*Lemna paucicostata*	26
Potamogeton nodosus	*Hydrilla verticillata*	30
Rorippa islandica	grasses, lettuce	76

Continued on next page

Table I. Continued.

Allelopathic plant	Affected plant(s)	Reference
Rorippa sylvestris	lettuce	77
Rumex crispus	*Amaranthus retroflexus* grain sorghum field corn	78
	Pisum sativum	79
Rumex obtusifolius	*Dactylis glomerata* *Lolium perenne* *Poa pratensis* *Trifolium repens*	80
Sagittaria graminea	*Hydrilla verticillata*	50
Sagittaria lancifolia	*Hydrilla verticillata*	30
	Myriophyllum spicatum	32
Sagittaria pygmaea	rice	81
Sagittaria subulata	*Potamogeton*	2
Schoenoplectus lacustris	*Equisetum limosum* *Phragmites communis*	52
	Potamogeton australis	51
Setaria sphacelata	lettuce	24
Solidago altissima	*Ambrosia artemisiaefolia* *Miscanthus sinensis* *Oryza sativa*	82
Solidago canadensis	*Acer saccharum*	25
Sparganium americanum	lettuce	83
	lettuce	8
Spirodela polyrrhiza	*Echinochloa crus-galli* *Lactuca sativa* *Triticum aestivum*	29
	Lemna gibba *Lemna minor* *Wolffia arrhiza*	63

Table I. Continued.

Allelopathic plant	Affected plant(s)	Reference
Thelypteris normalis	*T. normalis* (gametophyte) *Phelbodium* (gametophyte) *Pteris* (gametophyte)	*84*
Typha angustifolia	*Distichlis spicata*	*85*
Typha latifolia	lettuce, radish, tomato, cucumber	*22*
	Lythrum salicaria	*86*
	Anabena flos-aqua *Chlorella vulgaris*	*87*
	Typha latifolia	*88*
	Phragmites communis	*52*
	Acorus calamus *Equisetum fluviatile* *Glyceria maxima* *Phragmites australis* *Typha angustifolia*	*51*
Vallisneria americana	lettuce	*83*
	lettuce	*8*
	Hydrilla verticillata	*30*
	Myriophyllum spicatum	*32*
	Echinochloa crus-galli *Lactuca sativa* *Triticum aestivum*	*29*
Washingtonia filifera	lettuce, wheat, cabbage, cucumber seeds	*89*
Wolffia arrhiza	*Lemna gibba* *Lemna minor* *Spirodela polyrrhiza*	*63*
Zostera marina	*Staphlococcus aurens* micro-algae (8 species)	*90*

Bioassay Techniques for Allelopathic Activity

Two long range practical goals of work in the area of allelopathy are 1) the discovery and development of new, more environmentally acceptable herbicides, and 2) the development of selective planting techniques to introduce desirable plants, and to reduce or eliminate undesirable plants. Both of these goals require the determination of which plants are most allelopathic. One of the hindrances to the investigation of allelopathic activities is the lack of a standard assay system to measure potential activity. Many different assays have been used, ranging from co-planting of allelopathic plants with undesirable plants in large scale field trials to laboratory assays involving target plant growth inhibition by extracts from the identified allelopathic plant, or by plant-part extracts, or by leached organics from the allelopathic plant. Plant cell cultures have been used as a bioassay system. Compounds affecting plant growth may not ever leach or exude from the plant in nature and therefore might not be significant physiologically even though they may be significant in laboratory experiments. Although large scale field trials more closely mimic nature, these are also very time and labor intensive. Leather and Einhellig (*7*) have suggested that since various assays measure different influences, more than one assay method should be employed to determine allelopathic potential.

Bioassay Results of Aqueous Extracts of Hydrophytes

Work in our laboratory has involved the lettuce seedling and the *Lemna minor* assays (*8*) to identify those hydrophytes which are most allelopathic. We have subjected some 26 different aqueous plant extracts to these two assay systems. Equal weights of fresh plant material and distilled deionized water were blended, and the resulting extract was taken as representing plant material to calculate the parts per thousand (ppt) concentrations, even though most of the extract was deionized water, not plant extract. The values give a relative measure of inhibition. Of the 26 extracts, nine reduced lettuce radicle growth by 78% or more at 250 ppt as listed in Table II. Six of the extracts reduced *L. minor* frond number by 68% or more at 250 ppt as listed in Table III.

Attempts to rank the inhibitory activity of allelopathic plants by comparison of the results of bioassays depends on which results are compared. For example, Table II lists the nine extracts in order of their inhibitory activity at 250 ppt. Were the comparison made at 125 ppt for these same nine extracts, both *Ceratophyllum demersum* and *Eleocharis acicularis* would rank above *Vallisneria americana.* Comparison of inhibitory activities at 25 ppt would rank *C. demersum* third in inhibitory activity, rather than seventh. In our lettuce seedling bioassays, 26 of 26 plant extracts were inhibitory at 250 ppt, 23 of 26 were inhibitory at 125 ppt, and 11 of 26 were inhibitory at 25 ppt. The *Lemna minor* assay was more selective: 17 of 26 extracts were inhibitory at 250 ppt; 8 of 26 were inhibitory at 100 to 125 ppt; only 2 of 26 were inhibitory at 20-25 ppt. At 25 ppt, two of the plants listed in Table II, *B. schreberi* and *V. americana*, are stimulatory toward lettuce seedling growth, although the stimulation is not statistically significant. *Nymphaea odorata* is no longer inhibitory at this concentration. *Nymphaea odorata* (roots and rhizomes)

inhibited 72% of *Lemna minor* frond formation at 250 ppt, but it was stimulatory to *L. minor* at 20 ppt, although again, this observed stimulation was not significantly different from the control at $P \leq 0.05$ according to Duncan's multiple range test. We might expect stimulation of plant growth by aqueous plant extracts since these extracts may contain plant nutrients. We have observed stimulation in other extracts, but the stimulation was not statistically significant in any case. Others have suggested that many, perhaps most, allelopathic compounds would be stimulatory at low concentrations but inhibitory at higher concentrations (*9,10*).

Table II. Inhibition of Lettuce Seedling Radicles by Aqueous Extracts.

Extract source	% Inhibition		
	250 ppt	125 ppt	25 ppt
Nuphar lutea (roots & rhizomes)	100	100	100
Nuphar lutea (leaves)	100	100	100
Nymphaea odorata (leaves)	95	91	61
Juncus repens	86	81	52
Vallisneria americana	83	55	st *
Brasenia schreberi	82	55	st *
Ceratophyllum demersum	80	74	66
Eleocharis acicularis	78	62	32*
Nymphaea odorata (roots & rhizomes)	78	30 *	0

Means are significantly different from the control at $P \leq 0.05$ according to the Duncan's multiple range test unless noted with an asterisk. st = stimulatory

Table III. Inhibition of *Lemna minor* Frond Number by Aqueous Extracts.

Extract source	% Inhibition		
	250 ppt	100 ppt	20-25 ppt
Nuphar lutea (leaves)	100	100	62
Nuphar lutea (roots & rhizomes)	100	100	57
Nymphaea odorata (leaves)	98	78	21 *
Myriophyllum aquaticum	83	73	7 *
Nymphaea odorata (roots & rhizomes)	72	60	st *
Cabomba caroliniana	68	39	16 *

Means are significantly different from the control at $P \leq 0.05$ according to the Duncan's multiple range test unless noted with an asterisk. st = stimulatory

Promising Allelopathic Extracts

Of the 26 aqueous plant extracts thus far examined in our laboratories, *Nuphar lutea* is by far the most inhibitory (*11*). We found it to be almost ten times as inhibitory as the second most active extract in both the lettuce seedling and the *L. minor* bioassays. Lettuce seedlings were killed at extract concentrations of greater than 12.5 ppt; extracts of both leaves and roots and rhizomes at 2.5 ppt inhibited 70% of lettuce seedling radicle growth, and 22% of *L. minor* frond production. *Lemna minor* was killed by 100 ppt of either leaf or root and rhizome extract of *N. lutea.*

Aqueous extract from *Nymphaea odorata* leaves was the second most inhibitory of the extracts we examined. There have been reports of some chemical constituents of *N. odorata* (*12,13*) but no examination of its allelopathic capacity. We have successively extracted dried, ground leaves with solvents of increasing polarity: hexane, ethyl ether, acetone, and 95% ethanol. Each extract was subjected to lettuce seedling bioassay. The hexane extract was inactive; the 95% ethanol extract was the most active. This ethanol extract was separated into aqueous, ethyl ether, and ethyl acetate soluble fractions. The ethyl acetate fraction was the most inhibitory toward lettuce seedlings, inhibiting more than 50% of radicle growth at a concentration of 100 ppm (parts per million). We are presently pursuing a bioassay directed isolation of the allelochemicals from this ethyl acetate fraction.

Examination of the literature revealed *N. lutea* to possess both antibacterial and antifungal activity (*14*). It is also rich in alkaloids possessing a 3-furyl group attached to quinolizidine or piperidine ring systems (*14-17*). Because of the notably high allelopathic activity of *N. lutea* in our bioassays, we are in the process of re-isolating the alkaloids of *N. lutea* so that we may test them for allelopathic activity. None of these alkaloids are available commercially.

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Chapter 5

Identification of Allelochemicals in *Eucalyptus citriodora* and *Polygonum sachalinense*

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The relationship between the allelochemicals, *p*-menthane-3,8-diols (*cis* and *trans*)and the ontogenetic age in *Eucalyptus citriodora* was elucidated. The diols in the soil from a *Eucalyptus* grove were analysed by chromatography. On the other hand, the root exudates from *Polygonum sachalinense* in a recirculating system significantly inhibited lettuce seedling growth. Bioassay of the neutral-acidic fraction on the TLC agar plate showed the inhibitory activity corresponded to the two yellow pigments. Two compounds were isolated and identified as anthraquinone compounds; emodin and physcion. The results indicate that these anthraquinones are responsible for the observed interference in nature and are potent allelochemicals.

Among organisms, higher plants cannot change the location since germination, but they adapt themselves to the given environment, and are equipped with defensive and offensive mechanisms to protect themselves and promote the growth of their species. This defense mechanism has been acquired during the long process of evolution, and here chemical substances play an important role.

The search for allelochemicals not only illuminates plant ecology, but also provides the key to the development of herbicides (1,2). Generally, allelopathy is the phenomenon in which preventive influences are provided for another plant, either directly or indirectly, through the outflow into an environment of chemical substances produced by certain plants (including microorganisms). Since Molisch defined the term allelopathy in 1937 (3), a large number of scientists have been concerned with the exploration and exploitation of allelochemicals (4-12).

We have been interested in allelopathy in *Eucalyptus* groves and *Polygonum* shrubs. It is well known that some *Eucalyptus* species (Myrtaceae) are surrounded by bare (grass-free) ground. In the

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course of our research on *Eucalyptus* metabolites which exhibit biological activity, we have studied the allelochemicals from *Eucalyptus citriodora* (lemon-scented) leaves.

On the other hand, the shrubs *Polygonum* species grow vigorously and form colonies along roadsides and river banks in gravelly soils. The genus *Polygonum* has about 300 species and is distributed all over the world. Al Saadawi and coworkers demonstrated that *Polygonum aviculare* was allelopathic, and several phenolic compounds and long-chain fatty acids appeared to be responsible for this activity (8,13,14). *P. sachalinense* (Sachaline giant knotweed) is a perennial shrub found in native habitats in Japan. Judging from its ability to rapidly colonize, we hypothesize that allelopathy contributes to its aggressiveness.

This paper deals with the isolation, characterization and biological activities of allelochemicals in *Eucalyptus citriodora* and *Polygonum sachalinense*.

Experimental

***Eucalyptus* Plants and Soil Materials.** Seeds and leaves of *E. citriodora* Hook. were collected near Canberra in Australia with the aid of Dr.D.M.Paton, Department of Botany, the Australian National University. Seedlings were cultivated in the greenhouse of Hokkaido University, Sapporo.

Soil samples were collected at an *E. citriodora* grove in Queensland state, Australia by Dr. I.C.MacRae, Department of Microbiology, University of Queensland. The soil (*ca* 500g) without leaf trash was extracted with 900ml of MeOH at ambient temperature, for 2 weeks.

***Polygonum* Plants and Soil Samples.** Rhizomes with roots, aerial parts, and fallen leaves of *P. sachalinense* were collected at several wild places near the campus of Hokkaido Tokai University. Excavated rhizomes were rinsed with tap water and brushed lightly to remove soil, and air-dried. The rhizome (5.1kg) and aerial part (3.8kg) were sliced and steeped in separate containers with 80% acetone for two months at room temperature. The extracts were filtered and evaporated to remove acetone under reduced pressure. In another experiment, 100g of fallen leaves were extracted with 300ml of ether for 24hr, and the residues were removed by filtration.

Soil samples were collected in November 1990 from the center of a *P. sachalinense* community near the campus of Hokkaido Tokai University at the depth of about 10cm. Litter was removed as completely as possible by sieving and the soil was dried at room temperature overnight. A 500-g soil sample was stirred in 2 liters of acetone-1 N HCl (2:1, v/v) for 1hr at 50°C and further treated with ultrasonic energy for 1min. After leaving overnight at room temperature, it was filtered. The filtrate was evaporated to remove acetone and extracted three times with ether. The ether extract was evaporated in vacuo and fractionated by SiO_2 column chromatography using a hexane-$CHCl_3$-MeOH gradient as an eluent. Allelochemicals were identified by the interpretation of spectral data as mentioned later. The contents of emodin and physcion as allelochemicals were quantitatively determined by HPLC. The amounts of anthraquinone glycosides were determined by weighing the isolated

crystals of emodin-1-*O*-β-D-glucoside and physcion-1-*O*-β-D-glucoside (15).

Root Exudate Recirculating Systems. The evaluation of the effect of the root exudates as allelochemicals was carried out according to the root exudate recirculating system of Stevens and Tang (16). Lettuce seedlings were planted in the acceptor pot, while *P. sachalinense* was grown in the donor pot. Nutrient solution (17) from the donor pot was lifted through 8-mm-diameter glass tubing to the acceptor pot using an air pump. Nutrient levels were maintained, and pH was kept 5.5 with H_2SO_4 or NaOH. Recirculating solution was not changed during the experiment except for replenishing water and nutrient solution. After 10-14 days, the growing condition of lettuce (*Lactuca sativa L .*) seedlings was compared with that of the control growing under the same conditions without the donor plant. The experiment was carried out in a greenhouse.

TLC Agar Plate Bioassay. Thin-layer chromatography was carried out on 20 × 7-cm silica gel glass plate (Merck, Kieselgel 60 F254) 0.25mm thick using hexane-$CHCl_3$-MeOH (4:10:1 v/v/v) as developing solvents. The chromatogram was covered with a 0.5% agar layer (2mm), and the seeds of green amaranth (*Amaranthus viridis L.)*, timothy grass (*Phleum pratense L*), crabgrass *(Digitaria adscendens* Henr.), and Chinese cabbage (*Brassica campestris L .*) were sown in a row perpendicular to the bands on the chromatogram. The seeds were incubated in a moisture saturated growth chamber at 24-26°C with 14hr light and 10hr dark period. After four to seven days, seed germination and plant growth were examined.

Germination Test. Fractions (300 µg/ml, 2ml) or pure compounds (10-300µg/ml 12ml) dissolved in Me_2CO, were allowed to soak into filter papers in Petri dishes (dia.6cm). After the papers had been dried, Tween 80 solution (100µg/ml, 2ml) was poured into each dish which was then left overnight. Next day each dish was sown with 50 seeds of lettuce, garden cress, green foxtail, barnyard grass or rice and incubated at 22° for 1-5 days in the dark. The bioassay was repeated twice under the same conditions. Germinated seeds were counted and compared with control.

Growth Test. The sample (20-600mg) was dissolved in 10ml Me_2CO and the resultant solution (0.1ml) added to 20ml 0.35% agar solution. After the agar solution had solidified, 20 seedlings (lettuce, garden cress, green foxtail or timothy grass) were put on the agar in a deep Petri dish (60mm × 60mm dia.) and incubated at 22° for 7 days in 14hr light and 10hr dark. The average length of hypocotyl or leaf sheath and the longest roots of the testing plants was measured and compared with control.

Isolation and Identification of *Eucalyptus* Allelochemicals. Fractionation of an Me_2CO extract of fresh adult leaves was monitored by inhibitory activity against germinating seeds and seedlings of lettuce (*L.sativa*), garden cress (*L.sativum*), and green foxtail *(S. viridis)*. Active fractions were obtained by steam distillation and silica gel column chromatography using a hexane-Et_2O gradient

as an eluent. Rechromatography of active fractions gave two inhibitors as crystals.

***p*-Menthane-3,8-*cis*-diol (1).** MP 81.0-82.5° (crystallized from Et_2O -hexane), $[\alpha]_D^{23}$ ± 0° ($CHCl_3$; c 0.2). IR ν_{max}^{KBr} cm-1: 3240, 2930, 2900, 1450, 1420, 1250, 1160, 930; High resolution FIMS *m/z* (rel. int.): 173.1532 $[M+H]^+$ (16), 157 $[M-Me]^+$ (37), 154 $[M-H_2O]^+$ (100), 114 (9), 96 [M-OH-hydroxyispropyl]$^+$ (83), 77 (41), 59 (85); EIMS (probe) 70eV, *m/z* (rel. int.): no M^+peak, 157 $[M-Me]^+$ (1), 154 $[M-H_2O]^+$ (2), 139 $[M-H_2O-Me]^+$ (3), 121 (2), 111 (2), 96 (40), 81 [M-OH-hydroxyisopropyl -Me]$^+$(100), 68 (16), 59 (79), 55 (21), 54 (21), 43 (42), 41 (34); ^{1}H NMR (200 MHz, $CDCl_3$, TMS): δ0.87 (3H,*d*,*J*=6.4Hz,H-7), 1.22 (3H,s, H-9), 1.36 (3H,s,H-10), 4.41 (1H,*q*,*J*=2.4Hz,H-3); ^{13}CNMR (50MHz, $CDCl_3$, TMS): δ20.4 (t,C-5), 22.3 (q,C-7), 25.7 (d,C-1), 28.8 (q,C-9), 29.0 (q,C-10), 35.0 (t,C-6), 42.6 (t,C-2),48.4 (d,C-4), 68.1 (d,C-3), 73.3 (s,C-8).

***p*-Menthane-3,8-*trans*-diol (2).** MP 77.3-78.3° (from Et_2O -hexane), $[\alpha]_D^{23}$ ± 0° ($CHCl_3$; c 0.1). IR ν_{max}^{KBr} cm^{-1}: 3250, 2960, 2920, 1450, 1420, 1220, 1180, 1000, 910, 870; High resolution FIMS *m/z*(rel. int.): 173.1546[M+H]$^+$(47), 157 $[M-Me]^+$(11), 154 $[M-H_2O]^+$ (30), 114 (10), 113 (15), 96 [M-OH-hydroxyisopropyl]$^+$(54), 77(87), 59 (100); EIMS (probe) 70eV, (rel. int.): no M^+peak, 157 $[M-Me]^+$(1), 154 $[M-H_2O]^+$(1), 139 $[M-H_2O-Me]^+$(3), 121 (2), 111 (1), 96 (38), 81 (100), 68 (10), 59 (90), 55 (13), 54 (20), 43 (34), 41 (19); ^{1}H NMR (200 MHz, $CDCl_3$, TMS): δ0.92 (3H,*d*,*J*=6.4Hz,H-7), 1.22 (6H,s,H-9 and H-10), 3.72 (1H,dt, *J*=10.4,4.3Hz,H-3); ^{13}CNMR (50MHz, $CDCl_3$, TMS): δ22.0 (q,C-9), 23.8 (q,C-10), 27.1 (t,C-5), 30.1 (q,C-7), 31.4 (d,C-1), 34.6 (t,C-6), 44.7 (t,C-2), 53.5 (C-4), 72.9 (d,C-3), 75.0 (s,C-8).

Determination of the Diols. *E. citriodora* seedlings were grown at Hokkaido Tokai University, Sapporo. Fresh leaves (*ca* 30g) were harvested at each growth stage (5-21 months), and Et_2O extracts of the fresh leaves were analysed by glass capillary GC; SCOT column, 30m× 0.28 mm i.d. coated with PEG-HT; oven temperature, 100-180° at 2°C/min; flow rate, 1.25 ml/N_2/min. α-Terpineol and lauryl alcohol were used as internal standards for the determination of citronellal, citronellol and *p*-menthane-3,8-diols. Quantitative error was within 2%.

Isolation and Identification of *Polygonum* Allelochemicals. The inhibitory substances against seedlings growth were fractionated by SiO_2 column chromatography using a hexane-$CHCl_3$-MeOH gradient as an eluent, and the desired products were recrystallized from hexane-$CHCl_3$ to give two orange-needle crystals. The isolated compounds were identified from IR, UV, mass and NMR spectra (15).

Emodin (3): mp 255°C; IR (KBr) ν_{max}3400(H)(OH), 1630(C=0)cm^{-1}; UV(MeOH) λ_{max}221, 253, 265, 289, 437 nm; [^{1}H]NMR [$(CD_3)CO_2$]δ2.46 (3H, s,Ar-CH_3), 6.65 (1H,*d*,*J*=2.5Hz,H-2), 7.12 (1H,br s,H-7), 7.24 (1H,*d*,*J* =2.5Hz,H-4), 7.55 (1H,br s,H-5), 12.05 (1H,s,OH), 12.18 (1H,s,OH); HR-EI-MS *m/z* 270.0515 ($C_{15}H_{10}O_5$).

Physcion (4): mp 216°C; IR(KBr); ν_{max} 3400(OH), 1630(C=0)cm^{-1}; UV(MeOH)λ_{max}223, 254, 265, 287, 434 nm; [^{1}H]NMR($CDCl_3$) δ2.45 (3H,s, $ArCH_3$), 3.94 (3H,s,Ar-OCH_3), 6.68 (1H,*d*,*J*=2.5Hz, H-2), 7.08(1H,br s, H-7), 7.36 (1H,*d*,*J*=2.5Hz,H-4), 7.63 (1H,br s, H-5), 12.12 (1H,s,OH), 12.31 (1H,s,OH); HR-EI-MS *m/z* 284.0704 ($C_{16}H_{12}O_5$).

Isolation and Identification of Anthraquinone Glycoside. The rhizomes (5.1kg) with roots and the aerial part (3.8kg) of *P. sachalinense* were collected in June 1991. Immediately after cutting, they were extracted in EtOH by refluxing for 5 hr and the EtOH solution concentrated to one third of the original volume. The aqueous concentrate was extracted with ether to remove free anthraquinones and other ether-soluble constituents. $Ba(OH)_2$ solution (5%) was added to precipitate the organic acids. After filtration, the filtrate was adjusted to pH 3 and was extracted with *n*-BuOH. The *n*-BuOH layer was separated and evaporated in vacuo to give the crude glycoside fraction, which was further fractionated by SiO_2 column chromatography using a hexane-$CHCl_3$-MeOH as an eluent to obtain anthraquinone glycosides. Rechromatography and further recrystallization from MeOH gave two glycosides as orange-needle crystals. Identification was based on the comparison of the melting poing, R_f values, and spectrometric data with those in Kang and Woo (18) and Kato and Morita (19).

Emodin-1-*O*-β-D-glucoside (5): mp 193°C; IR(KBr)ν_{max} 3400 (OH), 1630 (free C=O), 1600 (chelated C=O)cm^{-1}; UV (EtOH)λ_{max} 221, 253, 265, 286, 421 nm; [^{1}H]NMR(CD_3OD) δ2.40 (3H, s, Ar-CH_3), 4.96(1H, *d*, *J*= 7.5Hz, anomeric H), 7.04 (1H, br s, H-7), 7.10 (1H, *d*, *J*=2.5Hz, H-2), 7.32 (1H, *d*, *J*=2.5Hz, H-4), 7.48 (1H, br s, H-5); FAB-MS(DMSO)*m/z* (NaCl addition)455 ($M + Na)^+$, 478 ($M + 2Na)^+$.

Physcion-1-*O*-β-D-glucoside (6): mp 240°C; IR(KBr); ν_{max} 3400(OH), 1640 (free C=O), 1600 (chelated C=O)cm^{-1}; UV(MeOH)λ_{max} 226, 273, 417nm; [^{1}H]NMR(CD_3OD)δ2.46 (3H, s, Ar-CH_3), 3.97 (3H, s, Ar-OCH_3), 4.96 (1H, *d*, *J*=7.5 Hz, anomeric H), 7.11 (1H, br s, H-7), 7.30 (1H, *d*, *J*=2.5Hz, H-2), 7.57 (1H, *d*, *J*=2.5 Hz, H-4), 7.60 (1H, br s, H-5).

Quantitation of Anthraquinones and Anthraquinone Glycosides. Plant samples were collected at several wild places near the campus of Hokkaido Tokai University. Fresh aerial parts, rhizome with roots, and fallen leaves were extracted with 80% acetome. After concentration, the aqueous residues were extracted with ether. The ether-soluble fractions were quantitatively analyzed by HPLC (JASCO System-800) and compared with standard emodin and physcion (purchased from Funakoshi Co.Ltd.). The analytical conditions are as follows: column-YMC-Pack ODS-AM (30cm × 10mm ID); solvent-MeOH/H_2O (80:20, v/v); flow rate-4 ml/min, wavelength of detection-245nm.

Spectrometers. IR spectra were recorded on a JASCO IR-810 as KBr disk for crystals. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. NMR spectra were measured on a Bruker AM-400 (400 MHz) spectrometer using TMS as an internal standard. Mass spectra were recorded using JEOL JMS-DX303HF GC-MS spectrometer.

Results and Discussion

Allelochemicals and Ontogenetic Age in *Eucalyptus*. The full details of the isolation and subsequent characterization of the *cis* and *trans* isomers of *p*-menthane-3,8-diol from adult leaves of *E.citriodora* are given in the Experimental.

The relationships between the amounts of constituents determined by GC and GC/MS and ontogenetic age in *E. citriodora* seedling are shown in Fig.1 (20). The diols were absent from ontogenetically juvenile tissue until 13 months or approximately 50 nodes. (±)-Citronellal, which is the major component of *E. citriodora* essential oils, gradually increased and after 13 months decreased dramatically. In contrast, the *cis* and *trans* *p*-menthane-3,8-diols increased with a ratio of 2 to 1 after 13 months. This suggested that the *cis* and *trans* diols are formed by cyclization of citronellal by a possible biogenetic pathway. However, it is not clear whether or not the diols are produced enzymatically from (±)-citronellal. The diols were not artifacts since the pH of the homogenized tissues was neutral.

***p*-Menthane-3,8-diols in Soil.** Soil samples were collected at an *E. citriodora* grove in Queensland state, Australia and extracted with methanol (see Experimental). The methanol extract was concentrated and re-extracted with ether. *p*-Menthane-3,8-diols in the ether soluble fraction were detected by GC/MS. The GC trace given by the total ion current showed very complicated peaks. However, the ions monitored at *m/z* 59,81 and 96 gave two big peaks on the gas chromatogram which were identified as the *cis* and *trans* forms of *p*-menthane-3,8-diols by comparisons of MS and GC data with those of synthetic standard compounds prepared by acid-catalysed cyclization of citronellal.

Furthermore, the concentrations of the diols were quantitatively determined by GC/MS. The sum of concentrations of the *cis* and *trans* diols was approximately 15 ppm although the the concentration of the *cis* diol in the fresh leaves was *ca* 4600 ppm. The low concentration of diols indicates that methanol extraction of these compounds from soil is inefficient and/or that they are partially transformed by microorganisms in the soil (20).

Germination and Growth Inhibitory Activity. In the previous paper (8), it was indicated that the germination inhibitory activity of the *cis* isomer was much higher than that of the *trans* isomer, and that the (+) synthetic *cis* isomer had a higher activity than the optical antipode. In this paper, the (+) synthetic *cis* isomer was used for germination and growth tests against several higher plants. Bioassays were made on filter papers or 0.35% agar solutions ranging in concentration from 10 to 300 ppm. The inhibitory activities against seeds and seedlings of several plants are shown in Figs. 2 and 3, respectively. Concentrations as high as 100-300 ppm (5.8×10^{-1} -1.7×10^{-3} M) were inhibitory to seed germination and hypocotyl growth in lettuce (*Lactuca sativa* L. cv. Wayahead), garden cress (*Lepidium sativum* L.), green foxtail (*Setaria viridis* L.) and barnyard grass (*Panicum Crus-galli* L.). However, these and higher concentrations of the diol had no inhibitory effects on germination and growth of *E.citriodora* itself and rice (*Oryza sativa* L.). In terms of herbicidal properties, it is interesting that the biological activity of the *cis* diol is very selective against higher plants.

Allelochemicals in *Polygonum*. The root exudates from *P.sachalinense* showed an inhibition on lettuce seedling growth using the

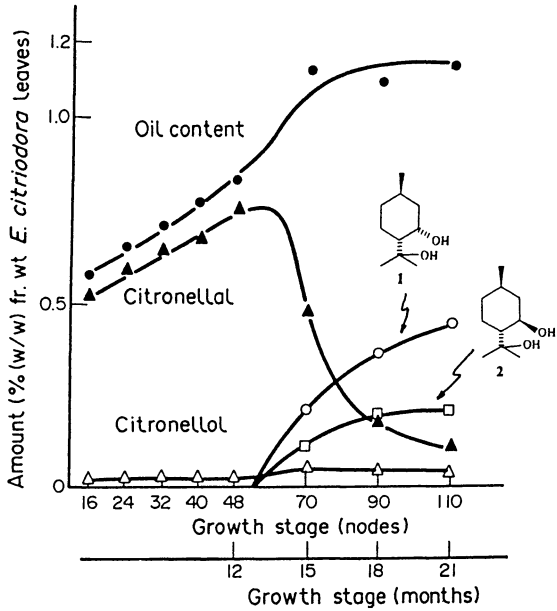


Figure 1. Relationship between amounts of constituents and ontogenetic age of E. citriodora. 1, p-menthane-3,8-cis-diol; 2, p-menthane-3,8-trans-diol. (Reproduced with permission from reference 20. Copyright 1984 Elsevier Science.)

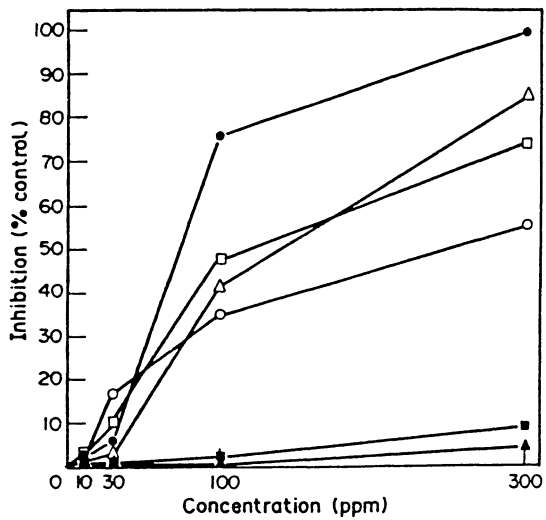


Figure 2. Germination inhibition of *p*-menthane-3,8-*cis*-diol (**1**) against seeds of several higher plants. Experimental error was within 7 % ●, Lettuce; △, garden cress; □, green foxtail; ○, barnyard grass; ■, rice; ▲, *E. citriodora*.

recirculating system. It is well known that organic compounds are exuded from healthy undamaged plant roots, and we expect that allelochemicals or their precursors are present in the plant tissue and also released from the rhizome and roots.

From the results of the TLC agar plate bioassay, growth inhibitory activity was shown in the two bands of yellow pigments against green amaranth and timothy grass on the chromatogram of the neutral-acid fraction (Fig. 4). Two corresponding orange needles were isolated and identified as anthraquinone compounds: emodin (1) and physcion (2) (Fig. 5).

Growth Inhibitory Activities of Anthraquinones. Emodin and physcion exhibited inhibitory activities against the seedling growth of several test species (Fig. 6). Emodin inhibited root and hypocotyl or leaf sheath growth over 100 ppm (3.7×10^{-4}M). It severely inhibited the growth of lettuce seedlings at 50 ppm (1.85×10^{-4}M). Physcion was less active and inhibited root and hypocotyl growth at 200 ppm (7.0×10^{-4} M).

The presence of anthraquinone glucosides was expected since they are common for transportation and storage and are less toxic in the plant. As a result, two glucosides were isolated from both the rhizome and aerial part of *P. sachalinense* and identified as emodin-1-*O*-β-D-glucoside (3) and physcion-1-*O*-β-D-glucoside (4) (Fig. 5). In plant growth bioassay, both emodin and physcion glucosides showed no phytotoxicity at 200 ppm against lettuce seedlings.

Role of Anthraquinones in Ecosystem. The concentrations of emodin and physcion, and their glucosides in the rhizome, aerial part, fallen leaves, and soil were quantitatively determined. Contents of emodin and physcion in the fresh rhizome were ca. 158 and 32 mg/kg fresh weight, and in the aerial part, ca. 72 and 22 mg/kg fresh weight, respectively. Large amounts of anthraquinones were still detected in dry fallen leaves more than four months after defoliation: emodin, 213 mg/kg dry weight; physcion, 180 mg/kg dry weight. In addition, contents of emodin and physcion in the soil were 55 and 30 mg/kg dry weight, respectively; these concentrations are enough to inhibit plant seedlings. These facts indicate that the anthraquinones are very stable in the ecosystem.

Allelopathic substances are released from plants in four ways (21):
(1) Litter of leaves and stems decomposes by physical or biological processes and the substance is released. (2) The active substance is released to the soil from the root directly by exudation or through the decay of dead roots. (3) Volatile material is vaporized from plants and acts on other plants through the air. (4) Rain and fog drips transfer toxic compounds from leaves to the soil.

We consider that the first two items above are associated with the allelopathy of *P. sachalinense*. It has anthraquinone glucosides in the plant tissue. We propose that either these glucosides or the aglycons are released from the rhizome. The glucosides are then decomposed to active forms-emodin and physcion-and subsequently exhibit plant growth inhibitory activities against other plant species. To confirm this hypothesis, isolating glucosides and aglycons from the root exudate solution is currently

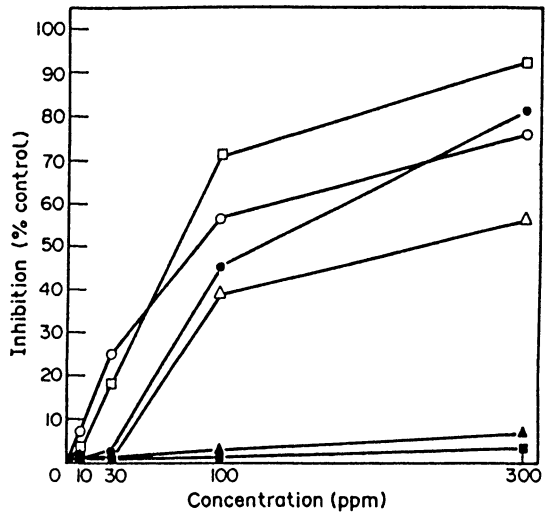


Figure 3. Growth inhibition of *p*-menthane-3,8-*cis*-diol (**1**) against seedlings of several higher plants. Experimental error was within 7 %. Symbols used are as in Figure 2.

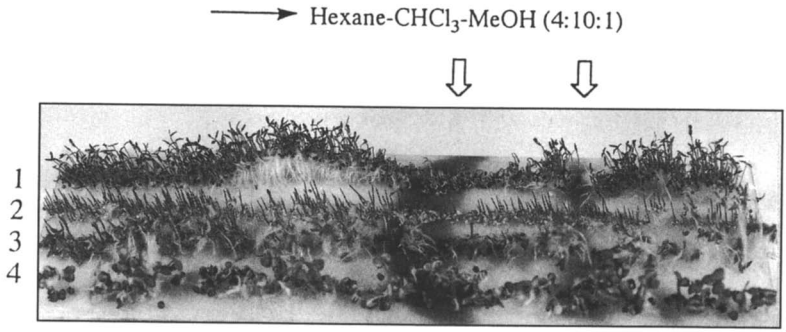
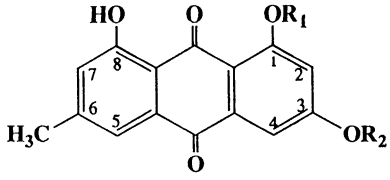


Figure 4. Bioassay of neutral and acid substances from Polygonum sachalinense by TLC-agar-plate method (5 days). Growth inhibitory activity is shown at pointed two yellow pigment parts by arrows. 1, green amaranth; 2, timothy grass; 3, crab grass; 4, Chinese cabbage. (Reproduced with permission from reference 15. Copyright 1992 Plenum.)



(3) R_1=H, R_2=H Emodin
(4) R_1=H, R_2=CH_3 Physcion
(5) R_1=Glu, R_2=H Emodin-1-*O*-β-D-glucoside
(6) R_1=Glu, R_2=CH_3 Physcion-1-*O*-β-D-glucoside

Figure 5. Chemical structures of allelochemical candidates from *Polygonum sachalinense*.

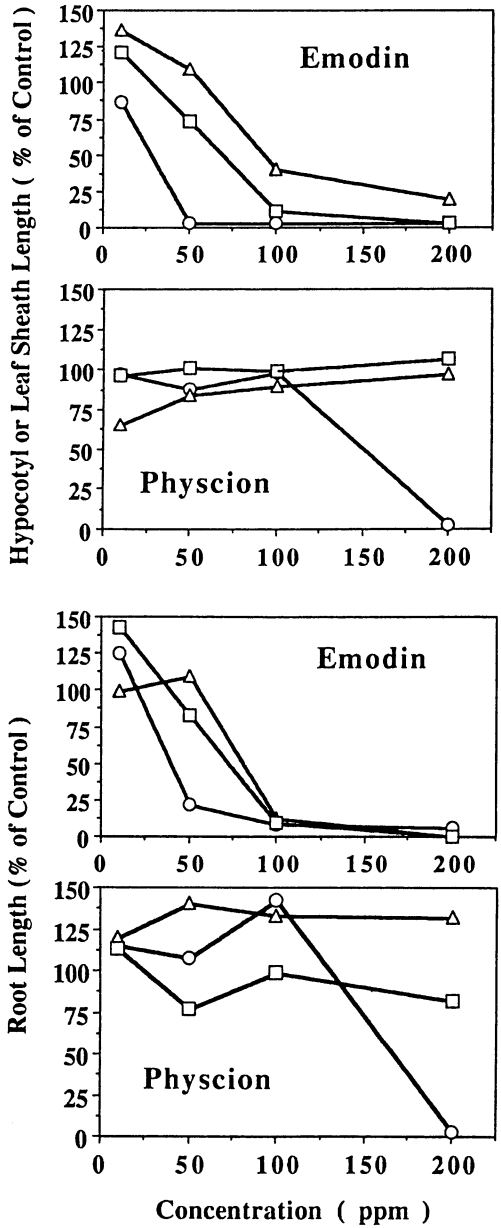


Figure 6. Growth inhibitory activities of emodin and physcion against plant seedlings. ○:lettuce, △:green amaranth, □:timothy grass.

under investigation. Furthermore, anthraquinones exuded from the fallen leaves of *P. sachalinense* accumulate on the soil surface and affects early seedling growth of nearby plants of the community.

There are reports about antimicrobial activities of emodin and physcion (22-24). Some antimicrobial activity by emodin and physcion may directly or indirectly affect the growth or other plants. Rice (25,26) and AlSaadawi et al. (8) pointed out that some weeds produce allelopathic chemicals that inhibited soil bacteria and changed the soil environment to their own advantage. While these anthraquinone compounds are potent allelopathic substances, the question of the extent of their impact against the flora in *P. sachalinense* is yet to be fully evaluated.

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Chapter 6

Quercetin and Quercitrin from *Pluchea lanceolata* and Their Effect on Growth of Asparagus Bean

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Two flavonols, quercetin and quercitrin (quercetin 3-O-rhamnoside), were isolated from the leaves of the rapidly spreading noxious perennial weed, *Pluchea lanceolata*. Quercitrin was also detected from the natural soils associated with the weed, while quercetin was not detected from the weed-associated soils. High performance liquid chromatography established that quercitrin concentration was higher in weed-infested soils with cultivation than in weed-infested, uncultivated soils. Aqueous extracts of $1x10^{-4}$ M, $5x10^{-4}$ M and $1x10^{-3}$ M concentrations of quercetin and quercitrin affected the seedling growth of the legume, asparagus bean (*Vigna unguiculata* var. *sesquipedalis*). Mixture of the two flavonols also were effective inhibitors. These data indicate that quercetin and quercitrin produced by *P. lanceolata* may cause allelopathic interference of crops associated with this weed.

Our previous research established that the weed, *Pluchea lanceolata* (DC.) C. B. Clarke (Asteraceae), interfered with growth of certain plant species and physiological parameters of asparagus bean through water-soluble compounds from the leaves of the weed *(1-3)*. A flavanone (hesperidin), a dihydroflavonol (taxifolin 3-arabinoside), and an isoflavonoid (formononetin 7-O-glucoside) found in the natural soils associated with the weed, inhibit seedling growth of certain plant species *(4, 5)*. These observations become more significant in explaining the interference potential of the weed under natural conditions as usually the above-ground parts of the weed are ploughed under in the cultivated fields prior to sowing. In view of the presence of flavonoids in the weed-associated natural soils *(4, 5)*, isolation and characterization of flavonoids of *P. lanceolata* leaves and its associated soils were undertaken to demonstrate their allelopathic potential.

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In spite of wide occurrence of flavonoids in seed plants *(6)*, flavonoids have not been widely tested for their allelopathic potential *(7)*. Stenlid *(8)* found that flavonoids lacking substitution on the B-ring are more potent allelochemicals. Flavonoids were reported to inhibit ATP production in mitochondria isolated from cucumber hypocotyls *(8, 9)*. Koeppe and Miller *(10)* found that kaempferol inhibited phosphorylation in corn mitochondria. Tanrisever *et al. (11)* detected a novel flavonoid, ceratiolin, in water leachate of fresh foliage of *Ceratiola ericoides*. However, ceratiolin had little effect on seeds of *Schizachyrium scoparium*, while its degradation product, hydrocinnamic acid, cause inhibition of seed germination and root growth of *Schizachyrium*.

The objectives of the present study were to carry out flavonoid analysis of *P. lanceolata* leaves and in soil associated with the plant, and to establish, if any, the allelopathic potential of flavonoids contributed by the weed.

Experimental

Leaves of the weed, *Pluchea lanceolata,* and its associated soils, were collected from the fields in and around Delhi (Long. 77.12 E; Lat. 28.38 N). Leaves were carefully cleaned and oven-dried (45 C). Soil samples were air dried and sieved (2 mm sieve). Both leaves and soil samples, were stored in paper bags until the phytochemical analysis.

Extraction, Purification and Identification of Quercetin and Quercitrin. Fifteen grams of *P. lanceolata* leaves, and 30 g of soils associated with the plant, were soaked for 72 h in 100 ml of double distilled water (DDW) at room temperature (25 $\pm$ 3 C). Leachates were then filtered and evaporated to dryness under vacuum, and residues were extracted with 10 ml of methanol. These extracts were loaded on Whatman No. 3 (46 x 57 cm) chromatographic paper and developed through descending chromatography using n-butanol:acetic acid:water (BAW, 4:1:5, upper phase) *(12)*. The developed chromatograms were then scanned under UV and UV+NH_3, and bands marked. Each band was eluted in 10 ml of methanol, concentrated under vacuum and again chromatographed on Whatman No. 3 and washed repeatedly with DDW through descending chromatography to purify these following Harborne *(12)*. Finally each band was eluted from chromatograms with methanol and used for spectral analysis, which included acid hydrolysis and acid-base shifts (NaOH, $AlCl_3$, $AlCl_3$/HCl, NaOAc, NaOAc/H_3BO_3) of parent as well as hydrolysed fractions. Fluorescence (UV and UV+NH_3) and R*f* values of each compound were recorded in four solvent systems: DDW, BAW, 15% acetic acid and forestal (conc. HCl:acetic acid:water, 3:30:10). The aqueous layer of the hydrolysed fraction was used for the identification of sugar moiety *(12)*. Additionally, the aglycone fraction was co-chromatographed with authentic samples of quercetin (Sigma Chemical Co., USA) and spectral data matched with that of authentic compound *(13)*.

High Performance Liquid Chromatography. HPLC analysis was carried out to study the variation in concentration of isolated flavonoids from cultivated and uncultivated *P. lanceolata* associated natural soils. Five grams of weed-associated soils from either uncultivated areas, rarely cultivated areas, or regularly cultivated

fields, were collected at two depths (topsoil and subsoil). The soil was shaken with 10 ml of methanol for 1 h at room temperature. Methanolic extracts of the soils and of authentic flavonoid were subjected to HPLC analysis. Reversed phase chromatorgraphy was carried out using a steel column (15 x 0.46 cm I. D.) containing a Zorbax C_{18} stationary phase and elution with methanol, the variable wavelength UV detector set at 275 nm, and a flow rate of 1 ml/min. For each analysis, 10 µl of compound and 30 µl of soil extracts were injected. On the basis of their retention time, flavonoid fractions were differentiated and identified *(14)*.

Test Solutions. Aqueous solutions of the identified flavonols were prepared for bioassays. Quercetin (Sigma Chemical Co.) and quercitrin (Extrasynthase, France) were initially dissolved in methanol, dried under vacuum, and dissolved in DDW to prepare the solutions of $1x10^{-4}$ M, $5x10^{-4}$ M and $1x10^{-3}$ M. Further, since the compounds cooccur in the leaf tissues, the effects of mixtures of these compounds were also investigated. For this purpose, equimolar mixed test solutions of each concentration of flavonols were prepared.

Growth Experiments. The commonly grown legume asparagus bean, *Vigna unguiculata* var. *sesquipedalis* (L.) Walp, was selected to assess the allelopathic potential of isolated flavonoids. Earlier studies established that the growth of asparagus bean was affected by *P. lanceolata* under experimental conditions *(3)*. Fifty seeds of asparagus bean were sown on filter paper (Whatman No. 1) moistened with 60 ml of DDW (served as control) or test solutions, and placed in 15-cm diameter Petri plates. To maintain the uniform moisture status in the Petri plates, a cotton pad soaked in either DDW or a test solution was placed below the filter paper. During the period of investigation, a temperature regime of 30 ± 5 C and light regime of 18.8 Klux was maintained. Root and shoot lengths were recorded on the seventh day. Treatments were arranged in randomized block design and repeated and replicated three times. Comparison of growth parameters were made through one-way analysis of variance.

Results and Discussion

Fluorescence, R_f values, and UV spectral data comparisons to the reference sample confirm the presence of flavonols, quercetin and quercitrin, in the leaves of *Pluchea lanceolata*, and of quercitrin in weed-associated natural soils (Table I).

Quercitrin was present both in *P. lanceolata* leaves and its associated soils. However, quercetin was detected only from the leaves of the weed and not from its associated soils. Presence of quercitrin in the soils could be explained as it is resistant to the fungal degradation *(7)*, whereas quercetin has been shown to be decomposed by soil fungi, especially *Aspergillus (7, 15)*. Padron *et al. (16)* reported that *Aspergillus*, when grown in medium with quercetin as the sole carbon source, produces an enzyme which degrades quercetin immediately. However, quercetin is likely to be functional in allelopathy because of its repeated availability through aqueous leachate. Grummer *(17)* reported quercitrin as an allelopathic compound in the leaves of *Artemisia absinthium*. Quercetin was reported

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Table I. UV Absorption Spectra of Quercetin and Quercitrin Isolated from *Pluchea lanceolata* Leaves and its Associated Soils[a]

Flavonoid	*Fluorescence[b]*		*Rf values[c]*				*UV Absorption Spectra*					
identification	*UV*	*UV+NH$_3$*	*DDW*	*BAW*	*HOAc*	*Forestal*	*MeOH*	*NaOH*	*AlCl$_3$*	*AlCl$_3$/HCl*	*NaOAc*	*NaOAc/H$_3$BO$_3$*
Quercetin	Y	Y	00	67	04	40	255 (255)[d] 371 (373)	254 (254) 325 (325) 428 (428)	270 (270) 333 (333) 431 (433)	269 (268) 360sh (360sh) 431 (427)	269 (267) 324sh 380 (380)	269 (269) 383 (386)
Quercitrin	Br	Y	55	22	48	47	255 (255) 352 (350)	271 (271) 331sh (330sh) 404 (399)	274 (274) 419 (423)	274 (274) 316sh (314sh) 396 (396)	269 (262) 358 (359) 396 (396)	266 (264) 373 (370)

[a]Quercetin was not detected in *P. lanceolata*-associated soils.
[b]Fluorescence key: Y, yellow; Br, brown.
[c]Solvent key: DDW, double distilled water; BAW, butanol-acetic acid-water (4:1:5, upper pahse); HOAc, 15% acetic acid; Forestal, conc. HCl-acetic acid-water (3:30:10).
[d]Values in parentheses indicate that of authentic reference compound.
sh, shoulder

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from ponderosa pine and associated soil which was found to have allelopathic effect against *Nitrosomonas (18)*. Wepplo *(19)* identified quercetin and parasorbic acid glycoside from the leaves of American cranberry (*Vaccinium macrocarpa*).

Table II. HPLC Analysis showing Trapped Concentrations (μg g^{-1}) of Quercitrin in soils from uncultivated areas, rarely and regularly cultivated fields infested with *Pluchea lanceolata*

Soil	*Trapped Concentration (μg g^{-1})*		
	Uncultivated area	*Cultivated fields*	
		Rarely	*Regularly*
Topsoil	8.09	13.83	11.08
Subsoil	5.91	7.01	15.01

HPLC analysis of topsoil and subsoil from *Pluchea lanceolata*-infested fields demonstrated that quercitrin concentration was higher in cultivated fields than in uncultivated areas (Table II). It is interesting that the concentration of quercitrin increased with cultivation, especially regular cultivation. Subsoils of regularly cultivated fields had higher quercitrin content than rarely cultivated fields, while this difference between cultivated sites was not found in topsoil (Table II). The fact that quercitrin concentration of topsoil of regularly cultivated fields was not higher than that of rarely cultivated fields could be due to the leaching of quercitrin from topsoil to subsoil. The higher concentration of quercitrin in the cultivated fields is probably due to more incorporation of leaves of the weed into the soil *(3)*. Inderjit and Dakshini *(20)* found that in comparison to uncultivated areas, soils from *P. lanceolata* infested cultivated fields had higher values for total phenolics.

The data suggest that concentration of quercitrin may vary in *P. lanceolata* soils under field conditions because of localized variations resulting from agricultural practices and irrigation. Other investigators have suggested uneven distribution of phytotoxic substances in the soil *(21-23)*. In fact, allelopathic effects depend upon the probability of exposure of roots of affected plants to pockets of toxins in the soil.

Compared to quercitrin, quercetin was a stronger inhibitor of the seedling growth of asparagus bean (Table III).This observation could be explained by the observations of Stenlid *(8)* that aglycone moiety of flavonoids are more active than glycosides. Quercetin significantly ($P < 0.001$) inhibited the root and shoot growth of asparagus bean at all the three levels of concentrations (Table III). However, quercitrin significantly ($P < 0.001$) inhibited the shoot growth of asparagus bean at the level of 5×10^{-4} M concentrations, and root and shoot growth at the level of 1×10^{-3} M (Table III). Furthermore, when present together in equimolar mixtures, these two flavonols inhibited the root growth at the level of

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Table III. Effect of Three Different Concentrations ($1x10^{-4}$M, $5x10^{-4}$M and $1x10^{-3}$M) of Quercetin and Quercitrin on Seedling Growth of Asparagus Bean

Flavonol	*Treatment*	*Seedling length[a] (cm)*		*Percent Relative Response[b]*	
		Root	*Shoot*	*Root*	*Shoot*
Control		6.7 ± 1.9	3.9 ± 2.7		
Quercetin	$1x10^{-4}$M	3.4 ± 1.5**[d]	2.7 ± 1.7	-48.28	-30.65
	$5x10^{-4}$M	3.7 ± 1.3**	1.8 ± 1.0**	-44.71	-55.02
	$1x10^{-3}$M	3.5 ± 1.6**	2.5 ± 1.4**	-47.84	-36.93
Quercitrin	$1x10^{-4}$M	5.9 ± 2.3	3.7 ± 2.8	-12.22	-05.52
	$5x10^{-4}$M	6.1 ± 2.5	2.0 ± 1.4**	-09.23	-49.49
	$1x10^{-3}$M	5.5 ± 1.9**	1.9 ± 1.5**	-18.48	-51.00
Mixture[c]	$1x10^{-4}$M	5.8 ± 2.3*	4.3 ± 2.8	-13.56	+07.28
	$5x10^{-4}$M	6.3 ± 2.2	3.3 ± 2.1**	-05.96	-17.33
	$1x10^{-3}$M	3.9 ± 2.3**	1.9 ± 1.8**	-41.28	-50.25

[a]Seedling length on seventh day of seed sowing.
[b]Average percent increase (+) or decrease (-) in seedling length as compared to control.
[c]Each compound in equal volume.
[d]The data are means of three replicates ± standard deviations of means and asterisk indicate significant differences between control and treatment values at the level of $*P < 0.05$; $**P < 0.005$.

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$1x10^{-4}$ M ($P < 0.05$) and $1x10^{-3}$ M ($P < 0.001$) concentrations. Shoot growth was inhibited significantly ($P < 0.001$) by the mixture treatments of $5x10^{-4}$ M and $1x10^{-3}$ M (Table III). In contrast to observation of Williamson *(24)*, Einhellig and Rasmussen *(25)*, Rasmussen and Einhellig *(26)*, Einhellig *et al. (27)* and Williams and Hoagland *(28)* on synergistic activity of allelochemicals, such an effect was not apparent in the present study. The two compounds appear to have an additive inhibitory effect. However, the experiment was not designed to separate additive, synergistic, or antagonistic actions.

Seedlings grown with either of the flavonols showed browning of root tip at the level of $1x10^{-3}$ M concentrations. Presently, it is difficult to conclude if this effect resulted from ascorbic acid reduction in xylem vessels *(7)* or due to oxidation of flavonols, and further study is needed.

The data presented is of significance as it has established that quercetin and quercitrin can bring about allelopathic interference to the growth of asparagus bean. Furthermore, the data are relevant to field conditions. Given the perennial nature of the weed, continuous availability and periodic replenishment of these flavonols in soil will occur. Regular cultivation which is common to agricultural fields appears to enhance quercitrin in the soil. In summary, quercetin and quercitrin may play a significant role in initiating the chain of allelopathic interferences observed with crop seedling in agricultural fields infested with *P. lanceolata.*

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Chapter 7

Mechanism of Action of Allelochemicals in Allelopathy

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The primary mode of action has not been established for any allelopathic compound, albeit some physiological actions are known. The array of compounds cuts across many chemical classes, and it is unlikely they have a common mechanism of action. Allelochemicals active against higher plants are typically characterized as suppressing seed germination, causing injury to root growth and other meristems, or inhibiting seedling growth. A primary action on ATP production is indicated for the two quinones, juglone and sorgoleone, since they inhibit chloroplast oxygen evolution (I_{50} = 0.2 and 2.0 μM respectively) and strongly affect mitochondrial functions. The chloroplast block by sorgoleone is in the photosystem II complex. Cinnamic and benzoic acid derivatives alter membrane potential and have several physiological effects that suggest membrane perturbations are their initial site of action. Their thresholds (100 to 1000 μM) for inhibition of seedling growth, singly or in combinations, correlate with impairment of plant-water relationships. These phenolic compounds also alter mineral uptake, chlorophyll content, photosynthesis, carbon flow, and phytohormone activity. Phytotoxicity of many allelopathic chemicals may be from a generalized cellular disruption rather than a specific mechanism. A case study of *Sorghum* allelopathy suggests that inhibition of a receiving species results from the joint action of a number of allelochemicals with different cellular sites of action.

Efforts to explain the phenomenon of allelopathy are ultimately challenged to identify the site, or sites, of action of the chemicals involved. However, this lofty goal has seldom come to fruition (*1*). Success has been greatest in localizing the subcellular target of activity for certain antibiotics and other microbial toxins (*2-4*).

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Antibiotics and those microbial compounds that influence growth of higher plants are a legitimate part of allelopathy, since the allelopathic phenomenon includes the spectrum of all chemical interactions among microorganisms and higher plants. However, in an effort to limit the scope of this chapter, the primary focus will be on allelopathic chemicals identified in higher plant communities. There also are many secondary plant compounds, including defense chemicals, which will not be considered in this discussion because they have not been implicated in either plant to plant or plant and microorganism interactions.

Several hundred compounds have been reported as agents in allelopathy, and newly identified organic constituents are regularly added to this list. These allelochemicals arise from many chemical classes, and they represent an even larger array of structural complexities. It is not realistic to expect that they may have a common mode of action. The literature provides some insights concerning mechanism of action of allelopathic chemicals, but there are significant gaps in our understanding of precisely how they alter growth in a receiving plant or microorganism. These deficits of information are the result of a number of problems, which range from difficulties in the methodologies to a lack of focus on this aspect of allelopathy.

Perhaps the two most significant limitations that have curtailed attempts to investigate how allelochemicals alter growth are the lack of sufficient quantities of a compound and the fact that radioisotope labeling of allelochemicals has been very limited. Any organic compound linked to allelopathy must be isolated in an amount adequate for identification, yet usually the expense of procedures for isolating these natural products has precluded obtaining enough compound to study effects on physiological processes and cellular mechanisms. Several of the phenolic allelochemicals are exceptions to this picture, and it is not surprising that they have received the most scrutiny.

Some coumarins and a few of the benzoic and cinnamic acids have been radioisotope labeled for use in metabolism, membrane, or translocation studies. Steck (*5*) used ^{14}C-labeled scopoletin, esculetin, caffeic acid, and ferulic acid to study leaf metabolism of these compounds. Other early work established that hydroquinone and arbutin readily enter root tissue by diffusion and active transport, respectively (*6*). Van Sumere et al. (*7*) reported that coumarin, cinnamic acid, caffeic acid, and ferulic acid were taken up and metabolized by yeast cells and germinating lettuce and barley seeds. Salicylic acid rapidly crosses root cell membranes with the rate highest at low pH, an environment where most of the compound is in the molecular form (*8*). Work with seedlings established that ^{14}C-labeled salicylic, ferulic, and *p*-hydroxybenzoic acids are readily removed from a nutrient medium and translocated throughout the plant (*9-10*).

Such uptake and translocation data are not available for more novel allelopathic chemicals. Likewise, subcellular sites of action have not been explored by use of radioisotope-labeled allelochemicals. Analysis of cellular activity is further complicated because of glucosylation of allelochemicals as they are accumulated (*11-12*), a situation that confuses the issue of effective concentration of compounds at cellular sites. Interestingly, Hogan and Manners (*13*) showed that two plant species differed in their ability to detoxify hydroquinone. Because of the complications that have been noted, much of what is known about how

even the more common phenolic allelochemicals alter growth is by inferences drawn from their activity on physiological processes.

Hints from Herbicides

The net result of allelopathic interference has several parallels in the action of herbicides. Hence, insight into the mechanisms of herbicide action can be valuable for predicting possible actions of allelopathic chemicals. By definition, herbicides destroy or inhibit plant growth, and the major focus in allelopathy has been on inhibitory interactions. The 1989 *Herbicide Handbook* contains information on 148 chemicals and a larger number of commercial products (*14*). The physiological and biochemical behavior of herbicides illustrates that some compounds act by altering an essential biochemical pathway; others act in a more generalized fashion on membranes or some phase of plant growth (Figure 1). Although this schematic is a simplified view and not a full spectrum covering all herbicides, it illustrates the diversity of herbicide injury. Several reviews of herbicide action provide a more in-depth view of specific herbicides and note additional mechanisms of action (*14-17*).

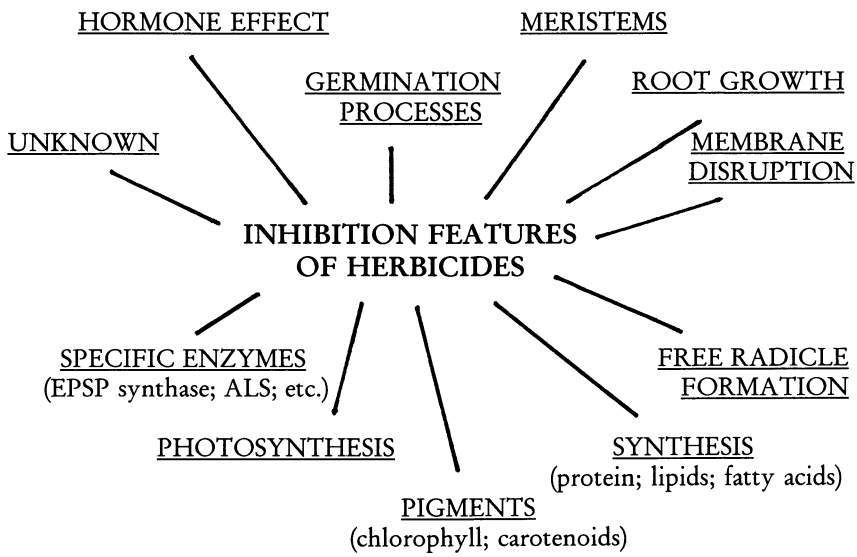

Figure 1. Mode of action of major herbicide molecules active in commercial formulations. (EPSP synthase = 5-enolpyruvylshikimate-3-phosphate synthase; ALS = acetolactate synthase)

At the onset, we recognize that even for some commercial compounds the inhibitory mode of action has remained elusive. Hormone-type herbicides, such as 2,4-dichlorophenoxy acetic acid (2,4-D), have been widely used without a good understanding of their specific mechanism of action. Other auxin-like herbicides include the chlorobenzoic acids. The dinitroanilines, inhibiting cell division, illustrate general growth-inhibitor functions. Several compounds injure membrane integrity and others have a membrane-based mode of action through inhibition of chlorophyll or carotenoid biosynthesis. Diphenylethers and paraquat, a bipyridylium, are membrane-active by enhanced production of toxic free radicals. Even when membrane activity is not a primary effect, most all the major classes of herbicides cause some disruption of membrane functions (*15*).

Processes of photosynthesis are a target of a wide range of herbicide molecules. Atrazine, the most widely used herbicide in the United States (*18*), inhibits photosynthesis on the reducing side of photosystem II. Triazines, phenylureas, nitriles, benzothiadiazoles, quinone analogs, and several others act on photosynthesis. The altered step is not the same for all photosynthesis inhibitors. Some impact photosynthesis by causing destruction of chloroplast membranes.

Herbicidal inhibition of enzymes is fairly common. Examples of this mode of herbicide function are evident in the blockage of specific enzymes in amino acid synthesis pathways by glyphosate, the sulfonylureas, and imidazolinones. A step leading to aromatic amino acids, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, is the target of glyphosate, while the latter two types of herbicides selectively block branch-chain amino acids through inhibition of acetolactate synthase (ALS). By comparison, although some allelopathic chemicals from higher plants are capable of altering the activity of certain enzymes, none have had their mechanism of action reported at this level of specificity.

Interrelationships of Allelochemicals and Plant Hormones

The first widely used organic herbicide was 2,4-D, a molecule with auxin-like activity that was developed during the 1940's. Hence, it is not surprising that some early evaluation of the more widely distributed allelochemicals focused on their effects on auxin activity. Scopoletin and isomers of chlorogenic acid inhibit oxidation of indoleacetic acid (IAA) (*19-20*). Assays performed in several investigations indicate that allelochemical phenolics may either reduce or promote IAA concentration (*21-23*). The monophenols, such as *p*-hydroxybenzoic, vanillic, *p*-coumaric, syringic, and phloretic acids, reduce available IAA by promoting IAA decarboxylation. In contrast, activity may vary but many diphenols and polyphenols (i.e., chlorogenic, caffeic, ferulic, protocatechuic) synergize IAA-induced growth by suppressing IAA destruction. Promotion of rooting in cuttings has been reported for several phenolic allelochemicals (*24*), but in a test of 21 compounds Kling and Meyer (*25*) found only salicylic acid interacted with applied IAA.

The hydroxamic acids 6,7-dimethoxy-2-benzoxazolinone (DMBOA) and 6-methoxy-2-benzoxazolinone (MBOA) modify the binding affinity of auxins to membrane-bound receptor sites and this activity correlates with inhibition of auxin-induced growth in oat (*Avena sativa*) coleoptile sections (*26*). Perez (*27*)

proposed that their phytotoxicity is likely due to interference with normal activity of auxins. Cutler (*28*) has successfully applied the etiolated wheat (*Triticum aestivum*) coleoptile section assay to screening for a broad range of natural products, and many herbicides exert activity in this assay (*29*). This coleoptile elongation bioassay was originally developed for growth regulators, such as IAA and abscisic acid (ABA), and its sensitivity for a variety of allelochemicals may indicate these compounds have some interactions with plant hormones.

Auxins stimulate ethylene production, but the current evidence has not linked allelochemical effects on auxin with changes in ethylene. Evaluation of this potential, as well as the possibility of allelopathic induction of ethylene as a stress response, should be explored. In an investigation of the effect of ferulic acid on ethylene production from velvetleaf (*Abutilon theophrasti*) seedlings, no consistent data were obtained, perhaps due to difficulties in methodologies (F.A. Einhellig and G.R. Leather, unpublished results). Cell suspension culture work of Leslie and Romani (*30*) showed that salicylic acid inhibits ethylene biosynthesis, but other related phenolic acids had no effect. The growth regulatory role of ethylene can factor into another aspect of allelopathy, since this volatile hormone may be released from decomposing residue (*31*).

A number of phenolic allelochemicals antagonize the action of two other phytohormones, gibberellic acids (GA) and ABA. Ray et al. (*32*) listed ten compounds, including coumarin and several flavonoids, that released growth inhibition of ABA on hypocotyl elongation and they suggested this was not through their regulation of IAA oxidase. Other studies show that normal GA stimulation of growth and amylase synthesis is blocked by tannins and some phenolic acids (*33-34*). However, Ray and Laloraya (*35*) found that low concentrations of phenolic compounds when present together with GA and ABA favored GA-induced growth by antagonizing the inhibitory effect of ABA.

The foregoing evidence suggests that disturbance of the delicate phytohormone balance contributes to some inhibitory effects of allelopathic phenols. The data also lead to speculation that endogenous phenolic substances may have some role in growth regulation.

Allelochemical Effects on Production of Metabolites

Effects on Enzymes. A number of chemicals that have been implicated in allelopathy can modify the function of specific enzymes. These include the previously noted effects of phenolic acids on IAA oxidase and inhibition of amylase by tannins and some other phenols. Tannins also block activity of cellulase, polygalacturonase, pepsin, proteinase, dehydrogenases, decarboxylases, and many other enzymes (*36*). Many of the cinnamic acid derivatives and related compounds inhibit activity of phenylalanine ammonia-lyase (PAL), suggesting they have a regulatory role in phenylpropanoid metabolism (*37-38*). Neither these reports nor others showing the function of a specific enzyme altered by an established agent of higher-plant allelopathy, have made a case that the enzyme blockage was the mechanism of action of the compound. However, it should be noted that several microbial phytotoxins studied for their herbicide potential act

by disrupting biosynthesis of amino acids (*2-4*). These include bialaphos, phosalacine, and phaseolotoxin. Certainly more specifics are known about the action of some of the antibiotics than are known concerning the working of allelochemicals produced by higher plants.

Biosynthesis and Distribution. Metabolism studies of Van Sumere et al. (*7*) showed coumarin and a group of compounds related to cinnamic and benzoic acids inhibited uptake and incorporation of phenylalanine by yeast, lettuce seeds, and barley embryos. Cameron and Julian (*39*) found 50 μM cinnamic and ferulic acids reduced protein synthesis in lettuce seedlings. Recently, Mersie and Singh (*40*) reported 1 μM ferulic acid caused 50% reduction in incorporation of leucine into protein in a cell suspension. Following labeled glucose in a cell-suspension culture, Danks et al. (*41*) found 100 μM ferulic acid reduced carbon flow into soluble amino acids, proteins, and organic acids, whereas carbon incorporation into the soluble lipid fraction increased. However, effects of cinnamic acid on the flow of carbon into cellular constituents were different than ferulic acid. Although none of these studies targeted a site of action, changes in cellular pools would influence overall growth. These and other studies illustrate the value of applications of tissue culture systems (*12-13,40-42*), and it is surprising that such systems have not been utilized more extensively to explore the mechanisms of action of allelopathic compounds.

Cell Morphology. Some of the earliest work on the action of allelochemicals showed that exposure to volatile monoterpenes, primarily cineole and camphor, reduced cell division and resulted in root cells of greater diameter, reduced length, irregular nuclei, and large internal globules (*43*). Coumarins cause similar changes in root cell width to length dimensions and cells are highly vacuolated compared to controls (*44-45*). Coumarin and scopoletin also decrease mitosis (*46*). Transmission electron microscopy in John Lovett's laboratory showed that root tip cells subjected to the alkaloids gramine and hordenine had damage to the cell walls, disorganization of organelles, increased cell vacuoles, and the appearance of lipid globules, indicating a slowing of metabolism of food reserves (*47*). Similar cellular changes have been reported from benzylamine and the tropane alkaloids, scopolamine and hyoscyamine (*48-49*). I expect further studies will reveal ultrastructural changes in cells subjected to allelopathic conditions, given the multiple effects some of these chemicals have on metabolic processes and membranes.

Allelochemical Interference with Photosynthesis

Effects on Intact Plants. Our data show that ferulic acid reduces photosynthetic rate in velvetleaf (*Abutilon theophrasti*) seedlings (Table I). Both 500 and 750 μM ferulic acid suppressed photosynthesis to approximately two-thirds the rate of controls, and there was a concomitant decrease in stomatal conductance and an increase in leaf resistance. The seedlings were grown in one-half strength Hoagland's (*50*) solution with summer glasshouse conditions. Plants were treated with ferulic acid-amended nutrient solution 19 days after germination, a stage

when the largest leaf was approximately 3-cm diameter. Net photosynthesis, conductance, and resistance were measured from the largest leaf by a transient technique using the Li-Cor 6000 Portable Photosynthesis System (LI-COR Inc., Lincoln, NB) fitted with a one liter chamber. These measurements were taken at midday full sun (1400 to 1700 μmol $m^{-2}s^{-1}$ PAR) with plants outside.

Table I. Effects of Ferulic Acid (FA) on Velvetleaf Photosynthesis (PS), Stomatal Conductance (SC), and Leaf Resistance (RS)

Days Trt.	Parameter	Treatment		
		Control	500 μM FA	750 μM FA
1	PS (mg CO_2 m^{-2} s^{-1})	0.74 A	0.49 B	0.45 B
	SC (cm s^{-1})	5.28 A	3.27 AB	2.35 B
	RS (cm s^{-1})	0.25 A	0.32 AB	0.46 B
2	PS	0.63 A	0.44 B	0.41 B
	SC	1.68 A	1.27 A	1.22 A
	RS	0.61 A	0.83 A	0.84 A
3	PS	0.72 A	0.50 B	0.53 B
	SC	2.39 A	1.85 A	1.55 A
	RS	0.44 A	0.65 A	0.68 A
5	PS	0.92 A	0.59 B	0.56 B
	SC	3.45 A	3.39 A	3.78 A
	RS	0.22 A	0.32 AB	0.33 B

Values (n = 4 to 6) in a row not followed by the same letter are different, $P < 0.05$, ANOVA with Duncan's multiple-range test.

Several other cinnamic and benzoic acids, plus scopoletin and chlorogenic acid, have been shown to inhibit photosynthesis in whole plants (*51-52*). Using *Lemna minor* assays, our laboratory tested over 20 phenolic allelochemicals and found the threshold concentration for inhibition of photosynthesis ranged from 100 to 2000 μM (*53-54*). Salicylic acid was the most toxic, while some compounds were inactive in the range tested. The relative strength of their effects on photosynthesis generally paralleled their degree of phytotoxicity. *L. minor* tests also revealed several allelopathic short-chain organic acids and sesquiterpene lactones interfered with photosynthesis (*53-55*). Artemisinin is a very active compound, reducing *L. minor* photosynthesis at 1 μM (*56*).

Isolated Cells. Assays in cell suspension cultures remove some questions about allelochemical transport to leaves, regulation of photosynthesis in whole plants,

and indirect physiological effects. Mersie and Singh (*40*) reported 100 μM ferulic, *p*-coumaric, chlorogenic, and vanillic acids inhibited photosynthesis from 33 to 65% in enzymatically isolated leaf cells of velvetleaf. These data show that inhibition of photosynthesis occurred at a lower allelochemical concentration in the cell suspension system than with intact plants, illustrating the buffering effects of the whole-plant system on photosynthesis.

Chloroplasts. Investigations of allelochemical action on chloroplasts eliminates the ambiguity of stomatal interference and effects on chlorophyll levels. Sorgoleone and juglone, two quite different quinone allelochemicals, are powerful inhibitors of chloroplast CO_2-dependent oxygen evolution, having an I_{50} action at 0.2 and 2.0 μM, respectively (*57-58*). Sorgoleone is the oxidized form of a *p*-benzoquinone found in *Sorghum* root exudates, and juglone is the naphthoquinone prominent in walnut allelopathy. Einhellig et al. (*57*) reported that the concentration of sorgoleone required to achieve inhibition of chloroplast oxygen evolution was the same as that found for a comparable effect from atrazine, a potent photosynthesis inhibitor on broadleaf plants. Studies using thylakoids and oxygen-evolving photosystem II membranes indicate the site of sorgoleone inhibition is blockage of the oxidation of Q_A by Q_B in the photosystem II complex (*59*). Localization of the affected chloroplast events has not been determined for juglone, but juglone did not appear to uncouple chloroplast electron transport (*58*). These data indicate that the photosynthesis process is an important site of action for these two quinones.

Moreland and Novitsky (*60*) found I_{50} values for effects of phenolic allelochemicals on chloroplasts ranged from 50 to 5000 μM. Of the six compounds they tested, the two flavonoids were on the lower side of this range, whereas vanillic and ferulic acids had high I_{50} concentrations. Although further study is needed, the relatively high phenolic acid concentrations required to significantly alter chloroplast oxygen evolution suggest primary events of photosynthesis are probably not the target site of these compounds.

In other studies, Andreo et al. (*61*) reported the indole alkaloid gramine behaves as an uncoupler of photophosphorylation. Thylakoid studies show zaluzanin C, a sesquiterpene lactone, inhibits photosystem II at the level of oxygen evolution (*62*). Work in progress in our own laboratory indicates that sesquiterpene lactones vary greatly in their activity on chloroplasts (A.M. Hejl and F.A. Einhellig, unpublished results).

Chlorophyll. Allelochemicals can affect photosynthesis in a receiving species by altering chlorophyll content. Vanillic, ferulic, and *p*-coumaric acids reduced chlorophyll in soybean (*Glycine max*) leaves, but grain sorghum (*Sorghum bicolor*) was not affected (*63*). No determination was made whether the reduced chlorophyll in soybean was from enhanced destruction of chlorophyll or reduced synthesis. Chlorophyll reduction is a common observation in *Lemna* bioassays of allelochemical activity (*54,58,64*), but this could be secondary to other cellular damage. The literature reports indicate reduced chlorophyll accompanying growth

inhibition from *Abutilon theophrasti, Vigna sinensis, Parthenium hysterophorus*, and *Tephrosia purpurea*, among others (*65-70*).

Allelochemical Interference with Respiration

Effects on Mitochondria. Tests with mitochondrial suspensions show that allelochemicals from a variety of classes perturb respiratory metabolism (Table II). These data should not be viewed as precise comparisons due to differences in test conditions and methodology, but they do indicate the toxicity of allelopathic chemicals to mitochondria varies by several orders of magnitude. Their activity by general class is quinones > flavonoids > coumarins > phenolic acids, with tests from alkaloids and volatile terpenes too limited to generalize.

Table II. Effects of Allelochemicals on Mitochondrial Oxygen Uptake

Compound	Source of Mitochondria	μM or Other Action	Reference
Sorgoleone	soybean, corn	0.5	*71*
Juglone	soybean	stimulation	*58,72*
Quercetin	mung bean	20	*60*
Naringenin	mung bean	110	*60*
Umbelliferone	mung bean	1,900	*60*
Ferulic Acid	mung bean	4,530	*60*
Salicylic Acid	soybean	6,000	*73*
Gramine	rat, bovine	10,000	*74*
Vanillic Acid	mung bean	17,670	*60*
Cineole	*Avena fatua*	[a]20,000	*75*

[a]Time-delayed effect.

The quinones, sorgoleone and juglone, are active on mitochondria at very low concentrations and this toxicity very likely contributes to their impairment of growth in conjunction with effects on photosynthesis. Juglone does not seem to block electron flow along the cytochromes, but induces some type of alternate pathway to oxygen (*58*). In contrast, our data show sorgoleone blocks mitochondrial electron flow between the cytochrome b and c_1 complex (*71*).

The flavonoids of Table II are the second most active class of allelochemicals inhibiting mitochondrial oxygen uptake. Using the firefly luciferase method, Stenlid (*76*) found that a variety of flavonoids inhibited the production of ATP in mitochondria. Small variations in the structure of the several flavonoids tested profoundly influenced their activity, but when applied at 100 μM a number of

these compounds reduced formation of ATP (*76*). While it is clear that more work is needed on the respiratory action of various flavonoids implicated in allelopathy, even a small interference with ATP production in the intact plant could impact growth.

Johansson and Hagerby (*77*) reported that phenolic acids lowered the ATP pool of growing fungal mycelia, but that relationship to growth was unclear. Table II data show the concentration of phenolic acids required to inhibit mitochondrial oxygen uptake is several orders of magnitude higher than flavonoids. At one micromolar treatments, Van Sumere et al. (*7*) found several phenolic acids and aldehydes lowered the ADP/O ratio in yeast mitochondria. This inhibition was not large and ferulic acid, *p*-coumaric acid, vanillin, and two coumarins were inactive. They also reported mixed results, ranging from stimulation to inhibition, when the phenolics and related compounds were tested on yeast, barley and lettuce respiration. Demos et al. (*78*) showed tannic, gentisic, and *p*-coumaric acids reduced respiration and released respiratory control in isolated mung bean (*Phaseolus aureus*) mitochondria. No activity on mitochondria resulted from ferulic, caffeic, *p*-hydroxybenzoic, and syringic acid treatments, yet these compounds reduced hypocotyl growth similar to that found with the other compounds. Interestingly, *p*-hydroxybenzoic acid is one of the four phenolic acids that Pellissier (*79*) found to inhibit respiration of mycorrhizal fungi at concentrations as low as 10^{-7} M.

Several reports indicate volatile monoterpenes and sesquiterpene lactones may perturb respiratory metabolism, either at the whole plant level or in studies with mitochondria (*56,75,80-82*). However, as with the case of the phenolic allelochemicals, it will take more substantial evidence to establish this effect as a primary mechanism of action that causes growth inhibition in allelopathy.

Allelochemical Action on Membrane Associated Processes

Upon first introduction to tissue, the phenolic compounds that are derivatives of benzoic and cinnamic acid have a profound effect on membranes. Glass and Dunlop (*83*) showed 250 μM treatments of these phenolic acids caused a rapid drop in membrane potential of barley (*Hordeum vulgare*) root cells, and the extent of this depolarization correlated with their lipid solubilities. Butyric acid, an allelopathic agent from straw decomposition, causes a similar effect. Even one micromolar treatment with these weak acids produce some plasma membrane depolarization (*84*). At relatively high concentrations, 10 mM, salicylic acid collapses mitochondrial transmembrane potential (*85*). Hence, the phenolic acids appear to cause changes in membrane structure and permeability.

Ion Accumulation. Phenolic acids have both a direct effect on ion uptake and a sustained action resulting in changes in tissue concentrations. Essentially all of the benzoic and cinnamic acids implicated in allelopathy inhibit the rate of phosphorus and potassium ion uptake by excised roots (*8,86-87*). This occurs at 250 to 500 μM, a concentration range that corresponds to their growth-inhibition threshold. Similar work illustrates that some of the flavonoids may inhibit

mineral absorption (*88*). Both altered membrane permeability and inhibition of ATPase activity may contribute to these reductions in mineral uptake (*89*).

In whole plant studies, ferulic acid reduced levels of phosphorus and potassium in grain sorghum shoots and roots after 3 and 6 days of treatment, and variations also occurred in tissue content of magnesium, calcium, and iron (*90*). The changes in mineral content occurred at the growth inhibition threshold for these seedlings, 250 μM ferulic acid. Lyu et al. (*91*) found that ferulic, vanillic, and *p*-coumaric acids in a mixture had an additive inhibitory effect on phosphorus uptake by cucumber (*Cucumis sativus*) over an 8 hr period. Booker et al. (*92*) reported some of the short-term (3 hr) effects of ferulic acid included inhibition of phosphate, nitrate, and sulfate uptake and promotion of potassium efflux from cucumber seedling roots. Even 20 μM ferulic acid depressed nitrate uptake. Additional work shows that ammonium uptake is less sensitive than nitrate uptake to inhibition by ferulic acid (*93*). At this time, localization of the specific action of ferulic acid on membrane transport processes has not been accomplished.

Other data show that the different phenolic acids implicated in allelopathy can alter mineral content of plants. Caffeic, syringic, and protocatechuic acids decreased nitrogen, phosphorus, potassium, iron, and molybdenum in cowpea (*Vigna sinensis*), but magnesium was not altered (*66*). Leaf content of nitrogen and phosphorus in *Parthenium hysterophorus* was reduced by 1000-μM treatments of chlorogenic, caffeic, vanillic, *p*-coumaric, and ferulic acids, and the latter three still reduced phosphorus at 100 μM (*68*). A growth study that extended over seven weeks showed that chlorogenic acid altered the mineral content of *Amaranthus retroflexus* (*94*). Chlorogenic acid reduced tissue levels of phosphorus, increased nitrogen, and did not affect potassium.

Balke (*89*) summarized a variety of investigations showing allelopathic residue, leachates, and extracts caused a change in mineral content in the receiving plant. It is not surprising that augmentation of fertility has been suggested as a way to ameliorate allelopathy. This inference has had conflicting evidence, but recent studies show additions of phosphorus or nitrogen did not overcome growth inhibition from ferulic acid (*95-96*).

Water Relationships. Plant-water status is a sensitive indicator of the resulting stress when plants are exposed to allelopathic phenolic acids and coumarins (Table III). Using the ratio of carbon isotopes ($^{13}C : {}^{12}C$) assimilated into leaf tissue as an indicator of water stress, recent experiments revealed that sustained exposure to phenolic allelochemicals at levels near the growth-inhibition threshold caused a chronic change in water-use efficiency. Results from treatments with single compounds were mimicked by equimolar combinations of several phenolic acids, showing that these compounds can work in concert (*97-98*). Although there is a consistency in the effects of the allelochemicals cited in Table III, this physiological action can not be automatically extrapolated to other classes of compounds. Rutin, a flavonoid, inhibited growth without a concomitant effect on plant-water balance (*98*).

Table III. Effects of Allelochemicals on Plant-Water Relationships

Species	[a]Compound	Threshold Effect - μM	[b]Parameter Altered	Reference
tobacco	CGA, SC	500	S	99
sunflower	CGA, SC	500	S	99
grain sorghum	*p*CA, CA	500; 1000	S	100
soybean	FA,*p*CA	250	S,ψ	101
soybean	CA,*p*CA,FA, GA,*t*CnA,VA	1000	S	52
grain sorghum	FA,*p*CA	250	S,ψ	102
cucumber	FA,*p*CA,VA	250	S	103
soybean	HQ	250	S,ψ ,C	97
soybean	UM,VN	500	S,ψ ,C	98
cucumber	FA	200	ψ	92
soybean	SA	150	S,ψ ,C	104

[a] CGA = chlorogenic acid; CA = caffeic acid; FA = ferulic acid; GA = gallic acid; HQ = hydroquinone; *p*CA = *p*-coumaric acid; SA = salicylic acid; SC = scopoletin; *t*CnA = trans-cinnamic acid; VA = vanillic acid; VN = vanillin; UM = umbelliferone.

[b] S = stomatal closure or diffusive resistance increase; ψ = lowered water potential; C = less ^{13}C discrimination

Research in Udo Blum's laboratory showed ferulic acid in the growth medium depressed root uptake of water, and this was paralleled by an elevation in the endogenous ABA level (*105*). Although there was some variation in these effects among the species studied, connecting water stress to an elevation of ABA is an important step in recognizing the complex involvement of phytohormones as part of the mechanisms of allelopathic action. Likewise, ABA is regulatory in stomatal functions, and stomatal interference from allelochemicals is strongly interrelated with photosynthesis, as suggested in the data of Table I.

Beyond studies with specific chemicals, changes in the water balance have been linked as part of the allelopathic effect of several plants. Seedlings whose growth was suppressed by extracts from *Celtis laevigata* and *Abutilon theophrasti* had a lower water content than control plants, and this was not due to any osmotic effect of the extract (*106,65*). Allelopathic interference from *Kochia scoparia, Helianthus tuberosus, H. annuus*, and *Xanthium pensylvanicum* (*X. strumarium* var. Canadense) causes water stress (*102,107-108*). Aqueous extracts from these plants resulted in significantly higher diffusive leaf resistance and lowered water potential in the receiving plant, and similar changes were noted in seedlings grown in soil amended with the allelopathic plant residue.

Action Model for Phenolic Allelochemicals

The data reviewed show that the cinnamic and benzoic acid allelochemicals, as well as the coumarins and some flavonoids, affect an array of physiological processes in higher plants. It seems unlikely that these compounds act in a specific manner on a single process, but multiple effects in expressing activity are indicated. However, my synthesis of the evidence is that some effects are more primary than others, and that their action on membranes is central to their disruption of most other processes (Figure 2).

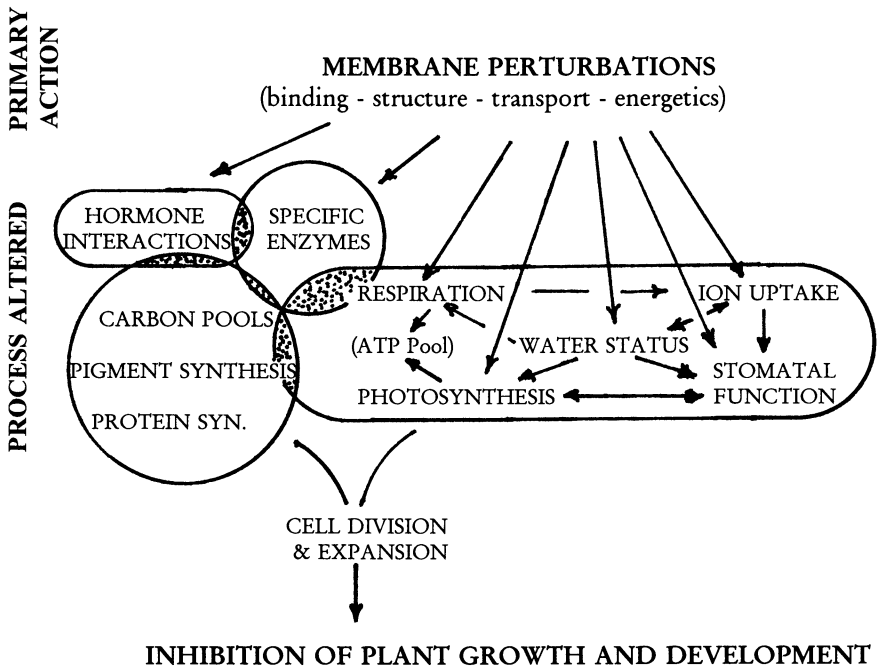

Figure 2. Proposed model relating the deleterious actions of phenolic allelochemicals. Arrows suggest a negative impact on the process and shaded overlaps imply likely interactions among domains. [Adapted from Einhellig (1)]

Permeability of the plasma membrane could be altered as allelochemical molecules partition into the matrix, thus disrupting structural associations, producing or modifying channels, and diminishing functions of enzymatic proteins like the ATPases. Such allelochemical actions would affect transmembrane ion transport and retention and water balance, which are factors impacting stomates

and the photosynthesis process. Dysfunctions from allelochemical entry into the special molecular associations of chloroplast and mitochondrial membranes may alter electron transport or uncouple this transport from phosphorylation, resulting in decreased ATP production. Possible deleterious effects from limited cellular ATP are extensive, including a feedback on molecular synthesis and other processes that maintain membrane integrity.

It has been postulated that several phytohormone actions are initiated from membrane reception sites or other membrane-mediated responses. Allelochemicals may interact with and alter these functions. Auxin synergism and antagonist activities of the cinnamic acids illustrate this potential. The action of plant hormones also may imply synthesis or activation of specific enzymes. These and other interrelationship were shown in Figure 2 by interlinkage of compartments.

The hypothesis of this model, that membrane perturbations are a common denominator in the action of several phenolic allelochemicals, should not be interpreted as defining a single site of action for these compounds. A more generalized cellular damage is postulated, and it is this collective detriment on multiple physiological processes that can be linked to inhibition of plant growth. I suspect the relative importance of different compartments of the model varies with different compounds and the concentrations of plant exposure. For a definitive insight into what are primary effects, data are needed on structural changes in membranes caused by the allelochemicals, and the relative concentration of a chemical in the plasma membrane, or other membranes, at the time phytotoxicity is expressed.

One can only speculate on how the action of nonphenolic allelochemicals may fit this interaction model. Pandey et al. (*70*) noted that the physiological effects of *Parthenium hysterophorus* residue included deterioration of membrane integrity. This allelopathic residue contains sesquiterpene lactones as well as phenolic acids, so both types of compounds may have contributed to the membrane leakage they observed. The allelochemical terpenoids and the two quinones, sorgoleone and juglone, will absorb into cell membranes because of their lipid solubility. Membrane dysfunctions from the presence of these nonphenolic allelochemicals certainly could initiate their actions, but no generalizations are feasible at this time.

Mechanism of *Sorghum* Allelopathy

The activity of *Sorghum* species can be used to develop an integrated view of how allelopathic inhibition may function. Weed and crop species of this genus that are allelopathic plants include *S. halpense, S. vulgare, S. sudanense*, and *S. bicolor* (*109-113*). Grain sorghum is an effective weed control cover crop, and field studies in Nebraska showed reduction in weed growth the season following grain sorghum cropping (*114*). Autotoxicity also has been reported from *Sorghums* (*115*).

A diverse group of compounds have been linked as the agents of *Sorghum* allelopathy. *Sorghum* species produce cyanogenic glycosides, tannins, several flavonoids, and phenolic acids such as ferulic, *p*-coumaric, syringic, vanillic, and

p-hydroxybenzoic acids (*116-125*). Recent evidence has added root exudate *p*-benzoquinones, known as sorgoleone, to the chemicals causing *Sorghum* allelopathy (*126-127*). Sorgoleone is the most toxic of the *Sorghum* compounds, but in the field situation no single compound is likely to be present in sufficient quantity to inhibit growth. The known complex of *Sorghum* allelochemicals, and perhaps others yet to be determined, undoubtedly work together in allelopathic interference. There is ample experimental evidence for additive or synergistic growth effects from combinations of allelopathic compounds (*95,128-129*). These compounds may also have additive inhibitory interactions with endogenous growth regulators like ABA (*130*).

Several primary sites of action at the cellular level are indicated for allelochemicals from *Sorghum* (Figure 3). The phenolic acids have multiple actions, as noted previously, but their impact on membrane structure and functions is central to most of these effects. Tannic acid has many generalized cytotoxic impacts, ranging from interference with enzymes to impacts on gibberellin activity. The known physiological actions of sorgoleone and flavonoids are on energy metabolism. Hydrogen cyanide, a breakdown product of cyanogenic glycosides, is a powerful inhibitor of mitochondrial function. Figure 3 does not illustrate a full list of metabolic interferences, but these and other cellular-level perturbations translate into inhibition of germination and growth that is evidenced in *Sorghum* allelopathy.

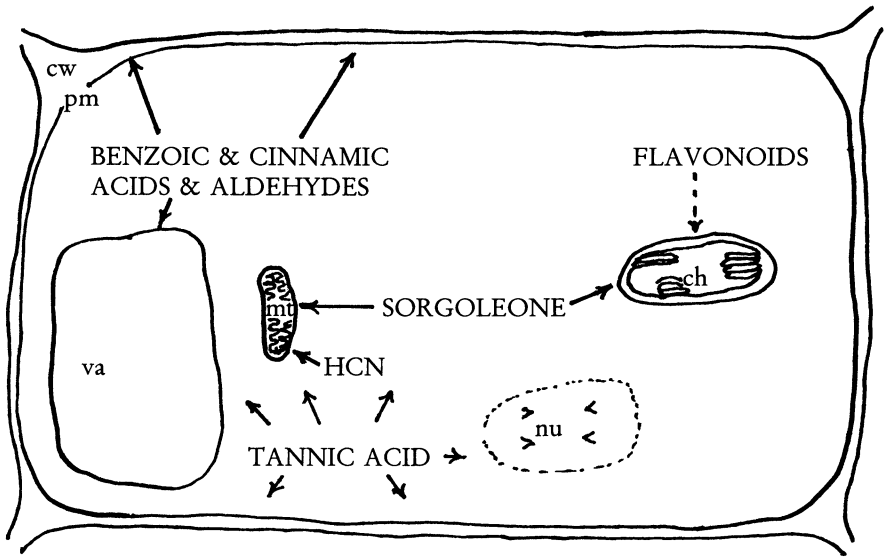

Figure 3. Model illustrating sites of *Sorghum*-allelochemical activity in cell physiology. Some primary sites of deleterious action are suggested by the arrows. cw = cell wall; ch = chloroplast; mt = mitochondria; nu = nucleus; pm = plasmalemma; va = vacuole.

The relative impact on plant growth by any one compound, or class of allelochemicals, must vary according to the soil microenvironment. Inputs of root exudates, such as sorgoleone, or phenolic acid releases from decomposing *Sorghum* tissue will not be uniform in a field. The allelochemical pool in the soil is also constantly changing through physical, chemical, and biological transformation of compounds, as well as removal of constituents by plants and microorganisms. In addition to variable exposures in the soil microenvironments, the extent of allelochemical damage will be magnified by temperature stress, moisture stress, and other suboptimal growth conditions (*95,131-132*). For example, inhibition of soybean seedling growth at 34° C requires less than half the concentration of ferulic acid that it does at 23° C (*131*). Such interactions between allelopathy and other stress factors could be extremely important in *Sorghum* allelopathy, since the species that have been listed are endemic in climates where temperature and plant-moisture stresses are common. In some cases, the joint action of allelochemicals and environmental stress is because both stresses alter the same process or structure. Certainly temperature and moisture stress, as well as phenolic acids, cause changes in the plasma membrane.

Conclusions

There is no simple equation for explaining the action of allelopathic chemicals that causes growth inhibition in higher plants. Likewise, since any specific case of allelopathic action in the natural environment may involve chemicals from several classes, we should recognize that different components of cell structure and physiology may be perturbed. The spectrum of data from the most studied allelochemicals, the phenolic acids and related compounds, indicates their activity on membranes triggers subsequent changes in physiology. However, it is likely that it is the combination of metabolic changes that impacts growth. As with a number of herbicides, the photosynthesis process is a sensitive target of quinone allelochemicals. Some flavonoids, sesquiterpene lactones and volatile terpenes also interfere with ATP generation, either photosynthetic or respiratory, but there is too little current data to infer much about their mechanism of action.

Further studies in this field should find it useful to evaluate cellular targets of herbicide action as potential points of allelochemical activity. However, most allelochemicals from higher plants are toxic to a broad spectrum of plants and microorganisms. I suspect the phytotoxicity of many of these compounds is not due to a localized site of interference, but instead, growth inhibition occurs from their action on multiple sites.

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Chapter 8

Allelopathic Properties of Alkaloids and Other Natural Products

Possible Modes of Action

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Out of a selection of 30 alkaloids and 26 other compounds 19 natural products were found with allelopathic properties and *in vitro* assays were carried out to elucidate their modes of action. Most compounds affect more than one molecular target: 8 compounds interact with DNA, 10 inhibit DNA polymerase I, reverse transcriptase, and protein biosynthesis and 3 lead to membrane leakage. It is suggested that the allelopathy observed is (at least) partly due to interaction of the compounds tested with these basic targets such as DNA and related processes, protein biosynthesis and membrane stability.

Plants compete with other plants for light, water and nutrients and have evolved complex strategies during evolution to cope with this problem. The production and accumulation of secondary compounds, which inhibit the germination or the development of other plants, is one way to enhance the fitness of a plant producing them. A number of plants and plant products with allelopathic properties have been reported (*1-3*), including many phenolics, terpenes and alkaloids (*1-4*).

Many secondary compounds are also toxic to animals and in many instances their modes of action have been elucidated already by biochemists, pharmacologists and toxicologists. This sort of knowledge and understanding is most often missing for natural products with allelopathic properties (*2*). Molecular targets which might be modulated in allelopathic interactions include membrane stability, enzymes, electron transport chains, photosynthesis, signal transduction, respiration, replication, transcription, protein biosynthesis, transport processes or hormone metabolism (*1-4*).

We have started to establish *in vitro* assays to elucidate possible interactions of a natural product with basic molecular targets whose intergrity is essential for survival and growth of plants (*4-7*) in order to understand the mechanisms of allelopathic activities. Here we have tested 30 alkaloids and 26 other compounds for allelopathic properties. For 19 active substances we have studied whether they interact with DNA, inhibit DNA polymerase I, reverse transcriptase, protein biosynthesis or destabilize biomembranes.

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ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Allelopathic Properties of Alkaloids and Other Natural Products

Using germinating seeds of *Lepidium sativum* we have assayed a selection of alkaloids and other natural products for their allelopathic properties (Table I). Since the biological variation was substantial we consider only deviations of 20% from untreated controls (which were run with each set of experiments under identical environmental conditions) to be meaningful. As can be seen from Table I radicle length was usually a more sensitive measure than hypocotyl growth.

If 1% solutions were applied we observed allelopathic effects for many compounds including inorganic salts, amino acids, and organic acids which are not considered to be specific plant growth inhibitors. To obtain more specific information we evaluated only the results of our natural products seen in 0.01 and 0.1% solutions.

Considering the inhibition of radicle elongation, allelopathic compounds (growth < 80% as compared to control; inhibitor concentration of 0.01%) are the alkaloids tryptamine, aconitine, colchicine, ergometrine, gramine, harmaline, papaverine, quinidine, quinine, salsoline, and the metabolic inhibitors cycloheximide and ethidiumbromide (Table I). For an evaluation of possible targets we have additionally included compounds which showed a 50% growth reduction in a concentration of 0.1%.: tannin, caffeine, cinchonine, lobeline, sanguinarine, theophylline, and essential oils of *Chamomilla, Mentha* and *Thymus*.

Interaction of Alkaloids with Molecular Targets

In a first approach we have chosen some basic targets whose integrity and function is essential for growth and development of all plants. These processes include interactions of allelochemicals with DNA and RNA, protein biosynthesis and membrane stability.

Replication and Interaction with DNA and RNA. We have established a number of assays to measure the potential interaction of a natural product with DNA. The measurement of the melting temperature of DNA (*8*) gave the most reproducible results (Latz-Brüning and Wink, in prep.). For the intercalating compounds mentioned in Table II, the melting temperature of DNA was augmented by more than 5 °C (Figure 1). A strong association between DNA and these natural products was also evident from assays with methyl green (*9*) or when DNA-alkaloid complexes were separated by gel filtration or agarose gel electrophoresis (Latz-Brüning and Wink, in prep.).

Compounds which strongly intercalate in DNA are usually inhibitors of DNA repair. As an experimental system we employed a modified "nick translation" assay (*10*) using DNA polymerase I (Figure 2). As can be seen from Table II, the intercalating compounds are indeed those which were inhibitory in this assay.

We did not employ a true transcription system but used a reverse transcription assay with poly A^+ mRNA and reverse transcriptase instead (Figure 3). All compounds which were found to intercalate in DNA and to block nick translation also inhibited reverse transcription (Table II). In addition, papaverine is an active compound in this context.

Inhibition of Protein Biosynthesis. As an experimental system we employed

Table I. Modulation of Radicle and Hypocotyl Growth of *Lepidium sativum* by Natural Products

	Effect (control = 100%)[a]					
	Hypocotyl Elongation			Radicle Growth		
Compound	0.01%	0.1%	1.0%	0.01%	0.1%	1.0%
Salts						
KCl	100	129	41	93	109	18
Na_2SO_4	94	94	44	108	81	11
NaCl	100	123	54	123	158	27
NaH_2PO_4	100	111	78	126	147	50
$NaNO_3$	94	113	50	72	62	19
Amino acids/amines						
Asparagine	89	95	84	106	91	28
Glycine	93	100	33	77	77	9
Lysine	111	100	28	122	83	22
Tryptamine	94	47	0	35	8	0
Sugars						
Sucrose	94	94	88	94	123	87
Phenolics						
Naringinin	100	100	100	114	116	108
Salicine	100	105	78	92	56	56
Tannin	107	60	27	89	27	9
Organic acids						
Ascorbic acid	94	106	75	103	103	52
Citric acid	100	100	64	109	147	22
Gibberellic acid	126	137	121	100	93	74
Tropic acid	105	113	0	100	49	0
Alkaloids						
Aconitine	125	0	.	75	10	.
Ajmalicine	116	104	.	104	110	.
Atropine	100	100	20	90	70	13
Berberine	101	38	.	79	16	.
Caffeine	92	31	0	72	17	0
Canadine	118	111	.	111	93	.
Chelidonine	119	117	.	114	71	.
Cinchonine	110	16	.	121	6	.
Colchicine	35	31	.	10	8	.
Cytisine	100	77	.	79	46	.
D-Ephedrine	115	105	.	87	64	.
L-Ephedrine	100	125	112	93	93	58
Ergometrine	115	65	.	68	29	.
Ergotamine	125	130	.	83	75	.
Gramine	94	70	.	70	44	.
Harmaline	70	1	.	19	8	.
Hyoscyamine	100	100	28	108	114	9

Continued on next page

Table I. Continued.

	Effect (control = 100%)[a]					
	Hypocotyl Elongation			Radicle Growth		
Compound	0.01%	0.1%	1.0%	0.01%	0.1%	1.0%
Lobeline	95	9	.	83	10	.
Narcotine	113	94	25	118	112	3
Nicotine	104	93	.	80	60	.
Papaverine	105	28	6	51	2	2
Quinidine	111	8	.	76	11	.
Quinine	100	18	0	73	7	0
Salsoline	89	64	.	50	13	.
Sanguinarine	86	57	.	82	15	.
Scopolamine	105	103	.	132	131	.
Sparteine	100	100	25	92	94	16
Strychnine	114	76	.	146	73	.
Theophylline	100	37	0	84	27	0
Tropine	100	131	61	94	100	33
Metabolic inhibitors						
Cycloheximide	0	0	0	0	0	0
Ethidiumbromide	42	0	0	11	0	0
Terpenes/Essential oils						
Balm mint	87	73	.	70	71	.
Chamomilla	89	0	.	106	0	.
Citrus	95	71	0	93	71	0
Eucalyptus	115	87	.	108	98	.
Foeniculum	104	89	.	89	85	.
Mentha	86	0	.	87	0	.
Picea	112	90	.	93	77	.
Saponin	105	94	35	89	31	9
Thymus	83	0	.	81	0	.

[a]50 seeds were employed in each assay and all experiments were performed in dublicates; values represent means; growth of controls, which were run for each compound, was set 100% (*5*); · = not determined.

Table II Molecular Targets Affected by Allelopathic Compounds[a] (of Table I)

Compound	DNA Inter-actions[b]	DNA Pol I[c]	RT[d]	Protein bio-synthesis[e]	Membrane leakage[f]
Phenolics					
Tannin		*	*	*	
Alkaloids					
Aconitine					
Berberine	*	*	*	*	
Caffeine					
Cinchonine	*	*	*	*	
Colchicine					
Cytisine					
Gramine					
Harmaline	*	*	*	*	
Lobeline	*	*	*	*	
Papaverine				*	*
Quinidine	*	*	*	*	
Quinine	*	*	*	*	
Salsoline				*	
Sanguinarine	*	*	*		*
Metabolic inhibitors					
Cycloheximide				*	
Ethidiumbromide	*	*	*		
Terpenes/Essential oils					
Saponin					*
Chamomilla					*

[a]Compounds which inhibited radicle or hypocotyl growth by more than 50% at a concentration of 0.1% were considered to be allelopathic.

[b]DNA-binding was assessed by measuring the melting temperature of DNA (*8*) and displacement of methylgreen (*9*).

[c]DNA polymerase I was tested in a "Nick translation" assay (*10*).

[d]Reverse transcription (RT) was tested in a "cDNA-assay" (*10*).

[e]For protein biosynthesis a reticulocyte lysate was employed (*10*).

[f]Membrane stability was determined in erythrocytes: released hemoglobin was measured photometrically.

* = significant activity

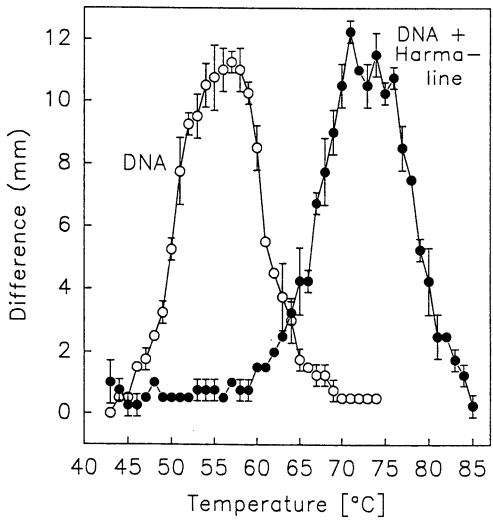

Figure 1 Influence of a DNA intercalating alkaloid (Harmaline) on melting temperature.

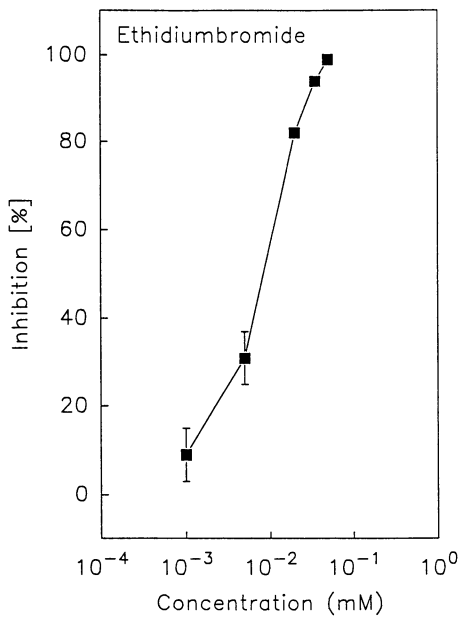

Figure 2 Inhibition of the "Nick Translation Assay" by ethidiumbromide.

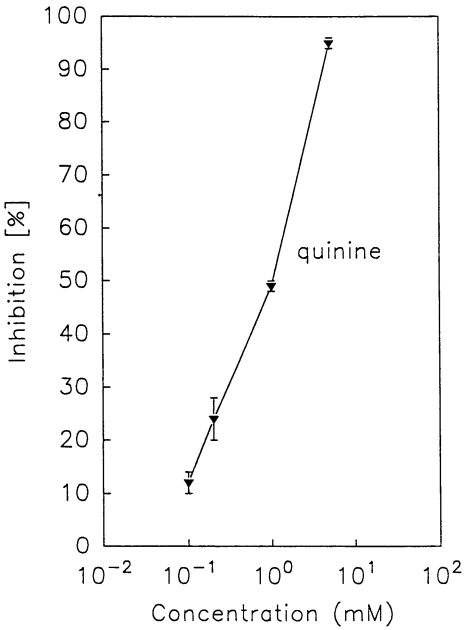

Figure 3 Inhibition of reverse transcription by the alkaloid quinine.

a reticulocyte lysate to which mRNA was added (Figure 4). As can be seen from Table II nearly all compounds which interfere with DNA and reverse transcription also inhibit protein biosynthesis substantially. Sanguinarine and ethidiumbromide which were active in the former assays are inactive in translation assays (but sanguinarine was tested at a low concentration only). Cycloheximide and to a minor degree salsoline are specific translation inhibitors. Tannin is a potent protein complexing agent which inhibits most enzymes; its activity in the DNA polymerase, reverse transcriptase and translation assay is therefore not surprising.

Induction of Membrane Leakage. We have chosen erythrocytes as an assay system and measured hemolysis (as an indicator for membrane leakage) photometrically (Figure 5). Only sanguinarine, saponin and essential oil from *Chamomilla* proved to be membrane destabilizing (Table II).

How to Explain These Allelopathic properties?

The allelopathic compounds berberine, cinchonine, harmaline, lobeline, papaverine, quinidine, quinine, sanguinarine and ethidiumbromide affect several molecular targets at the same time. Since these targets are basic for the functioning of a cell, it is likely that these interactions are responsible for the allelopathic effects observed. This does not rule out that additional targets are also envolved.

Cycloheximide and salsoline are translation inhibitors; at least for cycloheximide this target seems to be sufficient to explain the toxicity observed. The allelopathic effect of saponins and essential oils is probably due to interference with biomembranes, as measured *in vitro*.

Whereas tannin is an extremely potent protein inhibitor *in vitro*, its *in vivo* activity is only moderate. Tannin is a polar compound which cannot be resorbed easily by plant cells. Thus it cannot exort comparable detrimental effects *in vivo*.

For a few compounds, such as aconitine, caffeine, colchicine, cytisine, and gramine no active target was found in our assays. A few pharmacological properties have been reported for these compounds (*4*): Aconitine modulates Na^+ channels, caffeine inhibits phosphodiesterase, colchicine is a microtubuli blocker and thus prevents cell division, and cytisine binds to nicotinic acetylcholine receptors. It needs to be studied whether these targets are also relevant for allelopathy. Gramine might be an auxine modulator, because of some structural similarities.

In order to be effective in nature these compounds must be produced in high amounts by a plant and released to the soil either by active secretion from the rhizosphere or by leaching from leaves, stems, seeds or roots. Concentrations must be high enough in soil to reach inhibitory levels, but this depends on the type of soil, microbial degradation, thermal effects and drainage, only to mention a few variables which may affect allelopathy.

Our study shows that molecular targets can be identified for most compounds which plausibly explain their allelopathic activities. For most compounds more than one target seems to exist which is a common strategy for defence compounds of plants which have to protect against a wide variety of organisms ranging from microorganisms, other plants to arthropods and vertebrates (*3,4*). "Evolutionary molecular modelling" as we might describe this process obviously used several tar-

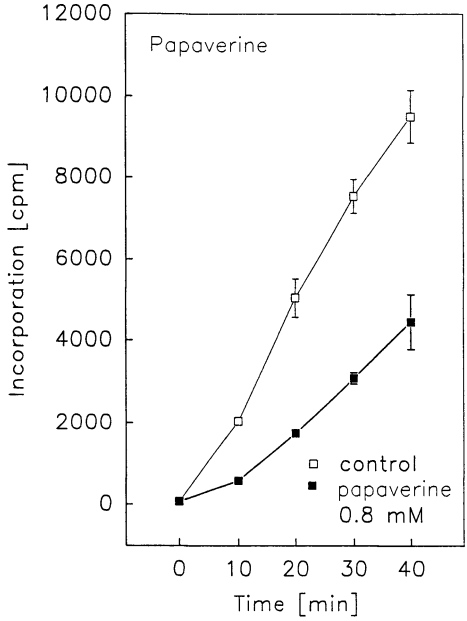

Figure 4 Inhibition of protein biosynthesis ("In Vitro-Translation") by the alkaloid papaverine.

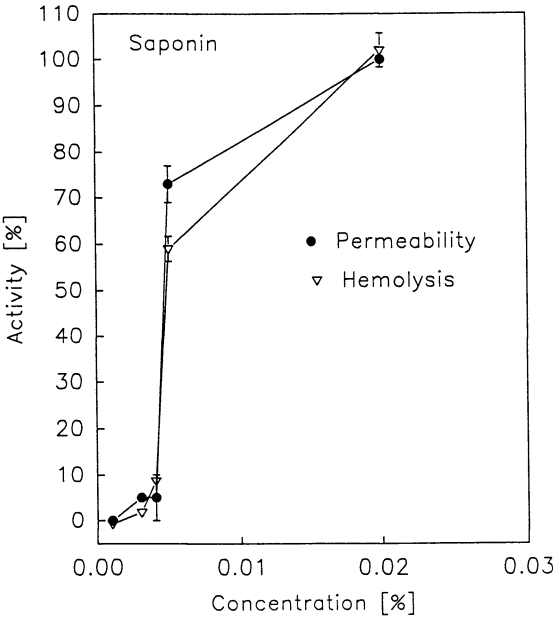

Figure 5 Induction of membrane instability (permeability and hemolysis) by the triterpen saponin.

gets to obtimize the structures of defence chemicals which is in contrast to modern chemical approaches where "molecular modelling" is orientated towards a single target.

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Chapter 9

The Value of Model Plant–Microbe–Soil Systems for Understanding Processes Associated with Allelopathic Interaction

One Example

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This review summarizes what happens to simple phenolic acids once they enter the soil environment and how plants and soil microbes growing in these soils respond to phenolic acids. This is a review of one plant-microbe-soil model system and what it tells us about the set of processes (e.g., source-sink relationships) that ultimately determine the available concentrations of phenolic acids in soils and thus how such processes may determine the magnitude of allelopathic interactions in natural and managed ecosystems.

The extensive literature of the potential roles of phenolic acids in plant-microbe-soil systems was summarized in three recent articles by Kuiters (*1*), Siqueira *et al.* (*2*) and Appel (*3*) and will not be repeated here. These three articles, in conjunction with earlier summaries (e.g., Rice, *4*), bring together information from a variety of systems, subsystems, environments, and species. They provide a listing and general overview of the set of processes that ultimately determine the allelopathic effects of phenolic acids in natural and managed ecosystems. The importance of these various processes and their interactions with each other in determining allelopathic effects of phenolic acids in natural or managed ecosystems will vary abruptly or continuously through time as a function of changes in the physicochemical and biotic environment. The unpredictable nature of natural and managed ecosystems led us to study a plant-microbe-soil system in the laboratory. Detailed studies of such a model system, we hoped, would help in characterizing the volatility or stability of individual processes in determining allelopathic interactions of phenolic acids in soils and thus might suggest what should be monitored in natural and managed ecosystems in order to detect and characterize allelopathic interactions. The techniques presently being used to determine the presence, magnitude, or absence of allelopathic interactions in natural and managed systems provide, at best, only circumstantial evidence, and some would argue these are inadequate.

The Model System

One purpose of studying model systems, which are simplified representations of natural systems, is to identify and to clarify important processes and to conceptualize how such

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processes may interact. One outcome of this modeling effort will be the identification and characterization of techniques that may be used to study such processes in nature. To achieve these goals (i.e., identification and characterization of processes and techniques) will require a model system composed of a phenolic acid sensitive species, and nutrient and soil culture techniques which provide an environment for rapid growth of this species. By proper manipulations of these system components, the effects of phenolic acid on seedlings and soil microbes may be studied in the absence (nutrient culture) and presence of soil materials. In this way individual processes (phenolic acid uptake by seedlings, microbial utilization of phenolic acids, soil sorption, etc.) and plant responses (nutrient and water uptake, growth, etc.) may be studied individually and in various combinations.

Our model system was composed of cucumber seedlings with a maximum age of 20 days (*5, 6 , 7*) and occasionally other species (*8 ,9, 10*), Portsmouth soil, and in some cases Cecil soil, materials (*11, 12*), resident soil microbes (*13, 14*), nutrient solutions (*15*), and various cinnamic acid (e.g., ferulic acid, p-coumaric acid) and benzoic acid (e.g., p-hydroxybenzoic acid, vanillic acid) derivatives. These components were chosen to maximize the chance of observing and manipulating the processes associated with allelopathic interactions.

Nutrient Culture. Seeds were germinated in the dark at 28 to 30 ^{o}C in trays containing sterile vermiculite and full strength Hoagland's solution. After 48 hr, the seedlings were transferred to 120-ml glass snap-cap bottles containing full-strength Hoagland's solution. Seedlings were suspended in nutrient solution through a hole in the cap of the bottles and held in place by a foam collar. The snap-cap bottles were placed under laboratory light banks which provided 130 to 150 μEinsteins/m^2/sec for 12 hr per day. The temperature under the light banks ranged from 21 to 30 ^{o}C. Seedlings were treated with phenolic acids dissolved in full-strength Hoagland's solution. Solutions were normally adjusted to pH 5.8. Solutions in bottles were replaced every other day.

Soil Culture. Seeds were germinated or transplanted into 155 ml plastic cups containing 150 g of soil : sand mixtures (1 : 2 by weight). Seedlings grown under the light banks were supplied with double strength Hoagland's solution every other day. Sufficient distilled water was added daily to bring the weight of any given cup (160 g for cup and soil) and its seedling to approximately 190 g. Cup weights, including seedlings, were always kept above 170 g. Phenolic acid solutions were added every other day and alternated with nutrient solution applications.

Based on studies using the above culture techniques and sterile and inoculated soil samples in test tubes we can make the following observations about the behavior of seedlings, microbes, soils and phenolic acids in our model system:

Seedling Behavior in the Presence of Phenolic Acids.

1. Effects of phenolic acids on plant processes (e.g., water utilization, transpiration, leaf and root growth) are concentration dependent and readily reversible once phenolic acids are removed from the root environment (*5, 6, 16, 17*).

2. Effects of cinnamic acid derivatives on plant processes are greater than those of their corresponding benzoic acid derivatives (*5, 18*).

3. Interactions with and uptake of phenolic acids by roots, and thus effects on plant processes, appear to be associated with the protonated and not the anionic form of the phenolic acids (*6, 7*). The pKa values of the cinnamic acid and benzoic acid derivatives are approximately 4.5.

4. Rates of phenolic acid uptake by seedlings vary with phenolic acid, phenolic acid concentration, and solution pH. Uptake of one phenolic acid may or may not be reduced in the presence of a second phenolic acid (*19, 20*).

5. The primary effect of phenolic acids appears to be to reduce hydraulic conductivity and net nutrient uptake by roots (*10, 20, 21, 22*). Reduced rates of photosynthesis and carbon translocation to roots (*17*), increased abscisic acid levels (*9*), reduced rates of transpiration (*5, 6*) and leaf expansion (*5, 16*) appear to be secondary effects.

6. Exposure of cucumber seedlings to chronic levels (i.e., low concentrations) of phenolic acids prior to acute level exposure (i.e., toxic levels) does not attenuate seedling growth responses to acute levels (*14, 16, 23*).

7. Reductions in growth, water utilization, and net nutrient uptake by phenolic acids are related directly to the proportion of the seedling root system in contact with the phenolic acids (*22, 23, 24*).

8. Effects of phenolic acids on cucumber seedling growth are not modified by different nutrient supplies (*16, 25*).

9. As the number of phenolic acids in the root environment increases, the concentrations of the individual phenolic acids in the mixture required to bring about a specific seedling response decline. Effects of individual phenolic acids in a mixture may be additive, partially synergistic, or partially antagonistic (*5, 18, 20, 23*).

10. The addition of either carbohydrates (e.g., glucose) or amino acids (e.g., methionine) in conjunction with phenolic acids to soil materials also reduces the concentration of phenolic acids required to bring about a specific plant response (*26*).

Soil Material and Microbe Behavior in the Presence of Phenolic Acids.

1. Addition of phenolic acids to sterile soil materials results in the immediate sorption of a fraction of the phenolic acid added. For cinnamic acid, but not benzoic acid, derivatives there follows a slow, continuous nearly linear sorption over time. The rates of sorption vary with the type of phenolic acid (*27, 28, 29, 30*).

2. The amount of sorption of phenolic acids in soil materials is directly related to the concentration of the phenolic acid added to the soil materials. Sorptions of individual phenolic acids in a mixture appear to be independent of each other (*30*).

3. Sorption of phenolic acids in soil materials is greatest under neutral or slightly basic conditions and increases with increasing organic matter and multivalent cation content of the soil materials (*27*).

4. Only a fraction of the sorbed phenolic acids are reversibly bound and available to soil organisms (*12, 30, 31*).

5. Microbial utilization and/or modification of phenolic acids, in the presence of adequate nutrition and moisture, is much greater than sorption by soil materials (*11, 13, 30*).

6. Addition of phenolic acids to low organic matter soil materials brings about an induction and/or selection of microbes which are able to utilize phenolic acids as a sole carbon source (*13*).

7. Populations of rhizosphere microbes increase when phenolic acids are added to soil materials at concentrations that are inhibitory to cucumber seedling growth (*14*).

What Does this Model System Tell us About the Processes Associated with Allelopathic Interactions?

Phenolic acids at inhibitory levels must be provided continuously over a sufficient time period and to a significant proportion of the root system to suppress seedling growth. If the time period is too short or the proportion of the root system in contact is too small, seedling biomass will not be affected or will recover fully once phenolic acids become sufficiently depleted within the rhizosphere. Depletion occurs via uptake, soil sorption and fixation, and microbial activity (leaching could not occur in this closed system). For a given concentration of phenolic acid, maximum suppression would appear to occur in acidic, low organic matter, and slightly nutrient- and moisture-limited soils. This, however, is only true when an independent supply of phenolic

acids is available, as was the case for our system. Since the major source of the phenolic acids in most soils is decomposing and leaching plant debris (although leaf leachates and root exudates can also be important), sufficient plant debris, adequate but not excessive moisture, and nutrition for the release of phenolic acids from debris is essential. Thus, the balance in source-sink relationships (i.e., fluxes), the distribution of roots, distribution of phenolic acid sources in the soil, the physicochemical environment, and the sensitivity of a seedling (stage of development (*32*); taxon (*9*)) are all important parameters for determining the level of allelopathic effects by phenolic acids. These foregoing conclusions are in general agreement with the literature summarized by Kuiters (*1*), Siqueira *et al.* (*2*) Appel (*3*) and Rice (*4*).

The debate about whether allelopathic effects of phenolic acids are important in natural or managed systems has primarily focused on the low available phenolic acid concentrations present in soils (*33, 34 ,35*) compared to the much higher concentrations of phenolic acids in nutrient culture (*5, 6, 16*) and soil culture (*11*) required for the inhibition of plant processes. Since most of the published bioassay studies were carried out under adequate to excellent growth conditions and with single phenolic acids (i.e., model compounds), the high concentrations necessary for inhibition of plant processes should not be entirely surprising. Natural or managed soil systems, however, are frequently growth limiting and contain complex mixtures of organic compounds, including phenolic acids, which may act in concert at very low individual concentrations to suppress plant growth. Such behavior for 2-, 3-, and 4-way phenolic acid mixtures and for mixtures of glucose or methionine with p-coumaric acid has been demonstrated in our plant-microbe-soil system (*7, 18, 26*) and to a certain extent with water-autoclaved extracts from no-till wheat soils (*31*). Concentrations of individual phenolic acids in these instances were similar or close to phenolic acid concentrations extracted from native or managed soils by water and calcium hydroxide (*34, 35 , 36*). Concentrations of phenolic acids in soil extracts, however, clearly will not provide an adequate means by which to determine the importance of allelopathic interactions in ecosystems. What is much more important, aside from the sensitivity of the plant taxon to phenolic acids, is the rate and timing of soil input or soil production of phenolic acids, and the "competitive nature" of roots as sinks for phenolic acids in soils (i.e., the rates of uptake and/or interaction). Residual phenolic acid concentrations in soil or even net changes of phenolic acids in soil solution will not provide insights concerning phenolic acid uptake or interactions with plant roots.

Definitive insight about the role of phenolic acids in allelopathic interactions clearly requires a systems approach. In general, data are needed on the turnover rates of phenolic acids in systems. More specifically, what are the factors that control and determine the rates of input and generation of phenolic acids in soils? How will the competitive nature of sinks (e.g., clays, organic matter, roots, seeds, microbes) for phenolic acids in soils change as physicochemical (e.g., types of phenolic acids and their distribution, pH, soil moisture, nutrition, organic compounds other than phenolic acids) and biotic characteristics (e.g., microbial populations, plant density, root distribution and surface area, plant maturity) of plant-microbe-soil systems change? Answers to these questions are not presently available, but these questions must be answered if we are to definitively demonstrate the importance of allelopathic interactions in natural and managed ecosystems. In this author's considered opinion, and given the difficulty of answering such questions in nature, the forgoing questions should first be studied in model systems where correlations between various system parameters and characteristics can be established. Thereafter, the insights gained should be applied to studies of natural or managed ecosystems. I am convinced this is the only way that the importance of allelopathic interactions to natural and managed systems will ever be resolved.

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Chapter 10

Characterization of the Mechanisms of Allelopathy

Modeling and Experimental Approaches

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Evidence merely showing that allelochemicals extracted from the roots of host plants could cause adverse effects on the root systems of surrounding plants does not constitute conclusive proof that these chemicals are the ones actually causing allelopathy under natural conditions. To establish the cause-and effect relationship, one needs to demonstrate that a sequence of events has occurred, as evidenced by production of these chemicals by the host plant, their transport from the host plant to the affected plants in the surroundings, and exposure of affected plants to these chemicals in sufficient quantity for sufficient time to cause the observed allelopathy. A key to deciphering the mechanisms of allelopathy could be through an understanding of such soil processes as retention and transformation which affect the fate and transport of allelochemicals. Modeling and experimental efforts in characterizing chemical transport must consider the biological and environmental factors affecting transport of chemicals and the unique plant-plant and plant-soil interactions at the scale specific for allelopathy to occur.

The phenomenon of allelopathy has been well recognized and widely reported (e.g., see *1-5*). The extensive literature attests to the progress made during the past decade. Most of the reports have provided gross evidence on adverse influence of host plants on affected or target plants in the surroundings. Less is known about the actual mechanisms involved in the cause-and-effect relationship between allelochemicals and allelopathy. Reported evidence implies that allelopathy is caused by transmittal of allelochemicals through the soil from the host to the target plants in the surroundings. For instance, specific chemicals have been extracted from the roots or the root environment of host plants, and when these chemicals are applied to the root systems of surrounding plants, adverse effects to these plants can be demonstrated. Other studies have shown that allelochemicals could also be transmitted through the air; or leached out of dead organic residues. Regardless of

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the sources of allelochemicals, the available evidence has not been able to provide a complete proof that the observed allelopathy was actually caused by the allelochemicals identified. To establish that the allelopathic effect is actually caused by specific chemicals, it will be necessary to demonstrate that a sequence of events must have taken place. It includes production of the specific chemicals by the host plant, transport of these chemicals from the host plant to the target plants in the surroundings, and exposure of target plants to these chemicals in sufficient quantity for sufficient time to cause the observed effect. Because chemicals can react with soil components in various ways and be prevented from reaching the target plants, a key to elucidating the mechanisms of allelopathy is through an understanding of the processes and influencing factors involved in the transport of allelochemicals from the host through the soil environment to the target plants.

Cheng (*6,7*) has proposed a conceptual framework for depicting the mechanism of allelopathy in the soil environment. The proposed model can be divided into three parts: (a) introduction of the allelochemical into the root environment, (b) transport of the allelochemical from the roots of the host plant to the roots of the surrounding target plants, and (c) exposure of the target plants to the specific allelochemical. Whereas Parts a and c have often been studied, this model emphasizes the need to consider Part b, concerning the fate of allelochemical in the soil environment during its transport from the host to the target plants in the surrounding, as critical in understanding the mechanism of allelopathy. The interactive nature of the retention, transformation, and transport processes and the influence of chemical, soil, climatic, and biotic factors affecting the fate of allelochemicals in the root environment are graphically presented in the conceptual model (Figure 1). This model clearly delineates how the transport process can be affected by the retention and transformation processes and provides the framework to develop both simulation models and experimental approaches applicable to characterize the fate of allelochemicals during their transport in soils.

Although what needs to be done to prove the existence of allelopathic relationship has been discussed extensively, how one can actually carry out a study to obtain such proof can still be problematic. For instance, many chemicals have been postulated to be allelopathic, but few have been monitored closely enough to show that their presence and participation in the sequence of events would meet the criteria for establishing the specific cause-and-effect relationship of allelopathy. If the identity of the allelochemical is uncertain, its transport and fate in the soil would be difficult to characterize. Few studies on allelopathy have been aimed to tackle the difficult problems in understanding the mechanisms of allelopathy. This paper is an attempt to outline considerations critical to designing models and experimental studies for characterizing the mechanisms of allelopathy in soils. The intent of this paper is not to decipher the mechanisms per se, but to provide some practical pointers on how to approach such studies for those intending to initiate studies on the fate of allelochemicals in soils and soil-related allelopathy research. To simplify the discussions, examples used will mostly be based on transport of allelochemicals from the rhizosphere of host to roots of target plants, although considerations should be similar for allelochemicals from plant residues.

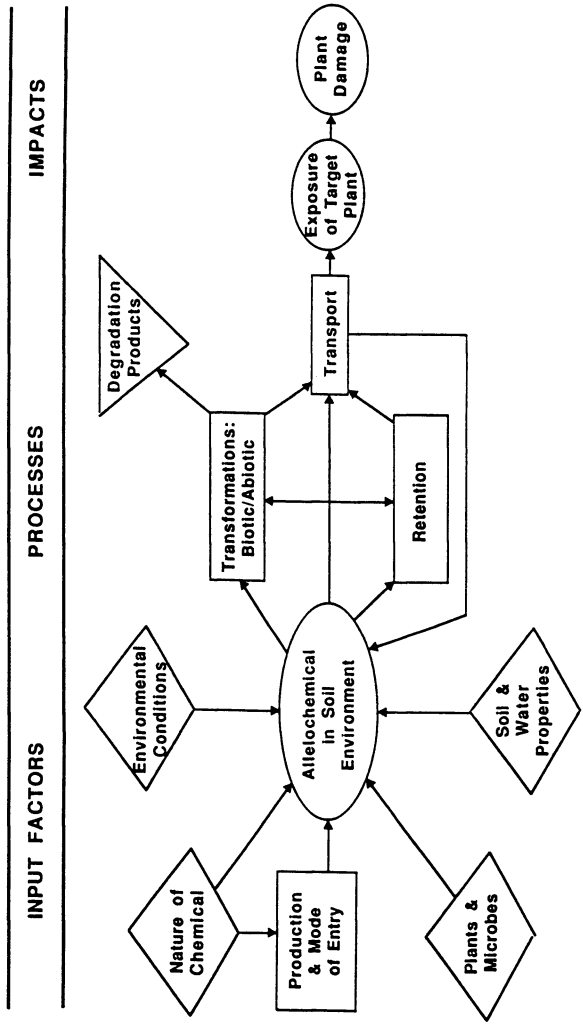

Figure 1. A conceptual framework depicting the relationship of the processes and factors affecting the transport of allelochemicals from the site of production to the target plant (Reproduced with permission from Ref. 6. Copyright 1989 Academia Sinica.)

Modeling Considerations

There is a wealth of literature on the fate and transport of organic chemicals in the soil environment. Although few of these studies were concerned with actual allelochemicals, studies dealing with trace organics and xenobiotics may be used as relevant guides to understand the behavior of allelochemicals. Especially useful are the literature on modern organic pesticides in the environment. Many models have been proposed to depict the various processes involved in the transport of pesticides in soils (see *8*). However, it should be recognized that although the principles developed for describing the fate of one type of organic chemicals in the soil may be applicable to describe the fate of other types of organics, there could also be significant differences in the behavior of these chemicals in the environment. Indiscriminant adoption and application of models without recognizing these differences could lead to significant errors. This discussion intends to point out some of the important differences between the behavior of allelochemicals and pesticides in the soil environment, and highlight precautions necessary to transfer knowledge from one field to the other.

Identity of Allelochemicals. A major difficulty in studying allelochemicals is the lack of knowledge of the identity of the chemicals involved in allelopathy. A large number of chemicals are known to be phytotoxic. Many of these chemicals have been extracted from root systems of host plants and, when applied to the root systems of target plants, they could cause damage to the root growth of target plants. Such evidence, however, does not exclude the possibility that certain undetermined chemicals are also present in the root systems, which may be the real culprit in causing the observed allelopathy. Often symptoms of allelopathy are reported in the literature in terms of the gross physiological responses of target plants, rather than the actual biochemical mode of action. The observed symptoms may be remote from the site of infliction, or delayed in time. As a result, it may not be easy to establish the identity of specific allelochemicals involved. Yet, knowing the identity of the allelochemicals is essential before any transport studies can be meaningfully carried out.

Entry of Allelochemicals Into Root Environment. The major difference between the introduction of pesticides and that of allelochemicals into the environment is their mode of entry. Pesticides are normally introduced in discrete entities (e.g., applied periodically to a specific volume of soil). Allelochemicals produced by plant roots could be exuded from different parts of roots sporadically throughout the rooting zone in response to specific triggering mechanisms or continually in an indiscriminant manner. Since pesticides are usually introduced into the soil in small quantities, except when they are accidentally spilled, their behavior is likely to be defined by the characteristics of the surrounding soil environment. In contrast, the allelochemicals can be exuded into the soil in sufficient quantities over time that they could exert a considerable influence on the surroundings or even modify the characteristics of the root environment, e.g., pH or the microbial population density, distribution, and activity. Thus, while pesticide behavior can be assessed by characterizing a representative sample of the soil, how the allelochemicals behave

must be studied in the specific environment in which the allelochemicals are present. It will be necessary to define and identify the loci of allelochemical production before soil samples can be correctly taken and analyzed, and before procedures for experimental verification of models can be designed and carried out.

Allelochemical Transport in Soil. Chemicals can be transported in the soil in the vapor phase or as a solute in the aqueous soil solution. As plant roots in the soil are surrounded by aqueous solutions and mucigels, the allelochemicals exuded by roots are likely to be solutes. Solutes in soil are transported either by mass flow as water moves in the soil or by diffusion in the soil solution along a concentration gradient, moving from higher to lower concentration areas. Since allelochemical concentration is understandably higher at the root surface, one can perceive that diffusion is involved in the transport of allelochemicals from roots to surrounding soils. On the other hand, roots normally absorb water from soil. Water in the vicinity of roots tends to move from the bulk soil to the roots, in opposite direction from the dispersal of allelochemicals. Thus, mass flow is not likely a dominant process for transport of allelochemicals in the soil. Consequently, many pesticide transport models based on water flow may not be useful or appropriate for depicting allelochemical transport in the soil.

Processes and Factors Influencing Allelochemical Transport. Many processes and factors in the soil can influence the transport of organic chemicals in the soil (*8*). The major processes are retention and transformation. The retention process may be reversible or irreversible. Irreversible retention is a sink that removes the chemical from being transported, whereas reversible retention merely retards the allelochemical transport. Most models assume the retention process as a partition of the chemical between the aqueous solution and the solid soil particle surface, implying that the process is reversible and in equilibrium. The kinetics of retention and the activation energy required to release the chemical from soil particle surface to soil solution are often ignored. If the assumptions are not valid, the model could be erroneous in simulating the actual occurrence.

Similarly, the transformation process can render a chemical ineffective in its allelopathic functions. Most attention has been given to the microbially-mediated transformation as the dominant process in the degradation of allelochemicals. The root environment is rich in microbial population and in energy and nutrient supplies. Whether a chemical can survive the attack by microbes during its transport from the host to the target plants must be taken into considerations in any modeling effort. The allelochemicals are by design highly reactive. Therefore, chemical reactions between an allelochemical and soil minerals are a distinct possibility. For instance, many phenolic acids are rapidly oxidized by manganese(IV) oxide, which are commonly present in soils (*9*). This type of chemical oxidation can transform these chemicals so rapidly that their allelopathic potential may not be expressed before the chemical is transformed and loses its phytotoxicity.

In addition to the retention and transformation processes that compete with the transport process, many factors can also affect the kinetics of the transport. Major among the factors are: the stability and reactivity of the allelochemical, physical and chemical properties of the soil (e.g., bulk density, hydraulic

conductivity, pH, cation exchange capacity, organic matter and clay contents), environmental conditions such as temperature and moisture, and the growing stage and biological activities of the plants and microbes present. The influence of soil organic matter contents on allelochemical stability was demonstrated by Lehmann and Cheng (*10*), who found that phenolic acids were more stable in the surface forest soils than in cultivated agricultural soils because of the high organic matter in the forest litter tends to reduce the reactive manganese oxide surface. This reactivity could be restored if the organic matter is removed by oxidation.

To understand which factors may influence the allelochemical transport process, one needs, moreover, to appreciate the special nature of the root environment or rhizosphere. To be of use, models must be able to depict the appropriate processes involved in the transport of allelochemicals under the specific environmental conditions and factors surrounding plant roots. In spite of its importance, the rhizosphere environment is seldom studied directly. The main obstacle has been the lack of appropriate tools and methodology for direct measurements and characterization of the rhizosphere environment. Consequently, effort to develop mechanistic models to simulate the rhizosphere environment also suffers because of lack of means for validating the model.

Spatial and Temporal Scale of Modeling. A key consideration in developing models to describe the mechanism of allelopathy is the scale of the sphere of allelopathic influence in relation to the retention, transformation, and transport processes shown in Figure 1. Little information is available on the distance an allelochemical is transported from the host plant to reach the target plants. One can only assume that the shorter the distance, the more influence the host plant will have on its surroundings. The farther a chemical has to be transported, the less is its potential to influence the target plants. If diffusion is the predominant process of allelochemical transport, the sphere of allelochemical influence will then be limited to the distance which these chemicals can diffuse within a reasonable time frame. If the chemical is retained by the soil to some degree during transport, the effective distance would be even shorter. This is also true in terms of the time required for transporting the chemical from the host to the target plants. The shorter the time, the more would be the impact, and vice versa. How far and for how long the chemical can be transported will depend upon how stable it is. If the chemical is transformed or degraded, the chemical's concentration would be decreased along the way, losing its allelopathic potential. Thus, all modeling efforts must consider both the spatial and temporal scale of the sphere of the allelopathic influence of the chemical.

Experimental Considerations

Before any experimental studies on the fate and transport of a chemical in the soil environment can be examined, it will be necessary to develop reliable analytical methods to determine quantitatively the amount of the chemical present in the system at any given time. Although this is an essential requirement in any experimental work, little attention has been given in the literature on the identification and quantification of the specific allelopathy-causing chemicals in the

soil. For proper methodology development, one first has to define what chemical to measure and then to know how to measure it. However, the identity of the specific chemicals may not be so easily established, since a large number of chemicals are present in the soil. A chromatogram of a soil extract may have innumerable peaks. Seldom have more than a few of the peaks been identified in any studies. Only when the identity of a specific allelochemical is known can such techniques as gas or liquid chromatography be useful. Chromatography in combination with various identification techniques, such as mass spectrometry, now available can be particularly useful in isolation and quantification of allelochemicals.

Many studies have been published describing analysis of likely allelochemicals. Most have concentrated on the determination step of the analysis but have not given much attention to the sampling and sample preparation steps before the determination step. Yet, the validity and usefulness of analytical results are affected by the appropriateness of the sampling and sample handling procedures as much as by the accuracy and precision of the determination step. Often not addressed are the difficult problems of how to collect the soil to obtain a representative sample; how to handle, treat, and store the soil sample to avoid or minimize alteration of the status of the soil; and how to extract the specific chemical from the soil for analysis without causing changes to the chemical.

Soil Sampling. First, it is necessary to decide what constitutes a proper soil sample. The more one can pin-point the sites of allelochemical production and transport, the better can one depict the mechanisms of allelopathy. As pointed out in the previous section, transport of allelochemicals may be limited to very short distances, e.g., in the rhizosphere of the host plant. Therefore, samples to be taken must be representative of the narrow zone of soil around the roots of the host plant. Once the sites of sampling are delineated, precaution should be given to preserving the existing conditions of the soil during the sampling procedure. Even then, what constitutes an appropriate sample may still be difficult to define. Rhizosphere soil offers a particularly difficult challenge, as it contains significant amounts of root fragments. Decisions have to be made whether these fragments should or should not be part of the soil sample.

Soil Sample Handling. Once an appropriate sample is obtained, one has to know how to preserve its natural state. Since allelochemicals by nature are very reactive, preserving the natural state of the soil during sample handling can be a demanding task. For instance, many of these chemicals participate readily in oxidative reactions and would be biologically stable only under anaerobic conditions. Furthermore, even under anaerobic conditions, chemical oxidation can still take place, as long as there are terminal electron acceptors existing at even lower redox potential. Lehmann et al. (*9*) and Lehmann and Cheng (*10*) have shown that such occurrence could be common in most soils. Therefore, results of allelochemical determinations based on analysis of soil samples which have been air-dried or exposed to oxidation may be questionable.

In Allelopathy; Dakshini, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Extracting Allelochemicals from Soil Samples. Extraction of allelochemicals is probably the most neglected aspect of research on the mechanisms of allelopathy. Water has been commonly used as the extractant for allelochemicals in the soil, under the assumption that only water soluble chemicals in the soil could cause allelopathy. Although allelochemicals are soluble in soil, it does not necessarily mean that they can be extracted readily by water. These chemicals may be reversibly retained on soil particle surfaces, but can be dislodged from sorbed sites, such as in a chromatographic partitioning process, or displaced by other chemicals in the soil solution, similar to an exchange reaction. The potential of many types of extractants has not yet been explored fully. Cheng (*11*) attempted to equate extraction efficiency with the ability of the extractant to break bonds between the chemical and the soil particle surface and postulated that the bioavailability of a chemical is related to the type of bonds which a chemical forms with the soil particle surface. Precautions are necessary to avoid alteration of the chemical during the extraction process. For instance, Cheng et al. (*12*) reported that many phenolic compounds can be readily oxidized during extraction, but such oxidation could be prevented by inclusion of a reducing agent in the extracting solution.

Measuring Allelochemicals in Soil Extracts. The common analytical approach for allelochemical determination is to use either gas or high-pressure liquid chromatography for separation and using various detection devices, including mass spectrometry and Fourier transformation infrared spectroscopy, for identification and quantification of the specific chemicals in question. If the identity of the allelochemical is known, use of ^{14}C-labeled radiotracers can be an effective technique to follow the fate and transport of specific chemicals in the soil environment. Since these procedures are standard and well documented, they will not be elaborated here further.

Characterizing Allelochemical Retention in Soils. Mechanisms of retention of chemicals by soils have been studied extensively. Koskinen and Harper (*13*) have summarized the major chemical bonds involved in the retention process. Experimentally, however, specific procedures for characterizing the various binding mechanisms have not been extensively evaluated. Commonly-used methods for retention characterization involve batch equilibration or flow-through procedures. While these methods cannot depict the different retention mechanisms, they can characterize the retention kinetics. The usefulness and applicability of these methods for allelochemical retention in rhizosphere soils has not been tested.

Characterizing Allelochemical Transformation in Soils. The kinetics of transformation of a chemical in soil is affected greatly by the nature of the soil environment. The stability of a chemical can be inherent in its structure or provided by its interaction with soil, such as by the retention process. There is increasing evidence that the plant root environment enhances the degradation of many organic chemicals (*14*). Kinetic studies of degradation of a chemical must consider both the enriched nutrient environment of the rhizosphere and the transient status of such environment in the course of root growth and development.

Characterizing Allelochemical Transport in Soils. The main difficulty in conducting allelochemical transport studies is to design these studies at the proper setting to reflect the process at the scale of actual occurrences for allelopathy. These studies need to be based on the boundary conditions defined by models so that results obtained can be coordinated with model studies.

Methodological Needs

This report has briefly discussed a number of experimental considerations which must be taken into account before a meaningful study on the transport of allelochemicals in the rhizosphere soil can be properly conducted. Not discussed are experimental techniques useful for studying the root environment in which transport of allelochemicals occurs. There is a dire need for innovative approaches to study rhizosphere directly. Recent efforts using mini-rhizotron and root observation boxes to examine roots directly in the soil environment (e.g., *15,16*) are illustrations of the challenges of the tasks confronting research in this area as well as promises for significant progress in future studies. More emphasis will have to be placed on microscale sensing and measuring devices and in-situ techniques to monitor the spatial and temporal variations in the concentration of allelochemicals present in the rhizosphere.

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Chapter 11

Plant Stress and Allelopathy

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Secondary metabolites in plants are known to contribute to the defense mechanism against herbivores and disease organisms. Concentrations of these compounds are often enhanced by biotic and abiotic stress and this reallocation of photosynthate is believed to be a part of the evolutionary adaptation. Similar rationale may be given to the possible enhancement of allelopathy under environmental stress. The increase of allelopathic phenolic and terpenoid compounds under environmental stresses has been well documented, and our work on purple nutsedge (*Cyperus rotundus* L.) showed that water stress enhanced phytotoxic secondary metabolites in both plant tissue and in the rhizosphere. While crucial questions remain unanswered, research on stress and allelopathy would provide valuable information in understanding both natural and agricultural ecosystems.

Allelopathy denotes biochemical interactions between plants including microorganisms. These interactions can be either beneficial or harmful, although allelopathy studies to date converge primarily on the harmful or inhibitory effects of a donor plant species against one or more acceptor plant species. An important point concerning allelopathy is that its effects depend on biochemicals, usually secondary metabolites released from donor plants to the environment. This is an unique feature when comparing with interactions between plants and pathogens (*1*), or between plants and herbivores (*2*), in which physical contact between the two interactive parties is involved. On the other hand, similar to antiherbivore defense and disease resistance, secondary metabolism also plays a key role in allelopathy (*3*).

Secondary metabolism is considered as part of the evolutionary adaptation, and the hypothesis of resource distribution in plant antiherbivore defense has been widely accepted (*2, 4*). Based on this hypothesis, under low resource environments natural selection favors plants with slow growth and high levels of defense compounds. This argument would serve equally well if allelopathy is regarded a means to competing for resources through biochemical modification of the environment.

Plant stress can be broadly defined as any external conditions unfavorable to its growth and survival. In nature, plant stress is usually unavoidable and may be caused by many biotic and abiotic factors (*5*), including water, temperature, radiation, mechanical force, man-made (e.g., pollutants, pesticides) and natural (e.g., ozone, heavy metal, allelochemicals) chemicals, herbivores and pathogens. In response, plants undergo a series of biochemical, physiological, and morphological changes. One of the common biochemical changes is the increased concentration of plant secondary metabolites (*6*). This response to stress might be a result of natural selection for increased resistance to herbivores or disease organisms at a time when damage could cause a particular great loss of fitness (*7*). Plants growing in poor nutrient habitat or

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under stressful conditions, therefore, often contain more secondary metabolites and population of animal species is negatively affected (*8*). On the other hand, few studies have been focused on the effect of stress on allelopathy. Scarcity of information is understandable since allelopathy by itself is a complicated phenomenon. Difficulties in collecting, isolating, identifying and quantitative determination of the responsible allelochemicals in the environment impose a formidable challenge to scientists. Yet, due to the omnipresence of chemical interactions in both agricultural and natural ecosystems, the impact of stress as expressed through allelopathy cannot be underestimated.

This review focuses on the effects of stress on the secondary metabolites in higher plants, especially those compounds which have been identified as allelochemicals. To demonstrate the enhancement of secondary metabolism and allelopathy by water deficit stress, our work on purple nutsedge is presented as an example. Purple nutsedge is considered as the world's worst weed (*9*). While the present review will not offer answers to important questions such as: What is the quantitative correlation between the stress-enhanced secondary metabolism and allelopathy? Would the acceptor plants which share the same stress in the environment become more vulnerable? What is the effect of biotic stress on allelopathy? And how do we evaluate the ecological and agricultural impact of these interactions under stress? We nevertheless hope that the present review will stimulate interest in this area of research and eventually lead to answers to these crucial questions in agricultural and natural ecosystems.

Stress and Allelochemicals

Water Stress. The most common form of stress encountered by plants is probably the water stress. Effects of water stress on secondary plant metabolites have been reviewed by Gershenzon (*7*). In general, water deficit stress increases the concentrations of secondary metabolites. Among them, phenolics and terpenes were most commonly studied. These two groups of natural products also represent most of the allelochemicals so far identified.

Phenolic Compounds. Early evidence on how stress affecting allelochemical concentrations comes from chlorogenic acid (CGA) and its isomers. It is noted that since much of this early work (see below) was performed prior to the wide use of HPLC, results of these important studies warrant re-examination using modern methodology.

Chlorogenic acid and its isomers have been identified in the natural leachates of sunflower (*Helianthus annuus* L.) leaves and *Eucalyptus* litter. CGA inhibited seedling growth both under natural and controlled conditions (*10, 11*). Factors affecting the concentrations of these compounds have been reviewed by Rice (*3*) and Einhellig (*12*). del Moral (*13*) studied in detail the concentrations of CGA and isochlorogenic acid (ICGA) in leaf, stem and root of sunflower under nitrogen deficiency, UV and drought stress. On a whole plant basis, water stress alone increased CGA 6-fold and ICGA 2-fold. Drought plus UV stress further enhanced the concentration of both CGA and ICGA. Drought plus nitrogen deficiency were often synergistic, producing 15 times as much CGA and 16 times as much ICGA as the non-stress treatments. Data also showed that increases in different tissues were caused by increased biosynthetic activities rather than reallocation of these phenolic compounds.

Recently, the interaction of water and UV stress on cowpea (*Vigna unguiculata* L. Walp.) was examined by Balakumar *et al.* (*14*). Increases of anthocyanins, total phenols and activities of some enzymes were observed. Healthy seeds were planted in plastic trays and the seedlings received three treatments: drought, UV irradiation, and both stresses. Anthocyanins showed 8% enhancement under water stress, and the total phenols were increased by 19%. Greater increases were observed when UV irradiation were also imposed; anthocyanins and total phenol increased by 25% and 63%, respectively. Since the increase in phenolic concentration could not be accounted for the

decrease in fresh or dry weight of the tissue, the enhancement likely was due to a net increase in the biosynthesis of these compounds.

Pot cultures of two varieties of marigold (*Tagetes erecta* L.), viz., African giant double yellow (V1) and African giant double orange (V2) were subjected to water deficit stress by withholding irrigation at transplanting and pre-blooming stages for a period of 12 days (*15*). Accumulation of free phenols were manifested in both varieties at both stages. Total free phenols showed a significant increase from 0.626 and 0.505 mg/g f. wt on day four to 1.049 and 0.851 mg/g f. wt on day 12 in V1 and V2, respectively, at the transplanting stage. Similarly, at the pre-blooming stage, free phenols rose from 0.969 to 1.009 mg/g f. wt in V1 and from 0.576 to 0.843 mg/g f. wt in V2. The study of Kumar *et al.* unfortunately did not examine thiophenes and benzofurans in the *Tagetes*. These pesticidal compounds have been determined in the undisturbed rhizosphere of *Tagetes patula* (*16*), and are possible allelochemicals.

Hydroxamic acids and benzoxazolinones are well known pesticidal compounds in certain cereal crop plants (*17*). The root exudates collected from rye (*Secale cereale* L.) inhibited the root growth of wild oats (*Avena fatua* L.) and the level of inhibition was determined by the volume of exudate tested. 2,4-Dihydroxy-1,4-benzoxazin-3-one (DIBOA) was identified in the exudates (*18*). DIBOA and its precursor, 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one (DIMBOA) were found in greater quantities in corn (*Zea mays* L.) under water deficit stress (*19*). In their experiment, corn kernels were germinated on solutions of polyethylene glycol corresponding to water potentials ranging from –0.01 to –1.07 MPa. While statistically insignificant, the general trend showed that the content of DIMBOA and DIMBOA plus DIBOA were greatest at an intermediate water potential of –0.47 MPa. In another experiment, seeds were first germinated in well-watered pots. Upon emergence of the coleoptile, daily water supply was withheld from half of the pots. The total cyclic hydroxamic acid content of stressed seedlings was between two to three times greater than the non-stressed seedlings after four days of stress treatment, primarily because of sharp decrease of DIMBOA in the non-stressed seedlings.

Terpenes. Many terpenes and terpenoids are phytotoxic and allelopathic compounds (*20*, *21*). These compounds are usually present in the essential oils of the plant tissues, but some phytotoxic and allelopathic terpenes are quite water-soluble (*22*). Increases of essential oils have been reported in plants growing in poor soil conditions and in many cases plant-herbivore interactions and allelopathic activity are affected (*23*, *24*).

Most water stress studies to date were limited to water deficit or drought stress. Recently, Kainulainen *et al.* (*25*) examined the effect of both drought and water logging stresses on monoterpenes in needle of *Picea abies* under greenhouse conditions. Five-year-old cuttings were transplanted in plastic containers. Water logging stress was induced by placing the container in a water bath, and drought-stressed plants were not watered for 8 weeks. Total terpenes and individual terpenes were determined by gas chromatography-mass spectrometry. Twenty terpenes were determined in needles. The total terpene content of drought-stressed trees was significantly higher than that of the control, but the effect of water logging was not significant. Among the 20 terpenes, tricyclene, α-pinene and camphene of drought-stressed trees were significantly higher than those in the control (Table I). Some of these terpenes have been identified as allelochemicals and phytotoxic compounds. These results suggest that water logging stress, unlike water-deficit stress, does not promote secondary metabolism.

Nutrient Deficiency Stress. Similar to those of water stress, the effects of various nutrient deficiencies have been demonstrated in early studies of plant phenolic compounds and terpenes.

Table I. Absolute amounts of terpenes in needles of cloned Norway spruce trees exposed to drought or waterlogging for 8 weeks.

	Control		*Waterlogging*		*Drought*			
Compound	*x*	*SD*	*x*	*SD*	*x*	*SD*	*F*	*P*
Tricyclene	23.8	6.2	24.0	8.1	35.3*	9.8	3.85	0.045
α-Pinene	82.4	20.7	88.8	24.2	128.6*	30.9	5.72	0.014
Camphene	204.0	45.2	213.6	51.0	302.8*	68.6	5.72	0.014
Sabinene	9.3	3.1	11.7	4.4	11.5	2.1	0.95	0.409
β-Pinene	9.4	3.3	13.9	8.9	17.2	12.0	1.20	0.328
Mycene	55.8	14.7	65.0	19.3	71.8	10.5	1.67	0.221
α-Phellandrene	1.8	1.1	1.6	2.2	2.0	0.5	0.11	0.896
3-Carene	0.5	0.8	0.4	0.8	tr	tr	1.02	0.384
α-Terpinene	0.2	0.5	0.2	0.5	tr	tr	0.50	0.615
p-Cymene	1.3	1.1	1.6	1.8	1.3	0.9	0.09	0.917
Limonene	132.9	59.9	133.5	47.1	177.6	72.6	1.07	0.369
Linalool	1.5	1.3	2.5	3.2	1.6	1.9	0.38	0.694
Camphor	36.0	20.3	32.5	30.5	24.9	23.5	0.31	0.741
Borneol	43.0	31.5	45.7	48.6	92.3	75.0	1.54	0.247
Terpinene-4-ol	0.7	1.1	0.7	1.7	0.5	1.1	0.05	0.951
α-Terpineol	16.3	5.3	21.9	14.8	22.6	8.5	0.66	0.531
Bornyl acetate	308.4	62.7	326.6	83.1	433.1	140.5	2.67	0.102
α-Copaene	0.6	0.7	0.9	0.7	0.5	0.7	0.50	0.617
Longifolene	2.7	2.9	2.2	3.5	4.2	1.6	0.77	0.479
β-Caryophyllene	11.1	6.7	7.3	7.6	2.2*	2.3	3.31	0.065
Total terpenes	948	221	1004	239	1333*	323	3.71	0.049

NOTE: The means (x) and standard deviations (SD) of six trees of each treatment are given as micrograms of terpene per gram of needle fresh weight and F and P values of analysis of variance.

tr indicates trace.

*Significantly ($p < 0.05$) different from control according to Duncan's multiple range test.

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Phenolic compounds. In sunflower on a whole plant basis, nitrogen deficiency alone increased CGA by 10-fold and ICGA 8-fold (*13*). Similar results were obtained in sunflower by Lehman and Rice (*26*). Experimental plants were irrigated with nitrogen, potassium, or sulfur deficient nutrient solutions, and plants were harvested weekly for 5 weeks. Under nitrogen deficiency, CGA concentration in old leaves was over 10 times as high as in the controls. CGA in S-deficient old leaves was also significantly higher by the second week of treatment. 4-Caffeoylquinic acid (band 510) and neochlorogenic acid (5-caffeoylquinic acid) were generally significantly higher each week in S-, and N-deficient old leaves. Potassium deficiency had little effect. In general, deficiencies of nitrogen, sulfur, and potassium all resulted in higher concentrations of CGA and its isomers in the decreasing order of effectiveness listed (*26*).

Koeppe *et al.* (*27*) observed a large increase in the concentration of the isomers of CGA in extracts of sunflowers grown under phosphorus-deficient conditions. Groups of 6 plants each were watered with three different nutrient media: (**a**) complete nutrient medium; (**b**) nutrient medium with only 1/10 the complete concentration of phosphorus; and (**c**) nutrient medium with no phosphorus. Three chlorogenic acid isomers in similar-aged leaves were compared and there was generally a 1.5- to 2-fold increase in chlorogenic acid concentrations in the 1/10-P plants and a 3- to 5-fold increase in CGA concentration in the plants grown without phosphorous.

Nitrogen deficient tobacco (*Nicotinana tabacum* L.) leaves accumulated CGA (*28*). By the end of one week of treatment, total concentration of caffeoylquinic acids was more than twice that of the controls. After 5 weeks, it had increased to almost 5 times higher than that in the control. These increases resulted mainly from the increases in CGA concentration. CGA comprised approximately 50% of the total caffeoylquinic acids in control leaves, but in the treated leaves after 5 weeks, CGA comprised almost 88% of these phenolic acids.

Scopoletin, another allelochemical, has been reported in a wide variety of plants. It inhibits the growth of tobacco, sunflower and pigweed, and it has been found to reduce net photosynthetic rate (*29*). Scopolin (7-glucoside of scopoletin) concentrations were found to be greater in the leaves of N-deficient tobacco plants than in those of the controls. These increases approximated the time of appearance of deficiency symptoms. Increases were also found in the roots of the deficient plants, especially after one week. The level reached in roots by week 5 was about twice that of the control (*28*).

Terpenes. Mihaliak and Lincoln (*30*) examined changes in yield of volatile leaf terpenes and plant growth pattern in response to nitrate availability in *Heterotheca subaxillaris* (Asteraceae). Plants were grown from seedlings through rosette stage in an environmental growth chamber. Four nutrient regimes, differing in the concentration of nitrate (0.5, 1.5, 5.0 and 15.0 mM) were used. The volatile leaf mono and sesquiterpene content in *H. subaxillaris* was highest among plants with low nitrate availability while leaf nitrogen content was the lowest in the same plants. The average leaf terpene content increased from 3.1 to 5.1 mg/g as nitrate supply declined from 15.0 to 0.5 mM. Similar results were obtained using $^{14}CO_2$ (*31*). Terpenoid content was greatest in young leaves of 0.5 mM plants. In a later experiment the same authors (*24*) observed that the increased quantity of leaf mono and sesquiterpenes under nitrate-limiting conditions enhanced plant defense against the generalist insect herbivore. In this experiment, seedlings were transferred into a potting medium. The first leaf sample was used for feeding trial and a matched leaf was used to determine the volatile terpene, nitrogen, and water content. Terpene content was higher under nitrate-limiting than under nitrate-rich conditions. Larval feed consumption, growth, and survival were lower on the leaves of nitrate-limiting plants. These results are consistent with the ecological hypothesis on increased carbon allocation to defense under nitrogen-limiting conditions (*2, 4, 30*).

While studying the effects of water deficit stress on a number of Mediterranean plant species, the effects of nutrient deficiency were also studied using potted plants under glasshouse conditions (*23*). The plants were fed a complete nutrient solution or solutions lacking nitrogen or phosphorous. Growth and development were followed over a period of 18 months. N- and P-deficient treatments increased in essential oil content by 29% and 18% respectively. Simultaneous withdrawal of both gave a 40% increase in essential oil yield.

Other Stresses. Effects of biotic stresses such as wounding by herbivores and diseases caused by pathogens have been known to induce higher secondary metabolite content in plants. Abiotic stress factors such as ozone, heavy metals, and herbicide may also enhance secondary metabolite production. To our knowledge, these stress factors have not been linked to the allelopathic activity of the affected plants. Future research is needed in this respect .

Several studies were carried out to determine the effect of UV irradiation on some well known allelochemicals in tobacco and sunflower plants. In both younger and old leaves of tobacco (*32*), the concentrations of CGA, neochlorogenic acid, and Band 510 were greater after UV treatment. In sunflower, scopolin was found to increase with increasing UV intensity. The accumulation is primarily found in the leaves subjecting to inhibitory or injurious UV intensities, with old leaves having larger buildup than younger leaves. Similarly, del Moral (*13*) observed a 2.5-fold and 1.5-fold increase of CGA and ICGA, respectively, by UV treatment. Recently, Balakumar *et al.* (*14*) determined that irradiation at 280-320 nm anthocyanins and total phenol contents of cowpea seedlings were increased by 36% and 58%, respectively.

Chilling injury occurs usually at 0–10 °C. Koeppe *et al.* (*33*) found that CGA, Band 510, and neoCGA were increased to 1.5 to 5 times that of the control in leaves, stems and roots of tobacco plant exposed to low temperature treatment.

Examples in Ecosystems

Results of greenhouse and controlled field work on stress-enhanced secondary metabolism coincide well with some ecological observations. For example, interactions between herbivores and plant phenolics were studied by McKey *et al.* (*34*) in two African forests. One site was in the Kibale Forest, Uganda, and another was Douala-Edea Reserve on the coast of Cameron. The major differences between the two study sites are the significantly lower contents in nitrogen, phosphorus, and ash, and acidic pH values in the Douala-Edea site. Mature leaves of many of the most common tree species in each site were collected and examined for their phenolic content. Mean values for the Cameroon (nutrient-poor) samples were significantly greater than those for the Uganda (nutrient-sufficient) samples. Mean concentrations of total phenolics in the Cameroon leaves were twice that of the Uganda leaves; condensed tannins and their monomers were both at least two times higher in the Cameroon leaves. For the 16 trees abundant in Cameroon site, mean content of phenolics was roughly equivalent to 7.6% (dry weight), and that of condensed tannins was about 4.6%. Plants growing in dense stands on exceptionally poor soil within the Cameroon site often contain higher than average phenolics. In contrast, mean concentration of phenolics in mature leaves of Uganda site was 3.5%, and that of condensed tannins was 2.6%. A variety of uncommon phenolics have been identified in samples from Cameroon site, including insecticidal *n*-propylcoumarins and xanthones. Although this study only concerned the antiherbivoral activity, some phenolic compounds identified in this study may also play a role in allelopathy, it is possible that a similar conclusion can also be reached on allelopathy in these African ecosystems.

The Mediterranean weather has relatively mild, wet winters and long, hot and dry summers. Variations of volatile oil concentration in certain aromatic shrubs due to

weather changes have been reported (*35*). By the end of spring and beginning of summer, the volatile oil concentrations in seasonally dimorphic plants are almost three-fold higher than their lowest winter values. These changes are well correlated with the lowest precipitation and highest temperature during the summer. The highest concentration of volatile oil is found in young leaves and this is probably a reflection of the carbon allocation and the investment in chemical defense to more valuable plant parts under stress conditions (*2, 4*).

In a study of allelopathy in desert ecosystem in the Negev Desert of Israel, near Sede Boquer, within a region boasting up to 100 mm/yr rainfall, Friedman (*36*) observed that the vegetative yield of annuals on south-facing slopes was 6-8 times that on adjacent north-facing slopes. They noted that the north-facing slopes were dominated by the aromatic semi-dwarf shrub *Artemisia herba-alba* (Compositae), whereas the south-facing ones by the nonaromatic shrub *Zygophyllum dusoum* (Zygophyllaceae). Counts made during germination time showed that density of the seedlings of annuals in the vicinity of *A. herba-alba* was only half that observed 100 cm from the canopy. When shoots of *A. herba-alba* collected in the desert were placed near seeds of various annual plants, germination was inhibited and such inhibition was highly reproducible. Major volatile inhibitors turned out to be terpenes and terpenoids, such as α-pinene, camphor, and cineole. When plants of *A. herba-alba* were transplanted in a more humid region in Tel Aviv, however, similar inhibitory effects were obtained only when 3-4 times as many shoots were applied. These results again suggest that ecological conditions which impose water and nutrient deficit stress and extreme temperatures would enhance allelopathy.

Biochemical and Physiological Aspects

Although the enhancement of secondary plant metabolism under stress has been well established, its biochemical and physiological mechanisms are not well understood. Indeed, mechanisms of the accumulation can be complicated, and may be varied in different situations. Activity of genes and their protein products must be involved in the accumulation. New compounds can be produced and accumulated by means of switching on new metabolic routes. A well studied and possibly related area is the production of phytoalexins in plants infected by pathogens (*1*). For example, Douglas *et al.* (*37*) found that the parsley 4CL (4-coumarate:CoA ligase) gene can be activated by elicitor and light treatments, in which case the elicitor may be considered as a stress signal. The substrate level is also important in the regulation of phenylpropanoid accumulation (*38*). Another example is the production of heat shock proteins in heat stressed plants (*39*). In allelopathy, however, most allelochemicals identified so far are pre-existing rather than induced compounds such as phytoalexins. Accumulation of allelochemicals therefore is likely a result of an enhancement in biosynthesis, and/or a decreased rate of catabolism of these compounds. Furthermore, allelopathy is usually caused by a mixture of compounds, and synergistic effect of these interactive compounds are likely to play an important role (*12, 40*).

Under stress conditions, some factors favor the production of secondary plant metabolites. The basic response of plants to stresses is to decrease growth rate. One explanation for the accumulation (*7*) is that these stresses may have hindered plant growth, but this only have minor effects on secondary metabolism. The net results an increased concentrations of secondary compounds. However, in many cases either there is a net increase of total secondary metabolites, or a clear ratio change occurred among individual compounds (*25, 28, 41*), suggesting changes in the biosynthesis. This could be a result of reallocation of photosynthate to carbon-based secondary metabolites under stress conditions (*2, 4*). It was proposed that resource availability in the environment is the major determinant of both the amount and type of plant defense (*2, 4*). The higher inhibitory activities of root exudates collected from the plants under water stress (*42*), and the leachate from stressed plants (*27*) imply elevated level of allelochemicals in

donor plants, although other possibilities may exist. For example, stresses may cause structural damage to the plant tissue and increase the leakage of cell contents.

Release of Allelochemicals under Stress Conditions. Although in general the concentration of secondary plant metabolites increases in plant tissues under stress conditions, it is less clear whether a corresponding increase of these compounds also appears in the environment. This question is naturally of great importance to the understanding of allelopathy.

Koeppe *et al.* (*27*) reported that more phenolic compounds were leached from living intact roots, dried roots, and tops of phosphorous-deficient plants than from phosphorous-sufficient plants. On a dry weight basis, P-deficient conditions led to increases of one- to four-fold in phenolic compound exudation by intact roots. Plant residues from P-deficient plants also released more phenolics per dry weight than did those from P-sufficient plants.

Plant residue often serve as an important source of allelochemicals. Hall *et al.* (*43*) observed that total phenolic compounds from the sunflower tissue, expressed as chlorogenic acid equivalents, increased with increasing nutrient deficiency stress (1/2 to 1/16 strength of Hoagland's solution treatment). When coarsely ground sunflower plant material from the stress treatments was added to soil, there was a significant depressive effect on germination of *Amaranthus retrofluxus* seeds. The germination was more closely correlated with total phenolic compounds (chlorogenic acid equivalents) added to the soil by the debris than with any other variable measured. The correlations were best for phenolic values of 200 μg/g soil or above.

In an extension of the study above, Hall *et al.* (*44*) investigated stress modification of the allelopathic effect of *H. annuus* L. debris, which was obtained from *H. annuus* plants grown in sand culture supplied with various strengths of Hoagland's solution. Two levels of debris, 2.86 and 7.14 mg debris/g of soil were thoroughly mixed into the upper half of the soil-sand mixture. Five plants per pot of *A. retroflexus* were established and plants were harvested 4 weeks later. Both total phenolics and the contents of N, P, and K were accountable for the significant variation of *A. retroflexus* plant weight. The most abundant phenolic compounds presented in *H. annuus* were chlorogenic acids which had been implicated previously as allelopathic agents (*10*). Four different levels of chlorogenic acid was substituted for *H. annuus* debris in the separate bioassay. *A. retroflexus* total plant biomass was also significantly reduced by increasing levels of CGA-amended media.

Although only limited information are available on the quantitative release of allelochemicals from living plants grown under stress conditions, these results are complemented by reports on the increased release of sugars, amino acids, lipids, and organic acids from roots of pine seedlings (*45*) and rape seedlings (*46*) under water stress. Reid (*45*) investigated the movement of ^{14}C-labeled compounds in pine seedlings and the resultant exudation of ^{14}C from the roots. Polyethylene glycol (PEG-4000) was used to decrease root solution water potentials by 0, –1.9, –2.6, –5.5, –9.6 and –11.9 bars in either aerated 0.25 strength Hoagland's nutrient solution or distilled water. Radioactive CO_2 was introduced to the seedling shoot by acidifying 10 mc of $NaH^{14}CO_3$ in a closed assimilation chamber before and after the plants were imposed water stress. In all treatments, there appeared to be a definite trend of an increasing proportion of sugars in the exudate as stress increased, with a marked change between 0 and –2.6 bars.

In study of the effects of water stress on the production of allelochemicals in the root exudates, we attempted also to use PEGs to control water potential in root exudates trapping system (*47*). However, it was found that the commercial PEGs contained various low molecular weight organic impurities which severely interfered with the XAD-4 trapping of hydrophobic root exudates. Assuming that these small molecule

contaminants in PEGs are harmless to the testing plants, it seems clear that PEGs would only be feasible if labeled metabolites are used for detection.

Svenningsson *et al.* (*46*) studied the effects of water-deficit stress on the release of amino acids, low molecular weight carbohydrates and lipids from rape seedlings with axenically cultivated roots. Rape (*Brassica napus* L.) seedlings were grown with axenic root systems in cuvettes with sand and nutrient solution. The plants were stressed for 24 hours by removing the nutrient solution but maintaining the air stream to the medium. Fresh nutrient solution was added and remained for another 3 days. The nutrient solutions containing root exudates were collected. The water-deficit stress caused a decrease in water potential (Pw) from Pw = -0.1 ± 0.1 MPa in the control plants to Pw = -1.0 ± 0.1 MPa in the stressed plants. The analysis of nutrient solution showed that the dissolved organic carbon of 4.4 mg/g root dry weight was released in stressed plants compared with 2.1 mg/g root dry weight in the control plants. The low molecular weight carbohydrates increased from 91 μg/g root dry weight for the control to 148 μg/g root dry weight for stressed plants. The fatty acids increased from 475 μg/g root dry weight for the control to 667 μg/g root dry weight for stressed plants. The only exception was amino acids which decreased from 982 μg/g root dry weight for the control to 543 μg/g root dry weight for stressed plants. A significant increase was found in the sterols β-sitosterol and campesterol in stressed plants. The stigmasterol also increased but not as high as the other two sterols. TLC showed that the number of different polar lipid components found in the extracts increased after stress. Since allelochemicals, like the phytosterols in the root exudates of rape seedlings, are often non-saponifiable lipids, results from the above study are instructive in considering the effects of stress on allelochemicals.

In a recent field experiment at the University of Hawaii Waimanalo Research Station, interactions between sweet corn (*Zea mays* L.) and purple nutsedge were evaluated at different irrigation levels (*48*). The effect of purple nutsedge on the reduction of sweet corn yield was most severe at the greatest water stress imposed (2 mm/day). This result warrants further study on the allelopathic activity of purple nutsedge in relation to water stress.

Four chemotypes (*i.e.*, H, M, O, and K type) have been identified based on the sesquiterpene composition of essential oils (*49, 50*). Among these four chemotypes, the H-type is strongest in allelopathy (*51*). The overall potency of inhibition is in the order of H > M > K > O type (Table II). Seven sesquiterpenes have been isolated, identified and their bioactivities are determined using lettuce (*Lactuca sativa* L.) and oat (*Avena sativa* L.) seed germination and seedling growth bioassays (*50*). Sesquiterpenes with ketone (i.e., α-cyperone and cyperotundone) or hydroxyl (*i.e.*, cyperol) groups are more inhibitory than the acetates and hydrocarbons (Table III).

Our recent work on the effect of water deficit stress on allelopathy of purple nutsedge provided some insight on the enhancement of allelopathic compound in the tuber and in the rhizosphere (*42*). Root exudates have been collected from the purple nutsedge grown under water deficit stress and their inhibitory activity determined by the effects on lettuce seed germination and seedling growth (*42*). Selected tubers of purple nutsedge were planted in silica sand in the 4 liter solvent pots with bottom removed. The pots were watered daily with 1/2 strength Ruakura nutrient solution for 60 to 70 days. At the end of normal growth, the plants were subjected to water stress using a series of measured amounts and strengths of the nutrient solution for different treatment levels. Water potential was measured and recorded at certain intervals using a pressure bomb. Significant water potential drop of the leave blades was observed about 3 weeks after withholding water supply. Intermediate levels of water stress in the experiments were not readily achieved, perhaps due to the capacity of the tubers to mitigate drought conditions. The dry weights of shoot and root decreased with the increase in levels of water stress (Table IV).

At the end of water stress treatment, all pots were connected with XAD-4 resin columns (*47*). Four liters of deionized water were allowed to pass through slowly each

Table II. Effects of essential oils from the four chemotypes of *C. rotundus* tubers on the growth of seedlings of lettuce and oat

	Concentration (ppm)	*Chemotype*			
		H	*M*	*K*	*O*
Radicle[1] (Lettuce)	1000	11.9c	13.9c	15.7bc	15.7bc
	500	13.5c	16.5bc	18.1b	16.9bc
	250	14.2c	16.0bc	21.9a	22.4a
Hypocotyl[2] (Lettuce)	1000	9.6d	10.2d	12.8c	13.6bc
	500	13.2c	15.8b	13.1bc	15.1b
	250	15.7b	17.0b	16.3b	20.8a
Radicle[1] (Oat)	1000	14.6cd	12.8d	16.6c	18.0c
	500	16.9c	15.8cd	12.9b	21.5b
	250	24.4b	23.5b	28.2a	30.7a
Leaf sheath[3] (Oat)	1000	8.0d	7.2d	12.6c	13.5bc
	500	8.9cd	11.4c	16.9b	16.8b
	250	17.2b	16.2b	23.2a	24.5a

NOTE: Means within the plant part (among chemotypes) followed by the same letter are not significantly different at the 0.05 level of probability as determined by Duncan's mean separation.

[1]Radicle length in millimeters (lettuce control = 22.7; oat control = 30.5).

[2]Lettuce hypocotyl length in millimeters (control = 20.3).

[3]Oat leaf sheath in millimeters (control = 23.7).

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Table. III. Effects of sesquiterpenes isolated from *C. rotundus* tubers on the growth of seedlings of lettuce and oat[1]

Treatment (mol x 10^4)		Sesquiterpene I	II	III	IV	V	VI	VII
A	50	9.0d	9.8d	9.1d	13.7c	15.1bc	15.6bc	14.6bc
	10	11.6cd	10.1d	11.2cd	19.1ab	14.5c	18.4ab	21.9a
	5	16.2bc	17.5b	17.3b	19.7ab	19.3ab	20.6a	20.5ab
B	50	3.4d	5.9cd	5.0d	10.1b	11.7b	8.6c	9.1c
	10	4.3d	7.8c	5.3d	12.6a	10.1b	12.2ab	10.3b
	5	10.4b	12.0b	12.7a	15.0a	14.1a	14.4a	14.3a
C	50	11.7d	14.2cd	13.9d	22.0d	22.0b	23.9b	21.7b
	10	17.4c	17.5c	20.1c	24.2b	24.2b	27.3ab	24.0b
	5	23.1b	24.6b	24.4b	30.0a	30.5a	31.9a	29.2a
D	50	7.4d	10.5cd	9.6cd	15.3b	13.7a	15.0b	14.3c
	10	8.1d	11.8c	11.7c	22.2b	19.5b	19.1b	19.0b
	5	16.8b	15.9b	17.7b	26.1a	24.9a	22.0a	21.9a

[1]A: lettuce radicle length in mm (control = 19.2). B: lettuce hypocotyl length in mm (control = 15.8). C: oat radicle length in mm (control = 30.5). D: oat leaf sheath length in mm (control = 23.7).Sesquiterpene: I: α-cyperone; II: cyperotundone; III: cyperol; VI: β-selinene; V: cyperene; VI: sugeonol acetate; VII: patchoulene acetate; Means within the plant part (among sesquiterpenes) followed by the same letter are not significantly different at the 0.05 level of probability as determined by Duncan's separation.

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Table IV. Effect of water stress on the dry weight of shoot and root and water potential of *C. rotundus*

Stress level[a] (ml/%)	Dry wt. of shoot (g)	Dry wt. of root (g)	Water potential (MPa) wk.1	wk.2	wk.3	wk.4	wk.5
250/20	10.6	102.1	–.3	-.3	-.3	-.3	-.3
150/60	7.7	78.1	–.3	-.35	-.35	-.35	-.35
100/80	6.6	74.0	–.3	-.35	-.45	-.5	-.5
50/100	5.3	70.6	–.3	-.4	-.5	-.9	-1.05
0	3.4	62.5	–.75	-1.5	*	*	*

[a] different stress levels were obtained by watering the plants with a series of measured amounts and strengths of the Ruakura nutrient solution. * indicates water potential > 1.8 MPa.

pot and the resin column. This procedure was repeated for three times using the same recycled water. Hydrophobic compounds in the rhizosphere were eluted from the silica sand medium and trapped by the XAD-4 resin column. The resins from columns of the same treatment were combined into a larger column, washed with deionized water and excess water was removed by forcing nitrogen gas through the column. Root exudates were eluted by methanol and concentrated under reduced pressure for bioassays. Results showed that water deficit stress enhanced the inhibitory activity of root exudates of purple nutsedge against lettuce seed germination and radicle elongation (Table V). In two separate experiments, the methanol soluble root exudates fraction was partitioned with hexane. The hexane fraction of root exudates was much less inhibitory to both lettuce seed germination and radicle elongation compared with methanol fractions (Tables VI and VII), suggesting that the inhibitory activities were mainly contributed by relatively polar, methanol soluble compounds.

Similar results were obtained with tuber extracts of purple nutsedge grown under water deficit stress. The plants were transplanted in the silica sand culture pots. After about 20 days, stress was imposed to the plants for 3 weeks by stopping water supply. Tubers were harvested two weeks later. Tubers under water stress were very dry compared with those without water stress. The tubers were ground and extracted with methanol immediately after harvest. The inhibitory activity was determined with lettuce seedling growth on the dry weight basis of tubers. Results (Table VIII) showed that the methanol extracts from tubers of purple nutsedge under water stress had greater inhibition than those from purple nutsedge without water stress. Such a result is in agreement with the results using root exudates of purple nutsedge. Methanol extracts of tubers from two chemotypes of purple nutsedge were also compared and the results (Table VIII) showed that H-type was more inhibitory than K-type under the same water stress status. The observation is in agreement with previous studies on chemotypes of purple nutsedge (*51*). Furthermore, since the inhibitory activities of both root exudates and tuber extracts are increased after water deficit stress, we suggest that the allelopathic potential of purple nutsedge can be enhanced by water stress through the increased biosynthesis of allelochemicals in the tubers. The bioassays showed that in both extracts and exudates, higher inhibitory activities were found in the methanol fractions, rather than hexane fractions which would contain bioactive sesquiterpenes previously identified in the essential oils of tubers (*50, 51*). Additional work is needed to identify those relatively polar compounds in methanol.

Effect of Stress on Root Morphology. In addition to the enhanced allelochemicals in the rhizosphere, allelopathic phenomenon may also be manifested by the changes in plant morphology. This effect was demonstrated by Callaway *et al.* (*52*). They investigated the effects of *Quercus douglassi* on the productivity of understory grasses and forbs in central California. Both positive and negative effects have been reported. A continuum between these two extremes was also observed. The biomass patterns were confirmed as positive tree understories were twice as productive as open grassland, and open grassland was twice as productive as negative tree understories. In general, the biomass of understory species tended to be the highest under positive trees, intermediate in the open grassland, and lowest under the negative trees. Since the biomass of individual species in different habitat within experimental site is in the same order, it is not likely to be caused by different species compositions. It was found that the most striking difference between positive trees and negative trees was the low biomass of shallow, fine oak roots under the former, and the high biomass of shallow, fine oak roots under the latter. The soil moisture under negative trees was lower than under positive trees and that in open grassland after senescence of the understory plants and the leafing out of the oak trees. Bioassay were conducted using oak root leachate on *Bromus diandrus* which is a dominant species under the negative trees. The dry mass of which was significantly lower comparing to those watered with Hoagland's solution (controls) passed through pots with no oak donor plant. When *B. diandrus*

Table V. Effect on lettuce seed germination and radicle elongation of the methanol fraction of root exudates from *C. rotundus* grown under water stress.

*Stress level**	*Germination (%)*		*Radicle elongation (mm)*	
	(2.5ml)	*(5.0ml)*	*(2.5ml)*	*(5.0ml)*
Control	96.0a**	96.0a	10.9a	10.9a
250/20	98.0a	96.0a	7.2b	6.0b
150/60	96.0a	92.0a	9.1b	8.3b
100/80	96.0a	96.0a	8.4b	8.1b
50/100	74.0b	78.0b	3.8c	2.9c
0	82.0b	44.0b	2.6d	1.0d

* Different stress levels were obtained by watering the plants with a series of measured amounts and strengths of the Ruakura nutrient solution. **Treatments with the same letter do not differ significantly by student *t* test at the P > 0.05 level.

Table VI. Effect on lettuce seed germination and radicle elongation of the hexane fraction of root exudates from *C. rotundus* grown under water stress

Stress level[1] *(ml/%)*	*Germination (%) (5 ml)*	*Radicle elongation (mm) (5 ml)*
Control	98a*	11.1a
100/25	89b	9.8b
50/50	94a	10.3a
25/75	72c	8.3c
0	79b	7.7c

[1] Different stress levels were obtained by watering the plants with a series of measured amounts and strengths of the Ruakura nutrient solution. * Treatments with the same letter do not differ significantly by student *t* test at the P > 0.05 level.

VII. Comparison of effect on lettuce seed germination and radicle elongation of methanol and hexane fractions of root exudates from *C. rotundus* grown under water deficit stress.

Stress[a] level (ml/%)	*Methanol fraction (10 ml)*		*Hexane fraction (10 ml)*	
	Germination	*Elongation(mm)*	*Germination*	*elongation(mm)*
Control	100a*	12.5a	100a	12.5a
100/ 50	92a	6.4b	92a	11.3a
50/100	84b	4.2c	99a	11.9a
0	65c	3.3d	90a	11.7a

[a] Different stress levels were obtained by watering the plants with a series of measured amounts and strengths of the Ruakura nutrient solution. * Treatments with the same letter do not differ significantly by student *t* test at the P > 0.05 level.

Table VIII. Inhibition of lettuce seedling growth by root methanol extracts of *C. rotundus* of different chemotypes grown under water deficit stress (mean in mm)[1].

solvent amount (ml)	*without water-stress*				*with water-stress*					
	control		*H-type*		*K-type*		*H-type*		*K-type*	
	R	*H*	*R*	*H*	*R*	*H*	*R*	*H*	*R*	*H*
1.0	17.9	20.3	no further measurable growth from pregerminated seedlings							
0.25	17.9	20.3	5.0ab	4.1ab	7.5a	6.0a	2.9ac	2.8abc	3.6ad	4.7ad
0.10	17.9	20.3	10.1ab	6.6a	13.3a	9.7a	6.0abc	4.9abc	9.0ad	7.8ab

a=Significantly different from the solvent control for the same plant and same volume at P<0.05 by student *t* test. b=Significantly different from K-type for the same plant parts, same volume and same water status at P<0.05 by student *t* test. c=Means for H-type under water stress are significantly different from H-type without water stress for the same plant parts and same volume at P<0.05 level by student *t* test. d=Means for K-type under water stress are significantly different from K-type without water stress for the same plant parts and same volume at P<0.05 level by student *t* test.

was watered with oak root leachate that had been filtered through the XAD-4 resin column, the final biomass was intermediate to the nonfiltered and the control treatments. These results suggest a causal relationship between shallow oak fine roots and inhibition of understory productivity. The interference of understory plant growth by oak in the field could be due, at least in part, to allelopathic root exudates. Root morphology appears to determine the relative importance of these effects.

In greenhouse conditions, Callaway (*53*) studied the responses of seedling root systems of three species of oaks in California to two experimental soil moisture regimes by comparing lateral root development, root and shoot weights, and root/shoot ratios. The results showed that lateral root development on the upper 30 cm of primary root increased significantly for *Quercus lobata* and *Q. douglassi* in the shallow water treatment. In this treatment, *Q.* douglassi lateral root number increased by twofold and lateral root weight by over six fold on the upper 30 cm of primary root.

Stressful conditions have been considered to promote root exudation in general (*54*). In the case of water stress, the development of abundant lateral roots may increase the total surface area of root system and, probably, an expended area of allelopathic interactions.

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Chapter 12

Chemical Communication Between the Parasitic Weed *Striga* and Its Crop Host

A New Dimension in Allelochemistry

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Adaptation of *Striga* to parasitism includes not only dependence upon a host plant for metabolic inputs such as water, minerals, and energy, but also for developmental signals. In this way parasite and host development are highly integrated. The early host-derived chemical signals *Striga* requires, for seed germination and for initiation of the haustorium by which it attaches to host roots, are exuded from host roots into the soil. After *Striga* penetrates the host root, subsequent developmental signals are apparently exchanged directly, through vascular tissue. Germination stimulants for most *Striga* hosts have been identified as strigol-type compounds (strigolactones). Sorghum genotypes which produce extremely low amounts of stimulant are resistant to *Striga*. The gene for this trait has been mapped and incorporated into improved sorghums being released for production. Subsequent host-derived signals required by *Striga* are being characterized for possible independent mechanisms of resistance.

Plants that have surrendered their independence and adopted a parasitic lifestyle generally obtain their water, minerals, and/or energy (in the form of photosynthate) from their host plant. But successful parasitism involves much more than dependence upon a host for metabolic inputs. In addition to these essentials, the parasite's growth, morphological development and even its manner of reproduction must be compatible with that of the host. In effect, the entire life cycle of the parasite must, to a significant extent, be integrated with that of the host. This integration is necessarily more intimate, more molecular, than the integration of plants which depend upon other organisms for pollination or seed dispersal with the lifestyles and characteristics of the organisms which provide these services.

Because their requirements are so specific and complex, parasitic plants would seem to be more vulnerable than independent, non-parasitic plants. Successful plant parasitism involves a series of stringent conditions and interactions, all of which

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must be satisfied. These include, but are not limited to, recognition and selection of an available, compatible, competent host plant at the proper stage of development in an appropriate environment. Given these requirements, it is no wonder that parasitism is a relatively rare lifestyle among plants. Yet some parasitic plants are so successful that they have become serious agricultural pests. How do these parasitic weeds manage to satisfy these requirements? To answer this question is to invoke a truism: parasitic plants are exquisitely adapted to their hosts and to their environment. We are beginning to see, at least with one particularly troublesome group of parasitic weeds, the witchweeds (*Striga* species), that this adaptation to the host reaches down to the molecular level, with communication by means of stereospecific chemical signals, and that it may extend throughout the parasite's life cycle.

Striga Biology and Life Cycle (*1*)

The genus *Striga* in the family Scrophulariaceae is composed of some 50 species, all holoparasites of tropical cereals or legumes. *Striga hermonthica* (Del.) Benth and *S. asiatica* (L.) Kuntze are the species which cause the most economically significant damage to cereals. *S. gesneroides* (Willd.) Vatke is the species most serious on cowpeas and tobacco. These witchweeds constrain production of important food crops such as maize, millet, sorghum, and cowpeas, particularly in Africa but also in India. A severe infestation can result in complete loss of the crop, and to abandonment of otherwise productive fields. The *Striga* problem in Africa seems to be worsening, due to intensive cultivation involving continuous monocropping of host crops in an attempt to produce sufficient food for the burgeoning population. Unfortunately, many improved crop cultivars which have been introduced have proved to be highly susceptible to *Striga*.

Striga seeds are minute (0.2 mm), numerous (up to 100,000 per *Striga* plant and up to 100 *Striga* plants per host plant), and long lived (there are reports of *Striga* seed viability up to 20 years). Their requirements for germination include a dormant after-ripening period of several months, then pre-conditioning in moist conditions for one to three weeks, and finally, exposure to a specific chemical signal produced by the host root. After germination, a second host-derived chemical signal induces the elongating radicle to differentiate into a specialized structure, the haustorium, by which the *Striga* seeding attaches to and penetrates the host root. Approximately half the *Striga* life cycle is subterranean, living completely parasitically on the host roots. Much of the damage to the host occurs at this phase, before the *Striga* plant emerges from the soil. The mechanisms by which the damage occurs are not completely defined, but diversion of host resources to the parasite accounts for only a small proportion of the damage. Once above ground, the *Striga* plant develops chlorophyll and becomes green (except for *S. gesneroides*), fixing some but not all of its own carbon. The flowers, which are purple, red, yellow or white depending upon the *Striga* species, develop rapidly and the numerous seeds are produced about the same time as those of the host crop.

Comprehensive information on *Striga* characteristics, distribution, and control may be found in the volume edited by Musselman (*1*). Selective information

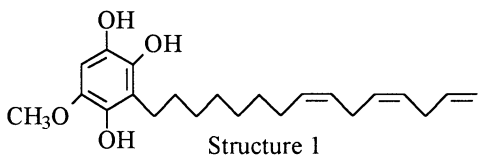
may be found in a research bulletin on crop breeding for enhanced resistance to *Striga* from Purdue University (*2*).

Host Control of Striga Development by Means of Chemical Signals

The first two stages of *Striga* development from seeds are controlled by chemical signals exuded into the soil around host roots.

Germination. Once the after-ripening and pre-conditioning requirements are met, *Striga* seeds respond within as little as 3 hours after exposure to germination stimulant produced by the host root. The earliest detectable response is production of ethylene; radicle elongation is detectable by 10 hours (*3*). Although ethylene appears to be the ultimate germination stimulant within the *Striga* seed, the nature of the germination stimulant exuded by host roots, which triggers ethylene production, has long attracted attention.

Sorgoleone. The first *Striga* germination stimulant isolated from a *Striga* host plant was a series of oily and unstable substituted hydrobenzoquinones (**structure 1**) collectively called sorgoleone, exuded as hydrophobic droplets from the tips of root hairs of sorghum (*4,5*). The low solubility in water of the active hydroquinones and their rapid oxidation to quinones, which are inactive as stimulants of *Striga* seed germination, suggested that only those *Striga* seeds close to the host root would be stimulated to germinate (*6*). This would be an advantage for *Striga* by leaving ungerminated and viable for another season those seeds too far away to reach the host roots. A more stable and mobile germination signal, capable of stimulating *Striga* seed germination farther from the host roots, would be less advantageous for *Striga*, because many more seeds would germinate and die, reducing the population of viable *Striga* seeds.



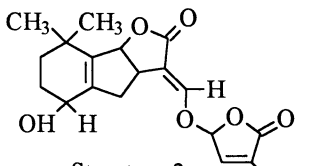
Structure 1
Sorgoleone

The limited water solubility of sorgoleone and its ready oxidation with loss of stimulant activity are not consistent with previous studies of *Striga* germination stimulants collected from hydroponically grown host plants (*7*). The amount of sorgoleone produced by several sorghum genotypes does not correlate well with their susceptibility or resistance to *Striga* (*8,9*). Moreover, sorghum and other *Striga* hosts produce other germination stimulants, more stable and more water-soluble than sorgoleone, in widely varying amounts that do correlate well with susceptibility or resistance in several cases (*8,10*). We have concluded that sorgoleone plays only a minor role, if any, in controlling *Striga* germination (*8*).

Sorgoleone, which has been synthesized (*11*), is a very active allelochemical, even in the oxidized (quinone) form which has no activity as a *Striga* germination

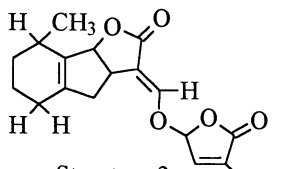
stimulant. It is a selective natural herbicide (*12,13*) inhibiting electron flow in mitochondria (*14*) and chloroplasts (*15*), active at concentrations as low as those of rotenone (*14*). Sorgoleone at least partially accounts for weed-inhibiting allelochemical effects long reported for sorghum (*15,16*). It is a powerful contact allergen (*12*), as expected from its structure which is similar to that of urshiol, the active component of poison ivy.

Strigolactones. The first naturally occurring *Striga* germination stimulant identified was the sesquiterpene derivative, strigol (**structure 2**). Strigol was originally isolated from root exudate of cotton (*17*), which is not a host for Striga, so the significance of strigol was long uncertain. The (+) enantiomer of strigol is active as a *Striga* seed germination stimulant at concentrations as low as 10^{-11} M (*18*), and concentrations as low as 10^{-16} M have been reported to be active (*7*).

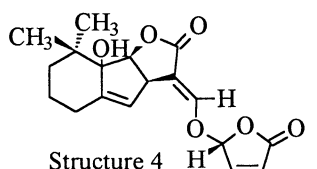


Structure 2
Strigol

The key to the identity of the host root-derived compounds which apparently do control *Striga* germination, and to the significance of strigol, was provided by the identification of sorgolactone (**structure 3**), a close analog of strigol, as accounting for most of the *Striga* germination stimulant activity produced by sorghum roots growing in water (*19*). The same group of investigators also identified alectrol (**structure 4**), another close analog of strigol, as the major *Striga gesneroides* germination stimulant produced by cowpea roots (*20*). Our group here at Purdue University subsequently showed that strigol itself is the major *Striga* germination stimulant produced by maize and proso millet (but not pearl millet, a common *Striga* host)(*21*). In each case, these *Striga* hosts produce three or more strigol analogs with germination stimulant activity. Sorghum, for example, produces a small (<1% of sorgolactone) amount of strigol and an even smaller amount of what appears to be alectrol (*21*), along with yet another apparent strigol analog. So with the exception of pearl millet, the major *Striga* germination stimulants produced by the common *Striga* hosts have been identified, and all are analogs of strigol or are strigol itself. For this group of sesquiterpene lactones active as *Striga* germination stimulants the collective name "**strigolactones**" is proposed.



Structure 3
Sorgolactone



Structure 4
Alectrol

Host-specific *Striga* strains have been reported, and the extent to which host specificity is accounted for by the unique mix of strigolactones produced by a particular host is of considerable interest. Strigol, to which virtually all *Striga* species (and others) respond, is produced by non-hosts as well as by most hosts. Alectrol stimulates germination of both *S. gesneroides* and *Alectra* (both of which parasitize cowpeas), as well as *S. hermonthica* and *S. asiatica* (neither of which parasitize cowpeas) (*20*). Although pearl millet is a common host for *S. hermonthica* in West Africa, its root exudate may be unique. Root exudates of the pearl millet genotypes we have tested do not stimulate germination of the same *S. asiatica* seeds which respond to strigol and the other strigolactones from sorghum, maize and cowpea. It seems that the nature of the stimulant produced may play a role in host specificity, but there must also be other determinants, as yet unknown.

A simple agar gel assay rapidly screens hosts and non-hosts non-destructively for low stimulant production, speeding the process of incorporating this resistance-conferring trait into crop cultivars for utilization in *Striga*-endemic areas (*22*). The sorghum gene controlling stimulant production is simply inherited, with high stimulant production dominant (*23*). This gene has now been mapped on the sorghum genome (Weerasuriya, Y., Purdue University, unpublished data).

The biological significance of the production of strigolactones by the roots of *Striga* hosts (and by non-hosts such as cotton) when *Striga* is not present has not been determined (*24*). Are they phytohormones? Are they phytoalexins? Are they actively exuded or are merely passively leaked out of host roots? There are some clues. Sorghum genotypes have been reported to differ qualitatively (*25*) and quantitatively (*26*) in root exudate production, and inoculation with nitrogen-fixing bacteria also has an effect on root exudate production (*27*). Sorghum genotypes differ by up to 9 orders of magnitude in the amount of strigolactones they produce, with those from China which have never been exposed to *Striga* generally producing 3 orders of magnitude greater amounts than the highest producers from Africa (*10*). The high stimulant producers from China are far more susceptible to *Striga* than the most susceptible genotypes found in Africa, suggesting there has been selection against stimulant production (and possibly other traits contributing to *Striga* resistance) in virtually all African sorghums. Very little is known about the effect of environmental conditions on the amount of these compounds produced by host roots. There is an unexplained but striking effect of light (*28*) and daylength on some hosts. For at least one of the high stimulant producing sorghums from China, production of germination stimulant is 6 orders of magnitude greater when the sorghum seedlings are provided 2 hours of light per day than when they are given 16 hours of light per day (*10*).

Other Germination Stimulants. In addition to ethylene, sorgoleone, and the strigolactones, many compounds from non-host sources have been shown to stimulate *Striga* seed germination, although generally at lower concentrations than stimulants from *Striga* hosts (*29-33*). Their biological relevance is not known. Characterization of the *Striga* seed's receptor site for germination signals might clarify structure-activity relationships between these molecules which are active as germination stimulants.

There have been many efforts to develop synthetic germination stimulants based on simple analogs of strigol (*34-38*). The lactone rings, the linkage between them and the substituents on them, seem to be of crucial importance for germination stimulant activity. The shape of the molecule affects activity (stereoisomers are non-equivalent) (*18*). A molecular mechanism for stimulation of *Striga* seed germination by strigol and analogs has been proposed (*39*). Treatment of strigolactones with a non-specific esterase enzyme results in loss of stimulant activity, apparently due to hydrolysis of the terminal lactone ring (*21*). Strigol and its synthetic analogs are short-lived in soil (*40*), presumably due to hydrolysis of the lactone ring. Natural stimulants must lose their activity rather quickly in the soil in order to define a particular time period for seed germination.

Haustorial Initiation Signal. A germinating *Striga* seed develops a radicle but does not differentiate further unless it receives another chemical signal, also derived from the host root and transmitted through the soil around the root (*41*). Unless this second signal is received within about 4 days of germination, the *Striga* seedling dies. If the radicle does receive the second signal, it rapidly responds by differentiating into a specialized attachment structure, the haustorium. Like germination, attachment is not host specific; if the seedling receives the signal, the resulting haustorium will attach to anything, including a glass plate (*41*). Elongation of the radicle apparently does not involve cell division (*42*), so the haustorial initiator is the first signal that results in cell division/differentiation. The germination signal and the haustorial initiation signal are independent in that neither has any activity of the other type (*43*). Several compounds from non-host sources have been shown to have activity as haustorial initiation signals (*41*). These include 2,6-dimethoxybenzoquinone, a degradation product of lignin, which is found in surface abraded sorghum roots exposed to an enzyme produced by *striga*, but not in undamaged roots (*44*). The natural haustorial initiation factor produced by sorghum roots seems to be less stable than 2,6-dimethoxybenzoquinone (Weerasuriya, Y., Purdue University, unpublished data), and its identity has not been reported.

Crop cultivars which produce *Striga* germination stimulant abundantly but which fail to produce haustorial initiation factor would be uniquely useful. In addition to their resistance due to failure of *Striga* to attach to host roots, they would stimulate germination of many *Striga* seeds which would die and diminish the seed population in the soil. The agar gel assay for screening crops for germination stimulant production has been modified to screen for production of the haustorial initiation factor. Preliminary findings suggest that the capacities for production of the germination stimulant and the haustorial initiation factor are inherited completely independently, and that sorghum genotypes differ relatively little in their capacity to produce haustorial initiation factor, compared to their wide differences in capacity to produce the germination stimulant.

Subsequent Signals Directly Communicated through Vascular Tissue. Once *Striga* is established on a host root, the vascular connections between host and parasite obviate any further need for host-parasite communication via chemical signals exuded into the soil. Host-parasite communication becomes direct but no less important, perhaps extending the limits of conventional allelochemistry.

There is evidence from *in vitro* culture studies that after establishment on a host root, the host plant provides the parasitic *Striga* plant not only moisture, minerals and photosynthate, but also additional chemical signals required for further growth and development (*45,46*). Depending upon the composition of the synthetic medium, *S. asiatica* cultured *in vitro* grows in a non-parasitic manner either as undifferentiated callus tissue or as roots without haustoria and without shoots. Growing parasitically on host roots, *Striga* forms mainly shoots, eventually forming adventitious roots which have haustoria. On media conditioned by previously growing detached sorghum roots or by adding extracts from roots and/or shoots of sorghum plants, *Striga* grows in the parasitic mode, developing shoots quite similar to those which grow on host roots (*46*). Supplementation of the medium with known growth factors and/or phytohormones cannot replace the requirement for host extract/exudate to obtain parasitic type growth. Significant differences were observed in the *in vitro* growth of *Striga* seedlings due to "signals" extracted from sorghum plants and between "signals" from *Striga*-resistant and -susceptible sorghum genotypes (Butler, L. G., Cai, T., Babiker, A. G. T., Ejeta, G., *Agronomy Abstracts*, in press). The results suggest that as yet unidentified host-derived signals influence both the rate of *Striga* growth and its differentiation and morphological development.

Disruption of Signals as a Means of Controlling Striga

No truly effective means of controlling *Striga* is available to the African subsistence farmers whose livelihood and family food security are so strongly affected by this parasite. Development of *Striga*-resistant host crops could provide relief for these farmers without requiring the use of herbicides or other relatively expensive and inaccessible inputs. Low stimulant producing sorghums which are strongly resistant to *Striga* have been developed and are being released in Africa (Ejeta, G., Purdue University, unpublished results), but similarly resistant maize and millet varieties are somewhat further in the future. In the meanwhile, the new understanding of *Striga*'s dependence upon its host for growth-promoting chemical signals suggests that control might be possible if a means of intercepting or disrupting the necessary signals could be developed.

An attractive rationale for *Striga* control involves cleanup of infested fields by treatment, in the absence of a host crop, with a synthetic germination stimulant. Germinated *Striga* seedlings do not survive for more than 4 days unless a host root is available. This approach presumes that a large proportion of the *Striga* seeds in the soil will be suitably conditioned to respond to germination stimulant when it is applied. In the US, this approach has been successful, with injection of ethylene gas into the soil to induce suicidal *Striga* germination (*47*). Injection of ethylene gas is not now a practical approach for African farmers. The laborious synthesis of strigol and its analogs has prevented adoption of this strategy with strigol analogs (*24*).

If synthetic stimulants are to be useful and practical in promoting suicidal germination to clean up infested fields, the synthesis must be inexpensive (and therefore simple) and the synthetic compounds must be active in the soil over a period of several weeks (in order to stimulate *Striga* seeds conditioned for germination at various times throughout the season). Natural stimulants must be short-lived in the soil in order to define a particular time for seed germination. If the

In Allelopathy; Dakshini, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

intact lactone ring proves to be an inviolable requirement for germination stimulant activity, there may be little hope of synthetic stimulants sufficiently stable for use in the field.

There may be a way to take advantage of the reactivity/instability of the lactone ring of the strigolactones. They are readily inactivated by non-specific esterase enzymes such as a commercial preparation from rabbit liver (*21*). It might be possible to create a transgenic form of a non-pathogenic bacteria that normally associates with the crop roots, genetically modified to overproduce and secrete large amounts of esterase or a more specific lactonase that inactivates strigolactones. When applied to the crop seed before planting, as *Rhizobium* is applied to legume seeds, growth of the bacteria with production of the degradative enzyme could result in germination stimulant being destroyed as fast as it is produced. Germination of *Striga* seeds might be selectively prevented or slowed. This may actually be the mechanism by which some of the so-called "bioherbicides" work.

A useful alternative approach to synthetic germination stimulants is the selection of agricultural chemicals already available on the market, already approved for use on crops, for their capacity to stimulate ethylene production by *Striga* seeds (*48*). Ethylene production, of course, leads to germination. A mixture of TDZ (a cotton defoliant) and 2,4-D looks promising in the laboratory, and is being tested in the field (*48*).

Germination Inhibitors. If *Striga* seeds have specific receptors for germination stimulant, structurally related molecules that bind to the receptor but do not trigger germination would act as germination inhibitors by blocking active stimulants from binding. Natural inhibitors of *Striga* germination do exist. The inhibitors and stimulants both present in host root exudate can be separated on a simple reversed phase chromatographic column (*10*). Assays of stimulant activity are much more straightforward and easy to interpret after removal of the inhibitors. The natural inhibitors have not yet been identified.

Quaternary salts of fatty amines strongly and irreversibly block *Striga* seed germination (Eplee, R., USDA/APHIS, personal communication, 1990) but are strongly bound by soil particles, which limits their effectiveness. Non-ionic detergents may inhibit *Striga* seed germination in soil more effectively because they are not so strongly bound by soil particles (Weerasuriya, Y., Butler, L. G., Purdue University, unpublished data).

If compounds specific for inhibition of *Striga* seed germination without inhibiting germination of the crop seed or its subsequent growth can be identified or developed, it may be possible to apply them as a crop seed treatment, as fungicides have long been applied, rather than as a soil treatment. Crop seed treatment technology is simpler and more accessible to subsistence farmers than is soil treatment technology.

Striga-derived Signals and Their Effect on the Host

Diversion of water, minerals, energy and/or carbon from the host to the parasitic *Striga* necessarily diminishes host productivity. But the degree by which host productivity is diminished by *Striga* is much greater than can be attributed to

resource diversion from the host (*49,50*). There have been suggestions of "toxins" produced by *Striga* that inhibit host plant growth and development, diminishing the shoot/root ratio, for example (*50-52*). A small proportion of ^{14}C-labeled CO_2 taken up by photosynthetically active *Striga* plants is eventually transferred to the host plant (*53*), possibly accounting for the "toxin". At Purdue University we are attempting to characterize the purported *Striga* "toxin". It is possible that chemical signals are exchanged in both directions between *Striga* and its host.

Conclusion

In adapting a parasitic lifestyle, *Striga* seems to have given up control of its own growth and development to an unusual degree. The array of chemical signals exchanged between *Striga* and its host plant, first through the soil and then directly through vascular tissue, extend the concept of allelochemistry beyond its conventional limits. *Striga*'s strong dependence upon its host plant for developmental signals may eventually lead to more effective control methods by disruption of the signals it requires. Resistance based on multiple mechanisms is likely to be more durable than resistance based on a single mechanism. Resistance based on stages later than germination would have the advantage of reducing the seed population, if normal levels of germination stimulant were produced. For investigations of plant development, *Striga* provides a great advantage over non-parasitic plants: its development can be precisely controlled, step by step, by externally applied chemical signals.

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Chapter 13

Allelopathy and Self-Defense in Barley

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Evidence for allelopathic activity in barley and the major temperate cereal crops is reviewed. We believe that the secondary metabolites, gramine and hordenine, produced by barley (*Hordeum* spp.) play a role in defending the producing plant against interference from other organisms. Our recent work has shown inhibitory effects on a fungus (*Drechslera teres*) and on armyworm (*Mythimna convecta*) larvae as well as on a number of plant species. This work and that of others showing activity against bacteria, aphids and mammals suggests a possible physiological resistance to these organisms which may be exploitable through plant breeding. A survey of 43 lines of barley including ancestral and modern types indicates that hordenine production by modern cultivars may already have been inadvertently favored by selection for agronomic traits while the ability to produce gramine may have been reduced or lost during this process.

Increasingly, the recognition of the defensive-come-communicational role of allelochemicals has led to investigation about their potential in natural resistance to pests and, hence, in crop protection, with special reference to biological control (see, for example, *1-5*). Activity of allelochemicals against insects and other organisms has long been identified with such widely distributed families of secondary metabolites as the alkaloids, terpenes, phenolics and cyanogenic glycosides (*6*). Notwithstanding the plethora of naturally-occurring compounds which is available, relatively few have been deployed in the cause of crop protection. Thus, in respect of activity against insects, Jacobson (*7*) noted that, from early Roman times to the middle of the present century only pyrethrum, rotenone, nicotine, sabadilla and quassin were widely used as insect repellents and toxicants, at least, in the Western Hemisphere. He concluded

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that the potential for producing further insecticidal compounds was large, especially in the families Meliaceae, Rutaceae, Asteraceae, Malvaceae, Labiatae and Cenallaceae but pointed to the necessity for such compounds to satisfy the following criteria before they could be widely deployed. They must be (i) safe for plant and animal life, (ii) biodegradable, (iii) come from plants which are readily accessible and/or cultivable, (iv) subject to critical determination of the active principle(s), or (v) amenable to synthetic production. These criteria will be applicable to the deployment of allelochemicals in any crop protection context.

Allelochemicals and Cereal Crops

Allelopathic potential has been identified in all the major temperate cereal crops, barley (*Hordeum vulgare* L.), oats (*Avena sativa* L.), rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.). Further, allelopathic activity in the cereals has been observed in the live plant, from residues and in soils following the presence of cereal crops.

Allelopathy and Living Cereal Crops. Allelopathy in living barley will be dealt with in detail, below. With oats, Fay and Duke (*8*) demonstrated the presence of the allelochemical scopoletin (6-methoxy-7-hydroxy coumarin), a naturally occurring compound identified as an allelopathic agent in each of 3 000 accessions of Avena spp. Twenty-five accessions of *A. sativa* were studied in detail. Accession PI266281 exuded more scopoletin on a weekly basis, when compared with cv. Garry, but there was no correlation between scopoletin level and magnitude of interference with *Brassica kaber* (DC) Wheeler var. *pinnatifida* (Stockes) Wheeler (wild mustard) in a soil system.

Barnes et al. (*9*) reviewed the substantial evidence for allelopathy against weeds in rye, in which hydroxamic acids may contribute to phytotoxic activity. The hydroxamic acid component of 55 accessions of *Triticum* spp. was determined by Niemeyer (*10*), who regards these compounds as natural resistance factors against a variety of organisms. Hydroxamic acids occurred in all accessions but were highest in wild diploid species. Such accessions are possible sources of high hydroxamic acid levels for wheat breeding programmes and could contribute not only enhanced resistance to attack by insects and plant pathogens but also differential tolerance to atrazine-derived herbicides. Increase in hydroxamic acid levels in wheat would pose no obstacle to human consumption as the compounds are not present in the grain (*10*).

Allelopathy and Cereal Crop Residues. Substantial research effort has been devoted to understanding phytotoxic effects associated with the release of compounds from residues of cereal crops. Particular interest has been shown in such release in conservation farming systems where crop residues are deliberately retained for their value in enhancing organic matter content of soils and in combating soil degradation.

Biologically-active compounds may be leached directly from cereal residues, liberated during decomposition or synthesized by microorganisms utilising the residue as a nutrient source. The production, accumulation, transformation and

ultimate destruction of these chemicals is influenced by a large number of environmental factors, including temperature, pH, availability of oxygen and moisture.

The work of Kimber (*11*), in Western Australia, pointed to stubble of wheat as a possible source of phytotoxins which might affect the germination of following crops. Subsequently, Lynch (*12*) has identified toxicity with organic acids, particularly, acetic acid, produced during decomposition. Lodhi et al. (*13*) identified five phenolic acids from wheat mulch and associated soil and discuss these compounds in the context of phytotoxicity in rotational systems. Purvis et al. (*14*) and Putnam et al. (*15*) are among workers who have demonstrated that phytotoxins from cereal and other residues may play a role in weed management in rotational systems.

Allelochemicals from Cereal Crops in Soil. Allelochemicals are commonly transferred from living plants, or their residues, by water to the soil. The identification of biologically-active compounds among the plethora of organics present in soil, however, presents formidable difficulties. Waller et al. (*16*) discuss long-term work at Oklahoma Agriculture Experiment Station in which inhibitory compounds were isolated from wheat soils worked under no tillage and conventional tillage systems. Cast et al. (*17*), in a development of this work, found a trend towards greater autotoxicity of wheat by wheat soils under no-tillage, concluding that accumulation of phytotoxins occurred because disturbance and dispersion by cultivation is reduced (Table I). Their data suggest that the allelochemicals responsible for inhibition in their experiments are products of microbial activity.

From this brief review of allelopathy among the cereal crops it is clear that a better understanding of allelopathic phenomena in cropping systems is needed in order that they can be harnessed to the benefit of crop protection. Allelopathic effects of the types reported often occur in concert with other stresses (*18*). Research into allelopathy has tended to concentrate on reduction of allelopathic stress on crops. However, there is substantial evidence in the literature to suggest that cereals (and other crops) have an allelopathic potential which could be developed to their advantage.

Allelopathy in Barley

Although, as indicated in the Introduction, a broad perception of the role of allelochemicals in plant communication and defence is still developing, there are numerous accounts in the literature of the nature of secondary metabolites of barley and their biological activity. Some examples are given here.

Barley Allelochemicals. The production of phenolic compounds, ferulic, vanillic and p-hydroxybenzoic acids, from cold water extracts of barley straw was documented by Börner (*19*). He also identified these compounds in methanol extracts of living barley roots. Growth of wheat and rye roots was affected at as little as 10 ppm of the pre-compounds in water culture. Two alkaloids, gramine and hordenine

(e.g.*20*) are also produced by barley. The gramine pathway of the barley shoot and the corresponding hordenine pathway of the barley root are discussed by Schneider and Wightman (*21*). Overland (*22*) found that the former inhibited the growth of chickweed even when present in low concentrations.

The occurrence, synthesis and biodegradation of gramine and hordenine, have received considerable attention in the literature (see, for example, *23*). Accumulation of gramine in leaves of barley grown under high-temperature stress has been reported by Hanson et al. (*24*). These workers noted that high gramine levels early in barley growth equate with the notion of secondary metabolites as agents of defence (*25*). Gramine levels normally fall in later life but, in their work, gramine accumulation in growing leaves was stimulated by high temperature. This may, again, represent a link between allelochemicals and other stress factors.

Vancura (*26*) examined the composition of root exudates of barley, identifying amino acids, organic acids, sugars and aromatic compounds. He speculated on the significance of these exudates and on the relationship between roots and microorganisms in the rhizosphere. Harper and Lynch (*27*) found that, under controlled conditions, metabolites of the bacterium *Azotobacter chroococcum* stimulated the extension of barley roots. This finding suggested that the presence of benign microorganisms in the rhizosphere could be to the advantage of barley plants. Subsequently, Liljeroth et al. (*28*) have studied root exudates of barley in relation to root growth, nitrogen fertilization and abundance of bacteria on the rhizoplane. Increased nitrogen was positively correlated with bacterial abundance, however, it was unclear as to whether the effect of nitrogen was direct or indirect, via change in growth and exudation from barley roots.

As with the other temperate cereals, residues of barley have been associated with phytotoxicity. For example, Read and Jensen (*29*) observed that, in bioassay, water-soluble substances present in methanol extracts of soil cropped with barley decreased seedling root length in lucerne (*Medicago sativa* L.), wheat and radish (*Raphanus sativa* L.). It is possible that such activity could be to the advantage of barley in competition with Brassicaceous weeds.

The potential for harnessing phytotoxins produced by rotting barley straw to limit the growth of algae, which can cause problems in aquatic systems has been identified by Welch et al. (*30*) and Gibson et al. (*31*). Observations of a disused canal show that the presence of rotting barley straw reduced contamination by filamentous algae. When microscope slides bearing algae were suspended downstream of the straw, algal growth was reduced by 90%. Follow-up laboratory studies confirmed these findings with filamentous and planktonic algae. Further, it was shown that microbial decomposition was essential to the production of growth inhibition. Fresh straw produced no inhibition and autoclaving barley straw destroyed the effect. The authors note that anecdotal evidence suggests that blue-green algae may also be susceptible to allelochemicals produced from straw.

The developing parallels between effects of allelochemicals in terrestrial and aquatic systems point to the increasing potential to employ knowledge of these compounds in managing pest organisms.

In Allelopathy; Dakshini, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

Barley and Plant Pathogens. Ludwig et al. (*32*) studied the resistance of young barley shoots to infection by the fungal pathogen helminthosporium (*Helminthosporium sativum*), finding that the resistance was lost after the first few days of growth. In subsequent work, Stoessl (*33*) and Stoessl and Unwin (*34*) have equated antifungal activity with the presence of hordatines, compounds more complex than the indole alkaloids, present in extracts of barley coleoptiles. These compounds and some of their derivatives were found to inhibit spore germination of a number of fungi at concentrations as low as 10^{-5}M. Grodzinsky (*35*) found that barley root exudates inhibited germination and germ tube growth of *Fusarium oxysporum* f. *vasinifectum*.

Peeters et al. (*36*) describe an evaluation of 40 barley accessions from the gene pools of Spain and Nepal in respect of their resistance to powdery mildew (*Erysiphe graminis hordei*). There was considerable variation in resistance but the authors noted, in particular, that the degree of resistance within a genotype could change over time and that material which was fully resistant in one environment might be fully susceptible in another. These are manifestations of the dynamic nature of resistance in host/pathogen systems. There were, however, a few lines with good overall resistance but there was no stated link between resistance and secondary metabolites in this example. Similarly, in a study of the pathogenicity of spot and net blotch (*Drechslera teres* f. *maculata* and *D. teres* f. *teres* respectively) in barley, Arabi et al (*37*) found two Middle Eastern lines with good overall resistance but did not link this resistance with secondary metabolites. Warham (*38*), however, in a study of resistance to Karnal bunt (*Tilletia indica*) in wheat, triticale, rye and barley, found that barley was immune to the disease and suggested that it, barley, may possess physiological resistance. However, she also pointed to a physical characteristic, close adherence of the lemma and palea to the grain, as being a possible contributor to resistance.

Gramine has, specifically, been identified as a compound which may decrease infection of barley leaves by *Pseudomonas syringae* (*39*). The effect of gramine on oxygen consumption by *P. syringae*, reflecting bacterial growth, was to stimulate at low concentration but to inhibit as the concentration increased (Figure 1). Bacterial growth was affected at concentrations of gramine similar to those found in barley leaves. Further, gramine content was inversely correlated with leaf damage caused by *P. syringae*.

Barley and Insects. There is a substantial literature on aphid pests of temperate cereals, including references to chemical factors affecting aphid predation of barley. For example, Juneja and Gholson (*40*) reported that barley varieties which were resistant to greenbug (*Schizaphis graminum*) contained free benzyl alcohol. Resistance to greenbug was, subsequently, equated with acidic metabolites of benzyl alcohol.

Leather and Dixon (*41*) and Kieckhefer and Gellner (*42*) have considered the effect of plant growth stage on cereal aphid reproduction, studying greenbug, English grain aphid (*Macrosiphum avenae*), bird-cherry oat aphid (*Rhopalosiphum padi*) and corn leaf aphid (*R. maidis*). The latter species showed greater fecundity on seedling

TABLE 1. ANNUAL MEAN ROOT AND SHOOT LENGTHS (mm)[a]

Treatment[b]	Aqueous extract	MeOH extract	Conc. MeOH extract
Root			
DW	27.56a	26.29a	26.29a
CT	28.83b	25.19b	24.49b
NT	26.04c	24.46c	23.09c
Shoot			
DW	7.42a	7.28a	7.29a
CT	7.47a	7.35a	7.54a
NT	6.97a	6.85b	6.84a

[a]Means with the same letter in a given column are not significantly different ($P > 0.05$) by Tukey's studentized range test.
[b]DW = distilled water; CT = conventional-tillage; NT = no tillage.
SOURCE: Reproduced with permission from reference 17. Copyright 1990 Plenum.

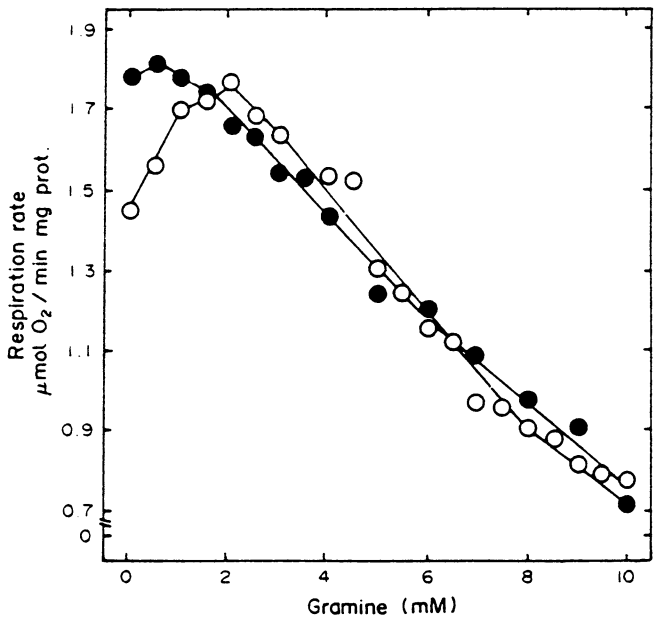

Figure 1. Effect of gramine on oxygen consumption by *P. syringae*. Points are means of three replicates. (○) Basal electron transport; (●) coupled electron transport with 3 mM ADP and 1 mM K_2HPO_4. (Reproduced with permission from ref. 39. Copyright 1990 Pergamon Press.)

barley than on later stages of growth, there being no significant differences between growth stages with the other species. Leather and Dixon (*41*), however, had found that *R. padi* showed a similar trend to that noted for *R. maidis* in the more recent work. These workers do not speculate on allelochemicals as modes of resistance or susceptibility to aphid attack, however, hydroxamic acids, phenolic acids and the indole alkaloid, gramine, all secondary metabolites, have been associated with resistance to grain aphids, the two former in wheat, the latter in barley (*43-45*). Hydroxamic acids have subsequently been found in species of wild but not in cultivated barley (*46*) and their concentration negatively correlated with aphid numbers (Figure 2). Similarly, Kanehisa et al. (*47*) and Rustamani et al. (*48*) found a negative correlation, in the field, between the abundance of *R. padi*, *R. maidis*, *S. graminum* and *Sitobion akeviae*, on barley lines which contained high gramine contents. These authors contend that gramine is one of the important resistance factors to aphids in this crop.

Interestingly, Leszczynski et al. (*43*) found that total phenols and hydroxamic acids, estimated in the flag leaf of wheat at anthesis, showed greater negative correlations with rate of natural increase of *S. avenae* than did the indole alkaloids. However, those cultivars which showed the greatest resistance to aphids were generally richer in all of the allelochemicals than susceptible cultivars, raising the possibility of synergism between the compounds.

A final example indicates that phytotoxicity between aphids and barley may be mutual, in that Riedell (*49*) postulates that phytotoxins injected into barley by the Russian wheat aphid (*Diuraphis noxia*) may contribute to a diminished capacity of aphid-affected barley plants to counter drought stress.

Barley and Mammals. Since it belongs to a group of alkaloids which can be toxic to mammals, gramine may be regarded as an "anti-quality factor" in barleys bred for forage use (*50*). In a survey of 24 genotypes of *H. vulgare* and *H. spontaneum*, these workers showed that considerable variation in gramine content occurred among genotypes and that gramine was present in some genotypes at concentrations which would be expected to depress performance in ruminants. They also observed that some *H. vulgare* cultivars were essentially gramine free, suggesting that they may lack the potential for synthesizing gramine.

Hanson et al. (*24*) noted that gramine can be viewed as a stress metabolite as high temperature tended to raise gramine concentration.

Barley and Weeds. The reputation of barley as a "smother crop" has been attributed to competition for environmental resources such as water and plant nutrients (*22*). However, in the absence of such competition barley still inhibits germination and growth of some weeds. Overland (*22*) showed that the inhibitory activity was selective among broad-leaved plants, chickweed (*Stellaria media* L.) being more severely inhibited than shepherd's purse (*Capsella bursa-pastoris* (L.) Medic.), and hypothesized that phytotoxins were involved (Figure 3). This was an early indication that allelopathic activity in barley might have potential for development in protection of the crop.

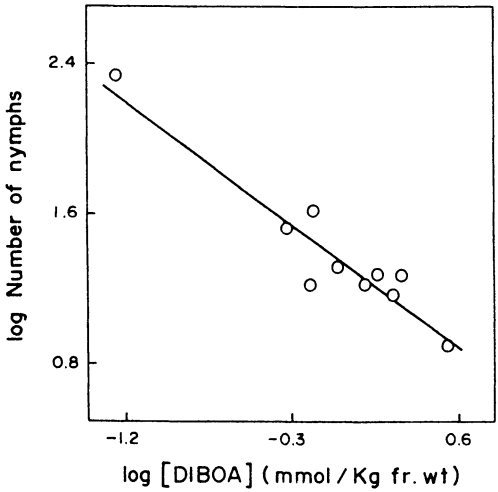

Figure 2. Correlation of DIBOA in 10 different barley lines taken from 6 species including *H.vulgare* (lowest DIBOA content) with performance of *R.padi* (number of aphids produced per new-born nymph living on a plant of a given species over a period of 16 days). Correlation coefficient 0.907. (Adapted from ref. 46.)

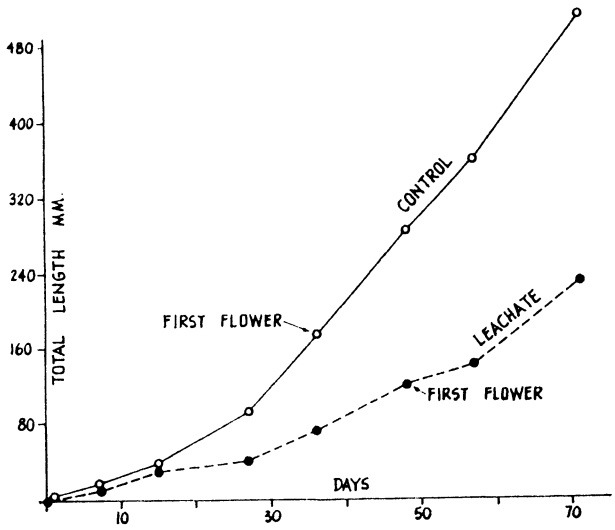

Figure 3. Effect of barley living root leachate on growth of *Stellaria media.* (Reproduced with permission from ref. 22. Copyright 1966 Am.J.Botany.)

More recently, Liebman and Robichaux (*51*) have discussed the competitive ability of barley monocultures and barley-pea (*Pisum sativum* L.) intercrops against two weedy species of mustard, *Brassica kaber* (DC) Wheeler and *B. hirta* (Moench). They determined that reductions in the access of mustard to light and nitrogen resulted from the presence of crop plants and, while acknowledging that allelopathy might have been a component of interference, were satisfied that competition for resources was important in explaining the reductions in photosynthetic performance, above-ground biomass and seed production of mustard, which they observed.

Biological Activity of Barley Allelochemicals : A Case Study.

The work of Overland (*22*) shows allelopathic activity by barley alkaloids against weeds, including members of the Brassicaceae. The initial work of our group explored the interactions between white mustard (*Sinapis alba* L.) (chosen for its synchronous germination and relative genetic homogeneity to represent a Brassicaceous weed) and these alkaloids and also the living barley plant (*52*).

The release of gramine and hordenine by barley roots was confirmed in bioassay and the compounds quantified. A concentration of 48 ppm hordenine, present after 4 days germination, significantly reduced radicle length in white mustard. The peak concentration of gramine, 22 ppm, also reduced radicle length in the test species (*53*). Release of barley allelochemicals was not, however, confined to the first few days of growth. Thus, where Hoagland's solution was recovered at intervals from a hydroponics system in which barley roots had been growing, the solution reduced radicle elongation of white mustard up to 75 days (*54*). The presence of hordenine and trace amounts of gramine in the hydroponics solution was confirmed by HPLC analysis. Hydroponics solution in which barley had not been grown did not affect radicle length of white mustard. Using a "stairstep" apparatus a negative effect on the growth of white mustard of root exudates of barley was demonstrated, over an extended period, in a system where competition was removed (*55*) and, in pot experiments, an attempt was made to apportion the contribution of competition and allelopathy to interference between barley and white mustard (*52*).

The response of radicle length of white mustard to gramine and hordenine followed the classic pattern in which an initial stimulation was followed by inhibition as concentration of the allelochemicals increased (*52*), a similar response to hordenine having been observed in *Amaranthus powellii* (*56*). Examination of white mustard root tips by electron microscopy showed evidence of increased vacuolation and cellular disorganisation, as is commonly observed in such cells when subjected to a variety of stresses (*4*). In recently completed work (Payne et al., unpublished data) we have demonstrated the ability of gramine to severely retard growth of white mustard in soil, under controlled conditions, at a concentration of 250 ppm. Similarly, hordenine at 1.5 mM in sand has been shown to depress the growth of prairie grass (*Bromus unioloides*) (*56*) and shepherd's purse (*Capsella bursa-pastoris*) (unpublished data).

Larvae of the common armyworm (*Mythimna convecta*), when fed diets containing gramine and hordenine, showed a similar response to that observed with

radicles of white mustard. The diameter of the mature portions of colonies of the fungus *Drechslera teres* followed a similar trend. Thus, a study which began by concentrating on allelopathy between barley and weeds developed to provide evidence of the wider activity of barley in suppressing pests, as reviewed in the earlier portion of this paper.

The potential value of enhancing levels of allelochemicals in crop plants as a means of developing the "self defence" implied by these findings has been identified by several authors (see, for example, *11*, *57*, *58*).

In the case of gramine and hordenine in barley we have begun to test the hypothesis that the content of allelochemical in crop plants has become attenuated as selection for other characteristics has progressed. In initial experiments 43 lines of Hordeum were investigated, including the ancestral forms *H. spontaneum* and *H. agriocrithon.*

Thirty lines were grown in a growth chamber (Experiment 1) and all lines were grown outdoors (Experiment 2), where environmental conditions differed, especially in respect of incident radiation. Samples of frozen leaf and root tissue from both experiments were extracted, purified and concentrated, and gramine and hordenine were quantified using HPLC (*59*).

Not all lines produced significant quantities of gramine but the data tended to confirm the hypothesis, in that gramine contents were higher in ancestral barleys or *H. vulgare* landraces than in cultivars (*60*) (Figure 4).

Among lines which produced gramine, the ranking from high to moderate production did not change materially when they were grown under different conditions. This implies genetic control (linear regression of Expt. 2 on Expt. 1 gave $r^2 = .9079$) (Figure 5). Of the Australian-grown lines, 4 produced small but detectable quantities of gramine only in Experiment 2, suggesting that the more favourable environmental conditions (especially light) allowed expression of the genetic potential to produce gramine.

The consistently greater gramine content of plants in this experiment implies competition between metabolic pathways, with diversion of substrate between "yield" and "defence" as a possibility.

A higher correlation for all 30 lines common to both experiments than from the 18 gramine-producing lines suggests a looser genetic control among the higher-producing lines .

Therefore, it is concluded that gramine production is the norm in barley but that, as a consequence of breeding for quantity and, perhaps, quality of yield, this ability has become attenuated (*60*). Along with it potential for biological suppression of pests ("self defence"), as discussed above, has also been diminished.

In contrast to gramine production, hordenine production does not appear to be under strong genetic control ($r^2 = .2370$), but is very sensitive to changes in the environment. Hordenine production was up to ten times greater under the lower light conditions of experiment 1. Hordenine was also found in all lines and tended to be found in greater quantities particlarly in the Middle Eastern bred lines but also in modern cultivars grown in Australia. We infer from this that hordenine production may confer defensive benefits on the barley plant and that hordenine production may

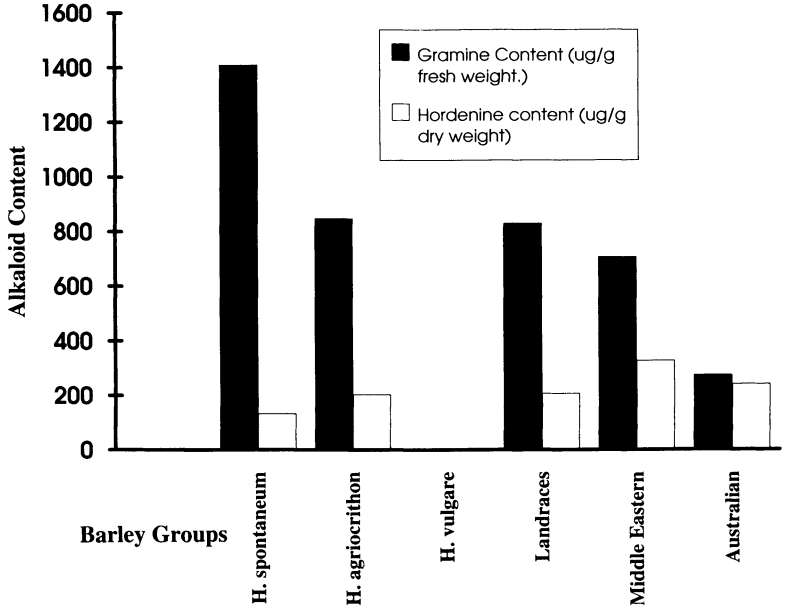

Figure 4. Mean gramine and hordenine content of five groups of barley lines.

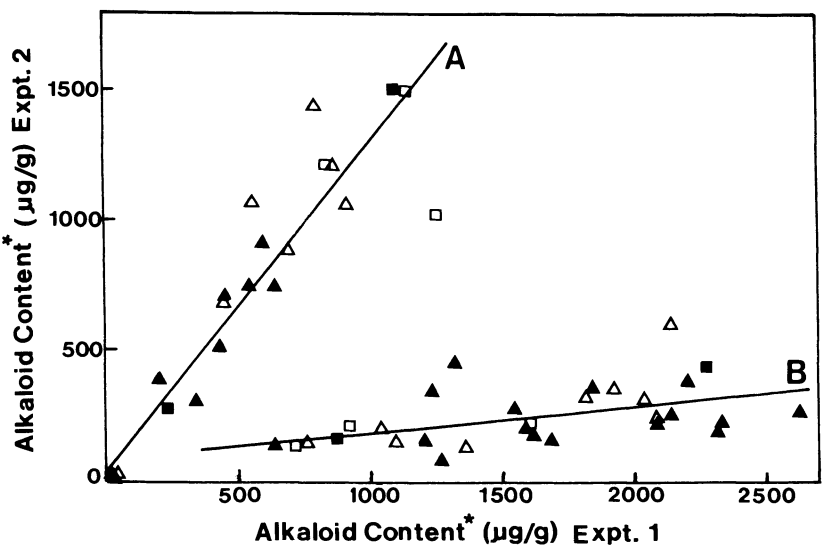

Figure 5. Comparison of gramine (Line A,) and hordenine (Line B) contents of 30 different barley lines grown in experiments 1 and 2.
□ *H. spontaneum*, ■ *H. agriocrithon*, △Middle Eastern cultivars, ▲Australian grown cultivars.
*Gramine expressed as µg/g fresh weight and hordenine as µg/g dry weight

have been favoured by selection for pathogen resistance, further, variation in hordenine production with environmental conditions could contribute to the dynamic nature of host/pathogen relations noted earlier. There appeared to be no relationship between gramine and hordenine production (Lovett and Hoult, *J. Chem. Ecol.*, in press).

Acknowledgments

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Chapter 14

Tillage and Allelopathic Aspects of the Corn–Soybean Rotation Effect

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Growth of young corn plants is reduced if their roots grow through a band of corn residue, but if the band is above the seed there is little toxicity. Water extract of residue or of decomposing residue in soil inhibit growth of corn seedlings, but if the extracts are filtered through a column of soil they have little toxicity. Under field conditions these residues have little effect on corn growth. The living roots of corn produce other types of water soluble chemicals which are more persistent in the soil. They are produced during the summer and decompose the following spring. Soybean growing in the field produces three chloroform soluble chemicals that stimulate corn seedling growth. Evidence suggests that corn leaves something in the soil that reduces grain yield of following corn and that soybean leaves something in the soil, in addition to avaiilable N, that increases yield of corn.

Crop growth and yield is determined by numerous environmental and genetic factors. Many of these factors interact, resulting in a very complex biological system. The biological complexity of this system is exemplified by crop growth and production response to crop or plant sequences. Many studies have reported the effect of previously grown crop plants on crop growth and yield (*1*). Others have addressed weed effects on crop yield, particularly as the weeds may influence crop performance beyond that of strict competition for resources (*2*). The impact of the previous plant species on plant growth is well documented, yet the factor(s) causing the response often remains unknown.

Crops grown in rotation often result in higher yields than crops grown in monoculture (*3*). Even with high fertility rates, the yield response favors the rotation. This has been termed the rotation effect. Studies have attempted to identify nonplant factors such as soil physical conditions (*4*), diseases (*5*), insects

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(*6*), and fertility (*7*) as causal agents. Generally, these factors fail to adequately explain the production differences between monoculture and rotations. The rotation effect, while partially caused by nonplant factors, is related to biological and/or chemical interactions between plant species. This biological interaction, allelopathy, may have significant implications with regards to crop management practices.

In the United States, conservation tillage systems are gaining popularity. On many highly erodible soils, continuous corn with no-till management is the only continuous row crop production system allowed due to soil conservation requirements. Continuous corn has repeatedly resulted in lower yields than corn in rotation, but why this occurs is largely unknown. Allelopathic interactions from the previous year's corn crop is highly suspected. Many concerns have been expressed about the allelopathic impact of corn residue on the succeeding corn crop with conservation tillage systems.

Tillage and Allelopathic Relationships

Various corn residue management factors may affect allelopathic responses of the new corn crop. Residue age, or previous weathering history, and placement of the residue with respect to the seed seem quite important. In a greenhouse study, placing fresh (unweathered) corn residues at the corn planting depth resulted in dramatic (45%) root growth reductions compared to no residue application or residue placement above the seed (8). Placement of fresh residue 5 cm below the seed likewise resulted in significant root growth reductions compared to placement above the seed. In this study soil temperature and water content were common for all treatments. Dry shoot weights at 49 days after planting were similarly affected.

In this same study, residue weathering, i.e., comparing effects of residue which remained on the soil surface over winter to that which was collected directly after harvest, was also significant. The greatest differences between residue effects occurred for those residues placed at or below the seed. Unweathered residue resulted in 69% of the root growth which occurred with weathered residue. Weathered residue resulted in 92% of the root growth which occurred without residue additions. No statistically significant differences between residue weathering effects were observed when residues were placed above the seed.

This study identifies three potentially important management considerations: 1) corn residue can have significant allelopathic activity on corn; 2) weathering residues seem to reduce their allelopathic activity; and 3) residues placed above the seed planting depth have less allelopathic activity than those placed at or slightly below the planted seed. Tillage systems which leave residues on or near the soil surface, from the allelopathic perspective, seem to be more favored than those which incorporate residues.

Some allelochemicals are water soluble. Thus surface placement of residues, as occurs with no-till, could result in rainfall "extraction" from these residues and movement with water through soil to the planted seed position. Various soil-residue extract interactions could occur, and thus influence the ultimate effect of this extract on corn seedlings. Yakle and Cruse (9) tested this "hypothetical"

situation in a laboratory study in which corn residues were incubated for 0, 15, or 30 days in soil. The residue/soil mixture was then extracted with tap water and either used to germinate corn seeds (for a 7 day period) or leached through 5 cm columns of soil before being used to germinate corn seeds. After 7 days, dry root and shoot weight of the seedlings were determined. Corn residue extract, soil extract, and tap water were compared. Generally, residue incubation in soil reduced the alleochemical activity (as determined by seedling growth), with activity inversely related to length of incubation time. Also, leaching the extract through soil reduced the effect of the extract on corn seedling root weight and shoot weight. These treatments did not have a significant effect on percent germination.

Garcia and Anderson (10) sampled soils during the growing season from a field of continuous corn with three tillage practices consisting of no-tillage, spring disc, and fall moldboard plowing; depths of sampling were 5, 10, and 20 cm, respectively. These are approximate depths that tillages incorporated the residue. The control consisted of a fallow soil stored at 5^0C. For water extraction, each soil sample was placed in a bottomless 0.5 L bottle. Drainage from the soil passed through an XAD-4 resin ion exchange column and was blown back to the soil surface for continuoous aerobic extraction for two days. Chemicals removed from the resin were used to wet germination paper for either corn or cress seed growth tests. At the May 1 soil sampling (Table 1) plots receiving all three tillage treatments contained chemicals that reduced seedling growth as compared to extracts of fallow soil.

Table 1. Growth of corn seedlings as affected by water extracts of soil samples collected during the growing season of second year corn with three tillage treatments (growth as a percentage of extracts from a fallow soil)

Tillage Treatment	Soils sampled on around the first day of each month					
	May	June	July	Aug	Sept	Oct
No-till	76	93	124	103	89	84
Disc	88	100	112	97	74	88
Plow	80	89	103	101	75	78

Toxicity also was present at the June 1 sampling, but by July the extracts were stimulatory as is common in decomposing residues (11). During the August and September samplings toxicity reappeared in the soil. The two types of bioassay gave similar results. The toxicity developing after July indicates that roots of living plants, and not the dead crop residue, produce the substances in the soil that were inhibitory to corn and cress seedling growth. Soil samples to a depth of 20 cm had as much or more inhibitory effect as soils from the top 5 cm which also indicates root involvement. The three soil tillage treatments had little effect on the disappearance of toxicity, the appearance of the stimulatory amount of activity, and the reappearance of toxicity later in the summer. In related studies, toxicity

In Allelopathy; Dakshini, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

developed in soil of corn fields with the soil covered by plastic sheets during the growing season.

These studies suggest the following. Leaching of allelochemicals from surface residue through soil is possible, although not likely to occur in sufficient quantities to cause early germination-growth problems. Chemicals leached from the residue into the soil more than a few days prior to corn planting will likely undergo transformation or breakdown processes in the soil reducing their inhibitory nature. Weathering of residues over winter will reduce allelopathic impacts of residue leachate in the spring. The greatest potential for allelopathy from corn residues seem to be from incorporation of corn residue shortly before corn planting. No-till management again seems favorable with regard to managing allelopathy from corn residues. The impact that corn residue allelochemicals has on corn yield has not yet been convincingly determined.

Allelopathic Aspects of the Rotation Effect

Throughout the middle western corn and soybean belt of the United States, corn grown following soybean yields greater than corn following corn with nitrogen fertilizer adequate for maximum yield. Results of a seven year study in Illinois showed a 17% greater yield of corn following soybean than corn following corn (12). The results in Table 2, from Schrader and Voss (13) in Iowa, illustrate the effects of soybean or meadow on corn yield vs. corn following corn. The magnitude of the rotation effect varies from year to year. During a droughthy and hot season of 1988 many farmers in southeastern Iowa reported corn after soybean yielded twice that of corn after corn. In contrast, corn after corn yielded greater than after soybean in 1989 due to corn essentially dying in late August of 1988 and thereby not using late August rainfall, whereas soybean recovered and used this moisture. The 1989 season also was dry and the extra stored soil moisture under corn was beneficial. In the deep soils of the midwest, crops of corn and soybean extract similar amounts of water. Table 2 indicates that at the highest rate of N corn partially remains influenced by the previous legume for two years.

Table 2. Grain yield of corn (tonnes ha^{-1}) in monoculture and in rotations with soybean or Corn-Corn-Oat-Meadow from a long term study in Iowa

N fert. kg ha^{-1}	Continuous corn	Corn-soybean	1st year CCOM	2nd year CCOM
0	2.45	5.20	6.60	4.75
54	4.60	5.90	6.40	5.75
108	5.65	6.40	6.60	6.00
162	5.90	6.50	6.45	6.20

It is difficult to determine if the rotation effect is due to toxicity left by the corn crop or if the soybean crop leaves a stimulatory effect. One method of determining if corn leaves something inhibitory to the following corn crop is to use continuous corn and determine if some hybrids leave a greater inhibitor effect than do other hybrids. Hicks and Peterson (14) in Minnesota, during a two year study, grew five hybrids in rotation and reported that hybrids varied slightly in how much inhibitory effect they leave. Dave Sundberg and I.C. Anderson (personal communication) in Iowa, grew six hybrids in all 36 possible combinations as previous and current hybrids during a four year study. Some of the results are reported in Table 3. All hybrids yielded less if grown following corn. All corn hybrids presumably left toxicity in the soil but Hybrid 4 left the greatest amount. Farmers frequently report that growing the same hybrid after itself appears to have a negative effect. In our study there was not a greater negative effect of a hybrid following itself. Note the magnitude of the rotation effect when Hybrid 3 followed soybeans.

Table 3. Grain yields of six corn hybrids grown with each of the six as previous corn crop (tonnes ha^{-1})

Previous hybrid	Current Hybrid 1	2	3	4	5	6	Mean
1	7.5	8.4	8.9	9.6	8.1	9.1	8.6
2	8.3	8.7	8.4	9.7	8.6	9.0	8.8
3	8.3	8.3	8.9	9.2	8.3	9.2	8.7
4	7.2	7.7	8.3	9.0	7.6	9.0	8.1
5	8.5	8.5	8.7	9.4	8.5	8.8	8.7
6	7.5	9.0	8.7	9.6	8.5	8.7	8.7
Soybean	-	-	9.7	-	-	-	-

Sarobol (15) at Iowa studied the effects of varying maturity and date of planting of both previous soybean and previous corn on the yield of the following corn crop (Table 4). In 1983 and 1984 five corn treatments were established: a very early hybrid planted May 15, an adapted hybrid planted May 15, June 30 and July 30, and a very late hybrid planted May 15. The five soybean treatments were similar in principle to that of corn. A control treatment of oat also was used. After the crops in each corn and soybean treatment matured or were killed by a frost, the grain was harvested and the crop residues were either left on the ground or removed. At the end of the fall the field was moldboard plowed to prevent any effects in the spring of variable surface residues on soil temperature and soil drying. In the spring the whole field was planted to a mid-season hybrid. Nitrogen fertilizer at a rate of 225 kg/ha^{-1} was applied before planting and an extra 55 kg/ha^{-1} applied before the last cultivation. The grain yield of corn following corn was less than corn following soybean. For corn following corn the least yield was from the corn following the very late hybrid. The next lowest yield was corn

following the adapted hybrid planted May 15. Both of these treatments produced considerably more growth than those of the other three previous corn treatments. The least amounts of corn growth in 1983 and 1984 were by the 30 July planted adapted hybrid and 15 May planted early hybrid which matured about 5 weeks earlier than the adapted hybrid planted 15 May.

The yield of corn following soybean was considerably greater than corn following corn, although the variation among previous soybean treatments was less

Table 4. Effect of previous crop, its maturity, and date of planting on grain yield of corn grown the following year

Previous Crop (1983 and 1984)			Grain Yield (tonnes ha^{-1})		
Type	Maturity	Planted	1984	1985	Mean
Corn	Early	15 May	9.02	7.71	8.37
Corn	Adapted	15 May	8.10	7.70	7.90
Corn	Adapted	30 June	8.94	7.63	8.29
Corn	Adapted	30 July	8.77	8.61	8.69
Corn	Late	15 May	7.22	7.42	7.32
Oat	-	-	8.59	8.07	8.33
Soybean	Early	15 May	9.55	8.40	8.98
Soybean	Adapted	15 May	9.71	9.59	9.65
Soybean	Adapted	30 June	9.23	8.65	8.94
Soybean	Adapted	30 July	9.14	9.29	9.22
Soybean	Late	15 May	9.47	8.69	9.08

than that with the previous corn treatments. The greatest yield of corn was following the adapted soybean cultivar planted 15 May and one of the least yields following the early soybean cultivar. The rotation effect between corn following the adapted soybean cultivar planted 15 May and the adapted corn hybrid planted 15 May was 21%. About 25% of this effects was an inhibitory effect of corn and 75% a stimulatory effect due to soybean compared with oat.

The mean effect of either removing or leaving previous crop residue had no effect on subsequent corn yield. Other studies (Crookson, 1982) showed similar results. These results agree with those reported in Table 1 by Garcia and Anderson (10) who proposed that allelochemicals from corn were being released by roots of the living corn plants into the soil. Neither the possible inhibitory effect of previous corn crop or possible stimulatory effect of the previous soybean crop (rotation effect) were due to the previous crop residues.

Kalantari (17) and Nelson (18) extracted soil from soybean fields after harvest with chloroform to test for stimulatory chemicals in the soil. The chloroform extract was taken to dryness, dissolved in isopropanol, and applied to a DEAE-sephadex column and eluted with isopropanol. Fractions were collected and bioassayed with the corn seedling assay (Kalantari), and by a Lemna minor assay

(Nelson). The results of a typical corn seedling assay is presented in Table 5. Stimulatory chemicals were found in Fraction 2, a slight inhibitory effect in Fraction 4, and a stimulatory peak in Fraction 12. The top of the column retained triacontanol that was stimulatory in the corn seedling assay. We made no attempt to identify the other two stimulatory chemicals. Chloroform extract of soybean straw showed activity in Fraction 2 but not in Fraction 12. Incubation of soybean straw with fallow soil for up to three weeks did not produce any activity in Fraction 12. Reis and Houtz (19) and Einhellig (20) added alfalfa meal to soil growing corn and other crops and reported a greater yield where alfalfa meal had been added than could be obtained with the highest rates of nitrogen fertilizer. They suggested that the response was due to triacontanol which could be isolated from alfalfa meal. Triacontanol, under certain prescribed conditions, stimulates plant growth.

Table 5. Corn seedling growth with column fractionated chloroform extracts of soybean soil (growth as a percentage of a water control)

Source of sample	Column fraction			
	2	4	12	top
Soybean field	123	102	148	139
Soybean residue	108	89	109	-
Fallow soil	95	97	112	-

Conclusions

1. Crop residue on soil surfaces slows the warming and drying of soil in the spring and that may have a negative effect on crop yield in cool temperate regions.
2. If seedling roots of corn grow directly into a soil area with concentrated corn residue, growth of corn plants are decreased. Incorporation of corn stover by spring tillage could leave spots of concentrated residue in the soil.
3. Water extracts of corn residue are toxic to corn seedling growth. Residue which has been weathered in the field had less effect. Water extracts of corn residue incorporated and incubated in soil were inhibitory to seedling growth. If these extracts, and those of residue itself were passed through a soil column toxicity was decreased.
4. Therefore, chemicals released from corn residue in the soil have only a small effect on corn seedling growth. The least toxic effect of corn residue would be if no-tillage were used.
5. Evidence indicates that the living roots of corn plants begin producing chemicals in the soil at about the anthesis stage of growth. These chemicals, or effects, accumulate and remain in the soil through the winter and are

degraded during the following spring. The degraded products appear to be slightly stimulatory to corn seedling growth. The chemicals from the living plant root are relatively long lived in the soil and probably are different from those released from corn residue.

6. The toxicity, or inhibitory effect, left in the soil by a previous crop of corn decreases the yield of corn the following year compared to following a crop of oat. Corn hybrids vary in the amount of toxicity left. Very early maturing hybrids have less toxicity to corn the following year than do adapted, or very late maturing hybrids.
7. The effects of either removing corn residue or plowing it under after harvest in the fall have similar effect on the yield of the following corn crop; this again indicates that corn stover residue does not contribute to the corn-soybean rotation effect.
8. Compared with a previous crop of oat, soybean leaves a substance in the soil that increases grain yield of corn. Soybean crop residue, either removed after harvest or left in the soil, does not effect the yield of the following corn crop. Adding soybean residue to corn fields in the fall does not overcome the toxicity left in the soil by corn residues.
9. Chloroform extracts of soil from soybean fields, after harvest in the fall, contain chemicals that stimulate the growth of corn seedlings. One of these chemicals is triacontanol. Triacontanol may contribute to, but probably has no major role for, the stimulatory aspect of soybeans in the rotation with corn.
10. The total rotation effect frequently is a 10-20% increase in corn yield following soybeans compared to corn following corn. It appears that both the toxicity left in the soil by corn and the stimulatory effect left by soybean contributes to the rotation effect. If our studies about 25% of the rotation effect was due to reduction by previous corn and 75% due to an increase from soybean.

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Chapter 15

Intercropping Allelopathic Crops with Nitrogen-Fixing Legume Crops

A Tripartite Legume Symbiosis Perspective

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Developmental and functional responses of the tripartite bean symbiosis were demonstrated to be indicators of allelopathy potential of a cabbage intercrop. A field experimental approach using a modified Nelder Fan design tested 3 intercrop proportions at 7 planting distances for a range of allelopathic interactions. Greater cabbage proportion generally reduced VAM infection and bean biomass, N and P but increased nodulation, except at the lowest planting distances. In addition, an intermediate proportion treatment at the higher distances produced positive effects of bean biomass, N and P which represented a potential over-yielding mechanism. The allelopathic basis for only the negative effects was corroborated in the greenhouse with plants grown in Leonard jars but not in a recirculating root exudate apparatus.

While the studies of allelopathy in agroecosystems have focused on crop-weed interactions, there have also been demonstrated crop-crop interactions, such as with rotations of monocultures (*1,2*). These crop-crop interactions will have a significant impact on the practice of intercropping which is "a cultural practice in which two or more crops and/or wild plants are grown in the same field with both temporal and spatial overlap" (*3*). Numerous crops have now been identified as having an allelopathic potential, e.g. oats, sunflower, alfalfa, celery, etc. (*1,4,5*), and their use as intercrops would promote greater occurrences of allelopathic interactions. This chapter presents the background and experimental approaches for evaluating the role of brassica crop-based allelopathy when intercropped with leguminous crops.

Allelopathy and Intercropping

The study of crop-based allelopathy is essential to ensuring successful intercropping because of its potential to impact important over-yielding factors of

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these production systems (*6,7*). Over-yielding means there is a yield advantage relative to monocultures, i.e. it would require a greater amount of land to produce equivalent yields by monoculture compared to intercropping practices, which is calculated by the land equivalent ratio (LER) (*8*). An important process of intercropping productivity that would promote over-yielding is the greater potential for mutualistic interactions between species, both direct and indirect (*6*). Mutualisms have also been ascribed to all levels of agricultural sustainability, e.g. organismal, agroecosystem, farm system, etc. (*9,10*).

The microbial components in sustainable agriculture (*11*) include important examples of mutualisms for the study of crop-based allelopathy with intercropping. To provide this data, research requires an expanded scope beyond just "direct" effects, i.e. from plant to plant, to "indirect" effects, i.e. from plant to plant-associated microbes which have important ecological functions as root symbionts, endophytes, and rhizosphere populations (*12*).

Legume Intercropping and the Tripartite Legume Symbiosis Perspective

Legume intercropping systems are receiving a great deal of support in sustainable agricultural development due to numerous examples of over-yielding (*13,14,15*). A critical factor involved in this trend is the capacity of many legumes (e.g. pulses, forages and trees) for biological nitrogen (N) fixation by a mutualism with the micro-symbiont *Rhizobium* bacteria, i.e. the conversion of atmospheric N_2 with nitrogenase enzyme to a form available for plant uptake (*16*).

More recently, the research perspective of the N_2 fixation system has been expanded to a tripartite legume symbiosis including vesicular-arbuscular mycorrhizae (VAM), as well as the legume host and *Rhizobium* bacteria (*17*). The tripartite symbiosis provides several mechanisms for yield advantages. For example, N complementation results from the improved partitioning of N sources between the legume and non-legume intercrops (*18*). Also N transfer has been demonstrated from the roots of legume to non-legume intercrop via VAM connections (*19*), and the use of the ^{15}N dilution technique has shown that N_2 fixation is the source of the transferred N (*20*). In addition, VAM-mediated transfer of P from a non-legume to the legume intercrop has now also been documented (*21*), thereby establishing the reciprocity required to describe an emergent property of a mutualistic relationship which would facilitate over-yielding.

Allelopathy and Legume Intercropping

Allelopathy represents a potential biotic factor affecting the legume's N_2 fixation system (*22*). For example, allelopathic inhibition of legume nodulation has been reported in old field succession (*1*), and the efficiency of agricultural N_2 fixation is reduced by allelopathic weeds (*2*). The literature on allelopathic interference on N_2 fixation is highly relevant to legume intercropping systems, particularly when including intercrops with known allelopathic potentials.

Although the role of belowground interactions as over-yielding mechanisms have been acknowledged in legume intercropping systems, there have been relatively few studies in general (*14*). And of the published allelopathy reports, the focus is either on the legume-*Rhizobium* symbiosis without mention of VAM levels (*23*) or on the legume-VAM symbiosis without consideration of N_2 fixation (*24*). There have been no studies reporting the responses of the tripartite symbiosis to allelopathic intercrops.

Case Study of a Legume-Brassica Intercropping

There is a growing interest with legume-brassica intercropping because of reported cases of over-yielding (*25,26,27*). A potential mechanism for over-yielding based upon N dynamics exists but would involve only N complementation. This exemption is due to the fact that brassicas are typically non-mycorrhizal (*28*), and would be incapable of receiving VAM-mediated N transfer from a legume intercrop.

The allelopathic status of brassica crops is based upon their production of secondary chemicals called glucosinolates (*29,30*). Upon enzymatic hydrolysis, glucosinolates form breakdown products, e.g. thiocyanates, isothiocyanates, nitriles, and sulfides, which have known biological activities (*31,32*). Brassica crops in rotations and as intercrops have been reported to adversely affect legume crop growth (*33,34*). A possible allelopathic mechanism is supported by published claims for a glucosinolate basis to brassicas' ability to reduce the VAM infection of mycorrhizal, non-leguminous crops (*35-38*), as well as explain their lack of endomycotrophy (*39*). The allelopathic potential of glucosinolate derivatives has been demonstrated in the laboratory on non-symbiotic bean growth (*40*), and VAM spore germination (*41*).

While examples of legume intercropping with brassicas have been reported worldwide (*42-45*), the majority involve cereal and root intercrops (*14*). Therefore, this case study is intended to contribute to the development of new applications of legume intercropping. The hypothesis evaluated was that allelopathy from a brassica intercrop included indirect effects on the tripartite legume symbiosis. A field methodology was developed for assessing this allelopathic potential, as well as compared to laboratory techniques. The responses of the tripartite legume symbiosis then were analyzed for the role of allelopathy on the over-yielding mechanisms of this legume intercropping.

Materials and Methods.

The test plants were beans, *Phaseolus vulgaris* L., (cultivar C-20 from Moran Seeds, Salinas, CA: a group III, indeterminate bush type, white navy dry type), and cabbage, *Brassica oleraceae* var. *capitata*, (cultivar Golden Acre from Niagra Seeds Co., Modesto, CA: a white, ball head type). The micro-symbionts were *Rhizobium phaseoli* ("D" Culture, peat formulation from Nitragin Co., Milwaukee, WI) as a seed inoculum, and VAM which was supplied in the field experiment from the background soil population (which had previously been

identified as *Glomus* spp.) and in the greenhouse experiments as a soil inoculum. Beans were intercropped with 4 week old cabbage transplants

Tripartite responses to cabbage plants of VAM infection, nodulation, and biomass was determined with a harvest at the R3 growth stage (*46*) in all of the studies described below. VAM as the percentage (%) of root length infected was quantified using the Phillips and Hayman staining procedure and the gridline scoring method (*47*). Nodules were picked by hand and measured for dry weight. Dry weights of all plant biomass was recorded after 48 hours of oven drying at 70°C.

In the field study, whole bean shoots were Wiley milled to 30 mesh for chemical analysis of total %N and %P at the Tissue Analysis Lab of Dept. of Land, Air and Water Resources, Univ. of CA, Davis, CA. Additionally in the greenhouse studies, the nitrogen fixation activity was measured in the greenhouse studies using the acetylene reduction assay (ARA) after an incubation of 1 hour (*48*).

Field Study. The experiment was conducted at the research farm of the Agroecology Program of the University of California, Santa Cruz, CA, from August to October, 1986. The climate is a cool summer, Mediterranean type, and the soil is an Elkhorn sandy loam (thermic Pachic Argixeroll)(*49*). Prior to planting, the soil was amended with 10 MT ha^{-1} of composted poultry manure (2-1-2) and 1.1 MT ha^{-1} of dolomitic lime, and soil tests indicated 22 ppm NO_3-N, 6 ppm P (Bray 1) and pH 6.1.

The experimental design was a Nelder Fan (50) with 3 replications. The whole plot was proportion with 5 treatments (i.e. 3 intercroppings and 2 monocultures). The split-plot was planting distance with 7 treatments (i.e. 28, 31, 35, 39, 44, 49 and 55 cm between intercrops).

The Nelder Fan was modified to produce a hexagonal configuration to the neighborhood of treatment plants around every sampled bean plant. A range of cabbage proportions was produced with a replacement series of the neighbors of bean plants in the bean monoculture to give the intercropping treatments. The resultant 5 whole plot treatments of proportion of bean (B):cabbage (C) are designated as 4:0, 3:1, 2:2, 1:3, and 0:4 for bean monoculture, 3 intercroppings, and cabbage monoculture, respectively (proportions based upon plant densities and not plant number per hexagon).

Statistical analyses were performed across the range of cabbage proportions and plant distances with regression ANOVA (*50*). To this end, orthogonal contrasts of the responses to increasing proportion and spacing treatments were calculated for linear, quadratic and cubic trends. Intercrop effects were also evaluated with ANOVA and class comparisons to demonstrate diversity as the true source of variation of recorded effects (*51*).

Due to the time constraints and physical requirements for sampling excavated roots for nodulation and VAM, not all treatments were sampled for them (see data tables). The specific treatments of these limited samplings were chosen for considerations of the described statistical analyses. On the other hand, all treatments were sampled for shoot biomass, height and width of beans because of the greater ease of sampling.

Greenhouse Studies. The experiments employed the methodologies described below and took place during March 1988 to August 1990 in the greenhouse of the Agroecology Program laboratory. Supplemental light was provided by HID, phosphorus-coated, grow lights (Geotechnology Inc., Santa Cruz, CA). The photoperiod was 14 hr (i.e. 6:00 to 20:00) with the grow lights turned off during the daylight hours of 10:00 to 16:00. Photosynthetic photon flux density (PPFD) at plant emergence level averaged 500 $\mu E\ m^{-2}\ s^{-1}$ with grow lights or daylight.

Bean and cabbage plants were grown in glass pots from brown reagent bottles which were cut at the bottom. The top half of each pot was filled with sand (30 mesh) mixed with non-sterile soil (1 mm sieved) and VAM inoculum in a 100:4:1 ratio while the bottom half of each pot was filled with sand only. The nutrient solution was a 1/2 strength Ruakuara formulation with 84 ppm N and 10 ppm P, pH 7.0, and an application rate of 300 ml $week^{-1}$ (*52*).

The experimental design for both greenhouse studies was a randomized complete block. The measurements were statistically evaluated by one-way ANOVA.

Leonard Jar System. Cabbage effects were studied using a standard Leonard jar (*48*) where beans are intercropped with cabbage in the same pot (4 L size). Moisture uniformity was maintained in the growth medium of the pot with multiple internal nylon wicks to a dH_2O filled reservoir from a cut-bottle bottom. Two neighbor plants (i.e. transplanted cabbages or planted beans) were placed 7 cm from and on opposite sides of the centrally planted target bean plant. Cabbage leaves that overshadowed the bean seedling were trimmed off in order to remove any possibility of light competition.

Cabbage effects were assessed at 2 different growth stages of bean, i.e. cabbage plants were transplanted 10 and 25 days after planting (DAP) bean plants due to preliminary Leonard jar studies which demonstrated bean mortality at earlier plant ages due to cabbage effects. There were 5 replications.

Root Exudate Recirculating System (RERS). Cabbage effects were studied using a stair-step RERS (*53*) which was modified for experimental use with a tripartite bean symbiosis. Here beans and cabbages were intercropped in separate pots (1 L size) which were connected by tubing which recirculated nutrient solution at a rate of 20 ml min^{-1}. There were 9 replications.

Results

Field Study. Nodule Dry Weight. Bean roots were nodulated at all sampled treatments (Table I). In the orthogonal trend analyses (Table III), the treatments overall (AVG#1) showed a significant positive linear trend in the proportion response but this trend was observed only at 44 and 55 cm distances (p=.08 at 44 cm). The treatments overall (AVG #2) did not show a significant spacing response, and it was not observed for each B:C treatment, except for 1:3 which had a significantly positive linear trend.

The class comparisons for diversity effects provided additional analysis of the observed proportion trend at 44 and 55 cm (Table IV). For example, at 55 cm

Table I. Cabbage Effects at Different Planting Distances on the Nodulation, VAM, and Shoot N and P of Bean Plants When Intercropped in Field Study.

B:C	*Distance (cm)*						AVG
	28	31	39	44	49	55	# 1[a]
Nodule Dry Weight (g plant^{-1})							
4 : 0	.093	.090	.077	.090	.103	.090	.090
3 : 1	----	.063	----	.073	.090	.097	.080
2 : 2	----	.110	.087	.130	----	.117	.120
1 : 3	.109	.087	----	.210	----	.267	.216
AVG # 2[b]	n/a	.090	n/a	.120	n/a	.143	
VAM Infection (%)							
4 : 0	71.4	56.1	61.2	58.7	64.0	56.5	57.1
3 : 1	----	63.0	----	56.7	57.5	63.7	61.1
2 : 2	----	46.6	40.3	41.1	----	51.4	44.8
1 : 3	27.9	31.3	----	32.4	----	39.2	34.3
AVG # 2[b]	n/a	49.2	n/a	47.3	n/a	52.7	
Shoot Total N (mg plant^{-1})							
4 : 0	792	881	1327	1381	1675	1497	1253
3 : 1	----	706	----	947	930	1085	912
2 : 2	----	694	918	1046	----	1373	1038
1 : 3	367	434	----	649	----	677	586
AVG # 2[b]	n/a	679	n/a	1006	n/a	1158	
Shoot Total P (mg plant^{-1})							
4 : 0	86	102	134	127	178	176	135
3 : 1	----	77	----	86	92	115	93
2 : 2	----	77	103	112	----	150	113
1 : 3	44	53	----	81	----	88	74
AVG # 2[b]	n/a	77	n/a	101	n/a	132	

[a],[b] = average calculated from pre-selected distances of 31,44 & 55cm
--- = data was not collected.
n/a = not appropriate calculation due to uncollected data for each cabbage proportion.

significant differences due to diversity were produced for only the proportion of 1:3. Similarly, at 44 cm the lack of significant diversity effect with the 2:2 intercrop suggested that only the 1:3 proportion was effective, although it could not be tested at this spacing due to limited treatments.

VAM. Bean roots had VAM infection at all sampled treatments (Table I). In the orthogonal trend analyses (Table III), the treatments overall (AVG #1) produced a significant negative linear trend in the proportion response but it was observed only at 31 and 44 cm. There was no significant spacing response.

The class comparisons for diversity effects offered insights to the observed proportion trends (Table IV). For example, the significant effects at 55 cm for both 2:2 and 1:3 disagreed with the lack of a proportion response at 55 cm. On the other hand, the significant diversity effect at 44 cm with 2:2 agreed with the observed proportion response at 44 cm.

Bean Shoot Biomass. Table IIa shows the bean growth at all the treatments. In the orthogonal trend contrasts (Table IIb), the treatments overall (AVG #1) produced a significant negative linear and cubic trend in the proportion response. This linear effect was significant at all distances, except 44 and 49 cm, but the cubic effect was significant only at 55 cm. The treatments overall (AVG #2) had a significant positive linear trend in the spacing response, and it was observed for each proportion treatment.

The class comparisons for diversity effects of shoot biomass further analyzed the observed trends in proportion response (Table IV). For example, the significant reductions in 3:1 at 49 and 44 cm disagreed with lack of a proportion response while non-significant differences in 2:2 at 55 cm agreed with the cubic trend. In addition, the significant diversity effect of reduction in 2:2 at 39 cm and non-significant differences in 3:1 at 39-31 cm demonstrated a density-dependence to the observed linear and cubic trends.

Bean Shoot Nitrogen. Table I shows the total N content of shoot biomass at selected treatments. In the orthogonal trend contrasts (Table III), the treatments overall (AVG #1) produced a negative linear trend in the proportion response, and it was also observed at 31, 44, and 55 cm. In addition, there was a significant cubic trend with AVG #1 and at 55 cm. The treatments averaged overall (AVG #2) and each proportion produced a positive linear trend in the spacing response.

The class comparisons for diversity effects confirmed the observed proportion responses, e.g. negative linear with significant reductions in 3:1 at 49 cm, and cubic with non-significant differences in 2:2 at 55 cm (Table IV).

Bean Shoot Phosphorus. Table I shows the total P content of shoot biomass at selected treatments. In the orthogonal trend contrasts (Table III), the treatments averaged overall (AVG #1) produced negative linear and cubic trends to the proportion response. When each spacing was examined, similar linear trends were found (at 44 cm, p=.14), and cubic trends at 44 and 55 cm. The treatments overall (AVG #2) and each proportion produced a positive linear trend to the spacing response.

The class comparisons for diversity effects corroborated the observed proportion responses with non-significant differences in 2:2 at 55 and 44 cm compared to 3:1 and 1:3 (Table IV).

Table IIa-b. Cabbage Effects at Different Planting Distances on Shoot Biomass of Bean Plants when Intercropped in Field Study. Orthogonal Contrasts for Response Trends to Proportion and Planting Distance.

a) Shoot Dry Weight (g plant^{-1})

B:C	*Distance (cm)*							AVG
	28	31	35	39	44	49	55	# 1
4 : 0	24.0	29.2	31.5	35.2	37.3	48.5	44.9	35.2
3 : 1	18.7	21.9	25.8	28.5	29.1	29.4	33.8	26.8
2 : 2	16.9	23.0	23.9	30.7	32.9	36.9	42.6	28.9
1 : 3	15.0	16.5	17.0	24.5	23.3	24.3	23.9	20.6
AVG # 2	18.6	22.6	24.6	29.7	30.2	34.8	36.3	

b) Orthogonal Contrasts for Response Trends

Proportion Response Trend[1]

	Distance (cm)							AVG
	28	31	35	39	44	49	55	# 1
L	.07	.06	.05	.05	ns	ns	.001	<.01
Q	ns	ns	ns	ns	ns	ns	ns	ns
C	ns	ns	ns	ns	ns	ns	.001	.05

Distance Response Trend[1]

	B:C				AVG
	4:0	3:1	2:2	1:3	# 2
L	.001	<.01	<001	<.05	<001
Q	ns	ns	ns	ns	ns
C	ns	ns	ns	ns	ns

[1] L = linear, Q = quadratic, C = cubic response trends
ns = F test was non-significant

Table III. Orthogonal Contrasts for Response Trends to Proportion and Planting Distance of Nodulation, VAM, and Shoot N and P of Beans When Intercropped with Cabbage in Field Study.

	Proportion Response[a]				*Distance Response*[a]				
	Distance (cm)			AVG	B:C				AVG
	31	44	55	# 1[b]	4:0	3:1	2:2	1:3	#2[c]
Nodule Weight[d]									
L	ns	.08	<.05	<01	ns	ns	ns	.001	ns
Q	ns	ns	ns	ns	ns	ns	ns	ns	ns
C	ns	ns	ns	ns	ns	ns	ns	ns	---
% VAM[e]									
L	.05	<.01	ns	<05	ns	ns	ns	ns	ns
Q	ns	ns	ns	ns	ns	ns	.09	ns	ns
C	ns	ns	ns	ns	ns	ns	ns	ns	---
Shoot Total N									
L	.05	<.05	<.01	<01	<.0	.01	<.01	<.05	<01
Q	ns	ns	ns	ns	1	ns	ns	ns	ns
C	ns	ns	.05	<05	ns	ns	ns	ns	---
					ns				
Shoot Total P									
L	<.05	ns	<.05	<05	<.0	.05	.001	<.01	<01
Q	ns	ns	ns	ns	1	ns	ns	ns	ns
C	ns	.10	.08	.05	ns	ns	ns	ns	---
					ns				

[a] L = linear, Q = quadratic, C = cubic response trends

[b,c] = see table I for legend.

[d] = square root transformation was necessary to meet the assumptions of ANOVA for data of 31-55 cm distances and 2:2 proportion.

[e] = arcsin transformation of all data was necessary to meet the assumptions of ANOVA.

ns = F test was non-significant.

--- = calculation not possible due to limited treatments.

Table IV. Class Comparisons for Diversity Effects on Nodule Dry Weight, VAM Infection, Shoot Dry Weight, and Shoot Total N and P of Beans Intercropped with Cabbage in Field Study.

B:C	*Distance (cm)*	Shoot	Nodule	VAM	N	P
3:1	31	ns	-----	-----	-----	-----
	35	ns	-----	-----	-----	-----
	39	ns	-----	-----	-----	-----
	44	<.05	-----	-----	-----	-----
	49	<.01	ns	ns	<.001	<.001
	55	.01	ns	ns	<.01	<.01
2:2	39	ns	ns	.08	ns	ns
	44	ns	-----	-----	-----	-----
	49	ns	ns	.05	ns	ns
	55	.10	-----	-----	-----	-----
1:3	55	<.05	<.05	<.05	.01	<.05

ns = non-significant F-test for diversity class effect
----- = orthogonal class contrast not performed due to limited treatments as explained in the text

Bean Seed Yield. Bean plants for seed yield were lost due to a gale windstorm at 99 DAP, and unfortunately seed harvest was not possible.

Greenhouse Leonard Jar Study. Beans of 10 DAP exhibited chlorosis and died 3-4 weeks after cabbage transplanting. On the other hand, beans of 25 DAP remained green when intercropped with cabbage until sampling time at 4 weeks after transplanting. Only 25 DAP responses are reported (Table V).

VAM. VAM infection was significantly reduced by cabbage.

Nodulation. Both nodule weight and number were significantly reduced by cabbage.

ARA. Only the bean control had N fixation activity.

Bean biomass and development. There were significant reductions in shoot and root dry weight due to cabbage. There was also a significant reduction in the number of nodes on the bean plant by cabbage. The neighbor bean plants of the control had reached the V4 growth stage at the time of sampling.

Greenhouse RERS Study. There was no significant cabbage effect on any measured parameter of the tripartite legume symbiosis (Table VI).

Discussion

Allelopathy of the Brassica Intercrop. The role of allelopathy was demonstrated from the field experiment by contrasting the bean's micro-symbiont responses to those expected from competition mechanisms for nutrients and light. For example, a greater potential for bean nodulation and VAM infection have been repeatedly shown in the literature with reduced soil N and P, respectively (*54,55*), and these nutrients would be affected by the factors of proportion and spacing. Therefore, nodulation and VAM were hypothesized to increase with greater proportions of cabbage but decrease with greater planting distances.

Nodulation but not VAM produced the expected proportion responses (Table III). Importantly, the observed reductions of VAM due to proportion (at 31 and 44 cm, as well) and due to diversity with 1:3 at 55 cm (Table IV) clearly indicated an interference mechanism not based upon nutrient dynamics. This conclusion was also supported by other results. First, there was the lack of a nodulation proportion response with 1:3 at 33 cm. Secondly, there was a significant spacing response of nodule increases in the 1:3 intercrop which was contrary to the other treatments and opposite to the expected trend.

Competition for light as an interference mechanism needed to be evaluated as a mechanism for the above unexpected proportion and spacing responses. Insufficient photon flux to the host for meeting the photosynthate demands has been shown to be a significant constraint in the development of the tripartite legume symbiosis (*56*).

The extent of shading here can be analyzed from plant height and width data. Figure 1 shows that beans responded to greater planting distances with height reductions in all of the intercrops, similarly to the monoculture. Such changes were expected if light was limiting due to greater shading by neighbor plants at the smaller distances (*57*). However, beans were always taller than cabbage

Table V. Canopy Overlap at Different Planting Distances of the Central Bean Plant with the Six Neighbor Plants

B:C	*Distance (cm)*							AVG
	28	31	35	39	44	49	55	# 1
				Canopy Overlap (cm)				
4 : 0	14.0	12.0	6.0	5.0	1.0	0.0	0.0	5.4
3 : 1	10.3	9.6	4.7	3.3	1.0	0.7	0.0	4.2
2 : 2	8.7	7.9	3.7	5.7	0.0	0.0	0.0	3.8
1 : 3	7.0	2.0	2.0	3.0	0.7	0.2	0.0	2.0
AVG # 2	10.0	7.9	4.1	4.2	0.7	0.2	0.0	

Calculation of canopy overlap at each distance based on following formula:

$$X = -\left(\sum_{i=1}^{6} (Y - (X_C + X_{Ni})) \div 6\right)$$

where

Σ = summation of 6 neighbors; X_C = radius of central bean

Y = planting distance; X_N = radius of neighbor plant

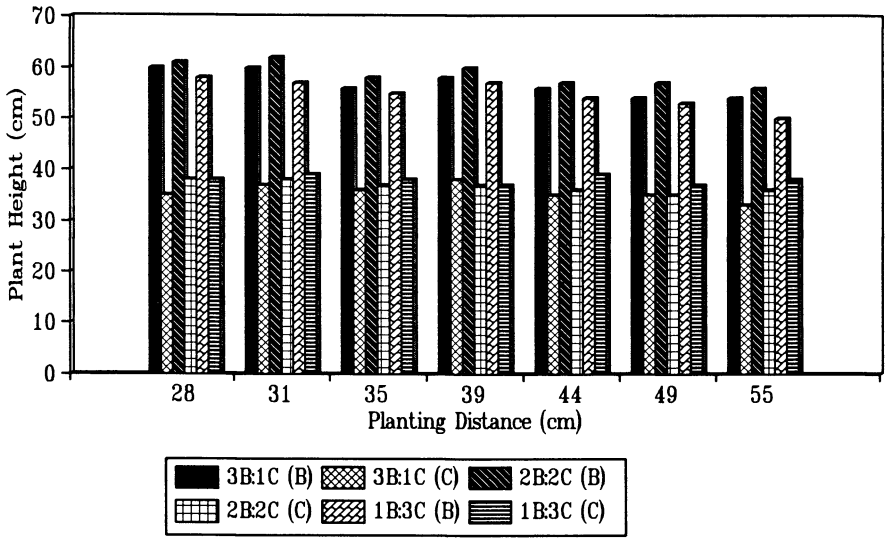

Figure 1. Bean (B) and cabbage (C) heights by intercrop proportion and spacing.

Table VI. Effects of Cabbage Compared to Beans on Tripartite Bean Symbiosis, using the Leonard Jar and RERS Experimental Systems.

Response of Tripartite Symbiosis	*Leonard Jar*[1]		*RERS*[1]	
	B	C	B	C
VAM Infection (%)[2]	64.8a	49.9b	68.0a	66.0a
Nodule Wt.[3] (g plant^{-1})	.044a	.004b	.489a	.540a
Nodule Number[3] (plant^{-1})	189a	28b	---	---
Total ARA[4] (μmol C_2H_4 plant^{-1} hr^{-1})	0.47*	0.00	19.07a	26.61a
Specific ARA[4] (μmol C_2H_4 g-nod^{-1} hr^{-1})	10.64*	0.00	39.00a	49.28a
Shoot Wt. (g plant^{-1})	3.486a	1.19b	4.62a	4.69a
Root Wt. (g plant^{-1})	1.13a	0.38b	1.14a	1.03a

[1] Values within an experimental system (i.e. Leonard jar or RERS) that have different letters are significantly different at the 0.05 level by ANOVA.
[2] ANOVA performed on all data after angular transformation.
[3] ANOVA performed on data after square root transformation.
[4] Test of significance by one-tail t-test of H_o: $\bar{u} > 0$.
* = significant at 0.05 level.
--- = data not collected.

neighbors, and, therefore, shading was progressively less a viable mechanism in the intercrops with greater cabbage proportion compared to the bean monoculture. Table V reports the degree of canopy overlap calculated from plant width data across the range of distances for each proportion treatment. These results demonstrated that canopy overlap existed as a potential constraint on growth only at distances less than or equal to 39 cm, and decreased with greater cabbage proportions. Similar trends were observed when canopy overlap was analyzed as a percentage of the canopy of the central bean (data not shown).

The trends from canopy overlap can explain the unexpected nodulation responses to spacing in 1:3. However, this analysis of shading does not account for other important responses of the micro-symbionts. For example, the unexpected VAM reduction due to proportion at 44 cm and to diversity with 1:3 at 55 cm was observed even when canopy overlap was eliminated at these higher spacing. In addition, the unexpected reductions of nodulation and VAM at 33 cm due to proportion actually occurred when canopy overlap was decreasing not increasing with greater cabbage proportion. Also there was no spacing response in nodulation or VAM in the other treatments compared to 1:3, although they had the greatest amount of shading at the lower distances to be eliminated at the higher distances.

The role of allelopathy was demonstrated by the micro-symbiont responses in the field which could not be adequately accounted by mechanisms of light and nutrient competition. The hypothesis of indirect effects was also clearly substantiated by the results of the Leonard jar experiment where light competition was removed and nutrient availability was standardized. At the same time, it must be emphasized in the field allelopathy functioned as a component in what can best be described as the interference complex from cabbage to beans because some of the data demonstrated that competition for nutrients and light was present.

Allelopathy and Over-yielding Processes. The unfortunate loss of bean seed yield prevented the complete LER calculations as originally planned. Nevertheless, the data presented several important aspects which established a framework for assessing the role of allelopathy on over-yielding in this intercropping.

Foremost, the significant cubic responses to proportion at the greater planting distances (Tables I and III) resulted in larger bean plants with increased nodulation and N level in 2:2 compared to 3:1 and 1:3 intercrops. These positive effects enhanced the potential for over-yielding from N complementation via a more effective N_2 fixation system. Interestingly, higher P levels which would also support N_2 fixation were observed when VAM levels were depressed.

In addition, important yield characteristics of legumes strongly support a potential for a yield advantage with the above positive effects. First, bean biomass is positively and strongly correlated with yields (*58*). Secondly, there is the ability of intercropped beans to utilize any patchiness of space by compensatory growth for increased yield (*14,59*). In this experiment, there was a temporal component in the bean/cabbage intercropping whereby the cabbage harvest created planned gaps (i.e. increased planting distances) for further growth

by beans. Thirdly, indeterminant beans can have higher yields with increased planting distances (*60*).

As the concept of over-yielding depends on the performance on both intercrops, it is also important to comment on the realized cabbage yields (data not shown). To summarize, cabbage showed no yield change from bean interference when bean growth was positively affected by cabbage. Similar responses were reported in broadbean-cabbage intercroppings where over-yielding resulted from broadbean but not cabbage yield advantages (*44*). The lack of a cabbage yield advantage must be weighed against the fact that there was negligible pressure from insect herbivory on cabbage due to the high predator populations typical to the organic management at the UCSC Agroecology research farm. This situation is not common in conventional production areas where a positive cabbage yield response would be expected because beneficial plant-insect interactions are usually reported for the brassica intercrop with the presence of a legume (45).

Allelopathy Research Methodologies. It must be emphasized that the demonstrated field experimental methodology was instrumental in documenting the occurrence of positive effects which were very restricted compared to the negative interactions on the tripartite bean symbiosis. The hexagonal configuration of the Nelder Fan was designed for reducing experimental variability by controlling the mechanisms of plant interference (*61*), and the range of cabbage proportions and densities was chosen to create a wide gradient of cabbage interference. The observed duality of negative vs positive effects corroborates the literature of brassica ecology which has reported contradictory effects on associated crops (*34*). This technique produced the experimental conditions which detected the dosage-dependent hormonal qualities (*62*) of glucosinolates, and which could be applied to other suspected crop-based allelochemicals.

The Leonard jar experiments produced convincing evidence for a root exudate-based allelopathic mechanism of the negative indirect effects on both nodulation and VAM observed in the field, as well as demonstrated the modifying effect of host plant age. These results are relevant to the published report of glucosinolate derivatives in the root exudates of an allelopathic brassica (*67*). In addition, the observed negative direct effects corroborated studies in the published literature. For example, cabbage root exudates have inhibited bean seedlings (*68*), and the herbicidal properties of glucosinolate hydrolysis products on beans include symptoms of foliar chlorosis (*69*) and a dependence on plant age (*70*). On the other hand, the RERS experiment did not duplicate the responses of the field or Leonard jar experiments. This result demonstrated difficulties of the RERS methodology for glucosinolate-based allelopathy, possibly due to the chemical nature of the suspected allelochemicals, i.e. glucosinolate derivatives, which are highly reactive, volatile, and hydrophobic (*71*).

Conclusion

The experimental results and approaches illustrated here provided an example of the role of allelopathy on the optimization of one important factor for over-yielding in legume intercroppings, i.e. the tripartite N_2-fixation system. Concerning legume-brassica intercropping, for example, these experiments demonstrated a quantitative and root exudate basis for brassica allelopathy. The data suggests that overyielding could be promoted by selection programs for bean, *Rhizobium*, and VAM tolerance to these root exudates, and for brassica varietal differences in root exudates. In addition, agronomic management (e.g. planting distances and dates) could be effective in modifying allelopathic interactions. Further application of these techniques with allelopathic, mycorrhizal intercrops could assess the impacts on the N transfer, as well as the N complementation, mechanisms of over-yielding in legume intercroppings.

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Chapter 16

Allelopathy and Sustainable Agriculture

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Allelopathy, a detrimental biochemical interaction between plants, plays an important role in weed control and crop productivity in Taiwan. The extracts of many dominant plants, such as *Delonix regia*, *Digitaria decumbens*, *Leucaena leucocephala*, and *Vitex negundo*, contain allelopathic compounds, including phenolic acids, alkaloids, and flavonoids. These can be used as natural herbicides, fungicides, etc. which are less disruptive of the global ecosystem than are synthetic agrochemicals. Many important crops, such as rice, sugarcane, and mungbean, are affected by their own toxic exudates or by phytotoxins produced when their residues decompose in the soil. For example, in Taiwan the yield of the second annual rice crop is typically 25% lower than that of the first, due to phytotoxins produced during the fallowing period between crops. Autointoxication can be minimized by eliminating, or preventing the formation of the phytotoxins through field treatments such as crop rotation, water draining, water flooding, and the polymerization of phytotoxic phenolics into a humic complex. By understanding and applying allelopathy we can sustainably maximize crop yields while minimizing disruptive and costly chemical input.

At the Sixth International Scientific Conference of the IFOAM, Allen and van Dusen (*1*) concluded that global agricultural development must focus on three important issues, 1) agricultural problems are global in nature, and so must be their solutions; this requires diverse perspectives on ways to strengthen agriculture, 2) the scientific disciplines can provide the much needed framework for a systematic, holistic approach to solving agricultural problems, and 3) sustainable agriculture is a long term goal. Sustainable agriculture is particularly important to human beings, who depend for their survival on agricultural products. Modern agricultural practice will, by applying an excess of fertilizers, herbicides, fungicides, and nematicides, etc, result in zeopadizing the physical chemical properties of the soil and polluting the soil and water, to the detriment of the global ecosystem. Sustainable agriculture, therefore, requires intensive organic farming methods that regenerate and conserve resources making efficient use of resources internal to the farm and relying on a minimum of purchased inputs, are biodynamic, utilize energy more efficiently, and have minimal impact beyond the boundries of the farm (*2*).

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To achieve the goal of sustainable agriculture, research is being done on plant breeding, soil fertility and tillage, crop protection, and cropping systems. Allelopathy, a detrimental biochemical interaction between plants (*3-5*), is directly or indirectly involved in each of these fields, and also plays an important role in crop productivity, conservation of genetic diversity, and maintenance of ecosystem stability (*6-9*).

Guenzi et al. (*10*) indicated that different cultivars of the same crop might release different amounts of phytotoxins. Bogdan (*11*) suggested that losses of vigorous strawberry stands may be partially due to allelopathy. Putnam and Duke (*12*) selected for allelopathic activity when breeding weed-controlling cultivars of cucumbers. They also made extensive reviews of the allelopathic influence on crop production of phytotoxins released from various plants or in the soil (*13*). Fay and Duke (*14*) tested many lines of *Avena sativa* and found that some lines produce allelopathic compounds, such as scopoletin, which suppress weed growth. Similar example were also demonstrated by Bell and Koeppe (*15*). Dzubenko and Petrenoko (*16*) found that *Lupinus albus* and *Zea Mays* could suppressed the growth of weed species. Liang et al. (*17*) conducted experiments to select aggressive, strongly weed-suppressant cultivars of pasture grass. Plant allelochemicals often have broad-spectrum biological activities involved with plant defense, including herbicidal, insecticidal, nematicidal, etc., and may be less likely than are man-made agrochemicals to damage the global ecosystem.

Autointoxication, another aspect of allelopathy, is the suppression of a plant's growth by its own toxic metabolites. Yield reduction in continuous monoculture of crops such as rice, wheat, sugarcane, asparagus, pasture grasses, and mungbean, is often ascribed to allelopathic autotoxicity (*18-26*). Alternate management systems, such as crop rotation, intercropping, and multicropping, can enhance crop productivity by reducing autotoxicity.

Since 1972, the author has investigated allelopathy in subtropical Taiwan, and has published a substantial body of findings. This paper summarizes much of the research done by the author and his co-workers on the role of allelopathy in sustainable agriculture.

The Role of Allelopathy in Weed Control

Many natural plant growth regulators, such as agrostemin, can be used to control weeds (*26*). Agrostemin is obtained from the corn cockle, *Agrostemma githago* L., a common weed in fields of wheat and other cereals. The compound has been widely used in eastern European countries and is harmful neither to animals nor humans. In addition, natural products from neem plants (*Azadirachta indica* A.) (*27*) have also been used extensively in India as herbicides, fungicides, and nematoticides. Other examples follow.

Allelopathic chemicals are natural herbicides. In leguminous plantations of *Leucaena leucocephala*, there is an absence of understory growth other than itself. This is due primarily to the phytotoxins, including eight phenolic acids, flavonoids, and mimosine, released from its leaves and litter. The compounds can suppress the

growth of many weeds and forest species, such as *Acacia confusa*, *Ageratum conzoides*, *Liquidambar formosana*, *Casuarina glauca*, *Mimosa pudica*, and *Alnus formosana* (*28*). It is notable that *M. pudica* was suppressed by *Leucaena* leaf leachate even though the leaves of *M. pudica* themselves contain relatively high levels of mimosine. Of eighty-four *M. pudica* seedlings tested, only two seedlings survived, showing that mimosine and other compounds, such as phenolics, can be of practical use in the control of field weeds.

Vitex negundo, a plant dominant in coastal vegetation, is widely distributed in the southern parts of Taiwan. Chou and Yao (*29*) found that the biomass and density of its associated understory is less than that in adjacent pasture. Field results showed that, compared to the rain-water control, the leachate of *V. negundo* significantly retarded the growth of *Digitaria decumbens* but stimulated the growth of *Andropogon nodosus*. The growth of *D. decumbens* in pots under greenhouse conditions was significantly retarded by watering with a 1% aqueous extract of *V. negundo*, but the growth of *Andropogon nodosus* and *M. pudica* was stimulated. The aqueous extract was phytotoxic to lettuce and ryegrass seeds. The aqueous effluents from a polyamide column chromatograph were bioassayed; some fractions inhibited the growth of lettuce and rice seedling radicles, whereas other fractions stimulated growth. The responsible substances, isolated and identified, included several phenolic acids and ten flavonoids (including 3' hydroxyvitexin) (*29*). These metabolites have potential usefulness as herbicides.

Recently, Chou and Leu (*30*) reported another plant, *Delonix regia*, which allelopathically excludes understory species. The aqueous extracts of the leaves, flowers, and twigs of *D. regia* revealed significant phytotoxicity (over 70%) against tested species such as *Isachne nipponensis* and *Centella asiatica*. The responsible allelopathic substances are 3,4-dihydroxybenzaldehyde and the acids: 4-hydroxybenzoic, 3,4-dihydroxycinnamic, chlorogenic, 3,4-dihydroxybenzoic, 3,5-dinitrobenzoic, gallic and L-azetidine-2-carboxylic. In addition, some unidentified flavonoids are present in theplant.

Pasture grasses can be used to control weeds. An increasing number of studies of the allelopathy of grassland species have been conducted in recent decades (*5, 31*). Most of the studies have been concerned with the interpretation of allelopathic phenomena in the field; only a few have explored allelopathy as a practical means of controlling weeds. Chou and Young (*32, 33*) evaluated the phytotoxicity of aqueous leaf extracts from twelve introduced subtropical forage grasses. Extracts of *Acroceras macrum*, *Cynodon dactylon*, *Chloris gayana*, *Digitaria decumbens* (pangola grass), *Eragrostis curvula*, *Panicum repens*, and *P. maximum* significantly inhibited radicle growth of the test plants. *Digitaria decumbens* had the highest phytotoxicity, even at an osmotic concentration below 10 milliosmols, at which no osmotic inhibition occurred (*32*). *D. decumbens* is an autotoxic species, and its productivity was significantly retarded after several years of planting (*8, 9*). With a sufficient application of nitrogen fertilizer, pangola grass forms pure stands in which almost no weeds grow. Growth performance and competitive ability of the grass varies with the cultivar. Liang et al. (*17*) selected eight varieties of pangola for field trials and laboratory assays. The invasiveness of cultivars A65, A255, and A254 was

highest at Hsinhwa, Hengchun, and Hwalien station, respectively, while cultivars A79 and A80 were inferior at all stations. Cultivars A84, A254, and A255 were the most phytotoxic; nine phytotoxic phenolics were identified. The interactions of grass in the field are very complicated; although allelopathy is not the only mechanism of its dominance, it is surely one of them.

The Role of Allelopathy in Crop Productivity

Reduction of rice yield is due to an adaptive autointoxication mechanism. Rice (*Oryza sativa*), the most important crop in oriental countries, is planted twice a year in a continuous monoculture system. For nearly a century it was noted that the yield of the second crop was about 25% lower than that of the first crop. This reduction of productivity has been particularly pronounced in areas of poor drainage (*18-20*). The rice cropping system in Taiwan is different from that in other countries. In the first crop season (from March through July) the temperature increases gradually from 15 to 30°C , but for the second crop (August through December) it decreases from 30 to 15°C. Between the two crops, there is usually a 3-week fallowing period (compared with a 10-week period elsewhere). The farmers always leave the rice stubble in the field after harvesting, and submerge these residues in the soil for decomposition during the fallowing time. Chou et al. (*18, 19*) conducted a series of experiments in which mixtures of rice straw and soil (100 g : 3 kg) were saturated with distilled water and allowed to decompose in a greenhouse for 1, 2, and 4 weeks. Soil alone was treated in the same manner, as a control. The rice seedlings grew normally in the control soil, but poorly in the straw-soil mixture. The roots of the retarded plants were dark brown and the root cells were abnormal and enlarged. In a subsequent experiment with a series of mixtures (0-100 g/3 kg), the phytotoxicity increased in proportion to the amount of rice straw. The toxicity persisted for 16 weeks after decomposition (*18*). The compounds present in extracts of rice residues decomposing in soil include the acids: *p*-coumaric, *p*-hydroxybenzoic, syringic, vanillic, *o*-hydroxyphenylacetic, and ferulic (*18*), and propionic, acetic, and butyric (*34*). In particular, *o*-hydroxyphenylacetic acid, which is toxic to rice at a concentration of 1.64×10^{-4} M, reached a concentration of about 10^{-2} M in soil containing decomposing rice residues.

There are environmental factors in the autointoxication of rice plants

Water-logged paddy soil is oxygen deficient. Although rice plants are not hydrophytes, they grow very well in water-logged paddy soil. Patrick and Mikkelsen (*35*) found that the oxygen level in paddy fields approached zero at 25 cm below the soil surface. We obtained similar readings in Taiwanese paddy soils. In many areas of Taiwan, such as Tsingshui (central Taiwan), Chiatung and Yuanlin (southern Taiwan), and Tungshan (the east coast), poor drainage or a high water table leads to oxygen deficiency. This is especially pronounced in the second crop season when the monsoon comes. During the decomposition of rice residues in soil, a significant quantity of phytotoxic substances, such as short-chain aliphatic

acids and phenolic acids, are produced, reaching maximum levels in the first month of fallowing. The toxins suppress root growth and panicle initiation, and thus reduce the rice yield (*20, 21, 34*).

Negativity of redox potential (Eh) in paddy soil is due to decomposing rice residue. The absence of oxygen in the soil below 25 cm results in an increasingly negative soil redox potential (Eh) (*35*). Chou and Chiou (*20*) found that the soil Eh ranged from -100 to 200 mV during the first crop season and from -200 to 100 mV during the second crop season in Nankang paddy fields. At the farm of the National Chung Shing University of Taichung, the Eh was remarkably negative, ranging from -500 to 100 mV during the second crop season. In pot experiments, we found that the soil Eh was below -300 mV in rice straw-soil mixture, and was above 100 mV in soil alone. Thus, the negativity of soil Eh was apparently related to the decomposition of rice residues in soil. The soil Eh was remarkable at the tillering stage (30-45 days after transplanting) and at the panicling stage (80-90 days after transplanting) (*20*). During this period, the growth of rice roots was retarded, the root cells swelled, and many adventitious roots developed. Wu et al. (*34*) postulated that the swelling of root cells could be an adaption mechanism to obtain more oxygen.

Soil microbes are involved in the decomposition of plant residues. The decomposition of plant residues in soil involves microbial activity (*36, 37*). Wu et al. (*34*) found that when rice residues were submerged in paddy soil, the denitrifying bacterium *Pseudomonas putida* became dominant in the rice rhizosphere, and the population of *P. putida* correlated positively to phytotoxin production and to poor drainage. They pointed out that in the well-drained soil of Tsaotun the population of *P. putida* was 2.5 x 10^7/g dry soil, while in the poorly drained soil of Lotung, Ta An, and Taichung, the population ranged from 207.6 to 229.5 x 10^7/g dry soil. The large population of *P. putida* in the poorly drained soil suggests that the organism uses the rice residues as its carbon source. Wu et al. (*34*) also found that the phytotoxic phenolics did not come from the metabolites of this microorganism, but were released from the decomposing rice residues. Wu et al. (*34*) found that the application of ammonium sulfate fertilizer to paddy soil is beneficial to the growth of *P. putida*, but may increase the formation of H_2S, which is toxic to rice. Chou and Chiou (*20*) corroborated these studies. They found that ammonium sulfate mixed with rice residues enhanced the phytotoxicity. They suggested that the addition of nitrogen fertilizer favors the growth of decomposer microorganisms, which expedite decomposition of the rice residues.

There is an interaction between phytotoxins and nutrients in paddy soil. Chou et al. (*21, 22*) determined that the level of phytotoxic phenolics varies directly, and that of leachable nitrogen varies inversely to the amount of rice stubble left in paddy soil, suggesting that the phytotoxins may interact with the nitrogen. They also found that the amount of leachable NH_4-N was about ten times as great as that of NO_3-N (*21, 22*). Chou and et al. (*22*) used ^{15}N-isotope tracer techniques to study the distribution of nitrogen in soil and in soil-rice residue mixtures under different

temperature regimes and sequences. In the absence of straw, most of the fertilizer N remained in the mineral form. The addition of straw moderately enhanced N immobilization. A gradual decrease in the proportion of fertilizer N in the mineral form was accompanied by a steady increase of fertilizer N in the amino acid fraction of organic N. Little accumulation of fertilizer N in the amino sugar or the insoluble humin fraction was found (*22*). Although the experiments did not show a distinct trend in relation to temperature variations, the temperature range of 25-30°C favored N transformation activities.

The quantity of available minerals might be affected by the decomposition of rice residues in soil, with a consequent alteration of plant growth. Chou and Chiou (*20*) studied the influence on cation dynamics of rice straw incorporated into soil. This revealed that, regardless of nitrogen fertilizer application, the concentrations of K, Cu, and Mn cations were higher in the first crop season and those of Na, Ca, Mg, and Zn were higher in the second crop season in Nankang paddy soil. Most of their findings agreed with those of Patrick and Mikkelsen (*35*). In flooded soil, the concentrations of reducible iron and manganese were relatively low. When the pot soil was mixed with rice straw and allowed to decompose, the amount of K was significantly higher than that in soil alone, but the concentrations of Cu, Fe, Mn, and Zn were, on the average, significantly lower. It is interesting to note that in several poorly drained areas in Taiwan, such as Changhwa, Taitung, and Pingtung, Zn deficiency is particularly pronounced during the second crop season.

Reduction of yield on sugarcane plantations has an allelopathic cause.

Phytotoxins are produced during the decomposition of sugar cane residues in soil. Inadequate germination and growth of ratoon cane are the two major problems on farms of Taiwan Sugarcane Corporation (TSC). The yield of sugar cane in monoculture has declined in many fields, but investigations have found no single cause. Wang et al. (*23*) demonstrated by field and laboratory experiments that the phytotoxicity of decomposing sugar cane residues in the soil is a factor. Five phenolic acids (*p*-hydroxybenzoic, ferulic, *p*-coumaric, syringic, and vanillic) and 6 short-chain fatty acids (acetic, butylic, oxalic, malonic, tartaric, and malic) were identified in decomposing sugar cane leaves in water-logged soil. A 3×10^{-4} M concentration of the phenolic acids in water culture inhibited the growth of young sugar cane root. The aliphatic acids were also found to inhibit the growth of ratoon sugar cane at 10^{-3} M. Wu et al. (*38*) found that the population of *Fusarium oxysporum* associated with the rhizosphere soil of poorly growing ratoon cane roots was much greater than that with productively growing ratoon, with newly planted sugar cane roots, or in plant-free soil. They found that fusaric acid, a secondary metabolite of *F. oxysporum*, was toxic to young sugarcane plants in vitro (*38*). When 10 ppm of fusaric acid was added to Murashige and Skoog's medium, the leaves of sugarcane wilted and became chlorotic (*38*).

Flooding with water leaches the phytotoxins and restores sugarcane yield. After growing sugarcane for several years, there is usually a significant reduction of yield (*23*). This is believed to be due to the phytotoxins produced during the

decomposition of sugar cane residues left in the soil and to an imbalance of the microbial population. An attempt was made to eliminate the phytotoxins and to improve the microbial balance by flooding the cane field with water (*23*). Before flooding, the population of *Fusarium oxysporum* was exceedingly high in the low-yield sugar cane soils, but decreased after flooding. In addition, the quantity of fusaric acid was significantly lower after flooding, suggesting that some of the phytotoxins had been leached out (*23*).

Asparagus plants are susceptible to autointoxication. *Asparagus officinalis* is a perennial ratoon crop widely planted in Taiwan. A significant reduction of yield and quality often occurs in old plantations, and is believed to be due to monoculture of the crop. Young (*39*) found about 40% reduction of yield in old plantations, and further showed that the exudates of asparagus root retarded the seedling growth of Mary Washington, California 309, and California 711 cultivars. Exudate collected by circular trapping with XAD-4 resin significantly retarded radicle and shoot growth of asparagus seedlings (*39*). Six phytotoxic phenolics, namely 3,4-dimethoxyacetophenone and the acids: β-(m-hydroxyphenyl) propionic, 3,4-dihydroxy benzoic, 3,4-dimethoxybenzoic, 2,5-dihydroxybenzoic, and 3,4-dihydroxyphenylacetic acids were found in the extracts and exudates of asparagus. The quantity of phytotoxins was significantly higher in the stem than in the root, and was well correlated to the phytotoxicity observed (25, 39). It is a reasonable conclusion that the reduction of asparagus productivity in old asparagus fields is due to phytotoxins released from the plants and produced during the decomposition of residues remaining in soil as well as other causes.

Reduction of mungbean production is due to allelopathy and pathogens. Mungbean (*Vigna radiata*), a species of legume, is an annual food crop in Taiwan. Field observation has shown that poor growth and reduced yield occurs after continuous cropping. The relative rates of inhibition of radicle growth by aqueous extracts of various mungbean plant parts is root > leaf > stem. A 1% aqueous extract of mungbean root inhibited 70% of lettuce seed root growth, indicating the allelopathic potential of the mature mungbean plants; aqueous extract of rhizospheric soil, however, did not show significant inhibition (*24*).

Pot experiments indicate that allelochemicals are the cause of 10 to 25% of the growth inhibition in successive crops of mungbean plants. Soil-borne pathogens cause injury to stems and roots of mungbean plants. Species of the fungus *Pythium* and other pathogens can also retard the growth of mungbeans. High contents of nitrogen in the soil may cause poor growth of mungbean plants, and even damping off of seedlings. The concentration of *p*-hydroxycinnamic acid was higher in the roots of plants slightly infected by the fungus but which maintained regular growth. The degree of fungus infection may be affected by this phenolic compound. The saponins present in mungbean were also studied. Partly purified saponins were extracted from seedlings using Soxhlet apparatus, chromatography, and other methods. Soyasaponin I was identified by mass spectrometry as the major mungbean saponin, representing about 40% of total saponins. High concentrations of saponin solution inhibited radicle growth of mungbean seed. Soil containing 0.2% crude mungbean saponins inhibited growth of mungbean plants.

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Polymerization of phytotoxins by humic acid is catalysed by clay minerals. Many phytotoxic substances can bind to clay minerals or other organic compounds, resulting in decreased toxicity. Wang and Li (*40*) and Wang et al. (*41, 42*) found that protocatechuic acid, one of the phytotoxins related to trans p-coumaric acid, can be polymerized with humic acid by using clay minerals as heterogeneous catalysts. In fact, humic acid can polymerize many substances, such as amino acids, flavonoids, terpenoids, aliphatic acids, and other nitrogen containing compounds, and thereby keep the soil free of active toxins. Unfortunately, the polymerization of phytotoxic phenolics fixed in humic substances can be reversed under certain environmental conditions, releasing free phenolic compounds (7). Thus, the organomineral complexes of humic acid are actually a pool of toxic substances rather than a permanent sink.

The Role of Allelopathy in Cropping Systems

In conventional agriculture, single-crop planting has often been used to maintain optimal biomass production conditions, such as fertilizer levels, water availability, light intensity. Continuous single-cropping, however, can cause soil sickness, such as an imbalance of soil microorganisms or an accumulation of toxins released from plants and decomposing plant residues (*13, 36, 43*). Intercropping, on the other hand, is an intensified form of multiple-cropping, where two or more crops are grown simultaneously on the same land (*44, 45*). In many parts of the world, intercropping is a common component of agroecosystem management (*2, 6, 7, 26, 31, 44*). Amador and Gliessman (*45*) compared biomass production of corn plants in polyculture with four densities of planting in monoculture at two sites in Cardenas, Tabasco, Mexico. They concluded that the biomass production was the highest one in all treatment of polyculture at C-34 site. However, in most treatments, the production was significantly higher in high intensity monoculture than in polyculture. They pointed out the importance of understanding the complex ecological interactions in polyculture so that ecologists can confidently arrange suitable combinations of crops. To clarify the role of allelopathy in intercropping systems, I will now briefly present several case studies.

Cover grasses intercropped with orchard plants can suppress crop growth. Wu et al. (*46*) examined the phytotoxicity of some cover grasses to orchard plants. Each of pineapple, banana, and mango was planted with each of *Centrocema* sp., *Indigofera* sp., and *Paspalum notatum* (Bahia grass). *Centrocema* and *Indigofera* caused significant suppression of growth, but *Paspalum* did not. The phytotoxicity of the grasses was further evalutated by bioassaying aqueous extracts of their leaves against pea, mustard, cucumber, cauliflower, canola, Chinese cabbage, mungbean, watermelon, tomato, and rice. They found that canola was most sensitive to the extracts. *Centrocema* and *Indigofera* were confirmed as the most phytotoxic of the three grasses. Moreover, the leachate of *Centrocema* inhibited the growth of banana. Currently, several cover crops, including *Bromus catharticus*, *Pennisetum clandestinum*, *Lolium multiflorum* (both chromosome 4X and 2X cultivars), *Paspalum notatum*, and white clover, are under investigation for allelopathic affect

on the productivity of apple and peach plantations in the Lishan area of central Taiwan (*8*).

Pasture-forest intercropping suppresses weeds and stimulates trees. Taiwan is an island, two thirds of which is mountainous, and its forests are extremely important for water conservation. The limited amount of agricultural land forces farming activities into the hills and to higher elevations. A forest-pasture intercropping system is thought to be a way to increase livestock production. Recently we have conducted several experiments in the forest area of Hoshe Experiment Station of National Taiwan University, located at an elevation of about 1200 meters (*8*). A one-hectare area was deforested. The leaf litter of the conifer tree *Cunninghamia lanceolata* was removed from part, and the rest was left unchanged, as the control. The half of the test plot adjacent to the control plot was planted with kikuyu grass (*Pennisetum clandestinum*) and the other half left open. The experiment was designed to determine the reciprocal interaction of fir litter and kikuyu grass, and to evaluate the allelopathic influence of the two plants on weed growth. The biomass of kikuyu grass invading the cleaned plot was significantly higher than that invading the control plot (*47*). In addition, the number of weeds that grew in the plot planted with kikuyu grass was lower than that in the control plot, indicating that the kikuyu grass may compete with and suppress weeds. The seedlings of fir regenerating in the deforested area grew well and seemed not to be affected by the neighboring newly planted kikuyu grass. The growth of kikuyu grass, however, was inhibited by the fir litter left on the unchanged plot in the first three months after deforestation. Bioassay of aqueous extracts showed that the fir litter extract exhibited higher phytotoxicity than did the kikuyu grass. Nevertheless, four months after deforestation the growth of kikuyu grass in the field was luxuriant, indicating that the phytotoxicity of fir litter disappeared (*47*).

In another experiment (*48*), a split plot design of eight treatments was set up after deforestation of Chinese fir (*Cunninghamia lanceolata*): open ground without planting (control), planted with kikuyu grass, planted with kikuyu grass and *Alnus formosana*, planted with kikuyu grass and *Zelkova formosana*, planted with kikuyu grass and *Cinnamomum camphora*, planted with *A. formosana*, planted with *Z. formosana*, and planted with *C. camphora*. Field measurements showed that weeds grew luxuriantly six months after treatment in plots which had not been planted with kikuyu grass. The growth of weeds was significantly retarded, but that of woody plants was not affected when the plots were planted with kikuyu grass. Compared to the tap-water control, the aqueous leachate of kikuyu grass stimulated the seedling growth of *C. camphora* and *A. formosana*, but the extract stimulated the growth of *C. camphora* and inhibited that of *A. formosana*. The aqueous extracts of three hardwood plants had varying degrees of inhibition on root initiation of kikuyu grass. The aforementioned extracts and leachates were bioassayed against seed germination and radicle growth of four test plants, including *Miscanthus floridulus*. The extract of *Z. formosana* revealed the highest phytotoxic effect on the test species while that of kikuyu grass showed the least affect. The phytotoxic phenolics were identified by means of chromatography. The quantity of phytotoxins present was highest in the extract of *Z. formosana* and was lowest in that of kikuyu grass.

The degree of phytotoxicity and quantity of phytotoxins showed good correlation, suggesting that a selective allelopathic affect was involved (*48*).

Crop rotation avoids many problems inherent in monoculture. Many monoculture fields have a soil-sickness problem resulting in decreased productivity, and which is assumed to be due to an imbalance of soil microorganisms, accumulation of soil toxins, mineral deficiency, or abnormal soil pH. Rotation of crops can avoid the problem or eliminate its cause. Although many successful examples of crop rotation have been reported, only a few studies have focused on allelopathy (*13*). Pangola grass (*Digitaria decumbens*), a dominant species in Taiwan, produces a highly productive pasture, but the productivity declines several years after planting. Chou found that pangola grass is susceptible to autointoxication (*8, 9*). The reduced productivity of this grass was particularly pronounced on the farm at Hengchun Experiment Station of Taiwan Livestock Research Institute, so a crop rotation system of pangola grass-watermelon-pangola grass was established. Watermelon was planted in the winter-season drought and pangola grass in the spring season following the watermelon harvest. The yield of pangola grass after a crop of watermelon was about 40% greater than that without the rotation. It is assumed that the increased grass production is the result of the disappearance of phytotoxins produced by pangola grass.

Conclusion and Discussion

The reduction of crop productivity in a continuous monoculture is due mostly to the deficiency of soil nutrients, pathogen infection, or allelopathy. It is clearly demonstrated in this review that allelopathy plays an appreciable role in the reduction of productivity in some major Taiwanese crops, such as rice, asparagus, sugarcane, pangolagrass, and mungbean plants. The nutrients applied to the field of crops mentioned were sufficient to prevent nutrient competition. The poor growth of the plants, however, could be caused by either pathogenic infection, allelopathy, or both. A soil-borne pathogen, *Fusarium oxysporum*, was found in the rhizosphere soil and roots of plants such as sugarcane and mungbean. An attempt has been made to determine its pathogenic effects on plants, yet no conclusive evidence was found to prove that the reduction was caused solely by fungal infection. On the other hand, toxins released from extracts of plant parts or from decomposing residues clearly had an allelopathic effect on the plant growth. In similar cases it was shown by Patrick (*36*) and Borner (*43*) that a replanting problem of peach and apple plants was due primarily to allelopathic compounds released by the plants or produced during the decomposition of the plant debris in soil, although some soil-borne disease was found in the soil. Patrick and Koch (*37*) pointed out that the biochemical and physiological changes, induced in the root tissues by toxins were conducive to fungal invasion and colonization, suggesting that phytotoxins may be an important host-conditioning factor in the development of root diseases. Of course, the combination of pathogenic infection and phytotoxic effect can result in a remarkable retardation of plant growth. In the present study, it is quite clear that phytotoxic effect plays a most important role.

Allelochemicals are naturally occurring plant growth regulators that are produced in different amounts when plants grow in various habitats (*4, 5, 7, 8, 9, 31*). It was found that the metabolic pathway of phenolic compounds or terpenoids can be altered at their intermediates, such as shikimic acid for phenolics and dimethylallylpyrophosphate for terpenoids (*49*). There is increasing evidence showing that plant growth stimulators, such as IAA, are produced in larger amounts when plants grow in a favourable environment; plant growth inhibitors, however, such as *p*-coumaric acid, are produced when plants grow under stressful conditions (*50, 51*). Waller and Nowacki (*52*) indicated that when plants grew in unfavorable environment, such as nitrogen deficient soil, the great amount of alkaloids was produced. Koeppe et al. (*53*) also found that *Helianthus annus* produced significantly larger amounts of chlorogenic and neochlorogenic acids when the plants were grown under phosphorus deficiency than when grown under normal conditions. The author also found that a significantly large amount of phenolic compounds was produced when rice plants were grown in a poorly drained paddy field.

The production of a larger amount of allelopathic compounds in a stressful environment than in normal conditions can be interpreted as an adaptive strategy to suppress the growth of competitors for nutrients. In addition, the growth inhibitors produced in a plant growing in unfavourable conditions may act as regulators to slow its metabolic process to save energy for survival. Another example is the allelopathic influence on the plant diversity of grassland communities dominated either by *Miscanthus floridulus* (*54*) or by *M. transmorrisonensis* (*55*). These two grasses are often distributed in nutrient-poor land, becoming dominant species; this indicates a strong allelopathic effect on the growth of other weeds sharing the same habitat, and a luxuriant growth of weeds appeared soon after clearing *Miscanthus* stands, suggesting the removal of a phytotoxin source (*49*).

In view of the tenet of sustainable agriculture that species diversity should be maintained, one may question whether the phytotoxins released by allelopathic plants might kill all associated species and result in decreased plant diversity. Most allelopathic compounds degrade rapidly to a non-toxic form; allelochemicals used as herbicides, fungicides, or nematicides would not have residual effects, as do 2,4D or 2,4,5T, and would not have a detrimental effect on the agroecosystem. In fact, allelochemicals may act as plant growth promoters when their concentration is relatively low. This dual function of allelochemicals is a great advantage in regulating species diversity without harmful effect on sustainable agriculture.

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Chapter 17

Allelopathy in Mexican Plants

More Recent Studies

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Research on allelopathy in Mexico has focused on plant species with ecological importance, some of them with medicinal use. Seeds, fungi and insects have been used in bioassays directed to assess activity of plant extracts and isolates. Allelochemicals can modify cellular structure and activities including respiration and division. Research priorities are based on the vast diversity of Mexican flora, its potential as a source of useful natural products, and the lack of knowledge in these areas.

Researchers of different disciplines have found allelopathy increasingly interesting because of potential application of allelopathic compounds as herbicides, pesticides or growth regulators. Allelopathy may also be used to further manage biotic resources.

The physiological effects of compounds with phytotoxic activity (allelochemicals, and/or herbicides) vary widely. Almost all of these compounds act with specificity on different groups of organisms. This characteristic renders them attractive in the search for new bio-active compounds (1).

We are currently conducting studies on different species of Mexican plants with ecological or medicinal importance. Traditional knowledge, so rich in Mexico, has frequently suggested where to look for active compounds. Knowing that allelochemicals may affect different life organization levels -communities, organisms, tissues, cells, organelles and metabolic processes- plant extracts and isolated compounds have been evaluated using seeds, phytopathogenic fungi and insects as bioassays. Germination, growth, development, reproduction and survival are first determined, followed by the evaluation of the possible modes of action of tested compounds on processes such as respiration, cell division, enzymatic activities, and structural arrangements of tissues and cells.

0097–6156/95/0582–0224$08.00/0

In Allelopathy; Dakshini, K., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1994.

We try to focus our studies bearing in mind that Mexican biodiversity ranks fourth in the world, and hence represents a great chemical diversity. Yet, our knowledge of Mexican flora and fauna is incomplete, and the rate of disappearance of many species threatens its completion. Biotic and chemical impoverishment are equivalent. The loss of a species means loss of natural plant products which probably are unique in nature. There are also economic and social reasons that justify a search of active secondary metabolites. Regional issues could be identified and used to set research priorities. Chemical exploration could become part of biological conservation (2, 3, 4).

Allelopathic potential of *Mirabilis jalapa*

Mirabilis jalapa L. Nyctaginaceae (four o'clock) is an endemic Mexican plant that grows in disturbed sites. Roots and seeds are used as cathartics and the alkaloids jalapine and convolvuline are probably responsible for these effects. Other secondary compounds have been identified in *M. jalapa* such as quercetin, caffeic acid, trigonellin, alkaloids, tiramine and dopamine (5). Aqueous leachates of the plant have been shown to protect other plant from viral attack (6).

Pelayo-Benavides (7) studied the effect of phytotoxic compounds of *M. jalapa* on cell division. The phytotoxic effects were determined by bioassays with several species of crops and weeds, using aqueous leachates and organic extracts. Alterations on cell division and root structure were evaluated in root tips of pea seedlings. The cell division was studied by measurements of mitotic index, phase index and cell cycle length. Cells were treated with colchicine to induced tetraploidy. The leachate of the dry aerial part (2% w/v) caused a significant decrease (31%) of mitotic activity. The duration of the cell cycle did not change, though fewer marked cells were found in the treated seedlings (Figure 1), probably due to a reduction in the number of meristematic cells in the roots.

Ratibida mexicana and Sesquiterpenic Lactones

The species *Ratibida mexicana* (Wats.) Sharp (Asteraceae) is an endemic Mexican plant that has a scattered distribution in inaccessible areas along the Sierra Madre Occidental mountains in the state of Chihuahua. Tarahumara indians call this plant "Howinowa". They use its roots for the treatment of rheumatism and as an antiseptic agent. Certain sesquiterpenic lactones are implicated in allelopathy and possess a wide biological activity spectra, but their effects on phytopathogenic fungi have been scarcely studied. (8-12). Mata and collaborators (Mata, R., et al., Universidad Nacional Autónoma de México, unpublished data) explored the effect of aqueous leachates and organic extracts of the root of *Ratibida mexicana* on the radicle growth of *Amaranthus hypochondriacus* (syn. *A. leucocarpus*) and *Echinochloa crusgalli,* and on the radial growth of some phytopathogenic fungi. Two sesquiterpenic lactones: Iso*allo*alantolactone and Eleme-1,3-11-trien 8,12-olide (Figure 2) were isolated from hexane extracts of this plant. Figure 3 shows that Iso*allo*alantolactone totally inhibited *A. hypochondriacus* growth at the three concentrations tested.

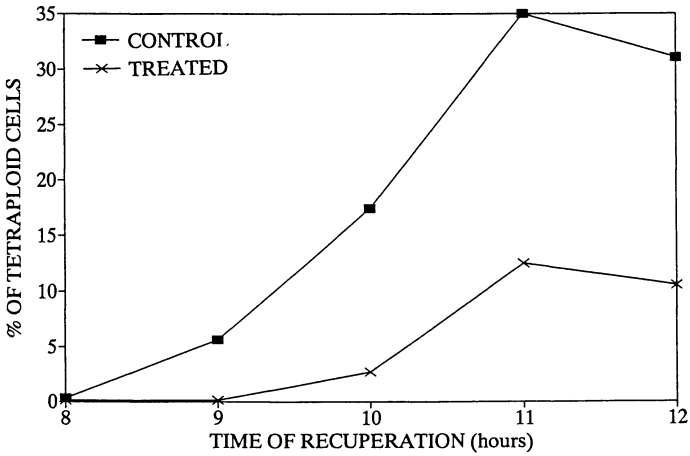

Figure 1. Effect of the aqueous leachate of the dry aerial part (2% w/v) of *Mirabilis jalapa* on the cell cycle in the meristem of pea seedlings roots.

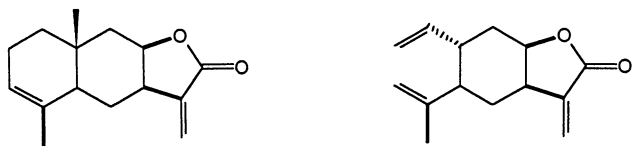

Figure 2. Chemical structure of the two sesquiterpenic lactones isolated from the hexanic extract of *Ratibida mexicana.*

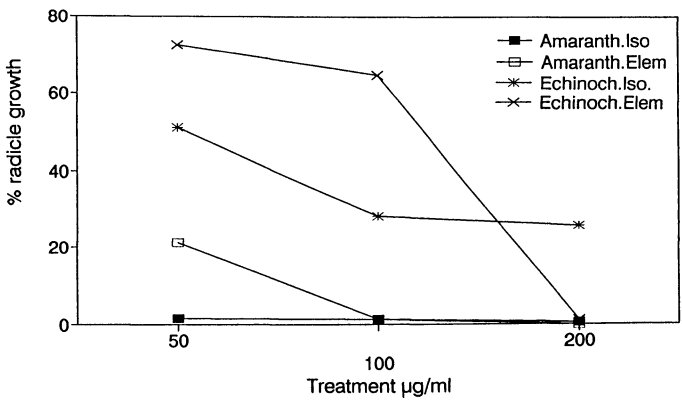

Figure 3. Effect of isoalloalantolactone and elemenodienolide on the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli.*

Elemenodienolide caused total inhibition of the radicle growth of this species at 100 and 200 μg/ml. *E. crusgalli* was more resistant to both compounds. Table I shows that Iso*allo*alantolactone and Elemenodienolide significantly inhibited the radial growth of *Helminthosporium* sp., and to a lesser extent, that of *Pythium* sp. Iso*allo*alantolactone (200 μg/ml) caused 50% inhibition of the radial growth of *Fusarium oxyporum;* this was the most resistant of the evaluated fungi.

Piqueria trinervia and Piquerols A and B

Piqueria trinervia L (Compositae) is a ruderal species of the firsts stages of secondary succession in crop fields. It is used in herbal medicine as an antipyretic, antimalarian, antirheumatic, and to combat typhus and gallblader stones (13, 14). González de la Parra et al. (15) demonstrated the phytotoxic activity of two diastereoisomers monoterpenes isolated by Romo et al. (16) from this species: Piquerol A and B. Jiménez-Estrada and collaborators (Jiménez-Estrada et al., Universidad Nacional Autónoma de México, unpublished data), tested two derivatives of Piquerol A: 3-isopropilen-2-methyl-1,4-benzenediol, and diacetyl-piquerol (Figure 4), and several synthetic phenols (Figure 5), on *Amaranthus hypochondriacus* and *Echinochloa crusgalli*. Table II shows the different effect of these compounds on growth. Piquerol A caused a significant stimulation of *A. hypochondriacus* at 10 μg/ml, and completely inhibited it at 100 μg/ml. In general, benzenediol and diacetyl-piquerol exhibited a stronger inhibitory activity on *A. hypochondriacus* compared with Piquerol A. *E. crusgalli* was less sensitive to these compounds. The synthetic phenols were less active compared with Piquerol A and its derivatives.

González de la Parra et al. (17), tested piquerol A and B on larvae and gravid females of tick (*Boophilus microplus*), and confirmed their acaricide activity. This activity can be compared with that of the organophosphoric acaricides against larvae and gravid females of ticks. Piquerol A and B produced 100% of mortality after 3 days of application. However, these compounds do not prevent oviposition in ticks, as almost all synthetic acaricides do.

Cruz-Ortega et al., (18) found that the H^+-ATPase activity of microsomes of *Ipomoea purpurea* radicle was inhibited (48.5%) by 500 μM diacetyl-piquerol; this inhibition was higher in the plasma (67.2%) than in the tonoplast membranes (31.6%). I*n vivo,* the plasma membrane ATPase might well be a more accessible target to the inhibitor than the tonoplast ATPase, given their respective cellular locations. These data suggest that the phytotoxicity of diacetyl-piquerol could be related to its effect on the plasma membrane H^+-ATPase.

Corn Pollen and Phenylacetic Acid

It has been shown that the practices used by some Mexican farmers to classify, select and use their weeds can be correlated with the allelopathic potential of these plants (19, 20, 21, 22). The allelopathic interaction between crops and weeds is carried out as a dynamic active release by aerial parts or roots and by decomposition.

Table I. Effect of Isoalloalantolactone and Elemenodienolide of *Ratibida mexicana* on the Radial Growth of Phytopathogenic Fungi

Treatments μg/ml	Isoalloalantolactone		Elemenodienolide	
		Radial growth (%)		
		Pythium sp		
	2 days	**3 days**	**2 days**	**3 days**
Control	100	100	100	
50	40.9*	27.7*	78.5*	82.2*
200	0*	0*	49.2*	54.7*
		Fusarium oxyporum		
	3 days	**8 days**	**3 days**	**8 days**
Control	100	100	100	100
50	94.4*	87.7	92.5	91.4
200	55.0*	59.0*	80.5*	86.0
		Helminthosporium sp.		
	3 days	**8 days**	**3 days**	**8 days**
Control	100	100	100	100
50	0*	0*	0*	0*
200	0*	0*	0*	0*

*$p < 0.05$

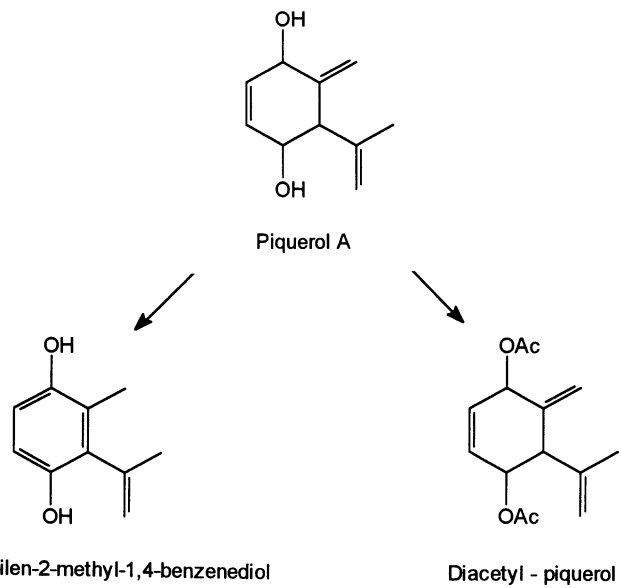

Figure 4. Chemical structures of Piquerol A, 3-Isopropilen-2-methyl-1,4-benzenediol and diacetyl-piquerol.

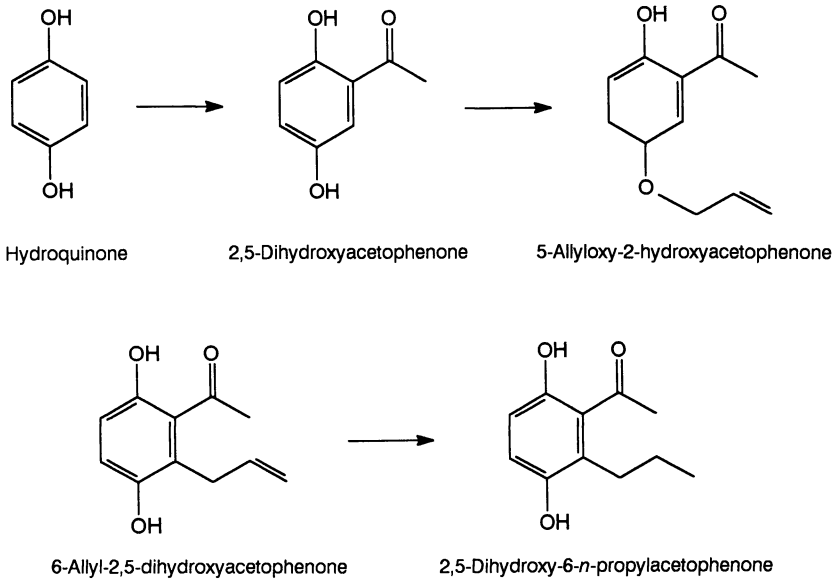

Figure 5. Phenols with chemical structures similar to Piquerol A.

Table II. Effect of Piquerol A and its Derivatives on *Amaranthus hypochondriacus* and *Echinochloa crusgalli*

Treatment	Radicle Growth (%)	
μg/ml	*A. hypochondriacus*	*E. crusgalli*
Piquerol A		
10	123.0*	82.0
30	70.6*	43.8*
100	0 *	17.6*
3-isopropilen-2-methyl-1,4-benzenediol		
10	50.5*	110.0*
30	28.5*	78.6*
100	0 *	59.5*
Diacetyl-piquerol		
10	45.8*	85.0*
30	35.3*	62.0*
100	0 *	25.0*

* $P < 0.001$

Corn (*Zea mays* L) produces secondary compounds in leaves, roots, and pollen (hydroxamic acids, phenylacetic, 4-phenylbutiric, benzoic, and o-hydroxy-phenylacetic acids). Growth regulators (brassinosteroids: catasterone, typhasterol and teasterone), flavonoids (quercetine, isorhamnetine and kaempferol), and β-carotene have been reported (23, 24, 25, 26). Some of these secondary metabolites posses allelopathic activity (27).

Dzyubenko and Petrenko (28) described that root excretions of corn inhibit the growth of some weeds such as *Amaranthus retroflexus*. Chou and Patrick (29) reported that phenylacetic acid (PAA) and other compounds produced during decomposition of corn and rye residues in soil were highly inhibitory to the growth of lettuce. Jimenez-Osornio and Schultz (30) found that weed growth decreased in the middle of the crop cycle when corn was mature and flowering. Jimenez et al. (31) showed that the sprinkling of corn pollen over the seeds of *Cassia jalapensis* in sterilized and non sterilized soil, vermiculite and sand inhibited their growth. In this regard, some farmers in Mexico assert that the fruiting of squash, bottle gourd, and watermelon is reduced, and leaves of beans are "burnt" when corn pollen falls over them (32). Jiménez et al. concluded that the accumulation of pollen in the soil and its phytotoxic effects, particularly upon weeds, can give some advantage to corn over its potential competitors, especially during flowering.

Some studies were conducted to identify the biochemical agents responsible for the allelochemical activity of corn pollen and their mode of action (32, 33). Cruz-Ortega et al. (33) found that an ethanolic extract of corn pollen acts as an inhibitor of the electron transport pathway, i.e., decreases oxygen consumption in watermelon mitochondria. A decrease of mitotic activity (more than 50%) of meristematic cells was also reported. The effects of a CH_2Cl_2 extract of corn pollen, and six chromatographic fractions of this extract on *Amaranthus hypochondriacus* are shown in Table III. All the treatments (except fractions 5 and 6) produced significant reduction of radicle growth of *A. hypochondriacus.* Fraction 5 produced a stimulatory effect (28 %). The GC/EI-MS of fraction 3 confirmed the presence of phenylacetic acid (PAA) in it. Table IV shows the effects of fraction 3 and PAA on *A. hypochondriacus* and *Echinochloa crusgalli.* Fraction 3 showed the highest inhibitory activity on *A. hypochondriacus,* and PAA on *E. crusgalli*. These differences are probably due to different sensitivity in the two species tested and to the presence of other compounds, in addition to PAA, in fraction 3. Phenylacetic acid content in corn pollen probably contributed to the observed allelopathic effects (32).

Fernández-Luiselli (Fernández-Luiselli, E., Universidad Nacional Autónoma de México, unpublished data) carried out bioassays to establish the minimal dose of PAA needed to cause a significant inhibition (MID) on radicle growth of some crops and weeds. An average of 300 seeds of the tested species were sown in Petri dishes with agar (1.5%) and different concentrations of PAA. After 24 to 72 hours, depending on the species, germination and radicle growth were evaluated. Table V shows the MID on the tested species. *A. hypochondriacus* exhibited the highest sensitivity.

Probably, the mode of action of PAA is related to its auxinic character. Pelayo-Benavides (Pelayo-Benavides, H.R., Universidad Nacional Autónoma de México, unpublished data) compared the effect of PAA to those of other auxinic

Table III. Effect of Methylene Chloride Extract of Corn Pollen and its Chromatographic Fractions on *Amaranthus hypochondriacus*

Treatment μg/ml	Germination (%)	Radicle Growth %
Control	100	100
Methylene chloride extract 100	80.0	70.0*
TLC fractions of CH_2Cl_2 extract 100		
1 R_f 0.05	80.0	44.2*
2 R_f 0.11	82.0	43.2*
3 R_f 0.22	76.6	23.2*
4 R_f 0.28	96.6	57.8*
5 R_f 0.51	90.0	128.4*
6 R_f 0.64	90.0	120.0

*P < 0.01

Table IV. Effects of a Thin Layer Chromatographic Fraction of CH_2Cl_2 extract of Corn Pollen (Fraction 3) and PAA on *Amaranthus hypochondriacus* and *Echinochloa crusgalli*

Treatments μg/ml	A. hypochondriacus Inhibition (%)	E. crusgalli Inhibition (%)
Fraction 3		
50	52.7*	21.8*
100	75.6*	47.5*
PPA		
50	30.6*	48.0*
100	62.2*	53.0*

* P < 0.05

Table V. Minimal Inhibition Dose (MID) of PAA for the Tested Plant Species

Species	MID	INHIBITION* %
Amaranthus hypochondriacus	7.35×10^{-6} M	13.66
Ipomoea purpurea	1.17×10^{-5} M	5.88
Cucurbita pepo	3.30×10^{-5} M	5.23
Zea mays	1.38×10^{-4} M	9.16
Echinochloa crusgalli	1.41×10^{-4} M	21.76
Phaseolus vulgaris	5.35×10^{-4} M	53.62
Cucurbita ficifolia	5.23×10^{-4} M	18.58

* Caused by MID, as compared to the control.

compounds: IAA, 2,4-D and HPAA on the development (first 48 hours) of *Amaranthus hypochondriacus*. Figure 6 shows the response of radicle growth to different molar concentrations of the compounds. There is a sigmoidal inhibitory effect of IAA and 2,4-D on the growth of *A. hypochondriacus*. On the other hand, the inhibitory effect of PAA and HPAA is exponential. In relation to the IC_{50} (Table VI), the order of activity was the following: 2,4-D>IAA>PAA>HPAA. This seems to be related to the water solubility of these compounds (HPAA>PAA>IAA>2,4-D). Depending on concentration, these compounds (except HPAA) produced morphological changes in the radicle and negative geotropism. PAA promotes some structural changes, for example, displacement of the maturation point of the xylematic elements.

Ipomoea tricolor and Tricolorin A

The genus *Ipomoea* (Convolvulaceae) includes about 600 species. Its richness in secondary metabolites with biological activity is perhaps its most remarkable characteristic. In Mexico, the seeds of *Ipomoea tricolor* ("tlitlitzin", "badoh negro" or "dondiego de día"), as well as those of the related species *Turbina corymbosa* (L.) Raf. ("ololiuqui" or snake plant), are used as hallucinogens in tribal rituals. This activity is due to their content of ergot type alkaloids. Other species of *Ipomoea* are widely used as powerful cathartics, an activity related to the presence of glycosidic resins. These glycosides have been reported as antimicrobials and antitumorals (34). The related species *I. aquatica* and *I. batatas* contain terpenoids and phenolic compounds with allelopathic activity (35).

Anaya et al. (36) confirmed that in tropical Mexico, some farmers use certain weeds to control the growth of others. Some *Ipomoea* species are used for this purpose. In the sugar cane fields of the state of Morelos, *I. tricolor* is grown as a cover crop from August to October. This plant eliminate all other weeds in two or three months. After this time, the plant is reaped and incorporated into the soil. Anaya et al. (op cit.) described the phytotoxic activity of *I. tricolor,* testing aqueous leachates and organic extracts of the plant on seedling growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli.* Bioactivity-directed fractioning of the active $CHCl_3$ extract of the plant led to the isolation of a phytotoxic compound, which turned out to be a mixture of the so-called "resin glycosides" of convolvulaceous plants. Pereda-Miranda et al., (37) elucidated the structure of tricolorin A as (11S)-hydroxyhexadecanoic acid 11-O-α L-rhamnopyranosyl-(1→3) - 0 - α - L - { 2-0- (2S - methylbutyryl) - 4 - 0 - (2S-methylbutyryl) } rhamnopyranosyl- (1→2) - 0 - β - D - glucopyranosyl - (1→2) - β -D-fucopyranoside-(1,3"-lactone) (Figure 7). Tricolorin A significantly inhibited radicle growth of the tested plants. Figure 8 shows the effects of tricolorin A and 2,4-D on both tested species. Radicle growth was evaluated after 24 h for *A. hypochondriacus*, and 48 h for *E. crusgalli*. *Staphylococcus aureus* was sensitive to the compound; the minimal inhibition concentration (MIC) was 1.8 μg/ml. This compound exhibited significant cytotoxic activity on cultured P-388 and human breast cancer cells (ED_{50}

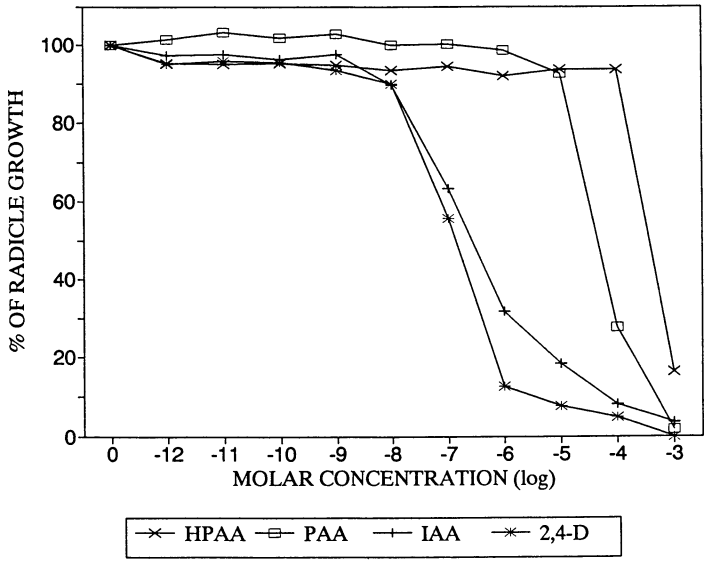

Figure 6. Effect of HPAA, PAA, IAA and 2,4-D on the radicle growth of *Amaranthus hypochondriacus.*

Table VI. IC^{50} of Tested Compounds on Radicle Growth of *Amaranthus hypochondriacus*

Compounds	IC50
HPAA	3.98 X 10^{-4} M
PAA	3.98 X 10^{-5} M
IAA	3.00 X 10^{-7} M
2,4-D	1.00 X 10^{-7} M

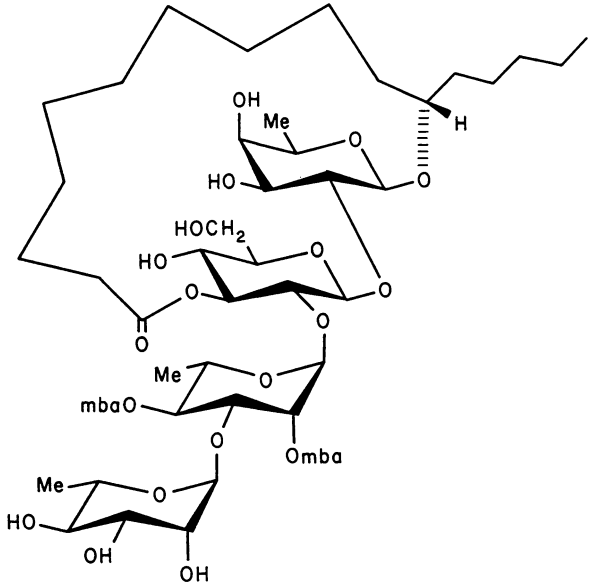

Figure 7. Chemical structure of Tricolorin A.

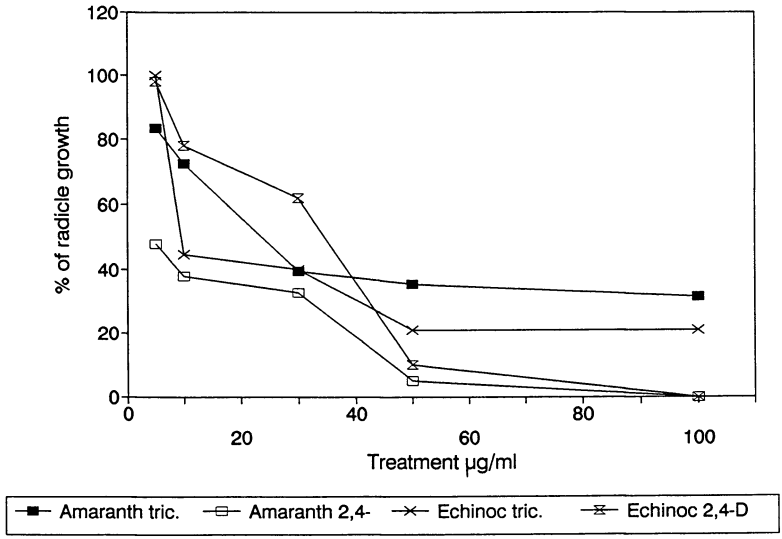

Figure 8. Effect of different concentrations of Tricolorin A and 2,4-D on the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli.*

2.2 μg/ml), and inhibited phorbol 12,13-dibutyrate binding when calf brain homogenate was used as a source of protein kinase C (IC_{50} 43 μM).

Calera (Calera, M.R., Universidad Nacional Autónoma de México, unpublished data), studied if the inhibitory effect of the resin glycoside from *Ipomoea tricolor* on radicle growth of *Echinochloa crusgalli*, was related to an effect on its plasma membrane H^+-ATPase activity. Table VII shows the effect of the resin glycoside on ATPase activity of vesicles at different levels of purification. In the U_3 fraction, which constitutes a highly purified (about 90%) plasma membrane fraction, the resin glycoside inhibited ATP hydrolysis in about 30 % as compared to an inhibition of 10% in the microsomal fraction or 18% in the U_2 fraction (less purified plasma membrane fraction). In consequence inhibition of ATP hydrolysis in the U_3 fraction was due to an effect of the resin on the plasma membrane ATPase, since it is the major ATP hydrolytic component.

Brassicaceae and Glucosinolates

Different species of Brassicaceae (Cruciferae) have been reported as allelopathic plants [*Brassica oleraceae* and *B. campestris*, (38)]. Plants of this family produce glucosinolates that do not have auto allelopathic effects (39). Glucosinolates of Cruciferae are of special interest because in this family there are several common edible plants as cabbage, broccoli and turnip. Glucosinolates are not volatile but their salts are with a characteristic strong odor. More than 20 volatile sulfur compounds have been identified in cabbage (isothiocyanates, sulfides, disulfides, trisulfides and mercaptanes) (40, 41, 42). These metabolites are toxic to *Colletotrichum circinans* and *Botrytis alli*. Mercaptanes prevent the germination of sclerotia of *Sclerotium cepivorum*. Isothiocyanates inhibit mycelial growth, and formation, motility and germination of zoospores in *Aphanomyces euteiches*. These compounds also modify larval activity of parasitic root nematodes *Meloidogyne incognita* and *Nacobbus aberrans* (43).

Celaenodendron mexicanum. Terpenoids and Flavonoids

Castañeda et al. (44) identified terpenoids and flavonoids (Figure 9) in *Celaenodendron mexicanum* (Euphorbiaceae), an endemic tree of the Pacific Coast of Mexico. The biological effects of aqueous leachates, a $CHCl_3$-MeOH extract and the isolated compounds of leaves and twigs (100 μg/ml) were evaluated on the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli*, and on the radial growth of phytopathogenic fungi. Flavonoids (bilobetin and amentoflavone) inhibited approximately 18-25% the growth of *A. hypochondriacus*, and triterpenes (friedelin, maytensifolin B) inhibited almost 40% the growth of *E. crusgalli*. In natural conditions, *C. mexicanum* causes a significant ecological effect on different organisms in the community. The almost year around bearing of foliage causes a constant light reduction that necessarily affects the growth of other species. Its richness in secondary compounds could contribute to its influence on the micro environment.

Table VII. Effect of the Resin Glycoside on ATP Hydrolysis from Purified Plasma Membrane Vesicles at Different Levels of Purification

	ATP HYDROLYSIS (nmol Pi min^{-1} mg $protein^{-1}$)		
	M.F.	U_2	U_3
Control	566.0 ± 0.5 (100%)	432.0 ± 1.9 (100%)	843.0 ± 1.3 (100%)
Vanadate	462.0 ± 0.5 (82%)	106.0 ± 0.9 (24%)	101.4 ± 2.3 (12%)*
Resin	506.5 ± 0.5 (90%)	358.8 ± 8.7 (83%)	576.8 ± 0.6 (68%)*

* $P < 0.05$, ANOVA.

Assays were conducted on microsomal fraction (M.F.) or U_2 and U_3 fractions of purified plasma membrane vesicles by succesive phase partitioning (1 μg of protein). ATP hydrolysis was measured as described under Materials and Methods. Each value represents the means of four samples ± SE.

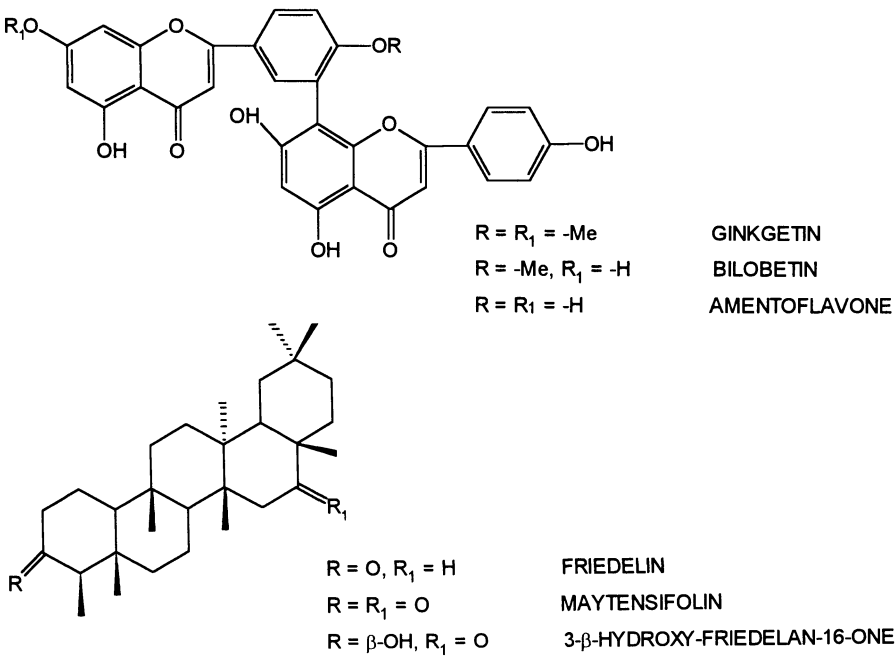

Figure 9. Chemical structures of terpenoids and flavonoids isolated from *Celaenodendron mexicanum*.

Swietenia humilis and Limonoids

Swietenia humilis Zuccarini (Meliaceae), locally known as "zopilote", "cobano", "caobilla", and "sopilocuahuitl" grows commonly in tropical Mexico. In some regions, the seeds of this plant are highly valued for their medicinal properties. Infusions of ground seeds are used as antihelminthic and to treat amebiasis. They are considered effective for treatment of chest pain, coughs, and cancer. *S. humilis* has recently been listed as an endangered species in need of protection. Segura-Correa et al. (45) isolated from the seeds of this species four new tetranortriterpenoids with the same limonoid type skeleton: humilinolides A, B, C, and D (Figure 10). Limonoids possess a wide range of biological activities, including insect antifeedant and growth regulating properties, a variety of medicinal effects in animals and humans, and antifungal, bacteriocidal, and antiviral activity. The effect of the MeOH extract of the seeds and the isolated terpenoids on the radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crusgalli* was evaluated. The radicle growth inhibitory concentration (IC_{50}) of the extract was 275.9 μg/ml for *A. hypochondriacus*, and 171.5 μg/ml for *E. crusgalli*. Humilinolides A and C inhibited the radicle growth of *E. crusgalli* with IC_{50} values of 99 μg/ml and 163 μg/ml respectively. *A. hypochondriacus* was less sensitive to these compounds with values of 199 μg/ml and 215 μg/ml respectively. When the MeOH extract of the seeds was administered to the third instar larvae of *Tenebrio molitor* on a diet containing 1% of the extract, both feeding and growth were significantly reduced. When the concentration of the extract was 0.5%, only a feeding deterrent action was obtained. No mortality was observed at these two concentrations tested.

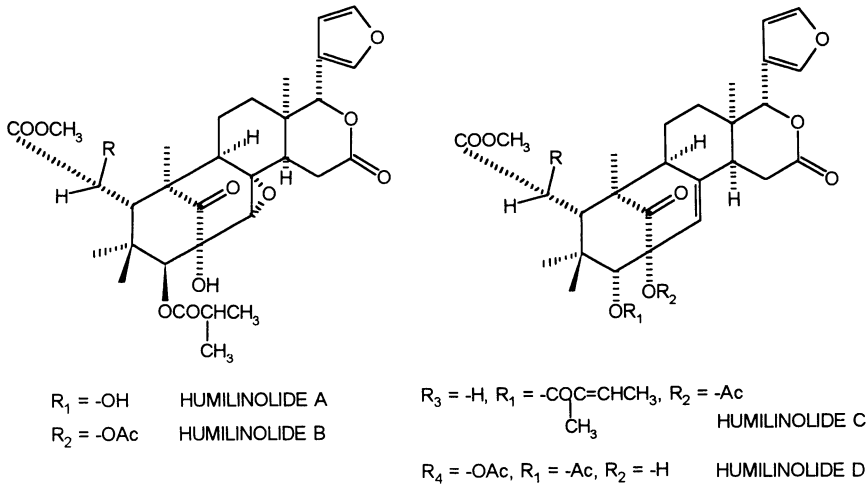

Figure 10. Chemical structures of limonoids isolated from *Swietenia humilis*.

Final Considerations

It was until 1989 that research on Chemical Ecology in México began to take more impulse. This is ironic because in the 60's Lincoln Brower's classic studies on the evolutionary ecology of toxic plants and their associates herbivores (46-47) were carried out with Mexican Monarch butterfly. This example of chemical interaction represents one of the most beautiful and spectacular in ecology and chemical evolution. Mexico should be a particularly propitious place for this kind of studies because of its great biological and ethnobiological richness which implies a diversity of management methods of natural resources practiced by the different ethnic groups of the country.(48).

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Chapter 18

Allelopathic Activity of Naturally Occurring Compounds from Mung Beans (*Vigna radiata*) and Their Surrounding Soil

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Continuous cropping of mungbean (*Vigna radiata*) presents a problem in certain parts of the world (such as Taiwan) where the plant is grown. Now we show that allelopathy may contribute as much as 10-25% of the growth inhibition of mungbean plants grown following mungbean plants. These plants have been found to be allelopathic, and their surrounding soil toxic also. Distribution of the phytotoxic activity showed it to be in the stems and the aerial parts (excluding the stems), with the roots causing little inhibition of the mungbean plant. Partitioning of the stem extracts with water and organic solvents showed that water extracts were most inhibitory to the mungbeans and lettuce; and the organic solvents were both inhibitory and stimulatory. Bioassay of compounds present in soils after mungbean harvest (72 h incubation) also showed inhibition of mungbean plants grown to maturity. The discovery of enhancement of growth of mungbeans by crude mungbean saponins was serendipitous; those plants showed quicker germination and enhanced growth; however, such treatment did not increase the yield.

Allelopathic chemicals are secondary plant metabolites that have roles in plant-plant, plant-soil, plant-disease, plant-insect, and plant-predator interactions that may be beneficial or detrimental to the plant. Mungbeans (*Vigna radiata* L.) planted in soil just used to grow mungbeans (plant-soil interaction) can encounter and produce such secondary metabolites. Mungbeans, a crop plant of economic significance in Taiwan and many developing countries of the world, were not known to have allelopathic activity until the recent finding of Tang and Zhang (*1*). Isolation of the inhibitory compounds produced isovitexin, which was the most active of three *C*-glucosyl flavanoids found. The concept of allelochemical spheres was introduced, which extended their observation from the germinating seed to the plant root system (*1*), and many biologically active metabolites occur at the root-soil interface; (*2*) thus the rhizosphere can be an allelochemical sphere in the environment

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In Allelopathy; Dakshini, K., et al.;
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surrounding the plant in the soil. The *C*-glycosyl flavanoids that were identified and bioassayed are present predominantly in the seed coat, not in the growing tissue of the mungbean plants; however, they possess only a slight inhibitory activity toward lettuce seedlings, and even less so for mungbean seedlings. Their role as allelochemicals in lowering the production of mungbeans remains unknown.

In 1980 the Asian Vegetable Research and Development Center (AVRDC) of Taiwan (*3*) noted that five continuous mungbean croppings showed lack of uniformity of growth patterns; the plants were smaller and produced fewer pods per plant, fewer and lighter seeds, and poor yields (only 25 kg/ha). By comparison, where mungbeans had not been grown for at least three cropping seasons, yield was 440 kg/ha. This led to the recommendation that a mungbean crop should not be followed by another such crop for at least three cropping seasons (*3, 4*). Among different crops (mungbean, soybean, tomato, Chinese cabbage, sweet potato, corn, crotalaria, sorghum, and buckwheat), mungbean was the most detrimental to a succeeding mungbean crop. In one instance, yields after mungbeans were 65 kg/ha compared to 346 kg/ha after tomato.

A series of experiments using a plant culture system designed to determine whether an allelopathic agent existed in the mungbean plant was performed (Young, C. C., National Chung-Hsing University, Taichung, Tawain, personal communication). The results strongly indicated that the mungbean plant produces phytotoxic substance(s) in its aerial parts and its root system; however, he did not follow up this lead. A few years later the mungbean root disease in the Philippines was described and it was reported that the primary cause was not fungi; however, the researchers did not mention that allelochemicals from the mungbean plant might have an effect (*5*).

Cheng (*6*) suggested that the establishment of a specific cause-effect relationship should be sought in allelopathic experiments, in which the following steps must occur: (a) a phytotoxic chemical is produced by a plant or from plant materials, (b) the chemical is transported from its source to the target plant, and (c) the target plant is exposed to the chemical in sufficient quantity and for sufficient time to cause damage. Presented data records an attempt to establish this cause-and-effect relationship for allelopathy in the mungbean plant-soil system. A preliminary account of this work has been published (*7*).

Materials and Methods

Soil and mungbean information. Control soil was collected from the Asian Vegetable Research & Development Center (Tainan, Taiwan), in December, 1991 and February, 1992; it was type AS-2, sandstone shale, older alluvial, 3-noncalciferous soil (*8*). No mungbeans had been grown in the soil for at least three years; the most recent crop was buckwheat (*Fagopyrum esculentum* Moench.) which had been planted during the preceding year as a green manure crop for the December soil, and fallowed during the past year for the February soil. The soils were stored at room temperature (16-40°C), and the times of collection are shown in Table I. The soil was air-dried, passed through a 1.5-cm^2 screen to remove large rock and plant debris, and pulverized; the small roots and stones were picked out, and the soil was passed through a 20-mesh (0.46 mm^2) sieve (Tokyo). The soil was thoroughly mixed and subdivided into appropriate pots or Petri dishes for experimentation. Five hundred grams of soil was placed in each pot, and 20-100 g were left to be divided among several Petri dishes experiments.

Vigna radiata L. (Wilzek) cv. Tainan No. 3 and Tainan No. 5 were grown to maturity (~ 90 days) in the plots designated.

Table I. Soils Used for the Allelopathic Experiments from AVRDS Collected from Plots During December 1991 - June 1992

Date Collected	Treatment of Soil	Soil Plot Numbers; Weeks After Harvest of Mungbeans (MB)							
		Control	1	4	7	14	16	19	27
Dec. 11, 1991	i) 3 yrs since MB were grown ii) plants left standing iii) MB plowed under	40, 41	38	33					
Jan. 23, 1992	i) MB plants left standing ii) MB plants left standing			34	38				
Feb. 1992	Fallow, since 1991	74, 75							
Mar. 10, 1992	MB plants plowed under					38			
Apr. 14, 1992	i) Planted in corn* & soybeans* ii) Plowed under iii) Planted in tomatoes**						34 38	33	
June 17, 1992	Plowed under								38

*Soil sample collected beside the two crops
**Samples collected from the tomato bed row

Mungbean Pot Experiments to Demonstrate Allelopathy

These experiments were designed to measure the effect of growing mungbeans on the same soil that was used to grow mungbeans. They were time-consuming (~ 90 days) and subject to considerably more variation than the 72-h bioassay that is frequently employed. Control soils in which no mungbeans had been grown for the past three years was used in all experiments (Table I, plots 40, 41, 74, and 75).

Allelopathic Effect of Mungbean Soils Used to Grow Mungbeans - 81 Days. This experiment was designed to get significant data from 12 replications in a random arrangement. The plants were rotated every second day so that they got approximately the same exposure to light and temperature. Four seeds per pot were planted; after one week the plants were culled to 2 per pot, grown in a warm room with 190 $\mu E/m^2/S$ at a temperature of 26-28°C daytime (14 h) and 17-20°C for night (10 h), for 85 days. The experiment was started in early February, 1992. Soils (Table I) used were: a) control soil from plots 40 and 41; b) 1-week soil (for simplicity this term is used, and analogous ones, to refer to soil collected at the specified time after harvest) from plot number 38; c) 4-week soil from plot number 33; d) 7-week soil from plot number 38.

Allelopathic Effect of Mungbeans Used to Grow Mungbeans - 41 Days. This was intended to repeat the pattern of the 81-day experiment using soils that had been exposed to atmospheric conditions at AVRDC for longer periods of time. However, it was not possible to use the identical soil since they change with respect to time. This experiment was begun in April, 1992. The soils (Table I) used were: a) control soil collected from plots 74 and 75; b) 1-week soil collected from plots 33 and 34; c) 4-week soil collected from plots number 33 and 34; d) 7-week soil collected from plot number 38; e) 14-week soil collected from plot number 38; f) 19-week soil collected from plot number 33; and g) 27-week soil collected from plot number 38.

There were 8 pots per treatment containing approximately 500 g soil each, 4 seeds per pot; plants were culled to two after 1 week, grown in a warm room with 190 $\mu E/m^2/S$ at 28°C (14 h) day time, and 26°C (10 h) at night, for 41 days. The conditions of the plants were observed and recorded; measurements of height in centimeters were made periodically, for 85 days.

Effect of Mungbean Plant Parts Mixed in the Control Soil. The control soil (plot numbers 40 and 41) was used to plant 4 seeds per pot. Mungbean plant parts (large stems 0.5%, roots 0.2%, and tops 0.9% (small stems, leaves, hulls from mungbeans, and seeds)) were mixed thoroughly in to 500 g soil, 2 pots per treatment. The plants were thinned to 2 plants per pot one week after planting, grown and cultivated in a plant growth chamber (Chang Kuang, Taipei, Taiwan) with 14-h light and 10-h dark with the temperature 28°C in the day and 24°C during the night. Plants were watered regularly. The plants were measured in height (cm) periodically, the condition of the plants was observed and recorded, and the experiment was terminated after 90 days.

Bioassays for 72-h Allelopathy

Bioassay experiments were designed to measure the early growth of mungbeans, lettuce, wheat, and tomato treated with extracts from mungbean plants by water and other solvents compared to a distilled water control.

Filter Paper Method for Aqueous Extracts. Modifications of the procedures of Chou and Young (*9*) and Wyman-Simpson, *et al.* (*10*) were used. One percent, 2%, 3%, 4%, and 5% of the aqueous extracts from mungbean plants were used. Mungbean, lettuce, and wheat seed bioassays were performed using twenty seeds with three replications.

Filter Paper Method for Organic Solvent Extracts. Four milliliters of the organic solvents containing the suspected phytotoxins and control organic solvents (hexane, ethyl ether, chloroform, 1-butanol, and ethyl acetate) were placed on one layer of Whatman No. 4 (12.5-cm) filter paper in a hood and left there until all the organic solvent was evaporated. The dried filter paper was placed in a polystyrene Petri dish, mungbean seeds were pre-wetted with the phytotoxic solutions for 10 h before placing in the dish, 4 ml of distilled water was added, the dish covered, and the assembly incubated as above. For the lettuce bioassay a 2-ml portion of each of the organic solvents containing the test compounds was placed on two layers of Whatman No. 3 MM 5x5 cm filter paper in a glass Petri dish, and allowed to dry in a hood, 2 ml distilled water was added, the dish covered, and the assembly incubated as above.

Soil Method. Soil (5 g) from AVRDC that had been used for growth of mungbeans and was suspected to contain phytotoxins was uniformly distributed on one layer of Whatman No. 4 (9-cm) filter paper in a glass Petri dish, seed arranged on the paper in a circle (mungbean, wheat, tomato, and lettuce), 4 ml of distilled water added, and the assembly incubated as in the filter paper method in above.

The radicle of the seedlings of mungbeans and lettuce were measured in millimeters. Plants showing any signs of fungal activity or bacterial contamination were discarded; however, they were few. Those with any changes in the appearance of the seedlings, such as root tip browning or necrosis, were also discarded, and they also were few.

Statistical Analysis of Data

Mean lengths per dish per treatments, and for the controls were calculated, and the Analysis of Variance (ANOVA) calculated. The difference between sample and the corresponding control was indicated by percent inhibition or stimulation as well as using Duncan's Multiple-Range Test (*11*).

Extraction and Isolation Procedures to Obtain Material for Bioassays

Distilled Water. Distilled water was used to extract mungbean powder (leaves, stems, and roots) at a concentration of 5% (w/v) in an Erlenmeyer flask; the mixture was shaken 2 h at room temperature with a Orbital Shaker (Model S-102, Firstek Scientific), filtered through Whatman No. 1 and 42 papers successively by using a vacuum created by a water aspirator, and diluted to 5, 4, 3, 2, and 1%. The osmotic pressure was measured and recorded in milliosmol/kg by using an osmometer (Model OS Osmometer Fiske Associates, Needham Heights, MA (USA)), and pH values were measured and recorded.

Organic Solvents. Extraction and isolation was patterned after the procedure of Weston, *et al.* (*12*) for possible mungbean phytotoxins. Aqueous extracts were made first by using distilled water at the same proportion as in the above section. The slurry was stirred at 4-7°C for 24 h, strained through muslin to remove most of the solid materials, and filtered through Whatman Nos. 1 and 42 paper, sequentially. The volume of the filtrate was reduced on the rotary evaporator, and by using a lyophilizer (Model No. 10-010, The Virtis Co., Gardiner, N. Y., USA), a brown dry powder was produced. Hexane, ethyl ether, chloroform, 1-butanol, and ethyl acetate were used (40 ml of each) to extract a portion (1 g) of the dry powder for 18 h; each extract was filtered through Whatman 42 filter paper, and diluted to 0.1% and 1.5% in preparation for the bioassay.

Saponin Isolation and Purification

Saponins were isolated, purified, and analyzed according to the procedure of Waller *et al.*, (*13*). The procedure used for those tests described in that paper were processed through the stage of extraction with 1-butanol and purified to 5-h dialysis treatment.

Plants for Allelopathy Tests

Lettuce (*Lactuca sativa* L., cultivar unknown) seeds were obtained from Taiwan Agriculture Materials Co., Taipei, Taiwan; mungbeans (*Vigna radiata* L. cultivars, Tainan 3, Tainan 5), and tomato (*Lycopersicon esculentum* Mill.) were obtained from Tainan District Agricultural Improvement Station, Tainan, Taiwan, and wheat (*Triticum aestivum*, CV Pioneer 2157) was obtained in Oklahoma, USA in March 1985 and stored at 4°C.

Results

Soils were used without additions of fertilizers, insecticides, fumigants, or other agents except when mungbean plant parts were mixed with the soil. The mungbeans were allowed to grow naturally. Values for pH and overall inhibition results are shown in Table II.

Table II. Determination of pH and Summary of Inhibition Results from the Pot Experiments with AVRDC Soils

Soil Plot Nos.	Age (from mungbean harvest)	pH	Summary of Inhibition Results
33	4 weeks	7.8	Yes
34	4 weeks	6.7	Very Slight
34	16 weeks	6.6	Very Slight
38	1 week	7.3	Yes
38	7 weeks	7.8	Yes
40, 41	Control	7.9	–
74, 75	Control	8.0	–

Both control soils as well as those of plots 33 and 38 were alkaline whereas the soil of plot number 34 was acidic.

Growth of Mungbean Plants on Soil at Different Times After Mungbean Harvest

Figures 1 and 2 provide evidence for allelopathy for plants grown of 40 and 81 days. The numbers on the pots (Figure 1) refer to one of the twelve plants grown in a particular plot of soil. The pH of plot number 33 was 7.8 where 65-70% inhibition occurred (Figure 2). There was no statistical difference between the soils collected from 1 week, 4 weeks, and 7 weeks after harvesting of mungbeans. This provides evidence that the alkaline soil promoting allelopathic activity. In contrast there was a dramatic difference in the growth of mungbeans in plot number 34, which showed a pH of 6.7. It had only a negligible amount of allelopathic activity. There was no apparent fungal attack on those plants grown in plot 34 (acidic soil), which indicates that the phytotoxic activity of the acidic soil was considerably less than in the alkaline soil. This pH effect on allelopathy of mungbean growth is repeatable and represents a new observation.

A 40-day experiment was run (Figure 3); however, the same soils were used that had been stored at room temperature (15°-40°C) from December, 1991 until July 1992. This storage at elevated temperatures permitted microorganisms to grow, using the naturally occurring phytotoxins as some of their substrates and thus becoming more active in the production of abnormal and normal metabolites. Plot 33 (pH 7.8) after 4 weeks showed greatest inhibition. Plot 38 (pH 7.3-7.8) (1W, 7W, 14W, 19W, 27W) showed that the soils were significantly different in their inhibitory activity.

Problems are always found in soils stored at room temperature since they are not exposed to similar conditions in the field. This makes the estimate of how long a period of time would be required before the soil returned to its normal state risky. At 27 weeks the soils still had a bout 25% inhibitory activity, so our best estimate is 1 year before the soil returns to the zero level to the allelopathic state. This is an improvement over the AVRDC recommendation which is 3 years.

Control Soil Mixed With Mungbean Plant Parts

Table III shows the overall result of comparing the height and weight of plants in the 81-day experiment with modified soil to that using the control soil. The stems showed inhibition of growth at 0.5% concentration; the roots showed almost the same amount at 0.2%, whereas the leaf or other aerial parts at 0.9% concentration caused about a 15% increase in plant height. The stems mixed with the control soil and the mungbeans were allowed to grow until maturity showed the greatest

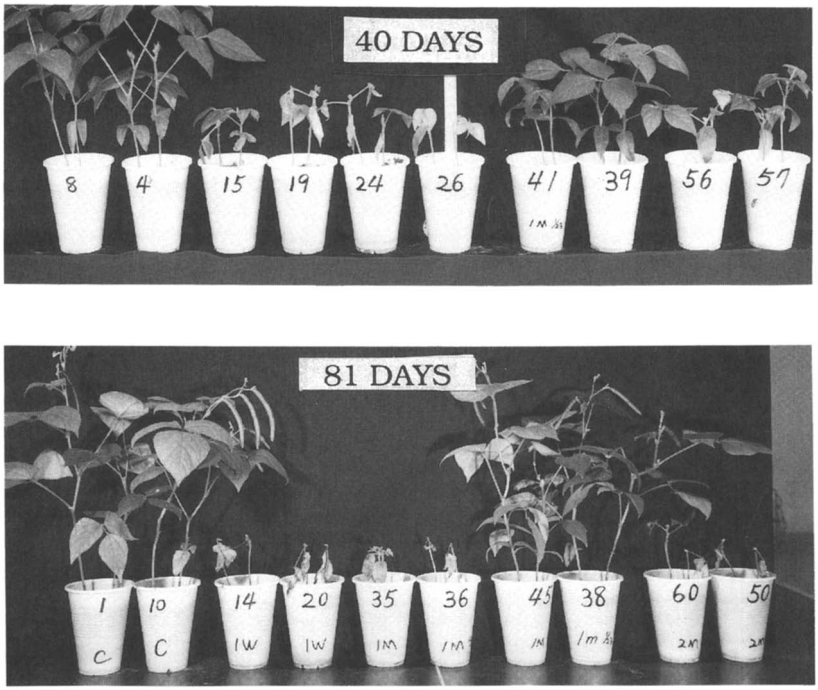

Figure 1. Allelopathy of mungbeans growing in AVRDC soil for 40 and 81 days. Legend: Control soil: plots 40 and 41 (mixed), 1 week (plot 33), Nos. 15, 19, and 14, 20; 4 weeks (plot 33), Nos. 24, 26, and 35, 36; 4 weeks (plot 34), Nos. 41, 39, and 45, 38; 7 weeks (plot 33), Nos. 56, 57, and 50, 60.

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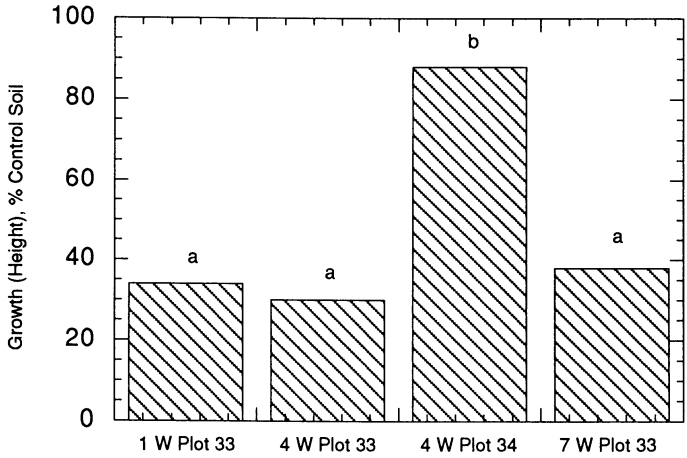

Figure 2. Allelopathy of mungbean plants grown in AVRDC soil following mungbeans. Legend: Duration of experiment (81 days); W = weeks following harvest of mungbeans; bars having different letters are significantly different, P = 0.05, ANOVA with Duncan's Multiple-Range Test.

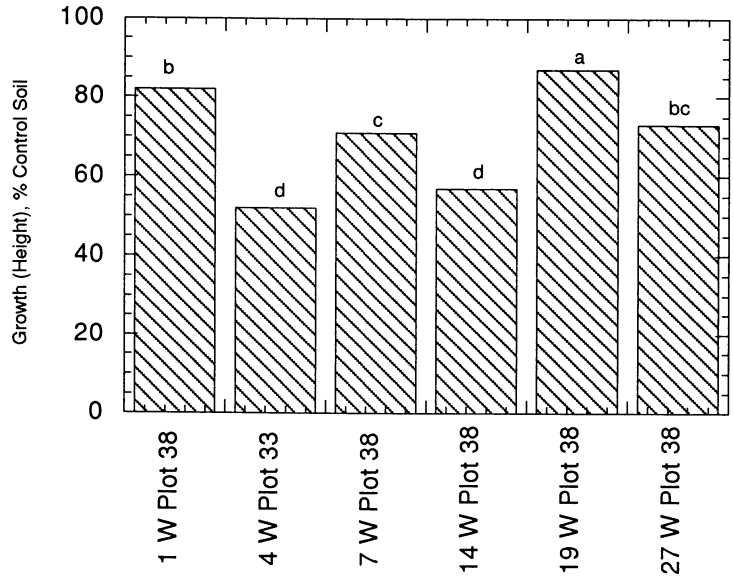

Figure 3. Allelopathy of mungbean plants grown in AVRDC soil following mungbeans. Legend: Duration of experiment (41 days); W = weeks; bars having different letters are significantly different, P = 0.05, ANOVA with Duncan's Multiple-Range Test.

inhibitory effect of the dry weight; the roots showed somewhat less, whereas the leaf/aerial soil mixture showed there was a 24% increase. It is clear that stems of the mungbean plant contain the predominant chemicals that exert their allelopathic activity toward mungbean plants. The plant material added was estimated, based on weights obtained from plants grown in the field just prior to harvest, to be about the normal amount plowed under in the field.

These data suggest that the complete mungbean plant when plowed under might have a negligible allelopathic effect on the new crop of mungbeans, since the effect of chemical compounds that can inhibit plant growth can be overcome by those which have stimulatory activity. As the plant material decays in the soil, residual compounds, sometimes in high concentrations do occur. Such compounds if allelopathic, could also stimulate or inhibit the growth of a crop of mungbeans planted soon after the harvest.

Table III. The Effects of Mungbean Residue Powder Mixed With Control Soil on the Growth of Mungbean at 81 Days

Treatment	Mungbean Growth and Weight, % of Control	
Mixing Rate (g/g)	Height	Dry Weight
Root Powder/Soil (0.2%)	98 ab	76
Stem Powder/Soil (0.5%)	87 b	33
Leaf/Aerial Powder/Soil (0.9%)	115 a	124

Values are the means of 12 replicates. The letters in a column not followed by the same letter are different, $P = 0.05$, ANOVA with Duncan's Multiple-Range Test.

Bioassay of Mungbean Plant Parts

Aqueous extracts of roots (Figure 4) showed a significant inhibition for lettuce and wheat seedlings, but only a small but typical inhibition for mungbean seedlings. The assay was run at the same pH (7.0 ± 0.3) as the plant parts extracted. The osmotic concentration varied from 16-82 mOsmol/kg for the 1-5% concentrations. A bioassay was run on mungbean and lettuce at 25 mOsmol/kg and it showed 12 and 27 inhibition respectively (*14*). Therefore we conclude that only a negligible effect was seen on the effect of mungbean roots on the early growth of mungbeans.

The bioassay of extracts of leaves from mungbeans is shown in Figure 5, which demonstrates a significant level of inhibitory activity for mungbean, lettuce, and wheat. The pH values for the extract were 6.0 ± 0.2, and the bioassay was run without change of pH. The osmotic concentration varied from 20-87 mOsmol/kg which was nearly the same value as for the roots. The stem values for inhibition were similar to those of the leaves (not shown); however, the extract had a pH value of 6.3 ± 0.3, and an mOsmol/kg that was almost identical to that of the leaf fraction.

These bioassay results from the roots agree with the findings on the whole plants grown for 81 days (Figure 2).

Bioassay of Compounds from Mungbean Stems Extracted into Water and Partitioned into Organic Solvents

The effects of extracts made with distilled water followed with partitioning with organic solvents are shown in Figure 6 (top and bottom) at 1% and 15% of the original plant weight. Mungbeans and lettuce showed mixed inhibition and stimulation; chloroform and hexane solutions showed slight stimulation of growth, whereas the water, ether, 1-butanol, and ethyl acetate extracts showed inhibition for mungbeans. The trend using lettuce seedlings was somewhat less at the 1% level of stems, but more inhibitory activity at 15% concentration.

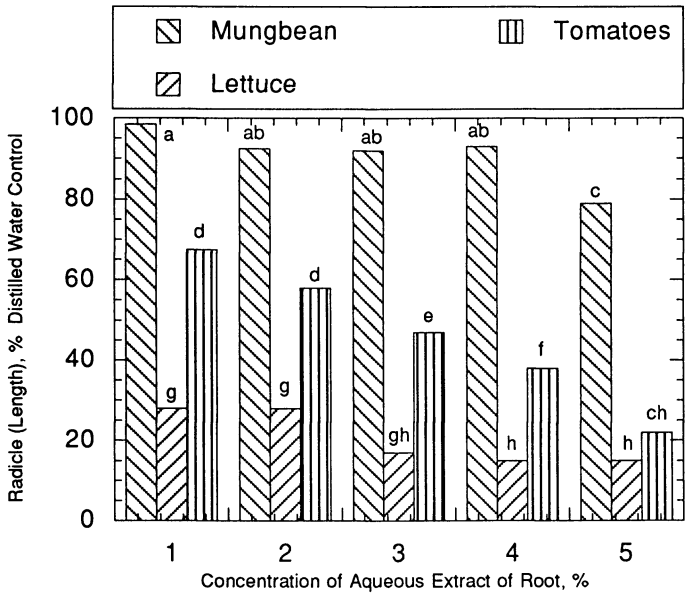

Figure 4. Inhibition by aqueous extractions of mungbean roots on mungbeans, lettuce, and tomato for 72 h. Legend: Bars having different letters are significantly different, $P = 0.05$, ANOVA, with Duncan's Multiple-Range Test.

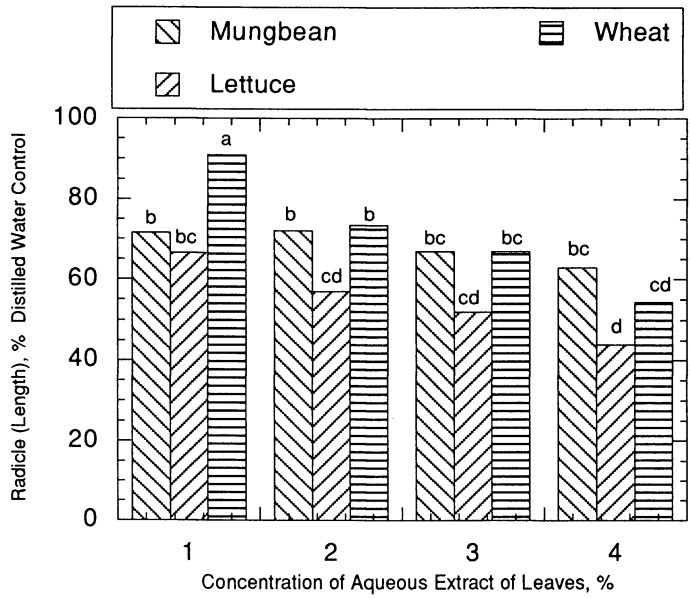

Figure 5. Inhibition by aqueous extracts of mungbean leaves on mungbeans, lettuce, and wheat, for 72 h. Legend: Bars having different letters are significantly different, $P = 0.05$, ANOVA, with Duncan's Multiple-Range Test.

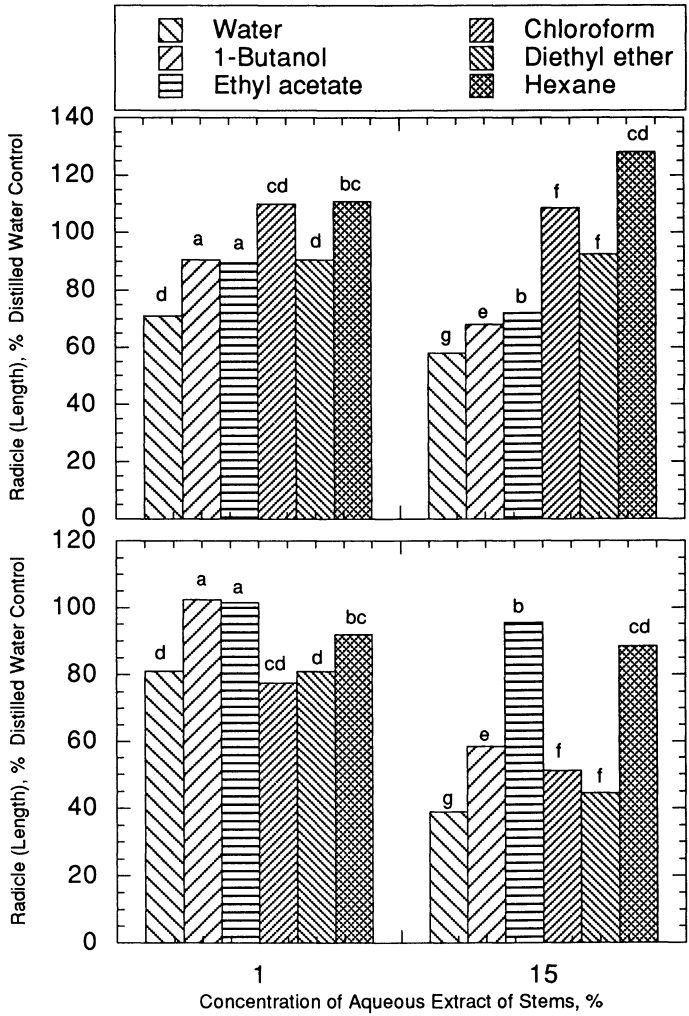

Figure 6. Allelopathic effects of extracts of mungbean stems in water and organic solvents on mungbeans (top) and lettuce (bottom) for 72 h. Legend: Bars having different letters are significantly different, $P = 0.05$, ANOVA, with Duncan's Multiple-Range Test.

It would appear that using this partitioning system could provide information on the compounds responsible for the increased growth effect described; however, this is not reported at the present time. A mixture of the saponins as well as some phenolic acids from mungbeans would be in the 1-butanol fraction. The water extract was the most inhibitory at both concentrations, which could be expected since it contains phenolic acids as well as other soluble chemical compounds from the partitioning of organic solvents which were both inhibitory and stimulatory.

Bioassay of Soils After Mungbean Harvest

Bioassays from soils of mungbeans are shown in Figure 7. These bioassay results tend to support the experiments with mungbean plants grown to maturity (Figure 3); however, the exception was the lack of allelopathic activity shown by the 1 week soil. The soil samples taken after 4 weeks from plots 33 (pH 7.8) and 34 (pH 6.7) showed approximately 8% and 18% inhibition respectively, where as at 7 weeks the inhibition was much greater (~32%) for mungbeans.

The effect on lettuce was surprising in that the compounds present in the soil were most inhibitory at 1 week and 4 weeks on the alkaline soils; however, the 4 weeks acidic plot and 7 weeks alkaline plot were slightly stimulatory. Clearly the compounds extracted from each soil were markedly different in their amounts and type.

Mungbean Growth Enhancement by Crude Mungbean Saponins

Crude mungbean saponins (*13*) were applied to the soil at concentrations of 15, 150, and 450 ppm, mungbean seeds were germinated in pots, the plants were allowed to grow until maturity, and the measure of height was recorded for several experiments; however, only the pertinent results are included in Table IV. The experimental plants as compared to the control clearly showed elongation of stems and other growth-enhancing effects when the 1-butanol extract followed by 5-h-dialysis-treated saponins were added; however, we cannot be certain that the active compounds are entirely saponins. Mungbean plants showed quicker germination and enhanced effects throughout their growth. The plants had leaves larger in size and darker green in color, which was an indication of enhanced photosynthesis; however, the number of seed pods was about the same, showing that the increased growth did not increase the yield. After 40 days all concentrations caused about 10% acceleration but after 67 days the two higher concentrations showed 10 and 15% growth enhancement respectively, while the effect of 15 ppm of saponin continued to drop to nearly zero. At the end of the experiment (84 days) the lowest concentration showed no effect, while 150 and 450 ppm saponins showed 20-25% enhancement of growth as measured by height of the plant. The preliminary indications are that 150 ppm of crude saponins (5-h dialysis) is required for the mungbean growth enhancement.

The crude saponins added to the soil may serve as a nutritional source, since little effort was made to remove inorganic elements, other than dialysis for 5 h which is a common technique to reduce the inorganic nutrients and small organic molecules by factors of 30-50-fold. These results should be considered as only preliminary; they should be repeated in the laboratory by others and reproduced in the field several times, with different concentration of crude saponins and also with pure saponins.

Attempts to recover added saponin immediately following its addition to the soil were unsuccessful by several extraction techniques; however, no humic or fulvic acid was isolated. This was interpreted to mean that the saponins added might become bound to the humus fraction of the soil.

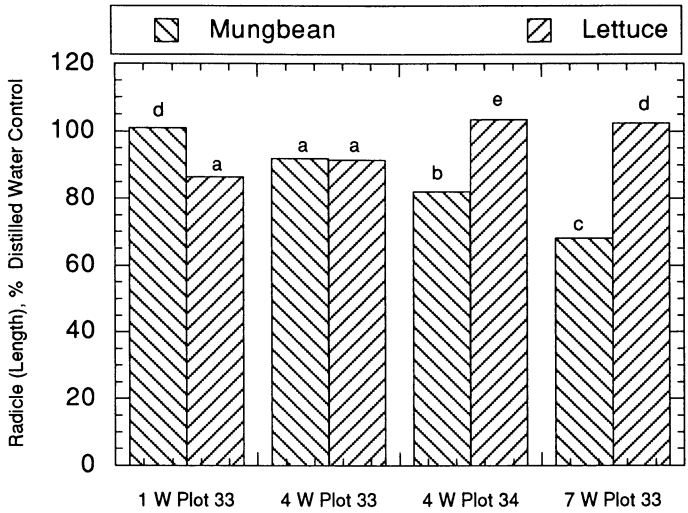

Figure 7. Allelopathy of soil toward mungbean and lettuce seedlings after 72 h. Legend: Soil (5 g) was placed on the filter paper and distilled water was added along with the seeds and set to incubate; bars having different letters are significantly different, $P = 0.05$, ANOVA, with Duncan's Multiple-Range Test.

Table IV. Enhancement of the Growth of Mungbeans by Soyasaponin I and Other Saponins Produced by Mungbean Plants Added to AVRDC Soil

Date 1992	Days	Observations of Pots[1,2,3,4] (Compared to Control Without Saponins)
Feb. 13	0	Experiment started
Feb. 21	8	Primary leaves, no trifoliate, growth enhancement with 88% germination as compared with 58% of the control
Feb. 25	12	First trifoliate, growth enhancement
Feb. 28	15	Second trifoliate, growth enhancement
Mar. 4	20	Third trifoliate, growth enhancement
Mar. 7	23	27% higher than control; more third trifoliate
Mar. 14	30	Less growth enhancement
Mar. 24	40	Plants average 10% higher than control; some flowering
Apr. 20	67	Plants average 13% higher than control; pods appear; maturity attained in a shorter time than for control
May 6	83	Plants 20-25% higher than control

[1]Saponins where extracted and purified with 1-butanol followed by 5 h dialysis with 3000 Mw tubing.

[2]Concentration of added saponins were 15, 150, and 450 ppm.

[3]Soils from Asian Vegetable Research and Development Center where no mungbeans were grown during the past three years; plots 41 and 42.

[4]Small differences of enhancement may be seen at: flowering, pod maturity, not pod number.

Roots and their rhizosphere have been the subject of research for more than a hundred years; however, their action on biological membranes and sites of enzyme action has not been thoroughly treated, although Tang *et al.* (*2*) alluded to the reactions that occur at the molecular level. Soil moisture, temperature, gases, humus, and inorganic (mineral) and organic compounds have important impacts on the development of the root system, and interact with each other in establishing the pattern of root development and function. The mungbean plant root rhizosphere can contain symbiotic associations with mycorrhizae and bacterial nodules where fungi and bacteria bring in mineral constituents in exchange for some of the plant (vitamins, carbohydrates, etc.). We suggest that saponins [(e.g., soyasaponin I (Figure 8), which is the predominant saponin present in 7-day old mungbeans (*13*)], can become attached to the root hair so that transport of water and nutrients is facilitated; the sugar portion of the saponin confers the maximum enhancement of mungbean growth; however, it could be hydrolyzed off by enzymatic cleavage and thereby produce another saponin molecule.

The saponins that occur in the soil rhizosphere around mungbean plants during the time required for maturity may undergo several changes in the microstructure of cell membranes (*15*). The rhamnose-galactose-glucuronic acid-soyasapogenol B [(soyasaponin I) Figure 8] is the dominant structure for the early germination of the seed, and the early plant growth enhancement occurs. As the plant develops, we suggest that hydrolysis catalyzed by enzymes produced by microorganisms in the soil cleave the sugars from the saponin in a manner as for medicagenic acid glycosides (*16, 17*) to give the aglycone (soyasapogenol B). Although the structural integrity of soyasapogenol B is not understood, this compound can further be broken down by microorganisms to serve as a carbon source. Figure 8 shows the hydrolysis that may progress through the sequential cleavage of sugars (which we think most likely), or cleavage could occur releasing the rhamnose-galactose-glucuronic acid as 2- or 3-piece fragments leaving soyasapogenol B. Since plant root hairs have a short lifetime, the process would be repeated many times throughout the life cycle of the mungbean plant making the root hairs continually exposed to saponin molecules.

All this discussion has been made on the assumption that the 1-butanol-extracted saponins, purified by dialysis for 5 h were pure; however, other compounds (e.g. steroidal glycosides) may be present. It is important to recognize that regardless of the saponin, whether a triterpenoid or steroidal glycoside, it has a strong detergent action.

Discussion

The inhibition of growth of mungbeans in pots, was found to be around 20-25% compared to those in control pots, and was often less, depending upon temperature, water, and soil characteristics. As the plant developed for 30-45 days the effect of phytotoxins from the mungbean plant almost disappeared, and the root pathogens grew with pronounced deleterious effects on the mature plants. There is evidence in the literature that Taiwan, the Philippines, and Kenya all have a problem with continuous cropping of mungbeans (*18*) associated with root pathogens. The present paper recognizes for the first time the role that allelopathy or allelochemicals have in causing damage done when mungbeans are planted in the same soil in which mungbeans were grown.

The inhibitory effect of mungbean stems that were processed through the partitioning showed that the naturally occurring phytotoxins were distributed throughout the water, 1-butanol, ethyl ether, and ethyl acetate fractions, which indicated that several types of compounds are represented.

We find that the mungbean plant grown under continuous cropping conditions in subtropical or tropical regions suffers reduction in height and yield of

mungbeans from allelochemicals under certain conditions. There is an allelopathic effect of phytotoxins from the mungbean plant that is present at all stages of growth. Soil acidity is an important factor: the more alkaline the soil, the more the microorganism population increases in metabolic activity. Each of these factors, and perhaps others, should be considered and appropriately recognized in growing mungbeans.

Mungbean saponins might interact with dioxins similar to the brassinosteroids (*19*). Early references [Heftman (*20*) and references cited therein; (*21*)] to the role of various saponins produced by plants showed that low concentrations promote germination, high concentrations inhibit the growth, and treating the seed had a lasting growth-promoting effect. Bisset (*22*) states that an advantage of saponins to the plant producing them is that they function as growth regulators as well as allelochemicals. This research provides the first definitive evidence that saponins produced by mungbean plants, when added to the soil enhance the growth of new mungbean plants as an allelochemical plant growth regulator.

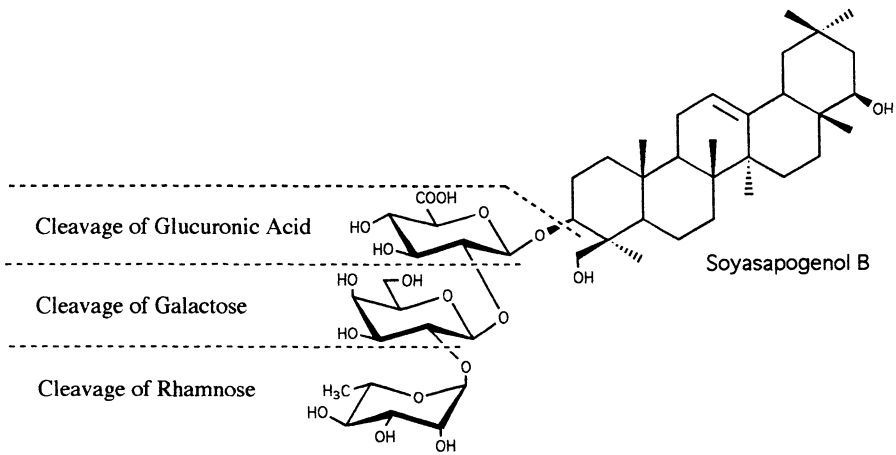

Figure 8. Suggested structural changes in soyasaponin I (3-O-[α-L-rhamnopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl] soyasapogenol B) that occur on the mungbean root surface and in the surrounding rhizosphere.

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Chapter 19

Potential of Hydroxamic Acids in the Control of Cereal Pests, Diseases, and Weeds

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Hydroxamic acids derived from 2-hydroxy-1,4-benzoxazin-3-one are secondary metabolites present in major agricultural crops such as wheat, maize and rye. Progress in research related to their ecological role in plants is summarized, as well as to potential uses in the control of pests, diseases and weeds. Problems presently limiting the exploitation of these compounds in plant protection are discussed.

In the last few decades, the dependence on fossil-fuel-based agrochemicals such as fertilizers and pesticides to produce agricultural and forestry products, has increased. This increased input of agrochemicals in arable crops can not be sustained in time, since agrochemicals pollute the environment and their production depends on non-renewable resources. Additionally, resistant strains of pest insects are emerging and herbicide tolerant weeds are appearing. Hence, new alternatives are needed which do not lead to the problems mentioned above and are of lower cost. Host plant resistance to pests, disease and weeds should play an increasingly important role in integrated pest management systems.

Cereals such as wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and rye (*Secale cereale* L.), which are major agricultural crops in the world, produce hydroxamic acids (Hx), a family of secondary metabolites discovered over three decades ago in relation to fungal diseases of rye (*1*), and thought to play a part in conferring resistance in some Gramineae to a wide range of pests and diseases (*2*). In addition, Hx have also been associated with detoxification of triazine herbicides (*3-5*) and with iron acquisition by plant roots (*6-8*). Hx are known to occur not only in maize, rye and wheat, but also in triticale and in several wild Gramineae (*2,9-14*), and to be absent from barley, oats and rice (*2*). Hx occcur in the plant as 2-ß-O-D-glucopyranosides which are hydrolyzed by endo-ß-glucosidases when the plant is injured

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In Allelopathy; Dakshini, K., et al.;
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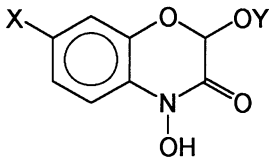
(*15,16*). The most abundant aglucone in wheat and maize extracts is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA). In rye, it is the demethoxylated analogue DIBOA (*2*).

Since Hx are present in the leaves, stems and roots of cereal plants, they can play a leading role in resistance against a variety of external agents. In this review, we will describe the potential uses of Hx in the control of pests, diseases and weeds of cereal crops, and discuss some of the problems that presently limit their exploitation in plant protection.



X	Y	Compound
H	H	DIBOA
H	Glucosyl	DIBOA glucoside
CH_3O	H	DIMBOA
CH_3O	Glucosyl	DIMBOA glucoside

Accumulation of Hx in Plants

Hx are not present in the grain but start accumulating as glucosides during the first stages of seedling development. The pattern of accumulation in aerial parts and roots varies between species and also between cultivars. The dynamics of Hx accumulation has been well studied in wheat and maize. In wheat, Hx are absent from the seed, increase upon germination (peaking at the young seedling stage) and decrease thereafter (*17*). In mature plants, the youngest tissue still retains a high concentration of Hx (*9-11,17-20*). Hx are present in all organs of the plant (*17*). Within the aerial parts of wheat and maize seedlings, they are present in the mesophyll as well as in the vascular bundles (*21,22*). Recently, it has been shown that the phloem sap of wheat seedlings, collected through excised aphid stylets, contained Hx glucosides (Givovich, A., Sandström, J., Niemeyer, H.M. and Pettersson, J., *J. Chem. Ecol.*, in press).

The information available on Hx accumulation in rye is still scarce. Barnes and Putnam (*23*) reported their presence in thirty five day-old greenhouse- and field- grown rye plants. Analysis of one rye cultivar grown under field conditions indicated that Hx are present throughout the life cycle of the plant (*24*). According to this limited information it seems that rye shows a pattern of Hx accumulation different from wheat and maize.

Furthermore, there exists evidence that higher levels of Hx may be induced by insect feeding in maize (*25*) and in wheat (*26*) and by artificial damage in maize (*27*), and that some abiotic factors such as light (*28*), temperature (*29*), water stress (*30*) and minerals (*6,7*) can modify the levels of Hx in maize. The mechanims involved in the regulation of Hx accumulation and degradation will be a major area of future research in this field.

Role of Hx in Insect Resistance

One of the first examples of the deliberate use of Hx in the control of a cereal pest involves the European corn borer, *Ostrinia nubilalis* Hübner, an important pest of maize in temperate regions. In the late 1960s, Hx were identified as a resistance factor against leaf feeding first generation larvae of this insect. Efforts were directed towards the production of hybrid maize with increased concentrations of Hx, which indeed showed increased resistance to the insect (*31,32*). The effect of DIMBOA on the life cycle of the insect has been studied in artificial diets (*33*). Studies on the mechanism of toxicity of DIMBOA towards the insect showed that DIMBOA acts primarily as a digestive toxin (*34*) and that the activity of detoxifying enzymes in the insect's midgut was modified by DIMBOA (*35*). Maize germplasm has been analyzed extensively for sources of resistance to the borer (*36*).

Artificial feeding of the borer *Sesamia nonagrioides* Lef., a pest of maize in the Mediterranean area, with diets supplemented with maize leaves or stems containing high levels of Hx, decreased the performance of the insect relative to diets without additive or diets supplemented with maize tissue with low Hx levels (*37*), suggesting a possible role for Hx in the resistance of maize to this borer.

The presence of Hx in the roots of maize has been related to resistance to the rootworm *Diabrotica virgifera virgifera* Le Conte. Performance of rootworm larvae was lower on maize accumulating high concentrations of Hx in its roots. Conversely, a high-Hx maize showed less damage due to rootworm than did a low Hx line (*38*). In choice tests, rootworm larvae 'preferred' to burrow into control maize roots rather than into roots treated with Hx, and Hx treatment of corn roots produced feeding deterrency in the larvae (*39*). Another report claimed that 6-methoxy-benzoxazolinone (MBOA), the main decomposition product of DIMBOA, acted as a volatile cue to the rootworm to find its host (*40*).

Recently, the presence of N-O-methyl-DIMBOA was demonstrated in maize whorl wax (*41*). The compound was toxic to the southwestern corn borer, *Diatraea grandiosella* Dyar, and was suggested as a possible resistance factor in maize to the insect (*41*).

Hx have been shown to be a major biochemical mechanism of resistance of wheat to aphids, acting through antibiosis and feeding deterrency. Thus, negative correlations have been described between Hx levels in the plants and growth rate and intrinsic rate of natural increase of cereal aphid populations (*18,20,42-44*), and mean relative growth rate of aphids (*45*). Population increase of aphids feeding on excised barley leaves (originally lacking Hx) into which different levels of DIMBOA had been incorporated, lead to similar negative correlations (*18*). Survival of cereal aphids in artificial diets decreased with increasing DIMBOA concentrations in them (*43,46*).

In choice tests, both winged and wingless *Rhopalosiphum padi* (L.) preferentially settled on seedlings with lower Hx levels (*47,48*). Electronic monitoring of aphid feeding behavior showed that in seedlings with higher Hx levels, fewer aphids reached the phloem within a given time, and they required

longer times to contact a phloem vessel (*48*). This feeding deterrency decreased transmission of barley yellow dwarf virus to wheat seedlings in the laboratory (*48*).

The developmental time of the predatory ladybird, *Eriopis connexa* Germar., was shorter and the number of aphids ingested higher when the beetle fed on aphids from a high Hx wheat cultivar as compared with an intermediate Hx cultivar (*49*), suggesting that higher levels in wheat could potentiate the beneficial effects of the predator.

Sub-lethal doses of an insecticide were more effective on aphids feeding on a high-Hx wheat cultivar than on a low one (*50*).

Although these are strong arguments for the inclusion of Hx in strategies for the integrated control of aphid pests in cereals, field experiments are needed to validate the laboratory results.

Role of Hx in Disease Resistance

Resistance of maize to Northern corn leaf blight caused by *Helminthosporium turcicum* Pass., was associated with the presence of Hx (*51,52*). DIMBOA inhibited the germination of spores of *H. turcicum* (*53*). Maize mutants lacking the Bx allele coding for the accumulation of Hx were more susceptible to infection than maize possessing it. Diffusates from young maize plants were more active in inhibiting spore germination and germ tube elongation of *H. turcicum* than diffusates from older plants (*54*). Differences in mycelial growth were noticeable when the mycelium reached the vascular bundles (*55*). Interestingly, the glucoside of DIMBOA has been found in the phloem sap of wheat (Givovich, A., Sandström, J, Niemeyer, H.M. and Pettersson, J., *J. Chem. Ecol.*, in press)

Correlations have also been reported between Hx concentration in a cereal plant and resistance of the plant to various fungal infections, such as those produced in maize by *Diplodia maydis* (Schw.) Lev. (*56*) and *Cephalosporium maydis* (*57*), and in wheat by *Puccinia graminis* var. *tritici* Erikss. and Henn. (*58*). It was recently reported that infection by *P. graminis* produced a substantial increase in the synthesis of the glucoside of the DIMBOA derivative methylated at the hydroxamic acid function, which may thus function as a phytoalexin inhibiting mycelial growth (*59*). However, evidence has been presented that Hx concentrations are not related to resistance of maize to *Colletotrichum graminicola* (Ces.) Wils. (*60*).

The presence of Hx in maize plants was associated with the inability of certain species of soft rotting bacteria of the genus *Erwinia* to attack them, the lag phase of bacterial growth being prolonged by DIMBOA (*61*). Further experiments indicated that Hx were not the sole factor responsible for bacterial resistance in maize (*62*).

The role of Hx in the interaction of cereals with fungi remains an important area where much research is needed.

Role of Hx in Allelopathy

Biochemical interactions among plants appears to be a fairly ubiquitous phenomenon, occurring in most natural and agricultural ecosystems (*63,64*). However, the occurrence of this phenomenon is not easy to demonstrate due to the complexity of the sequence of events involved. Host plants must produce allelochemicals which must, directly or indirectly, interfere with the target plants. The allelochemicals must be released to the environment by means such as volatilization from the living plant, leaching of water-soluble compounds by the action of rain, fog or dew, root exudation, incorporation of plant parts (*65*), or decomposition of plant residues (*64*). Finally, the allelochemicals must be available to the target plant in sufficient amount to produce the allelopathic effects. Additionally, allelochemicals can be transformed chemically or microbially in the soil (*59,66,67*), and soil itself can influence the transfer, transformation and retention of allelochemicals (*68*).

Phytotoxicity of Hx. Bioassays carried out with Hx indicate that they inhibit seedling growth and the emergence of several mono- and dicotyledoneous species (*23,69*). Bioassays based on a cress (*Lepidium sativum* L.) root growth assay were used to assess the phytotoxicity of residues and extracts of rye, most active fractions having been found in the ethereal extract. Further purification identified DIBOA, the main Hx found in rye and its breakdown product benzoxazolin-2-one (BOA), as the main compounds of the extracts (*70*). An assessment of Hx toxicity towards weeds from different families normally associated to Chilean cereal crops, showed in most cases that cotyledons and root growth were inhibited significantly by 1 mM DIMBOA (Pérez, F.J. and Ormeño, J., unpublished data). On the other hand, Hx showed no autotoxic effects on cereals producing them, such as maize, wheat and rye (*7*; Pérez, F.J. and Gonzáles, L., unpublished data). These observations make Hx interesting as natural herbicides, since many weeds are susceptible to their inhibitory effects, while cereals producing them are not affected.

Root Exudation of Hx. Hx are non-volatile compounds. They are not leached from leaves (*21*), but can be released to the soil by root exudation. Pérez and Ormeño-Núñez (*71*), using a continuous root exudate trapping system, reported that two cultivars of rye exudate DIBOA through their roots while three different wheat cultivars that accumulate Hx in their roots do not. In a recent report, Pethós (*7*), using hydroponic cultures and placing the plants in distilled water for 2 to 6 hours after removal from the nutrient solution, found that wheat and maize also exudate Hx through their roots. Both research groups found that in the root exudates Hx occur as aglucones, while in root extracts they occur as glucosides, suggesting their transformation previous to release. A reexamination of rye root exudates during the first 24 days after emergence, showed that the pattern of compounds exudated changes with the age of the plant and confirm that DIBOA was one of the main compounds exudated. However, HDIBOA-glucoside, the reduced form of DIBOA, was the main compound identified in the exudates (Pérez, F.J., unpublished data).

Spring-sown living rye reduced weed biomass by 93 % over plots without rye (*72,73*). Field experiments using a rye variety exuding Hx through their roots reduced total weed biomass by 83 and 76 % compared with wheat and forage oats. Moreover, the specific reduction of *Avena fatua* L. biomass, observed with the rye cultivar and not with the wheat and forage oat cultivar, correlated well with the phytotoxicity of Hx on *A. fatua* observed in bioassays (*24*). These results suggest that simply identifying roots with high contents of Hx is not adequate for the selection of varieties with allelopathic potential. Root exudate analysis will be also required.

Reduced Tillage and Cover Crops. The use of allelopathic cover crops in reduced tillage cropping systems may provide an ecologically and environmentally safe management strategy for weed control. In this regard rye has been extensively studied, and numerous reports show the phytotoxicity of rye and its residues (*72-81*). Rye residues reduced total weed biomass by 63 % when *Populus excelsior* was used as a control for the mulch effect (72). In a four-year experiment carried out to evaluate the effect of rye cover crop on weed control, soybean (*Glycine max* L.) yield and soil moisture, and control of giant foxtail (*Setaria faberi*, Herrm.), velvetleaf (*Abutilon theophrasti*, Medik.), smooth pigweed (*Amaranthus hibridus* L.) and common lambsquarters (*Chenopodium album* L.), the effect recorded was generally greater than 90 % in the rye mulch plots and better than the corn residue treatments. No differences in soybean yield were found between conventional tillage using herbicides, compared with a no-tillage system using rye mulch without herbicides. Herbicides improved weed control in the corn residue plots but did not do so in the no- tillage rye treatment, due to the excellent control by rye mulch. Weed control by rye residue treatments, regardless of herbicide treatment, was explained by the allelopathic effect of rye and the physical presence of the mulch on the soil surface (*80*). Since Hx are the main allelochemicals involved in allelopathic effects of rye (*24,73*), rye germplasm leading to high accumulation of Hx to be used as mulch in weed control seems desirable. On the other hand, the assessment of rye mulch in weed control of other crops such as wheat and maize represents an interesting possibility since Hx are not toxic towards these cereals (*7*; Pérez, F.J. and Gonzáles, L., unpublished).

Transformation of Hx in the Soil. It was reported that BOA, the breakdown product of DIBOA, was transformed by the action of soil microorganisms to 2,2'-oxo-1,1'-azobenzene (AZOB) a compound with herbicidal activity stronger than DIBOA or BOA (*82,83*). The same authors identified *Acinetobacter calcoaceticus*, a Gram-negative bacteria isolated from field soil as the factor responsible for the biotransformation of BOA to AZOB (*84*). However, Gagliardo and Chilton (*85*) did not find AZOB in incubations of soil with BOA, o-aminophenol or o-azophenol, and claimed that the red pigment obtained by Chase *et al.* (*83*) corresponded to 2-amino-3H-phenoxazin-3-one which can be formed by microbial hydrolysis of BOA to o-aminophenol which is oxidized to the corresponding aminophenoxazinone by air. Further

supporting this conclusion, the microbial transformation in the soil of two other naturally-occurring hydroxamic acids gave the corresponding aminophenoxazinones (*86*).

Clearly, more work will be needed to define precisely the nature of the compounds involved in field allelopathy by hydroxamic acids.

Prospects for Increasing Hx Levels in Cereals and their Use in the Control of Pests, Disease and Weeds

The decline in the concentration of Hx as the plant matures, as has been shown for maize and wheat, limits their action as a chemical defence, especially in the cases of organisms that invade the plant at its later stages of development. Little is known about the genetic expression and biosynthesis of Hx. This knowledge is highly desirable if Hx are to be used to their full potential in the protection of cereal plants. The first attempts to elucidate the biosynthesis of Hx were limited to feeding experiments using exogenously radiolabelled compounds (*87*). Anthranilic acid was identified as one of the precursors of Hx. On the other hand, the interconversion of the lactams and Hx was demonstrated *in vivo*, but not in a cell-free extract, suggesting that the substrates for the interconversion are the glucosides. Two Hx UDP-glucosyltransferases isolated from etiolated maize coleoptiles have been partially purified and characterized (*88*). Later, this activity was also identified in rye, wheat and *Hordeum lechleri* (Stend.) Schenk., a wild barley containing Hx (*12*). The enzyme in rye was partially purified and characterized, presenting a molecular mass of 43 Kda, a pI of 4.4 and K_m for DIBOA and DIMBOA of 73 and 82 μM respectively, while the corresponding lactams were not substrates for the enzyme (Leighton, V., Niemeyer, H.M. and Jonsson, M.V.L., *Phytochemistry*, in press). A cytochrome P-450 dependent N-monooxygenase which catalyzes the N-hydroxylation of HBOA, the lactamic derivative of DIBOA, to form DIBOA has also been identified in maize microsomal fractions. The enzyme required NADPH and was inhibited by sulfhydryl reagents, NADP, cytochrome c, cations, carbon monoxide and nitrogen (*89*) and did not recognize the 7-methoxylated analogue HMBOA nor the corresponding 2-O-ß-glucosides as substrates (Leighton, V., Niemeyer, H.M. and Jonsson, M.V.L., *Phytochemistry*, in press).

A single gene homozygous recessive mutant (bxbx) of maize resulted in an 8- to 10-fold reduction in Hx content of the seedlings (*3*). This bx locus was localized in the short arm of chromosome 4 by monosomic and B-A translocation analyses (*90*). Bailey and Larson found that the N-hydroxylase activity in homozygous (bxbx) maize seedlings was reduced in one half respect to heterozygous (Bxbx) ones, indicating that the Bx gene does not represent the structural gene for the N-hydroxylase activity, and suggesting that Bx could be a regulatory gene (*89*).

Hx could provide substantial benefits in the control of pests, diseases and weeds of cereals. The economic losses associated with the possible yield penalty for producing higher levels of defence chemicals in an annual crop may be compensated for by a reduction in the input of agrochemicals (*91,92*).

The establishment of breeding programs aimed at increasing Hx levels in wheat seems justified. Recent research has focussed on screening a wide genetic range of cultivars and species of the genus *Triticum*, in a search for germplasm useful for breeding programs aimed at increasing Hx levels. These studies have shown that there is more than an order of magnitude of variation in concentrations of Hx and suggests that there is scope to enhance the level of resistance to fungi and arthropod pests in modern wheat cultivars (*9,13,14,47,93*).

Alternatively, if techniques of genetic engineering are chosen which involve the use of *Agrobacterium tumefaciens* as a vector, care should be exercised since DIMBOA shows adverse effects on populations of *A. tumefaciens* and on the induction of virulence genes in the presence of acetosyringone (*94*).

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Chapter 20

Fungal Antibiosis in Biocontrol of Plant Disease

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The implication of antibiotics and enzymes of fungal origin in biocontrol has been extensively investigated, but only in a few cases has their role been conclusively elucidated. Production of the antifungal epidithiadiketopiperazine metabolites chaetomin and gliotoxin by the soil-inhabiting biocontrol fungi, *Chaetomium globosum* and *Gliocladium virens*, respectively, has been shown to be of importance in biocontrol. Recently, the antifungal properties of purified chitinolytic and glucanolytic enzymes from the biocontrol fungi *Trichoderma harzianum* and *G. virens* have been described, and evidence has been provided that these cell wall-degrading enzymes may act synergistically with antibiotics. Recombinant DNA technology, allowing the construction of genetically modified biocontrol agents will be useful for evaluating the role of specific compounds in biocontrol and for creating improved biocontrol organisms. Potential applications of this approach in future biocontrol practice is discussed.

Biological control of plant pathogens has received increasing attention as a promising supplement or alternative to chemical control, particularly of seed- and soilborne pathogens (*1*). In order to improve application and efficacy of biocontrol, a detailed understanding of the mechanisms of biocontrol agents is needed (*2*). Such knowledge will lead to substantial progress in selection of superior strains and appropriate formulation of biocontrol organisms. The mechanisms of biological control are generally classified as competition, parasitism/predation, and antibiosis (*2*). These modes of action have been investigated in detail in numerous studies (*3-8*). Most results indicate that, although one of the mechanisms is usually predominant in a given system, these modes of action are not mutually exclusive and, as will be discussed below, may frequently act in concert.

This chapter will discuss the implication of fungal antibiosis in biocontrol of plant disease. Antibiosis here is defined as antagonism brought about by metabolites of fungal origin, lytic enzymes, volatiles, or other toxic compounds (*9*). Only in a few cases has the role of antibiosis in antagonism to plant pathogens been conclusively elucidated, mainly because of the lack of appropriate methodologies or because extrapolation from *in vitro* results to the natural system proved to be elusive (*4,10*).

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Recent methodologies such as recombinant DNA technology have provided new tools to evaluate the role of biocontrol mechanisms and have already greatly advanced our understanding on the relative importance of antibiosis in bacterial systems (*8,11*); they are now increasingly applied also to fungal agents.

The role of antibiosis in biocontrol has been the subject of review elsewhere (*12*). Therefore, rather than merely reviewing the topic the present article intends to focus particularly on specific cases where the role of fungal antibiosis in biological control has been resolved to a great extent. Successful research strategies that have led to a better understanding of biocontrol mechanisms will be discussed, and an outlook will be offered on future directions in this rapidly evolving area of research.

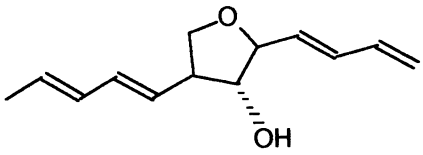
Fungal Antibiotics in Biocontrol

Antibiotics are generally defined as low molecular weight organic compounds produced by microbes, mostly soil inhabitors, that are inhibitory to the growth or other metabolic activities of other microorganisms at low concentrations. Antibiotics have been extensively exploited for pharmaceutical purposes. Several fungi used in biocontrol have been known for a long time to produce compounds with potent antibiotic activity. Particularly in the genera *Chaetomium* (*4,5,13,14*), *Gliocladium* (*6,15-18*), *Penicillium* (*19*), and *Trichoderma* (*20-25*), production of antifungal metabolites has been reported. The importance of these compounds in biocontrol of plant disease has long been questioned. However, recently substantial evidence has accumulated in certain fungal systems indicating that antibiotics play a fundamental role in biocontrol.

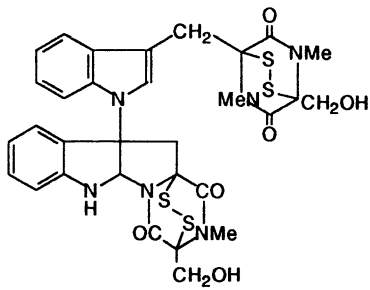
Some of the most potent antibiotics produced by biocontrol fungi belong to the class of the epidithiadiketopiperazines, including the metabolites chaetomin (*14*), gliotoxin (*26,27*), viridin (*28*), and gliovirin (*6*). These compounds show inhibitory activity against a wide range of microorganisms (*6,27-29*), and are particularly toxic to fungal pathogens belonging to the class of the Oomycetes (*5,6*). A disulfide group common to the epidithiadiketopiperazines is responsable for their antibiotic property (*29*). The two fungal biocontrol agents, *Chaetomium globosum* and *Gliocladium virens*, both of them producers of epidithiadiketopiperazine metabolites, provide excellent examples of cases where thorough studies of different laboratories have provided increasing and almost conclusive evidence for a crucial role of these antibiotics in biocontrol.

The saprophytic ascomycete, *C. globosum* is an effective antagonist of several soilborne and seedborne plant pathogens (*30-32*), and a role of antibiosis in its biocontrol activity has been suggested by several authors (*5,31-34*). The production of antibiotics by *C. globosum* is well documented. Different antifungal compounds from *C. globosum*, particularly chaetomin, were isolated from liquid cultures (*5,13,14*), pea seeds coated with ascospores (*34*), and apple leaves treated with an ascospore suspension of the fungus (*33*). In a recent study the role of antibiosis in biocontrol of the damping-off pathogen *Pythium ultimum* was investigated in detail (*5*). The authors found that different biocontrol isolates of *C. globosum* produced two compounds toxic to *P. ultimum* in liquid culture, chaetomin and 2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran (BHT). The composition of the culture medium determined which antibiotic was produced: chaetomin was only produced in corn steep medium, while BHT was produced in malt extract medium. The inhibitory activity of chaetomin against sporangial germination and mycelial growth of *P. ultimum* was 100 times higher than that of BHT, and was comparable to that of metalaxyl, a fungicide commercially used against oomycete plant pathogens (*5*). The authors found a strong correlation between chaetomin production and biocontrol ability of the *C. globosum* strains against *P. ultimum*. Furthermore, a spontaneous mutant that had lost the capacity for chaetomin production was isolated from an effective biocontrol strain. This mutant also was ineffective in controlling Pythium

damping-off of sugarbeet (*5*). Finally, chaetomin could be isolated from soil that had been inoculated with the parental strain effective in biocontrol, but not from soil inoculated with the ineffective biocontrol mutant (*5*). The results of this study strongly suggest an essential role of chaetomin production in soil by *C. globosum* in biocontrol of *P. ultimum*.



2-(buta-1,3-dienyl)-3-hydroxy-4-(penta-1,3-dienyl)-tetrahydrofuran (BHT)



chaetomin

However, while *C. globosum* is an active antibiotic producer in soil (*5*), this does not necessarily apply to other environments. A different study investigated biocontrol of *C. globosum* against the apple scab pathogen *Venturia inaequalis* on the phylloplane (*4*). The authors observed that up to 90% reduction in apple scab severity was observed consistently in growth chamber studies when ascospores of *C. globosum* were applied to apple leaves, apparently due to the presence of chaetomin on the phylloplane (*33*). However, in field trials only 0-25% disease reduction was achieved (*35*). A more detailed study by the same authors revealed that biocontrol in this system was merely due to the antibiotic compounds present in the ascospores of the antagonist, that diffused passively onto the leaf surface and inhibited infection by *V. inaequalis* (*4*). Lack of active metabolite production was confirmed by the fact that heat-killed ascospores that still contained chaetomin controlled scab as effectively as viable spores of the antagonist (*4*). The authors concluded that loss of biocontrol activity in the field was probably due to physical and chemical degradation of the antibiotic on the leaves. This example shows clearly that one has to be very cautious in extrapolating results obtained in a particular biocontrol system to similar systems, even if the same biocontrol agent is involved.

Another example where almost conclusive evidence for the role of antibiosis in biocontrol has been obtained, is provided by *G. virens*, one of the most promising and studied biocontrol agents that has been successfully used against different plant pathogens (*36-40*). Production of the antibiotic metabolites gliotoxin, gliovirin, and viridin by *G. virens* has been extensively reported (*6,15,16,18,25-28,38,41-43*), and has been described as the main biocontrol mechanism (*6,16,27,37,44*). Early reports on production of gliotoxin in soil (*18,42,43*), which have been confirmed recently (*16,41*), indicate that this metabolite plays a crucial role in biocontrol. Biocontrol effectiveness against damping-off of zinnia seedlings caused by *P. ultimum* and *Rhizoctonia solani* was strongly correlated with the amounts of gliotoxin detected in alginate prill substrate (*16*). Moreover, the importance of factors such as organic nutrient status, temperature, soil pH, and other chemical and physical soil characteristics for production and stability of the antibiotic in soil was clearly demonstrated (*16,18*).

Evidence for the role of gliovirin has been gained mostly from gliovirin-deficient and superproducing mutants of *G. virens* obtained by UV irradiation (*6*). The nonproducers did not control Pythium damping-off of cotton seedlings as well as the parental strain, while superproducers provided control similar to that of the parental, even though they grew more slowly. The authors concluded that gliovirin is an important factor in biocontrol of *P. ultimum* (*6*). This conclusion was confirmed later by the same authors. A study with seventeen *G. virens* strains revealed that disease-suppression efficacy against Pythium damping-off of cotton was closely associated with the capacity of the strains for gliovirin production (*38*). The whole body of evidence presented indicates that antibiosis is the main factor in biocontrol by *G. virens*.

Potential Nontarget Effects of Antibiotics

Antibiosis in biocontrol may be associated with nontarget effects, including harmful effects on plants, beneficial microflora, and humans. In particular antibiotic compounds may have undesirable side effects on nonfungal targets. Viridin, an antifungal metabolite produced by *Gliocladium virens* (*28*) is easily converted to viridiol (*45*) which is phytotoxic to crop plants (*46*). Ether-soluble fractions of culture filtrates of *Chaetomium cupreum* delayed germination of soybean seeds indicating the presence of a phytotoxic metabolite (*47*). Beneficial soil microorganisms also may be undesirable targets of compounds produced by biocontrol fungi. One of four *Rhizobium* spp. tested was sensitive to culture filtrates of *Gliocladium roseum* (*48*). On the other hand, no antagonism was observed between *G. virens* and plant symbiotic mycorrhizal fungi (*49*). Finally, the production of compounds toxic to mammals, particularly humans, may represent a severe drawback for registration and application of potential biocontrol agents. Several epidithiadiketopiperazine antibiotics including chaetomin and gliotoxin possess moderate toxicity to mammals (*13,29,50*). However, studies indicate that, under natural conditions, these compounds are rapidly inactivated by biological, chemical, or physical mechanisms (*4,51*). In a singular case, an indirect deleterious effect on ruminants caused by antibiotic-producing fungi has been reported. The antibiotic chaetomin from *Chaetomium globosum* (*13,50*) and certain isocyanide metabolites from *Trichoderma hamatum* (*52*) were shown to be toxic to symbiotic bacteria that digest cellulose in ruminants. Thus, these soil-inhabiting fungi have been implicated in ovine ill-thrift disease (*13,50,52*). This example shows that potential nontarget effects of compounds produced by biocontrol agents must be carefully evaluated before such organisms or substances are applied on large scale in agricultural practice.

Enzymes in Biocontrol

The role of enzymes in biocontrol can often be assigned to both mechanisms, parasitism and antibiosis. In particular cell wall degrading enzymes such as chitinases, ß-1,3-glucanases, and cellulases, are not only important features of mycoparasites for colonization of their host fungi (*53*), but also may exhibit considerable antifungal activity on their own. In order to evaluate the antifungal properties of cell wall degrading enzymes, they have to be available as isolated and purified proteins. This has recently been achieved for various chitinases and a ß-1,3-glucanase from the biocontrol fungi *Trichoderma harzianum* (*54-57*) and *Gliocladium virens* (*58*). The different enzymes were inhibitory *in vitro* to various plant pathogenic fungi, including *Botrytis cinerea*, *Fusarium solani*, *Ustilago avenae*, and *Uncinula necator* (*57-59*). The concentrations of enzyme required for fungal inhibition were considerably higher (factor 10 to 100, on a weight basis) than those usually observed for antibiotics (*57,58,60*). Nevertheless, the elevated quantities of enzymes produced by these fungi when grown on the appropriate substrates, and the fact that several types are usually

produced concurrently (*56,58,61*) suggest that they may play an important role in biological control.

Another example of the involvement of enzymes in biocontrol is provided by the biocontrol fungus *Talaromyces flavus* (anamorph *Penicillium dangeardii*) which has the capacity to suppress Verticillium wilt of eggplant under field conditions (*62*). The antagonist is known to parasitize plant pathogens, e.g. *Sclerotinia sclerotiorum* (*63*), but has not been observed to parasitize *Verticillium dahliae* (*64*). Instead, the fungus produces a compound able to kill microsclerotia of *V. dahliae* both *in vitro* and in soil (*65*). The antifungal activity was rapidly lost through separation procedures, but could be restored by recombining the fractions. This finding facilitated the identification of the critical compound as the enzyme glucose oxidase (*3,66*). The reaction of the enzyme with glucose produces peroxide which is toxic to microsclerotia of *V. dahliae*. However, the exact role of glucose oxidase in biocontrol and the question about the ability of its substrate to the antagonist under natural conditions still remain to be resolved (*12*).

Synergistic Interaction between Different Compounds in Biocontrol

As pointed out before, the strict separation of biocontrol mechanisms into antibiosis, hyperparasitism, and competition is purely theoretical (*2*). There is substantial evidence that the simultaneous occurrence of different modes of action under natural conditions is probably rather the rule than the exception. An excellent example for this point is again provided by the fungal biocontrol agent *Gliocladium virens*. As discussed above, production of fungitoxic metabolites, such as gliotoxin, has been proven to be the main mechanism of biocontrol in this fungus (*6,16,27,37,44*). Nevertheless, a recent study has provided new evidence for a subtle implication of cell-wall degrading enzymes in the process of biocontrol (*58*). An endochitinase isolated from *G. virens* not only possessed moderate *in vitro* antifungal properties (ED_{50} 125 $\mu g\ ml^{-1}$) but greatly enhanced the inhibitory effect of gliotoxin on the plant pathogen *Botrytis cinerea* (*58*). Thus, the ED_{50} of gliotoxin applied alone was 1.25 $\mu g\ ml^{-1}$, while it was only 0.5 $\mu g\ ml^{-1}$ when 50 $\mu g\ ml^{-1}$ endochitinase were applied simultaneously. The synergistic effect of the enzyme was probably due to partial cell wall degradation in the target organism which facilitated rapid diffusion of gliotoxin to its presumed action site on the cytoplasmic membrane (*44*). The authors concluded that synergistic interaction between lytic enzymes and fungitoxic metabolites may play a role in biological control (*58*). Similar antifungal synergisms have also been demonstrated between different chitinolytic and glucanolytic enzymes from *Trichoderma harzianum*, and between these enzymes and several antifungal compounds (*60*). All these data indicate that synergistic interactions between different biocontrol factors are occurring under natural conditions and play an important role in biological control.

Application of Molecular Genetic Technology to Antibiosis in Biocontrol

Molecular genetic technology is now applicable to a rapidly increasing number of economically interesting filamentous fungi (*67*). In biological control, these new techniques provide powerful tools for both studies on biocontrol mechanisms and molecular genetic improvement of potential biocontrol agents (*68,69*). The molecular genetic approach to studies on biocontrol mechanisms consists in cloning the genes encoding putative biocontrol factors; producing specifically deficient mutants of the antagonist by gene disruption; determining the loss or reduction of biocontrol ability of the mutants compared to their parental genotype; restoring ability to produce the factor by reintroducing the intact encoding sequence via transformation; and determining restored biocontrol ability in the transformants. This approach provides the most conclusive evidence at present available for the role of a particular factor in

biocontrol. In bacterial biocontrol systems, the molecular genetic approach has already led to complete elucidation of the role of antibiotics in biocontrol (*8,11*). Thus, the importance of the antibiotic phenazine-1-carboxylate from *Pseudomonas fluorescens* in biological control of take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* has been definitely confirmed (*8*). Mutants of *P. fluorescens* defective in phenazine synthesis were generated by transposon Tn5 insertion, and their ability to suppress take-all was compared to the parental strain. Six independent mutants provided significantly less control of take-all on wheat seedlings than the parental genotype (*8*). Antibiotic synthesis and suppression of take-all were coordinately restored in two mutants complemented with cloned DNA from a genomic library of the parental (*8*). These results indicate that the DNA sequences for phenazine production present in the bacterial genome are necessary for biocontrol function, and consequently support the importance of the antibiotic in suppression of take-all disease.

In principle, a similar approach is also feasible in fungal biocontrol systems. However, the lack of efficient transformation systems for filamentous fungi and the increased size and complexity of fungal genomes have hampered successful application of molecular genetic techniques for a long time. Also, antibiotic production in fungi is usually accomplished via complex metabolic pathways and the identification and cloning of genes involved is rather laborious. So far, only in the pharmacologically important genera *Aspergillus* and *Penicillium* there has been considerable progress in molecular genetic analysis of antibiotic production (*70*). Recently, successful genetic transformation of the biocontrol fungi *Trichoderma harzianum* and *Gliocladium virens* has been reported, using protoplasting (*71,72*) and biolistic techniques (*73*). The antagonist *G. virens* may be the first biocontrol fungus where molecular cloning and characterization of genes involved in antibiotic production will be successfully accomplished. Several polypeptides associated with *G. virens* strains that produce the antibiotic gliotoxin have recently been identified (*74*). Although the causal involvement of these proteins in gliotoxin synthesis remains to be demonstrated, their purification makes future cloning of the encoding genes feasible (*74*).

An example where fungal genes encoding putative biocontrol factors have already been isolated, is provided by the cell wall degrading enzymes of the biocontrol fungus *T. harzianum*. As mentioned earlier, different chitinases and a ß-1,3-glucanase have been recently isolated from this fungus (*56,57*) and have shown considerable antifungal activity (*57,59*). A cDNA sequence encoding for an endochitinase from *T. harzianum* has now been isolated and characterized (*75*). This sequence will be of use for creating specifically endochitinase-deficient mutants of *T. harzianum* via homologous gene disruption, in order to determine the exact role of the enzyme in biocontrol.

Besides studies on biocontrol mechanisms, molecular techniques can also be employed to create genetically improved strains of biocontrol agents (*69*). Protoplast fusion has been used to combine genetic traits desirable for biocontrol from different strains of *T. harzianum* (*68*). Fusion of protoplasts derived from two efficient biocontrol strains of *T. harzianum* resulted in the recovery of a progeny strain with greatly improved biocontrol ability (*76*). However, since the genetic events during protoplast fusion are still largely unknown (*68*), selection of improved strains is extremely laborious, and directed improvement is not possible with this technique. Directed transfer of fungal genetic sequences encoding for factors important in biocontrol will be possible as soon as such sequences are available. This has been achieved in the case of an endochitinase gene from *T. harzianum* mentioned earlier (*75*). Since the *T. harzianum* endochitinase exhibits antifungal activity (*59*), it is likely that transfer of the encoding gene will confer improved biocontrol ability to the receptor strain.

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Ultimately, genetic sequences encoding antifungal compounds produced by biocontrol agents can be directly transformed into plants where they are supposed to confer improved resistance to fungal pathogens. Transgenic tobacco plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani* have been produced which contain a chitinase gene derived from bean under the control of a constitutive promoter (*77*). Insertion of foreign biocontrol genes in plants is especially attractive for compounds that should have no undesirable side effects on humans or nontarget organisms, such as chitinolytic enzymes or inhibitors with specifically fungal targets. Technically, this approach is already feasible and will undoubtedly be followed by many laboratories in the next future. The results of these efforts will show if transgenic plants harbouring fungal biocontrol genes do indeed possess improved resistance to pathogens. In addition, the technical potential of this approach can only be exploited if public acceptance and legislation will allow its application in agricultural practice.

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Chapter 21

Weed Control with Mycoherbicides and Phytotoxins

A Nontraditional Application of Allelopathy

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Keys to the development of biologically-based agents such as mycoherbicides and phytotoxins as effective and practical components of weed management systems are the advancement of practical, reliable, cost-effective methods for their production, stabilization, formulation, and application. Some of the advantages of mycoherbicides over traditional chemical herbicides are their specificity for the target weed; absence of adverse effects on humans, wildlife or domestic animals; rapid degradation and absence of residues in surface or ground water, crops, soil or food chains. However, there are several intrinsic limitations which are common to nearly all biological agents which must be overcome before they will be widely acceptable for practical use.

This chapter will discuss recent developments and advancements in inoculum production, formulation, and application technology of mycoherbicides and phytotoxins which show promise as weed control agents. Commercially produced mycoherbicides will also be discussed.

The term 'allelopathy' coined by Molisch (*1*), originally described the biochemical interactions, both harmful and beneficial, which occur between microorganisms and plants. Its common usage now refers to detrimental effects of higher plants of one species (the donor) upon the germination, growth, or development of another species (the receptor) (*2*). Biologically-based weed control using plant pathogenic fungus called 'mycoherbicides' (*3*) or with secondary fungal metabolites called 'phytotoxins' may be considered forms of allelopathy which combine aspects of both the original definition and the modern concept, whereby the microorganism or its phytotoxin serves the role of the donor, while the target weed is the receptor.

Chemical herbicides play an indispensable role in averting crop losses, and are responsible for much of the increased crop productivity that has occurred since the

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end of World War II (*4*). However, the high costs involved in developing and registering chemical herbicides, and recent trends in environmental awareness concerning pesticides in general have prompted researchers to investigate alternative systems of weed control. For an alternative system to be acceptable, it must provide economical weed control at or near the same levels as that achieved with chemical herbicides, while at the same time not posing a threat to either the environment or to non-target organisms (*5*).

Plant pathogenic fungi are the most effective biologically-based alternatives to chemical herbicides that have been extensively evaluated. Because these fungi kill are applied to weeds in much the same manner as are chemical herbicides, they are commonly called 'mycoherbicides' (*3*).

In addition to the potential use of certain phytopathogenic fungi as mycoherbicides, some plant pathogenic fungi also produce chemical compounds known as phytotoxins that are responsible for some disease symptomatologies during pathogenesis. Similarly, some saprophytic fungi may also produce phytotoxic compounds under certain conditions. Both types of phytotoxins may have potential as microbially-based weed control agents, or mycoherbicides.

Production of Mycoherbicides

Infective Units. For practical and economic reasons, infective units, usually spores, of the candidate mycoherbicide must be produced in a timely and cost-effective manner. Although there are several types of spores, asexually produced spores, or conidia, are generally the easiest to produce under experimental conditions (*6*). Since spores provide the most common method for natural disease dispersal, they should, logically, serve as the best candidates as infective units of mycoherbicides.

Mycelial fragments may be used in place of spores for some fungi that do not produce spores or produce them only sparingly (*7,8,9*). Mycelial fragments are more difficult to quantitate, are less readily separated from the culture medium, and are less infective than spores (*9,10*). Additionally, the durability, longevity, and viability of mycelium are generally much lower than that of spores (*9*).

Selection of Culture Medium. For small-scale inoculum production where economics are not a primary concern, relatively expensive materials such as V-8 vegetable juice and agar culture have been used successfully to induce sporulation and obtain inoculum of several mycoherbicides (*11,12.13*). However, for mass-production of mycoherbicides on a larger scale, such as in pilot test studies and by industry, the candidate mycoherbicide must be produced as inexpensively as possible while the quality and quantity of the final product is maintained. Fermentation materials for scaled-up testing include crude agricultural products that are readily available at low costs and in unlimited quantities. Protein sources, such as soybean flour, corn steep liquor, distiller's solubles, brewer's yeast, autolyzed yeast, milk solids, cottonseed flour, linseed meal, corn protein, and a variety of fish

meal are some of the materials that may be used. The carbon sources that are commonly tested include cornstarch, corn flour, glucose, hydrolyzed-corn-derived materials, glycerol, and sucrose (*9*).

A growth medium with a balanced ratio of carbon and nitrogen generally produces vegetative (mycelial) growth. It may be necessary to alter this ratio or to amend the medium with other nutrients such as calcium, chelating agents, and various amino acids to induce sporulation (*14*).

Carbon sources that do not yield maximum vegetative growth may enhance sporulation. To optimize growth and sporulation, the carbon, nitrogen, and mineral levels may require precise balancing (*9*). In addition to the effect on growth and sporulation, the carbon:nitrogen ratio may also affect the viability, longevity, and virulence of the fungus. For example, the vegetative growth of *Fusarium solani* f. sp. *phaseoli* was increased by a high carbon:nitrogen ratio, while virulence of the fungus on *Phaseolus vulgaris* was decreased. Conversely, a low carbon:nitrogen growth medium resulted in decreased vegetative growth and increased virulence (*15*). Phillips, et al. (*16*) found the spore volume, nuclear number and virulence of *Botrytis cinerea* to hybrid rose *(Rosa hybrida*) increased linearly in response to increasing glucose concentrations. More recently, Jackson and Bothast discovered that conidia of *Colletotrichum truncatum* produced in a growth medium with a carbon:nitrogen ratio of 10:1 were more virulent against the weed hemp sesbania (*Sesbania exaltata*) than were conidia that were produced in media with 30:1 or 80:1 carbon:nitrogen ratios (*17*).

The inoculum density may also affect fungal sporulation. Slade et al. (*18*) found that high inoculum density of *Colletotrichum gloeosporioides* (2.5 x 10^6 spores per ml) caused formation slimy masses of conidia called "slime spots" on several commonly used growth media used at standard strength. Slime spots are associated with "microcyclic conidiation", where sporulation occurs directly after spore germination with little or no mycelial growth. Conversely, reduced inoculum concentrations or use of concentrated growth media dense, vegetative mycelial growth developed, and microcytic conidiation did not occur *(19)*.

Solid substrate fermentation. The technology of culturing fungi on solid substrates is not as well-developed in the West as it is in Japan (*6*). Several inherent problems associated with solid substrate fermentations include high labor costs, difficulties in maintaining sterility, lack of control on fermentation conditions, and difficulty in recovering spores from the substrates (*9*). These problems are responsible partly for the development of a highly developed submerged culture fermentation industry in the West (*6*). Solid substrate fermentation may offer the only method of spore production if the spores cannot be produced using liquid fermentation. Various cereal grains and vegetative residues have been used to produce simple, expensive inocula for a number of plant pathogenic fungi (*14*). Hildebrand and McCain (*20*) used wheat straw that was infested with *Fusarium oxysporum* f. sp. *cannabis*, to control marijuana (*Cannabis sativa*). Boyette and co-workers used oat seed which was infested with *F. solani* f. sp. *cucurbitae* to control Texas gourd (*Cucurbita texana*) (*12*). These types of bulky materials are difficult to sterilize, inoculate, and store until they are ready to

be used in the field. They are also difficult to apply using conventional equipment.

Combined solid substrate and submerged fermentation. Several mycoherbicides have been produced using combined solid and submerged fermentations techniques. Mycelium of *Alternaria macrospora* for controlling spurred anoda *(Anoda cristata)* was first mass-produced by culturing mycelium of the fungus for 48 hours in liquid fermentation. The mycelium was collected, blended, and mixed with 1000 g of vermiculite, spread into foil-lined pans, and exposed to either fluorescent light, or direct sunlight to induce sporulation after 24 hours. After air-drying, the mixture was sieved, packaged, and stored at 4 C. Each gram of culture medium produced a dry weight of approximately 4 g of spores. Spore yields were approximately 1 x 10^5 spores/g of dried mycelium (*21*). This procedure has also been used to produce inoculum of *Colletotrichum malvarum*, a mycoherbicide for prickly sida *(Sida spinosa)*, and *Fusarium lateritium*, a mycoherbicide for spurred anoda, prickly sida, and velvetleaf *(Abutilon theophrasti)* (*22*).

A modification of this technique was used to produce spores of *A. cassiae* for use as a mycoherbicide against sicklepod (23). Mycelia grown in submerged culture for 24 hours were collected, homogenized and poured onto foil-lined trays and subjected to 10 minutes of ultraviolet light treatment every 12 hours for 3 to 5 days to induce sporulation. The surface of the mycelia sporulated profusely while the mycelia dried. After 72 hours, the spores were collected by vacuum, dried over $CaSO_4$, and stored at 4 C. Approximately 8 g of spores were produced per liter of growth medium with this simple technique, with each gram of spores containing approximately 1 x 10^8 spores (*23*). Sufficient quantities of *A. cassiae* spores were produced using this technique to conduct field efficacy tests for a five state regional test, and for a two year pilot test study (*24*). This technique has also been used to produce spores of *A. crassa* for jimsonweed *(Datura stramonium)* control (*25*); *A. helianthi* for cocklebur *(Xanthium strumarium)* and wild sunflower *(Helianthus annuus)* control (*26*), and *Bipolaris sorjicola* for johnsongrass *(Sorghum halepense)* control (*27*).

Submerged culture fermentations. From both practical and economical standpoints, fungi that sporulate in liquid culture are favored over those that require additional steps to induce sporulation. This factor alone may prove to be the essential requirement for commercial development of a fungus as a mycoherbicide (*28,29*).

For early evaluations in small scale experiments, ample inoculum can usually be produced in shake flasks. However, with shake flasks it is difficult to control many of the parameters, such as pH, temperature, agitation, and aeration that may influence mycelial growth or sporulation. For larger quantities of inoculum and more precise control of growth parameters, laboratory-model fermenters are essential. Some models monitor and control several environmental factors such as temperature, agitation, dissolved oxygen and pH, all of which may affect growth and sporulation of the organism being cultured.

Slade and co-workers (*18*) developed a simple method to predict spore production of *Colletotrichum gloeosporioides* in liquid culture using microplate

assays of the fungus on various solid media. This system could possibly be used to provide an accurate, rapid, and inexpensive means to screen growth media for their spore production potential.

The commercially-produced mycoherbicides COLLEGO (Ecogen Inc., Longhorn, PA) and DeVine (Abbott Laboratories, North Chicago, IL) are both produced using submerged culture techniques. The formulated products of each will be discussed later.

Formulation and Application

Formulation is the blending of active ingredients such as fungal spores, with inert carriers, such as diluents and surfactants, in order to alter the physical characteristics to a more desirable form (*9*). This may include diluting to a common potency, enhancing stability and/or biological activity, improving mixing, sprayability, incorporation into granular matrices, and possibly integrating the mycoherbicide into a pest management system.

Colletotrichum gloeosporioides f. sp. *aeschynomene* (CGA) for northern jointvetch control was the first fungus to be evaluated as a mycoherbicide (*11*). In collaboration with researchers at the University of Arkansas and the U.S. Department of Agriculture, the Upjohn Company was able to mass-produce CGA, and market it under the trade name COLLEGO for use as a mycoherbicide to control northern jointvetch *(Aeschynomene virginica*) in Arkansas and Louisiana rice fields (*30*). The formulated material consists on an active component (dried CGA spores) and an inert rehydrating agent used for wetting the spores and plant surfaces, and to improve spore germination. These components are packaged separately, and are added to the desired volume of water immediately before application (*28,29*).

BioMal (Philom Bios, Saskatoon, Sask., Canada) contains the spores of fungus *Colletotrichum gloeosporioides* f. sp. *malvae*. The fungus is pathogenic to round-leaved mallow *(Malva pusilla*). A commercial formulation of BioMal using a silica gel carrier has routinely provided over 90% control of this weed in the field. The wettable powder formulation of this hydrophilic fungus disperses easily in water and is applied as a spray to its target weed (*31,32*).

Phytophthora palmivora was the first fungus to be marketed as a mycoherbicide. The fungus infects and kills stranglervine *(Morrenia odorata*), a problem weed in Florida citrus groves. For early field evaluations, highly infective chlamydospores were produced in V-8 juice medium contained in shaken flasks (*33*). Abbott Laboratories, in cooperation with Florida researchers, developed *P. palmivora* as a mycoherbicide marketed under the trade name DeVine. The formulation is not highly stable with a shelf life of only about 6 weeks and must be handled much like fresh milk (*34,35*). However, the marketing area is small enough to make refrigerated distribution and custom-order sales possible (*35,36*).

Abbott Laboratories also developed an experimental formulation of *Cercospora rodmanii* for controlling water hyacinth *(Eichhornia crassipes*) in Florida waterways. The experimental formulation, called ABG-5003, consisted of mycelial

fragments and spores and was applied as a wettable powder formulation (*8,37*). Although biological control of the weed was achieved, the efficacy was less than is required for commercialization, largely because of the restrictive environmental requirements of the fungus (*8,38*). More recently, Charudattan demonstrated that control of water hyacinth by *C. rodmanii* can be enhanced by using it in combination sublethal doses of various chemical herbicides, such as 2,4 dichlorophenoxy acetic acid, or with certain insects such as *Arzama densa (39)*.

The method of production of mycoherbicides may determine the method of application. Mycoherbicides are applied much in the same manner as are chemical herbicides, and often with the same equipment. Tanks and lines on the spraying system must be clear of chemical residues in spray tanks may be detrimental to a mycoherbicide. A slurry of activated charcoal and liquid detergent can be used for this cleansing (*10*). Similarly, pesticides, especially fungicides, applied to mycoherbicide-treated areas may reduce effectiveness of the mycoherbicide. For example, the fungicide benomyl and the herbicide propiconazol applied sequentially 7 and 14 days after COLLEGO was applied, suppressed disease development on northern jointvetch (*40*). Similarly, the efficacy of DeVine was reduced if the fungicides Aliette and Ridomil were used within 45 days following application of the mycoherbicide (*36*).

During initial evaluations, spray volumes and inoculum concentrations are very high, usually about 900 to 1000 L/ha with 10^5 to 10^7 spores/ml. These volumes and rates can generally be reduced to more practical levels following experiments to determine threshold levels. Recent work has shown that the volume required to control hemp sesbania with *C. truncatum* can be reduced to less than 5 L/ha, using a corn oil emulsion (Boyette, unpublished).

Although the simplest mycoherbicide delivery system is the fungus suspended in and sprayed in water, many weeds are covered with a waxy cuticle that prevents water from spreading evenly. This prevents an equal distribution of the mycoherbicides. Surfactants aid in wetting the plants, and also aid in dispersing the fungal spores throughout the spray mix. Because spores of mycoherbicides are finite units, it is of paramount importance that the surface area be covered with the material as evenly and equally as possible. A number of surfactants have been used in mycoherbicide research. Since some surfactants may be detrimental to the growth and/or germination of fungi, preliminary experiments should be conducted to determine the effect of the surfactant on the candidate mycoherbicide. For example, *Alternaria cassiae* spores do not germinate consistently in either Tween-20 or Tween 80 surfactants but readily germinate in .02% to .04% non-ionic nonoxynol surfactant. The spray coverage of plants is increased resulting in enhanced control of sicklepod (*23*). Some of the mycoherbicides applied in liquid based formulations are listed in Table I.

Granular formulations. With some exceptions, liquid formulations of mycoherbicides are best suited for use as post-emergence sprays, and are used primarily to incite leaf and stem diseases. Conversely, pathogens that infect at or below the soil surface are probably delivered best in a solid, or granular formulation. Granular formulations often are better suited for use as preplant or

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Table 1. Liquid-Based Mycoherbicide Formulations

Weed host	*Pathogen*	*Formulation*	*Reference*
Velvetleaf (*Abutilon theophrasti*)	*Fusarium lateritium*	Water + Tween-20 surfactant (0.02%)	(*22*)
	Colletotrichum coccodes	Water + sorbitol (0.75%)	(*41*)
		Experimental formulation; water	(*11*)
Northern jointvetch (*Aeschynomene virginica*)	*Colletotrichum gloeosporioides aeschynomene*	Commercial formulation; component A:dried spores component B:rehydrating agent + surfactant	(*28,29*)
Spurred Anoda (*Anoda cristata*)	*Alternaria macrospora*	Water + nonoxynol surfactant (0.02%) sucrose (5% w/v)	(*21*)
Giant Ragweed (*Ambrosia trifida*	*Protomyces gravidus*	Water	(*42*)
Field Bindweed (*Convolvulus arvensis*	*Phomopsis convolvulus*	Water + gelatin (0.1%)	(*43*)
Jimsonweed (*Datura stramonium*)	*Alternaria crassa*	Water + nonoxynol surfactant (0.04%)	(*25*)
Florida beggarweed (*Desmonium tortuosum*)	*Colletotrichum truncatum*	Water + nonoxynol	

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Table I. Continued.

Weed host	*Pathogen*	*Formulation*	*Reference*
Sicklepod *(Cassia occidentalis)*	*Alternaria cassiae*	surfactant (0.04%); paraffin wax mineral oil, soybean oil, corn syrup, lecithin	(*23*)
Common purslane *(Portulaca oleracea)*	*Dichotomophthora portulacaceae*	Water + Tween-20 surfactant (0.02%)	(*44*)
Horse Purslane *(Trianthema portulacastrum)*	*Gibbago trianthemae*	Water + Tween 20 surfactant (0.02%)	(*45*)
Hemp sesbania *(Sesbania exaltata)*	*Colletotrichum truncatum*	Water + nonoxynol surfactant (.20%; paraffin *wax; mineral oil, soybean oil* lecithin; unrefined corn oil	(*46*)
Eastern Black Nightshade *(Solanum ptycanthum)*	*Colletotrichum coccodes*	Water + Tween 80 surfactant (0.02%)	(*47*)
Stranglervine *(Morrenia ordorata)*	*Phytopthora palmivora*	Commercial formulation; chlamydospores in water	(*36*)

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preemergence mycoherbicides than are spray formulations because: 1) the granules provide a buffer from environmental extremes; 2) the granules can serve as a food-base for the fungus, resulting in a longer period of persistence; 3) the granules are less likely to be washed away from the treated areas than are spores.

A cornmeal-sand formulation of *Fusarium solani* f. sp. *cucurbitae* was used to produce mycelium and a mixture of microconidia, macroconidia, and chlamydospores (*12*). The ratio of these spore types can be altered by addition of various nutrients to the basal medium (*48*). Almost complete control (96% overall avg) of Texas gourd was achieved with preplant and preemergence applications with granular formulations of this fungus (*12,49*).

Another solid substrate which has been effectively used is Vermiculite. Walker (*21, 50*) grew mycelium of *Alternaria macrospora* in liquid shake culture and mixed the mycelium with vermiculite. The fungus sporulated profusely in the mixture and after air-drying was applied both preemergence and postemergence to spurred anoda providing 75-95% weed control. The control achieved preemergence with the fungus-infested vermiculite formulation was as good as the control achieved postemergence with foliar sprays of *A. macrospora*.

Granular formulations of several biocontrol fungi have also been made using sodium alginate (*51,52)* a method adapted from work with time-released herbicide formulations (*53*). In this method, fungal mycelium is mixed with sodium alginate and various fillers, such as kaolin clay, and dripped into 0.25 M $CaCl_2$. The Ca^{++}ions react immediately with the sodium alginate to form gel beads. The beads are allowed to harden in the $CaCl_2$ solution for a few minutes, and can then be collected, rinsed, and air-dried. The granules are of fairly uniform size and shape, and can then be used in a manner similar to preplant or preemergence herbicides, or rehydrated and exposed to UV light to induce the fungus to produce spores, which can then be collected and used as post-emergence sprays.

A pasta-like process was used to produce granules of several different mycoherbicidal fungi, such as *C. truncatum* for hemp sesbania control; *F. lateritium* for velvetleaf control; and *F. oxysporum* for sicklepod control. The granules are produced by mixing semolina wheat flour and kaolin clay with fungal propagules contained in a liquid component (either water or residual liquid growth medium). The mixture is kneaded into dough, rolled into thin sheets with a pasta-press, and air-dried for 48 h. The sheets are then milled and sieved to obtain uniformly-sized granules, and stored 4 C. These granules, called 'PESTA' controlled weeds 90-to-100% in greenhouse tests (*13,54)*. In field tests, 'PESTA' granules containing *C. truncatum* have controlled 80-85% of hemp sesbania over a 3-yr testing period (*55*). Table II lists some mycoherbicides that have been produced in solid substrate formulations.

Table II. Solid-Based Mycoherbicide Formulations

Host Weed	*Pathogen*	*Formulation*	*Reference*
Velvetleaf	*Fusarium lateritium*	Sodium alginate-kaolin granules	(*22*)
Spurred anoda	*Alternaria macrospora*	Vermiculite	(*50*)
Texas gourd *(Cucurbita texana)*	*Fusarium solani cucurbitae*	Fungus-infested oats; Cornmeal/sand; sodlium alginate-kaolin granules	(*12*)
Marijuana *(Cannabis sativa)*	*Fusarium oxysporum* var. *cannabis*	Fungus-infested Wheat straw	(*20*)
Hemp sesbania	*Colletotrichum truncatum*	*Fungus-infested wheat* gluten/kaolin clay (PESTA)	(*54,55*)
Sicklepod	*Fusarium oxysporum*	Fungus-infested wheat-gluten/kaolin clay (PESTA)	(*54,55*)

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Formulations That Improve Mycoherbicide Efficacy Or Overcome Constraints

Various adjuvants and amendments have been used to either improve or modify spore germination, pathogen virulence, environmental requirements, or host preference, all of which greatly influence the bioherbicidal potential of a candidate microorganism.

The addition of sucrose to aqueous suspensions of *A. macrospora* resulted in increased disease severity on spurred anoda (*21*). Also, increased spore germination and disease severity of Florida beggarweed *(Desmodium tortuosum)* anthracnose was reported when small quantities of sucrose and gum xanthan were added to aqueous spore suspensions of *Colletotrichum truncatum (56*).

Disease severity on johnsongrass infected by *Bipolaris sorjicola* was significantly increased by adding 1% Soy-Dex to the fungus spray mix (*57*). Similarly, when sorbitol was added to spray mixes of *C. coccodes*, there was a 20-fold increase of viable spores re-isolated from inoculated velvetleaf. When this amendment was used, three 9 h dew periods on consecutive nights were as effective as a single 18 h dew treatment (*58*).

Most pathogens being evaluated as mycoherbicides require free moisture (usually dew) in order for germination, penetration, infection, and kill of the target weeds to occur. This period of time ranges from 6 hours to more than 24 hours, depending upon the pathogen and the weed host (Table III). Recent work has indicated that invert (water-in-oil) emulsions provide a potential method to retard evaporation and trap water in the spray mixture thereby decreasing the amount of free moisture required for spore germination, and infection to occur (*59-61*). In these studies lecithin was used as an emulsifying agent, and paraffin oil and wax were used to further retard evaporation and help retain droplet size. Specialized spraying equipment was developed to deliver this viscous material (*60,62*). Greenhouse and field results indicated that excellent control (>95%) of sicklepod with *Alternaria cassiae* could be achieved with little or no dew (*59*). Recently, this system was also used to enhance control of hemp sesbania in the field with *Colletotrichum truncatum*. The control (95%) achieved was comparable to the control (96%) that occurred in plots treated with acifluorfen. Less than 10% control of hemp sesbania occurred in plots treated with the fungus alone (*46*).

Most mycoherbicides have a limited host range. From a standpoint of safety and registration for use this is beneficial. However, from an economic standpoint, this could have a negative effect on the practical use of a candidate mycoherbicide since only one weed species rarely predominates in row crop situations (*4*). The simplest way to overcome this limitation is by applying mixtures of pathogens to mixed weed populations. For example, two rice weeds, northern jointvetch and winged waterprimrose *(Jussiaea decurrens*), were simultaneously controlled with a single application of CGA and *C. gloeosporioides* f. sp. *jussiaea* (*69*). A mixture of these two pathogens along with *C. malvarum* also effectively controlled northern jointvetch, winged waterprimrose, and prickly sida (*57*).

It may also be possible to alter the host selectivity of some mycoherbicides through formulation. Recently, it was discovered that the host selectivity of *Alternaria crassa*, a mycoherbicide for jimsonweed can be altered by the addition of water-soluble filtrates of jimsonweed or dilute fruit pectin spore suspensions. Several plant species

Table III. Dew Requirements of Selected Mycoherbicides

Pathogen	*Weed Host*	*Dew (h) Required for Maximal Weed Kill*	*Ref.*
Alternaria macrospora	Spurred Anoda	12	(*21*)
A. cassiae	Sicklepod	8	(*23*)
A. crassa	Jimsonweed	10	(*63*)
Colletotrichum coccodes	Velvetleaf	20	(*41*)
Colletotrichum gloeosporioides f. sp. *aeschynomene*	Northern jointvetch	12	(*64*)
C. gloeosporioides f. sp. *jussiaea*	Wing waterprimrose *(Jussiae decurrens)*	12	(*65*)
C. malvarum	Prickly sida *(Sida spinosa)*	18	(*66*)
C. truncatum f. sp. *desmodium*	Florida beggarweed *(Desmodium tortuosum)*	18	(*56*)
C. truncatum	Hemp sesbania	6	(*46*)
C. orbiculare	Bathurst burr *(Xanthium spinosum)*	24	(*67*)
Fusarium lateritium	Prickly sida; Velvetleaf	12 16	(*22*)
Phomopsis convolvulus	Field bindweed *(Convolvulus arvensis)*	*>24*	(*43*)

that were either resistant, or exhibited a hypersensitive reaction to the fungus alone, exhibited various degrees of susceptibility following these amendments. Among the important weed species that were highly susceptible to infection following addition of these amendments were hemp sesbania, eastern black nightshade *(Solanum ptycanthum)* cocklebur and showy crotalaria *(Crotalaria spectabilis)*. Several solanaceous crop species, including tomato (*Lycopersicon esculentum*), eggplant (*Solanum melonegra*), potato (*S. tuberosum*) and tobacco *(Nicotiana tabacum)* also became susceptible to infection because of the amendments. With proper timing of application, it is possible that the amendments be used in a practical method to enhance the weed control spectrum of *A. crassa (70)*.

Amsellen and coworkers (*71*) found that the host specificities of *A. cassiae* and *A. crassa* were greatly expanded, and that a saprophytic *Cephalosporium* species became pathogenic when these fungi were formulated in an invert emulsion. Similarly, the host ranges of *C. truncatum* and *C. gloeosporioides* f. sp. *aeschynomene* (COLLEGO) were also expanded when spores of either pathogen were formulated in an invert emulsion. Hemp sesbania was immune both to inundative and wound-inoculations of COLLEGO when conidia were formulated in water only. However, when conidia were formulated in the invert emulsion, hemp sesbania became highly susceptible to the fungus. A similar response occurred with *C. truncatum*. Aqueous inundative or wound-inoculations with aqueous spore suspensions of *C. truncatum* had no effect on northern jointvetch, but susceptibility to infection was induced when the fungus was formulated in the invert emulsion. In rice field plots, over 90% of hemp sesbania plants were controlled by the COLLEGO/invert treatments, while COLLEGO alone had no effect upon hemp sesbania (*72,73*).

Disease severity of several plant pathogens are known to increase in response to various chemical herbicides (*74*). The addition of sublethal rates of the herbicides linuron, imazaquin, and lactofen to *A. cassiae* spores in an invert formulation resulted in significantly increased control of sicklepod (*75*). Control of velvetleaf was significantly improved by sequential applications of the herbicide 2,4 DB and spores of *F. lateritium*. However, fungal germination and disease severity were greatly reduced when the fungus and herbicide were tank mixed (*76*). Biocontrol of velvetleaf was also improved significantly by adding field rates of the cotton defoliant thiadiazuron to an aqueous spray mixture of *C. coccodes (41)*.

The rust fungus, *Puccinia canaliculata*, is erratic in infecting and controlling its host weed yellow nutsedge *(Cyperus esculentus)* when uredospores are applied alone even under optimal environmental conditions (*77*). However, sequential applications of the herbicide paraquat, followed by *P. canaliculata* spores resulted in a synergistic disease interaction, with almost complete control of yellow nutsedge occurring, compared to only 10% and 60% control, respectively, for paraquat, or the fungus alone (*78,79*).

Khodayari and others (*80*) demonstrated that the weed control spectrum of CGA can be extended by mixing with aciflourfen an excellent herbicide control for hemp sesbania, but is ineffective in controlling northern jointvetch. In these tests, both weeds were effectively controlled in soybeans by a single application of the mixture, providing the microenvironment was favorable for infection to occur on northern jointvetch.

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The Role of Toxins in Fungal Pathogens

Fungi also produce natural products that are responsible for phytotoxicity. In these cases, symptoms produced by the fungus and its phytotoxin alone are identical. Weeds can be controlled by using either the fungus or the phytotoxin. An example of this that we have studied is *Alternaria alternata* and its phytotoxin, AAL-toxin. *A. alternata* f. sp. *lycopersici* was initially described as a pathogen of susceptible tomato varieties. These varieties have the recessive genotype *asc/asc*. The toxin responsible for the pathogenesis was discovered to be AAL-toxin (Figure 1), which was also thought to be exclusively phytotoxic to *asc/asc* tomatoes (*81,82,83*).

A. alternata itself has not been found to be pathogenic to other weed and crop species (*Abbas, unpublished*). However, AAL-toxin has been shown to have a broad host range including jimsonweed, black nightshade, prickly sida (*84*), duckweed (*Lemna minor*) (*85,86*), and hemp sesbania (*87*). Several important crops are not significantly affected by AAL-toxin including cotton (*Gossypium hirsutum*), corn (*Zea mays*), and soybean (*Glycine max*) (*Abbas, unpublished*). AAL-toxin has been patented for use as a herbicide (*88*).

Destruxin B (Figure 2) appears to be responsible for the effects of *Alternaria brassicae*, the causal organism of blackspot disease of oil rape (*Brassica napus*) (*89-91*). *Alternaria alternata* f. sp. *tenuis* was originally isolated as a weak pathogen of cotton (*92*). It produces tentoxin (Figure 3), a cyclic tetrapeptide that is a potent, chlorosis-producing compound. When used in greenhouse experiments it has shown some promise as a pre-emergence, soil-incorporated herbicide against ivyleaf morningglory (*Ipomoea hederacea*), johnsongrass, sicklepod, and cocklebur (*87, 93-96*).

As described above, many phytotoxins isolated from fungi such as AAL-toxin and tentoxin have effects on other weed species than the ones from which they were isolated. Also, non-pathogenic species of fungi may produce phytotoxins when grown under certain conditions that are effective as weed control agents.

An example of this is fumonisin B_1 (FB_1) (Figure 4) that is produced by *Fusarium moniliforme* when fermented on rice. *F. moniliforme* is a saprophytic fungus isolated from jimsonweed. Although no symptoms are produced on jimsonweed by *F. moniliforme*, FB_1 is phytotoxic to jimsonweed and other weeds including black nightshade, prickly sida, and hemp sesbania. FB_1 is closely related chemically to AAL-toxin and has a similar host range (*84*). Use of FB_1 as a herbicide is limited by its mammalian toxicity (*97,98*).

Fusarium solani, a saprophyte isolated from sweet potato (*Ipomoea batatas*), produces a toxin or toxins that are phytotoxic to velvetleaf, sicklepod and various species of morningglories when grown on rice (*99*). The toxin(s) have not yet been identified but preliminary investigation has shown that none of the common phytotoxins were present (*100*).

Fungi may be pathogenic as well as produce phytotoxins. This is demonstrated by *Fusarium oxysporum* Schlect. emend Synd. and Hans. This fungus was isolated from diseased sicklepod plants and conidia infected sicklepod, coffee senna, and hemp sesbania (*13*). When *F. oxysporum* was fermented on rice, moniliformin (Figure 5) was produced. Symptoms produced by moniliformin on jimsonweed were nearly identical to those produced by *F. oxysporum* when grown on rice, and dissimilar to the symptoms caused by fungal spores (*Abbas, unpublished*). It is unlikely that the fungus is pathogenic by its production of moniliformin *in vivo*.

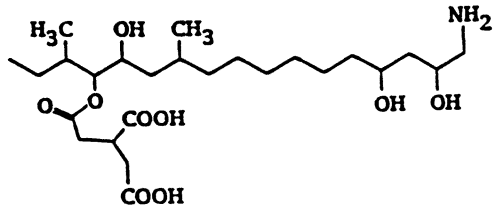

Figure 1. AAL Toxin.

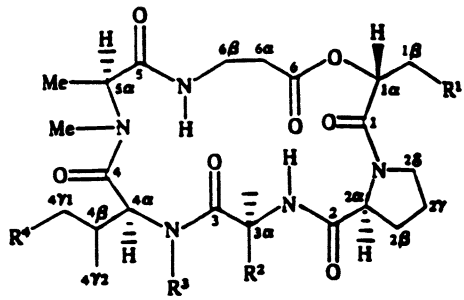

Figure 2. Destruxin B. (R^1 = $CHMe_2$; R^2 = CH(Me)Et; R^3 = Me; R^4 = H).

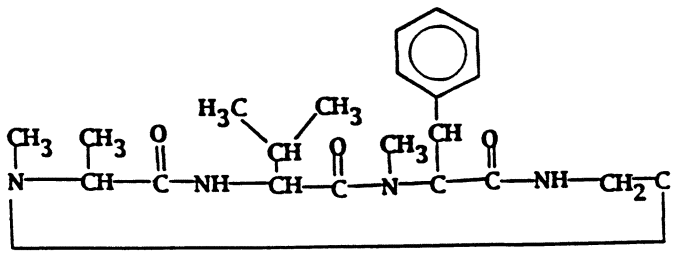

Figure 3. Tentoxin.

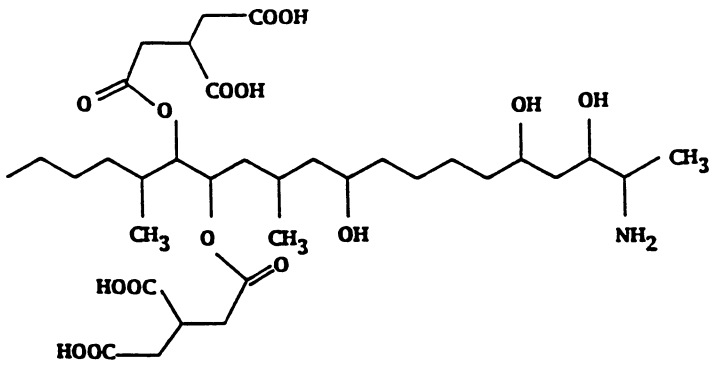

Figure 4. Fumonisin B_1.

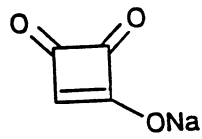

Figure 5. Moniliformin.

Conclusion

This review demonstrates that fungi have an expanding role in weed control. Fungi have been used directly by inoculation of target weeds with spores and mycelia or indirectly by production of secondary metabolites. Both methods can be enhanced by formulation in order to modify host ranges and to overcome environmental restrictions. Phytotoxins may avoid some of the environmental problems associated with living organisms, and there is less chance of environmental spread. However, some of the toxins are themselves potentially hazardous. In addition, because phytotoxins are chemicals, registration of these compounds would not be as relatively easy as registration of living organisms such as fungi. Much work remains to be done in the use of fungi for weed control. Only a few products are currently commercially available. It is likely with further refinement of techniques and closer cooperation among plant pathologists, weed scientists, formulation chemists, and agricultural engineers, this field will provide fertile sources of alternative methods to weed control.

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Chapter 22

Biological Control of Plant Pathogens by Antibiotic-Producing Bacteria

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Natural biological control phenomena are very complex systems consisting of integrated biotic and abiotic factors which are influenced by seasonal changes. Mechanisms such as competition, hyperparasitism, cross protection and antibiosis, may explain the pathways of the phenomenon of biological control. Antibiosis occurs when compounds produced by certain microorganisms have harmful effects on others. Selection for antibiotic producing bacteria begins with *in vitro* tests but ought to be followed by *in vivo* experiments. Purification, identification and application of the antibiotic as compared to the application of the antagonist itself, reveals the importance of antibiosis as part of the whole antagonistic phenomenon. Isolates of biocontrol agents which lack the gene for the antibiotic and are less effective against their target pathogens, strengthen the significance of antibiosis. Unfortunately, the correlation between results of *in vitro* tests and of field experiments is very small. Bacteria are less resistant than other biocontrol agents (yeasts, filamentous fungi) to adverse environmental conditions such as humidity and UV radiation.

Antibiosis and allelopathy consist of biochemical warfare between organisms. Allelopathy relates to the production and release of phytotoxic metabolites by plants (*1*) and was regarded as biochemical interactions among various types of plants (*2*). The term antibiosis applies to the production and release of such metabolites by microorganisms. In other studies, allelopathy and antibiosis are considered as synonyms (*3, 4*). The potential of this phenomenon, as part of biological control, can be used in agriculture to minimize the presence of pathogens or undesirable weeds. Cook and Baker (*5*) defined biological control as "the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man".

A few modes of action may explain the way biological control occurs. Competition takes place when two populations simultaneously share the same environment and have similar trophic requirements (*6, 7*). Nutrition, space, oxygen, water and iron are the main objects for competition when limited. Hyperparasitism is

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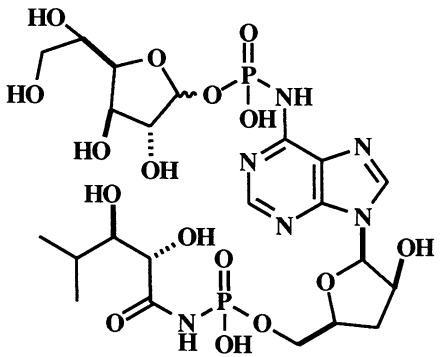
defined as a direct attack on a pathogen by a parasite (in anthropomorphic terms - "a biocontrol agent"). One of the attack strategies of hyperparasites is the use of extracellular lytic enzymes such as proteases, pectases, lipases, chitinases, glucanases and glucose oxidase (*8-14*). Kranz (*15*) indicates that hyperparasites can be efficient as biocontrol agents in diseases with high damage thresholds and in incipient diseases with slow progress (e.g.: slow rusting diseases). Cross protection and induced resistance are also potential strategies to achieve biological control. In these cases the host's resistance towards pathogens is enhanced by exposure to the biocontrol agent (*16, 17*).

Since bacterial antibiosis and allelopathy appear to be two sides of the same coin, a few antibiotics of microbial origin will be compared to allelochemicals produced by plants.

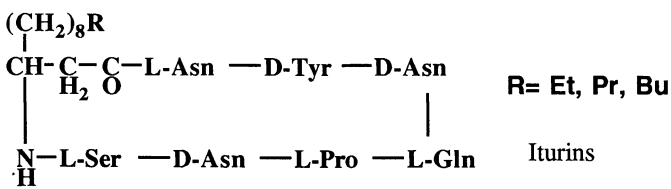
Bacterial Antibiotics

To prove the role of antibiosis in any biocontrol scenario, one has to isolate, identify and demonstrate by bioassays that inhibitory compounds are produced by one organism and inhibit another. The production of inhibitory compounds *in vitro* is known to be influenced by culture conditions such as medium composition, aerobic or anaerobic conditions, light, pH, etc., while natural conditions are subject to wide variations.



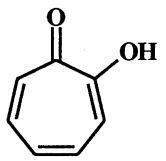
Agrocin 84

Despite numerous R & D efforts to develop biological materials for the control of plant diseases, only a few reached the stage of commercial application. One of the most successful applications was achieved by the use of *Agrobacterium radiobacter* pv. *radiobacter* strain K84 (producer of the bacteriocin, agrocin 84), against *A. radiobacter* pv. *tumefaciens*, the cause of crown-gall disease (*18, 19*). Application of a mutant strain which was unable to produce agrocin 84 and still reduced disease symptoms indicated that, possibly, competition with the pathogen on the host receptor site was also important (*20*). A wide range of antibacterial and antifungal activities has been found among *Bacillus* spp. (*21-25*). The most investigated is *Bacillus subtilis*, the producer of the antibiotic, iturin (*26-29*).

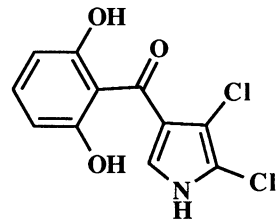


Iturins

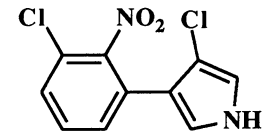
Fluorescent pseudomonads (FPs) are considered to be the most promising biocontrol agents among bacteria. FPs are known for their ability to produce a wide spectrum of inhibitory compounds (*30*). The antibiotics tropolone, pyoluterin and pyrrolnitrin isolated from *Pseudomonas* spp. were found to be effective against phytopathogenic fungi (*31-33*). Phenazine derivatives are secreted by different bacteria, but most investigations were performed with pseudomonads (*30, 34*).



Tropolone

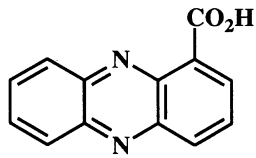


Pyoluteorin

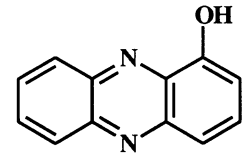


Pyrrolnitrin

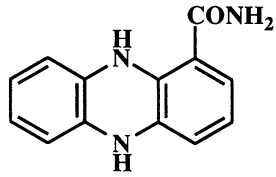
Pseudomonas fluorescens, strain 2-79, was found to suppress the take-all disease of cereals caused by the fungus *Gaeumannomyces graminis*. Its suppressive activity was attributed to the ability to produce phenazine-1-carboxylic acid, which also inhibits, *in vitro*, the growth of several other fungi and bacteria (*35, 36*). Mutants defective in phenazine synthesis, derived from the same strain of *P. fluorescens*, were less active against *G. graminis* (*37*). *Pseudomonas aeruginosa,* strain LEC 1, a suppressor of fungal leaf diseases (caused by *Septoria tritici* and *Puccinia recondita*) is a producer of two phenazine derivatives: 1-hydroxyphenazine and chlororaphin (*38, 39*). Oxychlororaphine and 1-hydroxyphenazine secreted from *P. aeruginosa* showed strong activity against green microalgae and cyanobacteria (*40*).



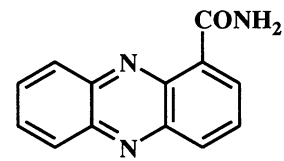
Phenazine-1-carboxylic acid



1-Hydroxyphenazine



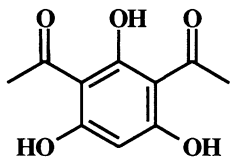
Chlororaphin



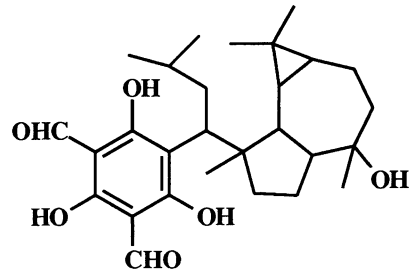
Oxychlororaphin

Other effective antibiotics against phytopathogenic bacteria and fungi are phloroglucinol derivatives. Currently there is an increased interest in the compound

2,4-diacetylphloroglucinol (Phl) which shows great promise as an antibiotic (*41*). While Reddy and Borovkov (*42*) reported that Phl exhibits strong antibiotic activity against gram-positive bacteria and actinomycetes and weak activity against gram-negative bacteria, fungi and yeasts, Garagulya et al. (*43*) found that Phl was the best antifungal substance among ten tested pseudomonads metabolites. Levy et al. (*44*) demonstrated antibiotic activity of Phl against both bacteria and fungi.



2,4-Diacetyl phloroglucinol

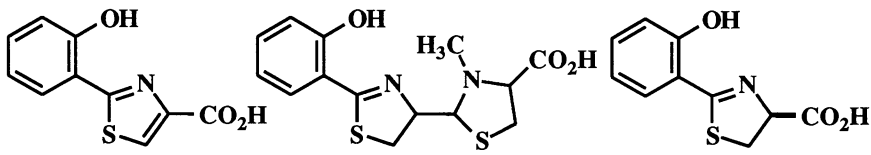


Macrocarpal A

Phloroglucinol derivatives found in plants were reported as allelochemicals. They were isolated from *Sedum sediforme* flowers (*45*), from the ferns *Dryopteris ardechensis* and *D. corleyi* (*46*), and from the wood of *Garcinia subelliptica* (*47*). Recently, it was found that the phloroglucinol derivatives, macrocarpals, isolated from *Eucalyptus macrocarpa*, inhibit aldose reductase, a key enzyme of the polyol pathway (*48*). Bacterial biodegradation of phloroglucinol was detected in a species of *Rhodococcus*, grown on phloroglucinol as the sole source of carbon and energy. Extracts of cells grown in such conditions showed phloroglucinol hydroxylase activity (*49*). The ability to detoxify and utilize phloroglucinol is surely an advantage for this bacterium in environments where phloroglucinol derivatives are common biochemical weapons.

In environmental conditions of iron limitation, certain organisms may produce organic compounds characterized by high affinity for Fe^{+3}. These compounds, named siderophores, enable producers to obtain iron more efficiently. In such situations, iron becomes more limited and may inhibit the growth of other microorganisms, including plant pathogens (*50, 51*). The antagonistic effect of siderophores is lost when iron is added to the medium. The antifungal activity of 275 strains of pseudomonads was studied by using media devoid of iron or supplemented with 10 μg/mL of $FeCl_3$ (*52*). Thirty three percent of the cultures showed lower activity against phytopathogenic fungi in the presence of iron. Addition of iron to phenazine and phloroglucinol derivatives (isolated from pseudomonad cultures) produced colored complexes but did not affect the antifungal activity of the later compounds. This observation might be the result of a weak bonding between iron and these compounds or of an improper stoichiometric ratio between iron and the chelating molecules, which may result in an excess of iron in the medium. However, addition of iron markedly lowered the antifungal effect of some crude, unidentified antibiotics and fluorescent pigments (*52*). This might be explained as hindering of the siderophoric effects of these compounds. Three siderophores have been described from fluorescent pseudomonads: aeruginoic acid (*53*), pyochelin (*54*), and (+)-S-dihydroaeruginoic acid, inhibitor of *Septoria tritici* and other phytopathogenic fungi and bacteria (*55*). Fourteen strains of *Bradyrhizobium* (nitrogen fixing bacteria) were divided into two groups characterized by iron requirements and siderophore production. One group produced siderophores under iron starvation, while the second group had a lower iron requirement and did not

demonstrate any siderophore production (*56*). The ability of one organism to utilize another's siderophores can be of selective advantage, as has been found in *Bradyrhizobium japonicum* (*57*).



Aeruginoic acid Pyochelin (+)-S-Dihydroaeruginoic acid

Siderophore production has been found in higher plants as well, e.g. in *Frankia* sp. under iron limitation (*58*). Oats produced the phytosiderophore avenic acid. Interaction between six siderophore producing bacteria and two oat cultivers (a phytosiderophore producer and a non-producer) was studied. It was found that inoculation of oat roots with the siderophore producing bacteria had little or no effect on iron acquisition by the plants (*59*).

Some other similar groups, such as phenolic compounds, quinones, peptides and volatile compounds are identified as allelochemicals or antibiotics (*3, 30, 60-68*).

Combined Effect of Several Modes of Action

It is logical that more than one factor is involved in allelopathic interactions among organisms in nature. Experiments designed to reveal a particular factor responsible for antibiosis are performed in defined laboratory conditions while in the non-controlled, natural environment these conditions usually fluctuate and change during growth. *Pseudomonas aeruginosa* strain LEC 1 produced, on King's medium B (*69*) two phenazine derivatives: 1-hydroxyphenazine (PhOH) and chlororaphin (*39*). In tryptic soy broth without dextrose (*70*) LEC 1 produced high amounts of proteases as well. The antibiotic properties of these compounds were assayed on cultures of the fungal plant pathogen *Septoria tritici*. Addition of PhOH resulted in decreased fungal growth, increased production of the antioxidative enzymes, catalase and superoxide dismutase, and production of melanin-like compounds. Addition of the LEC 1 proteases had no effect on fungal growth. However, addition of the antibiotic together with proteases had a synergistic effect on the fungus' production of melanin (*39, 71*). It was also observed that heavy melanization and loss of pathogenicity were associated events in old cultures of *S. tritici* (isolate ISR 398A1). The association of aging with increased melanization and loss of pathogenicity is a phenomenon known to occur in other fungi as well (*72, 73*). As the combination of proteases and PhOH from strain LEC 1 induced high melanin production, it seems likely that decreased virulence would also be a probable effect of this combination. These observations suggest that in certain natural circumstances this biocontrol agent may reduce the virulence of the pathogen rather then kill it.

The roles and importance of extracellular enzymes, as factors in biological control, were not thoroughly investigated (with the exception of mycoparasitism). Testing the effects of enzymes from a biocontrol agent on a biocontrol target organism, in laboratory conditions, may lead to incorrect conclusions if natural stress conditions are not taken into account. For example, native proteins are usually resistant to proteolytic activity. Oxidative stress conditions like exposure to peroxides, superoxides, etc., modify proteins by increasing their susceptibility to degradation by proteases (*74, 75*). One of the explanations given regarding the toxicity of PhOH is that it mediates production of toxic, oxygenated metabolites (*71, 76*). The combined

effects of the two components produced by the same bacterium are, therefore, both toxicity (of the antibiotic itself) and enhanced proteolysis. Moreover, it was also found that phenazine derivatives induced production of peroxidases and phenolic compounds in plants (*77*), both of which are considered signs of induced resistance. In this case, induced resistance of the plant host may be another interaction involved in the complex relationship among plant-host, pathogen and biocontrol agent. Bacteria generally grow faster than fungi. Therefore, in humid conditions they are the first to colonize new ecological niches and may deplete nutrients before pathogens become established. In cases where antibiotics and/or lytic enzymes were considered to be the main biological control factors, competition might still be an additional, secondary factor. The resistance of microorganisms to antibiotics is greater in nutrient-rich media (*66*). However, in natural conditions nutrients are usually scarce. All those factors which seem to have small or negligible effects in *in vitro* bioassays, may have, in combination, additive effects in field conditions. Einhellig (*78*) reviewed a few environmental stress factors (temperature, moisture, mineral nutrition) that seemed to have additive or synergistic effects when combined with allelochemicals. Einhellig also mentioned that allelochemicals seldom act alone and concentrations at levels considerably below inhibition thresholds in bioassays may be biologically active under natural conditions. In nature, where conditions are rather unstable, a biocontrol agent which acts through several mechanisms stands a better chance to survive and prosper.

Application Methods

Understanding the modes of action by which a biocontrol agent suppresses its target pathogens is very important for developing strategies of bioformulation and application. A few studies emphasized the importance of pH in bioformulations. The antibiotic phenazine-1-carboxylic acid (PCA) produced by *P. fluorescens* strain 2-79 is the factor responsible for the inhibition of the pathogen *Gaeumannomyces graminis* and other pathogenic fungi and bacteria (*35, 37*). The activity of this compound is pH-dependent (active only in acid media) (*79*). Therefore, the latter authors concluded that the effectiveness of the antibiotic depends on the pH of the soil and rhizosphere. However, it was demonstrated that certain plants extrude H^+ through their roots. The rhizosphere acidification resulting from H^+ extrusion increases the availability of limited resources such as P, Mo, and Fe (*80-82*). In these cases the antibiotic PCA may be active at the root surface where, in fact, the interaction between the pathogen and its biocontrol agent occurs. Thus, the whole picture, including the pathogen, its biocontrol agent and the host should be considered in the application of biocontrol agents. Addition of chitin increased the antagonistic activity of the mycoparasite, *Trichoderma hamatum*, whereas cellulose tended to decrease it (*83*). Chet (*13*) reviewed a few studies which demonstrated that acidification of the soil enhanced the antagonistic activity of *Trichoderma* spp. Biocontrol formulations usually contain, beside the active microorganism, inert carriers, surfactants, glues, protectants, and nutrients. These materials should be tested and verified as non-stimulating factors for the pathogen (*5*).

Future Prospects

Although bacteria, due to their impressive antibiotic production, seem to be wonderful candidates for biocontrol, very little success has been achieved in the field. In comparison to other microorganisms, (yeasts and filamentous fungi) bacteria (except spore forming ones) are less resistant to hostile environmental conditions such as high temperature, UV radiation and moisture. Blakeman (*84*), in his review on the ecological succession of phylloplane microorganisms, pointed to bacteria as the first colonists of the phyllosphere in temperate climates. Second in succession are yeasts

that continue to colonize leaves even under prolonged periods of high temperatures and absence of rain (*85*). Filamentous fungi are found particularly at the end of the season.

Considering on one hand the fore mentioned advantages of bacteria as biocontrol agents, and on the other hand their susceptibility, especially to the unsteady conditions of the phyllosphere, bacterial bioformulations should be developed with built-in protective ingredients. Until such appropriate bioformulations are developed, biocontrol attempts with bacterial agents should concentrate on controlled environments like greenhouses and plastic tunnels, to enhance their survival.

Schroth & Hancock (*86*) suggested that diseases where the plant hosts are susceptible for a short period of time are the ideal ones for biocontrol. For example, blossom blights of mango and citrus, caused by the fungus *Colletotrichum gloeosporioides*, occur during blossom (*87, 88*). Fire blight of pear and other Rosaceae, caused by the bacterium *Erwinia amylovora*, also occurs primarily at blossom time (*89*). Following the suggestion of Schroth and Hancock these diseases are ideal to suppress by biocontrol agents including bacteria which are known as poor survivors. Success was achieved using the bacteria *Erwinia herbicola* and *Pseudomonas fluorescens* as biocontrol agents of fire blight (*89, 90*). Seedlings attacked by damping off pathogens also need protection for a short period time that can be achieved by bacterial biocontrol agents (*32, 33, 91*).

Fluorescent pseudomonads are distributed in the phyllosphere, rhizosphere and aquatic environments. Their widespread distribution and colonization potential apparently stems from their ability to produce antibiotics. This hypothesis, concerning the role of antibiotics is still in controversy. It is difficult to believe that such energy wasting mechanisms survived evolutionary pressures without having useful functions. There are thoughts to create "super antibiotic producers" by genetic-engineering manipulations. Turner & Messenger (*34*) demonstrated that mutant strains of *P. phenazinium*, that lost the ability to produce phenazine, grew larger colonies and remained viable longer than the phenazine-producing wild types under laboratory conditions. On the other hand, in mixed cultures the wild type population overcame the mutants. To extrapolate from these experiments, hypothetical "super producers" might have a shorter life span than the wild type. It is logical to assume that antibiotic production and viability are in a certain equilibrium. Antibiotic production from zero to a certain optimum increases the producer's antagonistic potential, while further increase decreases its viability due to self intoxication. The work done by Howell and Stipanivic (*92*) with a superproducer mutant of *Gliocladium virens* appears to corroborate the above mentioned hypothesis. They found that the superproducer grew slower and had no higher antagonistic effect than the parental strain in *in vivo* tests. If this superproducer had a shorter life span (as it was found in *P. phenazinium* strains), then such mutants are not fit for long term defence strategies. These hypotheses should be tested with each new "super producer".

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Chapter 23

Allelopathy in the Search for Natural Herbicide Models

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The existence of allelopathy has been well documented during the past few decades on natural as well as in agroecosystems. The potentiality that allelopathy can offer useful clues in the search of new natural herbicide models, more specific and less harmful than those synthetic, at present used in agriculture is discussed. Three different strategies are presented considering allelopathic studies on natural ecosystems, agroecosystems and natural product models as natural herbicides with applications in agronomical important crops such as barley, oat, wheat, grapes, etc. The bioactivity levels of the present synthetic herbicides and those of several allelochemicals reported in the literature are compared and discussed.

The weed problem supports an important part of the agriculture research. As a consequence of this research, many chemicals have been developed since the 50's, and their utilization are widely extended (*1*). There are about 250 plant species sufficiently troublesome in agriculture to termed weeds (*2*). In spite of modern control methods, even in developed countries that rely heavily on chemical herbicides for control, losses due to weeds, including efforts to control them plus losses in yield and quality, are relatively high.

Herbicides will continue to be a key component in most integrated weed management systems in the future. Nevertheless, the increasing of the chemical control has become overwhelming economical border, and more important, it could pose a serious threat of the public health and of the environment as it has been proved in recent studies (*3,4*) from which the following facts are concluded: a) a considerable decreasing of the crop yields; b) the appearance of highly resistant species to commercial innocuous products traditionally used; c) a clear and evergrowing pollution on the phreathical layer. During last few decades the extensive use of synthetic herbicides and pesticides has been the cause of concern from both environnmental and health considerations. Most of the synthetic chemicals are more hazardous due to their long

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In Allelopathy; Dakshini, K., et al.;
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persistence, non-target toxicity, pollutive, carcinogenic and mutagenic activity (*5*-8). In addition some problems as crop injury, increased cost of discovering and developing new herbicides, enhanced soil biodegradation, and container disposal, are receiving increasing attention and concern.

Because of these problems much attention is being focused on alternative ways for weed control. Allelopathy, which studies biochemical plant-plant interactions including positive and negative effects (*9*,*10*), has been proposed as a possible alternative weed management strategy (*11*-*13*).

The multidisciplinary character of this science, where biologists, plant physiologists, ecologists, edaphologists, agricultural chemists, agronomical engineers, natural product chemists, etc. play an important role, can offer additional routes for weed control (*12*-*16*) by developing new techniques involving allelopathy for weed suppression, such as:

1. The use of natural or modified allelochemicals as herbicides.
2. Genetic transfer of allelopathic traits into commercial crop cultivars.
3. The use of allelopathic plants in crop rotation, companion plantings and smother crops.
4. The use of phytotoxic mulches and cover crop management for weed suppression, especially in conservation and no-tillage crop production.

The potentiality that allelopathy can offer helping in the search of natural herbicide models, more specific and less harmful than those synthetic at present used in agriculture, will be discussed in this chapter.

Developments in Weed Control

Developments in weed control can be divided into three periods. The first, before 1945, was marked by inorganic and organic herbicides having very low activity and no selectivity i.e. copper sulphate and dinitro-ortho-cresol "DN". The basic idea behind the research of the early herbicides was to spray a group of plants with a compound in order to kill the weeds leaving the crop unharmed. This type of compound was called post-emergence herbicide.

The modern era began in the mid 1940's with the discovery of the phenoxy herbicides, followed during the next 30 years by substituted phenylureas, triazines, glyphosate, and others. They allowed for the first time selective pre- and post-emergence weed control in seeded crops. With this second generation compounds came the finding that many different steps in the plant's biochemistry are susceptible to chemical exploitation (*17*). Those pathways that are different from other forms of life are primary targets for attack in the design of new agrochemicals (*18*).

The discovery of the sulfonylurea herbicides in the mid 1970's by Levitt (*19*-*21*) signed the start of the present low dose era of chemical herbicide, which is characterized by selective weed control on crop at very low use rate.

Strategies in the Search of New Herbicides

Strategies to search novel herbicides can be divided into four basic approaches:

a) **New Structural Types**: Synthesis of new compounds for broad biological screening motivated purely by structural novelty. It is not possible to establish any direct relationship between structural complexity and activity. Many more "simple" molecules of high activity can be found, considering the almost infinite ways in which the elements can be combined.
b) **New Ideas in Known Areas**: It depends on other approaches for its starting point but in the hands of creative chemists adds significant new value to existing classes of chemicals. Seldom is the initial product the last word for any area.
c) **Biochemically Directed Synthesis**: "Biorational herbicides". The common problem of poor correlation between "*in vitro*" enzyme inhibition and whole-organism activity should begin yielding to better understanding of xenobiotic penetration movement on metabolism. This strategy can be supported from the outset by biochemistry and biotechnology. Specific new modes of action can be sought intentionally.
d) **Natural Products**: As traditional methods of discovering new herbicides become more difficult and expensive, interest in natural products as sources of new herbicide chemistry increases. Natural products are an attractive source of potential leads to new natural herbicides, not only for the diversity and novelty of chemical structures produced by living organisms, but also for the potential specificity of biological action and the greatly reduced likelihood of harmful bioaccumulation and/or soil and ground water residues. Indeed, natural products can be used in any of the other three strategies mentioned above.

Allelopathy, which studies biochemical plant-plant interactions, can offer an excellent opportunity to help in the search of new natural herbicide models. Knowledge of chemistry and biology of allelochemicals is necessary to their exploitation in biocontrol programmes.

The knowledge of the allelochemicals involved in one specific interaction, their mechanisms of action and the receptors can allow us to develop new strategies in the search of natural herbicides models. Learning from nature how a specific plant can biochemically interact with another we can focus our attention to the natural products isolation based on the corresponding bioassay in order to found new structural types of herbicides more specific and less harmful than those synthetic at present in use in agriculture.

The sources for allelopathic agents can be classified into three groups:

1.- Secondary metabolites from species belonging to the own studied ecosystem (natural or agroecosystem).
2.- Secondary metabolites from other ecosystems, not necessarily related with the studied one. (i.e. from marine organisms).
3.- Synthetic analogues of the allelochemicals above mentioned.

Consequently three different strategies can be formulated depending on the origin of the allelopathic compound:

- The search of natural herbicide models from a particular ecosystem (natural or agroecosystem) with application on the own ecosystem.

- The search of natural herbicide models from a particular ecosystem with application on a different one.

- The synthesis of analogues of the previous allelopathic compounds in order to establish the structural requirements needed for a specific bioactivity.

With these concepts in mind and with the notion that allelopathic compounds have a wide diversity of skeleton type we have initiated several years ago two different and complimentary research projects: "*Natural Product Models as Allelochemicals*" and "*Allelopathic Studies on Cultivar Species*". We have initiated a systematic allelopathic activity studies on natural and agroecosystems as well as with synthetic bioactive natural product models in order to evaluate their potentiality as allelopathic agents and consequently as natural herbicides models.

The plant material selection is based on field observations and on preliminary bioassay of the crude water extract using 1:10, 1:20 and 1:40 (v:v) dilutions. After the first chromatographic separation a second bioassay is performed and the fractions are selected on the basis of their bioactivity. Each pure compound resulting from the separation is tested using a series of aqueous solutions at 10^{-4}-10^{-9} M in order to establish a structure-allelopathic activity relationship. Finally the synthesis of analogues is carried out in order to establish the specific structural requirements needed for this bioactivity.

In this chapter two examples related with the two first proposed strategies using allelopathic studies are presented. The first one belonging to a natural ecosystem: *Melilotus messanensis* (L.) All. and the second one from an agroecosystem: cultivar sunflowers (*Helianthus annuus* L.) var. SH-222.

Allelopathic Studies on Natural Ecosystems: *Melilotus messanensis* (L.) All.

Melilotus messanensis (L.) All. is a small shrub (less than 50 cm tall) endemic to the Mediterranean Basin (*22*), it is one of the 23 species that belonging to the genus *Melilotus* (Fabaceae). Recently, the potentiality of different *Melilotus* species such as *M. segetalis* or *M. messanensis* (L.) All. ecotypes from SW Spain, able to grow in saline soils, has been evaluated as forage resources, green manure and as source of biocide compounds (*23*,*24*). It must be pointed out that about 15 million ha in the Mediterranean Basin are affected by salinity and a diversified use of these "marginal land" must include extensive grazing.

Following the general proposed strategy a preliminary bioassay of *M. messanensis* (L.) All. crude extract was performed. It shows to be active on germination (stimulation) and radicle length (inhibition) of *Lactuca sativa* L. Results on the germination, root and shoot length (Figure 1) are expressed on % units over the control, consequently 0 means that the observed value was identical to the control and obviously no effect, a positive value means stimulation and a negative value represent inhibition.

Extraction of the fresh *M. messanensis* aqueous extract with Dichloromethane (DCM) afforded, after chromatography following the levels of bioactivity exhibited by the fractions over *Lactuca sativa,* on the second bioassay (Figure 1), six lupane-type

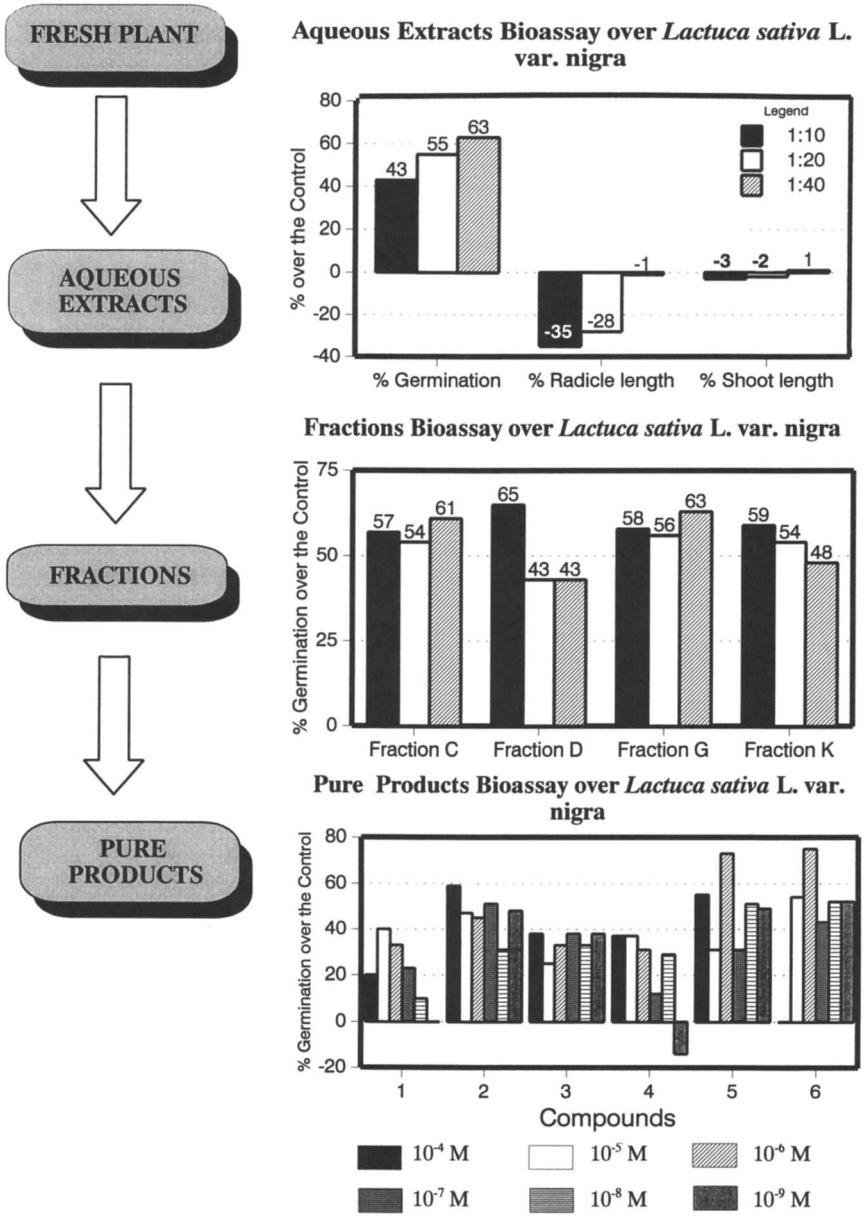
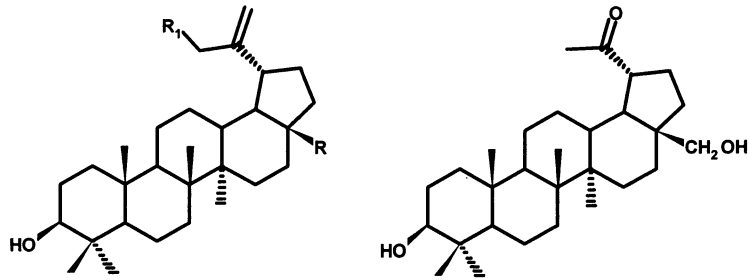


Figure 1. Strategy for isolation of allelopathic agents from *Melilotus messanensis* (L.) All.

triterpenes of increasing polarity, the known lupeol (**1**) (*25,26*), betulin (**2**) (*27*), betulinaldehyde (**3**) (*28,29*), betulinic acid (**4**) (*30*) and **6** (*31*) and a new nor-lupane messagenin (**5**).They were identified and characterized by spectroscopic techniques (IR, MS and 1D, 2D NMR experiments) and chemical correlation (*32*) (Figure 2).



1 R = CH_3 R_1 = H **2** R = CH_2OH R_1 = H **5**

3 R = CHO R_1 = H **4** R = COOH R_1 = H

6 R = CH_2OH R_1 = OH

Figure 2. Allelopathic lupanic triterpenes isolated from bioactive fractions of *Melilotus messanensis* (L.) All.

In order to evaluate their potential allelopathic activity and to obtain information about the specific structural requirements needed for their biological activity, we have studied the effect of a series of aqueous solutions at 10^{-4}-10^{-9} M of six natural lupanic triterpenes **1-6** on germination, root and shoot lengths of *Lactuca sativa* seedlings (dicotyledons) (Figure 2) and *Hordeum vulgare* and *Triticum aestivum* seedlings (monocotyledons) (*32*).

In the literature, only oleanic-type triterpenes as ursolic acid (*33*), medicagenic acid and their glycoside derivatives (*34*) or soyasapogenol B (*35*) have been reported to influence the growth of surrounding plants where the effect was inhibition for the acid derivatives and stimulation for soyasapogenol B.

As observed with the water extract and the fractions **C**,**D**,**G** and **K** (Figure 2) from which they were isolated, **1-5** showed a high stimulatory activity on the germination of *Lactuca sativa* seeds in high and low concentration, pointing out **2**, **5** and **6** (10^{-4},10^{-7},10^{-9} M, **2**: +38%; 10^{-6}M, **5**: +73%; 10^{-6}M, **6**: +75%) (Figure 2). The effects on the radicle and shoot length are, in general, of little or no significance.

These compounds have low effects on the germination and seedlings growth of *Hordeum vulgare* L. and *Triticum aestivum* L. (*32*), except for **3** over *H. vulgare* L. Compound **3** has an inhibitory effect on the shoot length (10^{-6}M, -42%; 10^{-7}M, -44%) and there are stimulatory effects on germination promoted by **3** (10^{-9}M, +30%).

These data suggest that the bioactivity of these compounds can be related with the presence of a free hydroxyl group at C-3, a -CH_2OH at C-17 as shown by **2**, **5** and **6** and this is increased when a methyl and ketone groups or CH_2OH and methylene is attached at C-20.

The concentrations of compounds **1-5** in the 1:10 aqueous extract are in the same range as those that were active in the bioassay (*32*). The above findings suggest that the lupane triterpenes are very likely responsible for the allelopathic activity of *M. messanensis* aqueous extract with a certain specificity over some dicotyledons species.

This is a very interesting example where the profile of activity shown by the original crude aqueous extract and the fractions **C**,**D**,**G** and **K** from which triterpenes **1-6** were isolated is perfectly correlated with the corresponding of pure compounds. The stimulatory effects shown over germination of some dicotyledons species suggest that these lupane triterpenes are excellent candidates to be used as a pre-emergence herbicides at very low doses (10^{-4}-10^{-9}M).

Allelopathic Studies on Agroecosystems: Cultivar Sunflowers (*Helianthus annuus* L.*) var SH-222*

Cultivation of sunflowers is predominantly performed to produce oil and plays an important role in southern parts of Europe. Biochemical investigations on sunflower reveal that this species (*Helianthus annuus* L.) is a rich source of sesquiterpenoids (*36*,*37*) and other plant metabolites with a wide spectrum in biological activities (*38*), nevertheless little is known about the function of its compounds. Recent investigations have shown that sunflowers can actively influence the growth of surrounding plants (*39*,*40*), but the mechanism of these allelopathic effects is unresolved.

In Andalusia region (Spain), 26 different varieties of sunflowers for crop production are used. Following the proposed strategy, we perform a preliminary bioassay with those varieties during four different plant development stages in order to establish which variety shows a better significative profile of activity and when is the best stage for use the plant material (fresh leaves) without injury the plant for the main crop production. As result of this previous bioassay *H. annuus* var. SH-222 during the third plant development stage (plants 1.2 m tall with flowers, 1 month before harvest) was one of the selected varieties (Figure 3).

The subsequent bioassays with fractions obtained from the first chromatographic separation, where fraction **G** (Figure 3) shows a good correlation respect to the crude extract (inhibition of the germination of lettuce seeds, -37%), guided to the isolation of the active principles.

From fraction **G** five new guaianolides named annuolides **A-E** (**7-11**) were isolated (*41*) as well as a sesquiterpene heliannuol **A** (**12**) the first member of a novel class of sesquiterpene (*37*) (Figure 4).

In order to evaluate their potential allelopathic activity, we have studied the effect of a series of aqueous solutions at 10^{-4}-10^{-9} M of compounds **7-12** on root and shoot lengths of *Lactuca sativa* L. and *Hordeum vulgare* L. seedlings.

There are several contributions about the regulatory activity on the germination and plant growth of sesquiterpene lactones (*42*,*70*,*72*) where it is reported that the activity is clearly affected by the conformation of the molecules and the accessibility of groups which can be alkylated as an α-methylene-γ-lactone moiety.

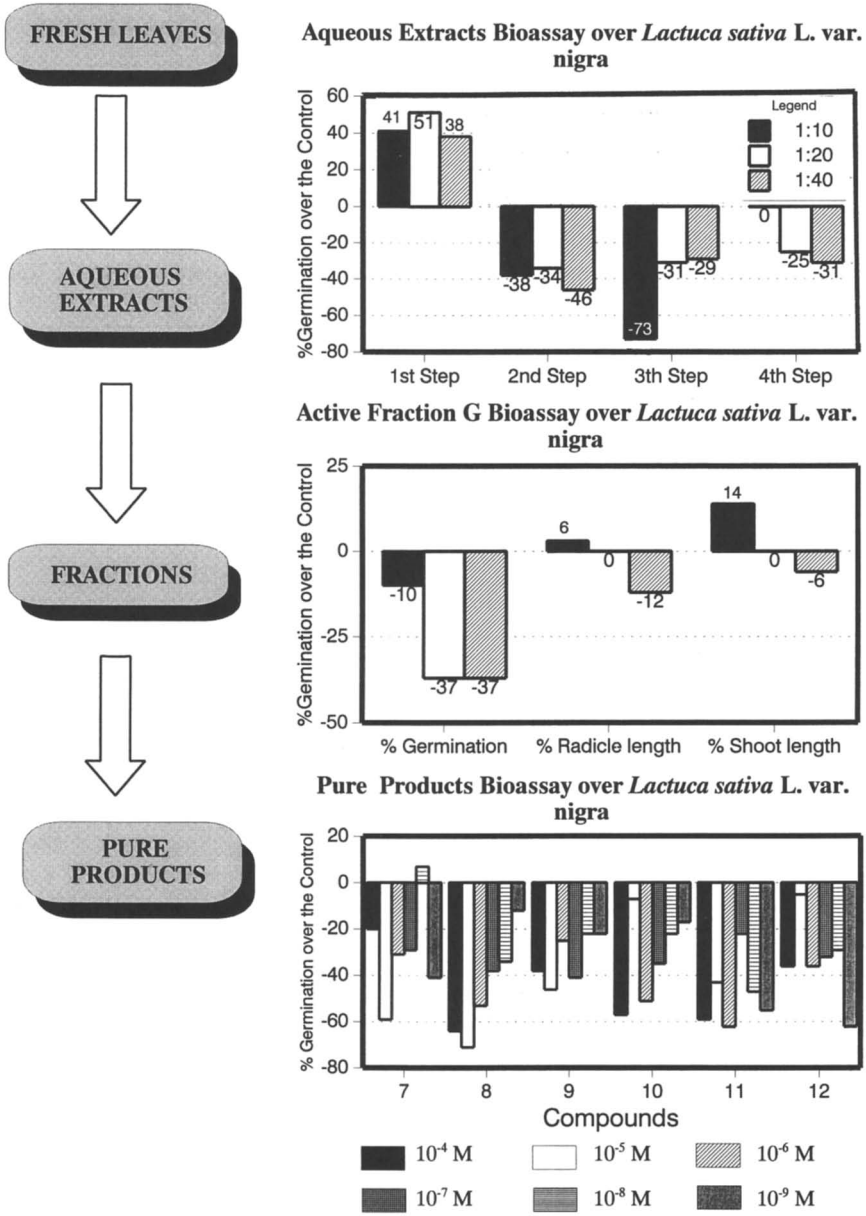
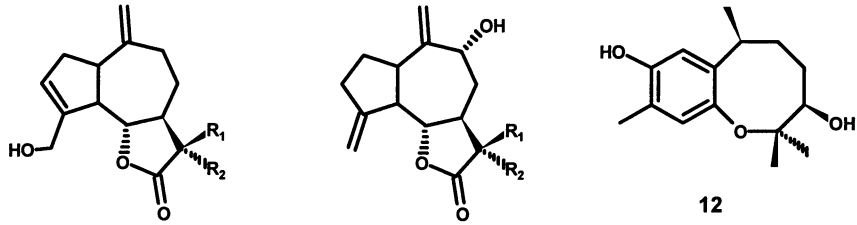


Figure 3. Strategy for isolation of allelopathic agents from *Helianthus annuus* L. var. SH-222.



7 $R_1, R_2 = CH_2$
8 $R_1 = H$ $R_2 = CH_3$

9 $R_1, R_2 = CH_2$
10 $R_1 = H$ $R_2 = CH_3$
11 $R_1 = CH_3$ $R_2 = H$

Figure 4. Allelopathic agents isolated from bioactive fractions of *Helianthus annuus* L. var. SH-222.

As observed with the fraction from which they were isolated, **8** and **11** showed (Figure 3) a high inhibitory activity on the germination of lettuce seeds in high and low concentrations (10^{-5} M **8**: -71%; 10^{-6} M **11**: -62%). The effects on the radicle and shoot length are, in general, of little or no significance, as well as with germination and growth of barley seeds (*41*), except for **7** and **10**. There are stimulatory effects on germination promoted by **7** (10^{-5} M, 27%) and **10** (10^{-5} M, 17%; 10^{-6} M, 23%).

The novel sesquiterpene **12** shows a homogeneous inhibitory profile of activity with an average of 40% of inhibition on the germination of lettuce from 10^{-4}-10^{-9} M and small effect on the germination and growth of barley seeds.

The above findings suggest that the guaianolides **7-11** and the heliannuol **12** are likely to be significantly involved in the allelopathic action of cultivar sunflowers with certain specificity over some dicotyledons species.

The inhibitory effects shown over germination of some dicotyledons species as well as the stimulatory effects shown over germination of some monocotyledons species suggest that these guaianolides and the heliannuol are excellent candidates to be used as a pre-emergent herbicides on a monocotyledons crop at very low doses (10^{-4}-10^{-9}M) where even some stimulation over the germination of the monocotyledons can be expected. This suggestions are in agreement with field observations in southern parts of Mexico (unpublished data) where the corn field present an important number of *Helianthus maximiliani* (*43*) and *H. microcephalus* (*44*) plants with positive effects over the crop, from which guaianolides similar to annuolides **A** (**7**) and **D** (**10**) has been isolated .

Experiments for Bioassays

Seed germination bioassay. Seeds of *Lactuca sativa* L var. nigra., *Hordeum vulgare* L. and *Triticum aestivum* L. 1991 crop, were obtained from Rancho La Merced, Junta de Andalucía, Jerez, Cádiz, Spain. Seeds were selected for uniformity of size and damaged seeds were discarded.

Germination bioassays consisted of germinating 25 lettuce seeds for 5 days (3 for germination and 2 for root and shoot growth) and 5 *Triticum aestivum* L. and *Hordeum vulgare* L. seeds for 3 days, in the dark at 25°C in 9-cm plastic Petri dishes containing a 10-cm sheet of Whatman no. 1 filter paper and 10 ml of a test or control solution for lettuce and 5 ml for barley and wheat.

Test solutions of water extract were prepared by diluting the original extract to 1:10, 1:20 and 1:40 (V. extract: V. H_2O) using deionized H_2O and for the fractions by diluting the appropriate amount of each fractions to obtain a similar concentration to 1:10, 1:20 and 1:40 aqueous extract. Test solutions (10^{-4} M) were prepared using deionized water and test solutions 10^{-5}-10^{-9} M were obtained by diluting the previous solution. There were 3 replicates for *L. sativa L.* and 19 for *H. vulgare* L. and *T. aestivum* L. of each treatment and of parallel controls. The number of seeds per replicate, time and temperature of germination were chosen in agreement with a number of preliminary experiments, varying the number of seeds, volume of test solution per dish and the incubation period.

All the pH values were adjusted to 6.0 before the bioassay using MES (2-[N-Morpholino]ethanesulfonic acid, 10 mM). The osmotic pressure values were measured on a Vapor Pressure Osmometer WESCOR 5500 and are on a range between 30-38 m osmolar. The germination, root and shoot length values were tested by the Student's t-test; the differences between the experiment and the control were significant at value of P=0.01.

Natural or Analogs Allelopathic Compounds as Herbicides

There are excellent reviews about the potential use of allelochemicals as herbicides where allelochemicals from higher plants and microbes (*12*,*45*,*46*) from microbes (*47*,*48*) or plants (*49*,*50*) with a wide diversity of structural types are proposed. Their potential use is discussed on the basis of their stability in the soil (some of the degradation products are more active than their precursors, particularly phototoxins), the environmental safety (biodegradability), the site of action (it has only begun to tap potential sites of herbicide action that can provide a start for biorational herbicides design around these sites), the accessibility (knowledge of the location of active principles within the plant might be crucial to determine the real possibilities of practical applications on agriculture) and the level of activity (to be successful, natural phytotoxins might be active at lower concentrations in comparison with synthetic herbicides).

In this section only allelochemicals from higher plants and particularly their level of activity are discussed. The focus here is to compare the level of activity between the traditional candidates, the more recent allelochemical discovered, which may impact weed science, and the synthetic herbicides. Specifically these are compounds released either by crops, weeds, their residues or synthetic analogues.

The dose of synthetic herbicides used for weed control has been changed along the different periods mentioned above. Trichloroacetic acid, corresponding to the first period, was used for non-selective weed control at rates of 55-225 kg/ha, ca. $5.5x10^4$-$2.25x10^5$ ppb (soil weight basis, distributed 10 cm deep).

Table I. Selected Allelopathic Compounds with Potential Use as Natural Herbicides

Name	Activity range (ppb)	Target species	Type of activity[a]
Simple acids and esters			
L-β-Hydroxybutyric ac. (*51*) (**13**)	$2.2x10^3$-$8.3x10^4$	*Chenopodium album* L.	Growth(-)
		Amaranthus retroflexus L.	Growth(-)
Ethyl propionate (*52,53*) (**14**)	$7x10^2$-$3.4x10^3$	*Allium cepa* L.	Germination(ret.)
		Daucus carota L.	Germination(-)
		Lycopersicon esculentum Miller	Germination(ret.)
Ethyl 2-methylbutyrate (*52*) (**15**)	$8.9x10^2$-$4.4x10^3$	*Allium cepa* L.	Germination(-)
		Daucus carota L.	Germination(ret.)
		Lycopersicon esculentum Miller	Germination(ret.)
Polyacetylenes			
Trans-DME (*54*) (**16**)	10^3	*Echimochloa crus-galli* (L.) Beauv.	Growth(-)
Cis-dihydro-ME (*54*) (**17**)	$5x10^3$-$5x10^4$	*Oryza sativa* L.	Growth(-)
Long chain fatty acids			
Arachidic ac. (*55*) (**18**)	$5x10^3$	*Cynodon dactylon* (L.) Pers.	Germination(-)
Behenic ac. (*55*) (**19**)	$5x10^3$	*Cynodon dactylon* (L.) Pers.	Germination(-)
Myristic ac. (*55*) (**20**)	$5x10^3$	*Cynodon dactylon* (L.) Pers.	Germination(-)
Alkaloids			
BOA[b] (*56*) (**21**)	10^5	*Echinochloa crus-galli* (L.) Beauv.	NA
		Lepidium sativum L.	Growth(-)
		Cucumis sativus L.	Growth(-)
		Phaseolus vulgaris L.	Growth(-)
AZOB[b] (*57*) (**22**)	$5x10^4$	*Echinochloa crus-galli* (L.) Beauv.	Growth(-)
		Lepidium sativum L.	Growth(-)
		Cucumis sativus L.	Growth(-)
		Phaseolus vulgaris L.	Growth(-)
Caffeine (*58*) (**23**)	10^5-$4x10^5$	*Lactuca sativa* L.	Growth(-)

[a]Results denoted by (+) = stimulation; (-) = inhibition; (ret.) = retarding; NA = Non Active; (+,-) = stimulation or inhibition pending on the concentration; ac.= acid.

[b]BOA= 2(3H)-Benzoxazolinone; AZOB= 2,2-Oxo-1,1-azobenzene.

Table I. continued

Name	Activity range (ppb)	Target species	Type of activity[a]
Benzoic acid derivatives			
p-Hydroxybenzoic ac. (*59-61*) (**24**)	<6.9x10^4 inactive	*Lactuca sativa* L.	Growth(-)
		Chamaenerion angustifolium Scop.	Germination(-)
		Deschampsia flexuosa Trin.	Growth(-)
		Scrophularia nodosa L.	NA
		Senecio sylvaticus L.	NA
		Lolium multiflorum Lam.	Growth(-)
Vanillic ac. (*61,62*) (**25**)	<7.6x10^4 inactive	*Saccharum officinarum* L.	Growth(-)
		Lactuca sativa L.	Growth(-)
		Chamaenerion angustifolium Scop.	Germination(-)
		Deschampsia flexuosa Trin.	Growth(-)
		Scrophularia nodosa L.	Germination(-)
		Senecio sylvaticus L.	Growth(-)
		Lolium multiflorum Lam.	NA
			Growth(+,-)
			Growth(-)
Cinnamic acid derivatives			
p-Coumaric ac. (*61-63*) (**26**)	<8.2x10^4 inactive	*Lactuca sativa* L.	Growth(-)
		Raphanus sativum L.	Germination(-)
			Growth(-)
		Lolium multiflorum Lam.	Growth(-)
		Chamaenerion angustifolium Scop.	Germination(-)
		Scrophularia nodosa L.	Growth(-)
		Deschampsia flexuosa Trin.	NA
		Senecio sylvaticus L.	Germination(-)
		Imperata cylindrica (L.) Beauv.	Growth(-)
			Growth(-,+)
			Germination(-)
			Growth(-)
Ferulic ac. (*61,62*) (**27**)	9.6x10^4	*Lepidium sativum L.*	Growth(-)
		Chamaenerion angustifolium Scop.	Growth(-)
		Scrophularia nodosa L.	Germination(-)
		Deschampsia felxuosa Trin.	Germination(-)
		Senecio sylvaticus L.	Growth(-)
		Lolium multiflorum Lam.	Growth(-,+)
		Imperata cylindrica (L.) Beauv.	Growth(-)
			Germination(-)
			Growth(-,+)

[a]Results denoted by (+) = stimulation; (-) = inhibition; (ret.) = retarding; NA = Non Active; (+,-) = stimulation or inhibition pending on the concentration; ac.=acid.

Continued on next page

Table I. continued

Name	Activity range (ppb)	Target species	Type of activity[a]
Coumarins			
Xanthotoxin (*64,65*) (**28**)	$<10^4$ inactive	*Anastatica hierochuntica* L. *Lactuca sativa* L.	Growth(-) Germination(-) Growth(-)
Bergapten (*65*) (**29**)	$<10^4$ incative	*Lactuca sativa* L.	Germination(-) Growth(-)
Xanthoxyletin (*65*) (**30**)	$<1.3x10^4$ inactive	*Lactuca sativa* L.	Germination(-) Growth(-)
Monoterpenes			
Tujone (*66*) (**31**)	$3.3x10^3$	*Lactuca sativa* L.	Germination(-)
Carvone (*66*) (**32**)	$8x10^3$	*Lactuca sativa* L. *Leptochloa dubia* Nees *Schizochyrium scoparium* L. *Lactuca sativa* L.	Germination(-) Germination(-) Germination(-) Germination(-)
Camphor (*66*) (**33**)	$2.7x10^4$	*Leptochloa dubia* Nees *Schizochyrium scoparium* L. *Lactuca sativa* L. *Leptochloa dubia* Nees	Germination(-) Growth(-) Germination(-) Growth(-) Germination(-) NA
1,8-Cineol (*66,69*) (**34**)	$2x10^6$	*Schyzochyrium scoparium* L.	Germination(-) Growth(-)
Quinones			
Emodin (*67*) (**35**)	10^4-10^5	*Lactuca sativa* L. *Amaranthus viridis* L. *Phleum pratense* L.	Growth(-) Growth(-) Growth(-)
Physcion (*67*) (**36**)	10^4-10^5	*Lactuca sativa* L. *Amaranthus viridis* L. *Phleum pratense* L.	Growth(+,-) Growth(+,-) Growth(-)
Juglone (*68,69*) (**37**)	$<9x10^3$ inactive	*Alnus glutinosa* (L.) Gaertner *Rudbeckia hirta* L. *Lactuca sativa* L.	Growth(-) Germination(-) Germination(-)

[a]Results denoted by (+) = stimulation; (-) = inhibition; (ret.) = retarding; NA = Non Active; (+,-) = stimulation or inhibition pending on the concentration; ac.=acid.

Table I. continued

Name	Activity range (ppb)	Target species	Type of activity[a]
Sesquiterpenes			
Guayulin A (*70*) (**38**)	$3.5x10^2$	*Amaranthus palmeri* L.	Germination(-)
		Amaranthus retroflexus L.	Germination(-)
		Daucus carota L.	NA
		Lactuca sativa L.	Germination (+)
Heliannuol A (*40*) (**12**)	$2.6x10^2$-$2.6x10^3$	*Lactuca sativa* L.	Germination(+) Growth(-)
		Hordeum vulgare L.	NA
Strigol (*71*) (**39**)	0.3	*Striga lutea* Lour.	Germination(+)
Sesquiterpene lactones			
Soulangianolide A (*72*) (**40**)	0.25-$2.5x10^4$	*Lactuca sativa* L.	Germination(+)
Melampomagnolide A (*72*) (**41**)	0.25-$2.5x10^4$	*Lactuca sativa* L.	Germination(+)
Annuolide A-E (*41*) (**7-11**)	0.25-$2.5x10^3$	*Lactuca sativa* L.	Germination(-)
		Hordeum vulgare L.	NA
11,13-Dihydro-burrodin (*73*) (**42**)	2.5	*Striga lutea* Lour.	Germination(+)
Parthenolide (*73*) (**43**)	0.25-$2.5x10^3$	*Striga lutea* Lour.	Germination(+)
		Lactuca sativa L.	Growth(+)
α-Santonin (*72,74*) (**44**)	0.25-$2.5x10^4$	*Phaseolus vulgaris* L.	Growth(+)
Sulfured compounds			
Hirsutin (*75*) (**45**)	$2.2x10^3$-$4.4x10^4$	*Lactuca sativa* L.	Germination(-) Growth(-)
Camelinin (*75*) (**46**)	$5x10^4$-$2.6x10^5$	*Lactuca sativa* L.	Germination(-) Growth(-)
Triterpenes			
Betulinaldehyde (*32*) (**6**)	0.5-$5x10^4$	*Lactuca sativa* L.	Germination(+)
		Hordeum vulgare L.	Germination(+) Growth(+)
Messagenin (*32*) (**5**)	0.5-$5x10^4$	*Lactuca sativa* L.	Germination(+)
		Lepidium sativum L.	Growth(+)
		Hordeum vulgare L.	Germination(+)
		Triticum aestivum L.	NA
Soyasapogenol B (*76*) (**47**)	10	*Agalinis purpurea* L.	Germination(+)

[a]Results denoted by (+) = stimulation; (-) = inhibition; (ret.) = retarding; NA = Non Active; (+,-) = stimulation or inhibition pending on the concentration.

The second generation membered by phenoxy herbicides, substituted phenylureas, triazines, glyphosate, etc., offered broad spectrum weed control at 1%-5% of the application rates of trichloroacetic acid and the inorganic herbicides with use rates generally ranging from 250-4x10^3 g/ha. ca. 250-4x10^3 ppb.

The present low dose era of herbicide chemistry started with the discovery of sulfonylureas, which is characterized by crop selective weed control at use rates of <100 g/ha. The sulfonylureas provide crop selective weed control achieved at use rates of 2-75 g/ha. ca. 2-75 ppb.

What is the situation with allelochemicals from higher plants? In most of the cases the available data are very confusing due principally to the lack of standard bioassays and, consequently, results do not provide homogeneity.

In order to establish a first approach to the potentiality that allelochemicals from higher plants can offer, a review on allelopathic compounds with potential use as natural herbicides, based on their level of activity and specificity, was performed. This review is referred to 13 classes of compounds, following Rice classification (*10*) on Table I and the structures on Figure 5. Table I presents the most representative members, natural or analogues, of each classes with the level of activity expressed on ppb in order to compare with the usual level of synthetic herbicides. The target species, in order to analyze the specificity, and the activity, expressed by germination and growth, when is related with root or shoot length, are included.

The analysis of Table I shows that in general the most promising allelochemicals are reduced to seven classes: Simple acids as ethyl propionate (**14**); polyacetylenes as *trans*-DME (**16**); long chain fatty acids as myristic acid (**20**); monoterpenes as tujone (**31**); sesquiterpenes as strigol (**36**); sesquiterpene lactones as annuolide A (**7**) and triterpenes as messagenin (**5**). It is important to notice that allelochemicals belonging to those families, in general, has been discarded *a priori* as potential natural herbicides, based on their presumed "low solubility in water". Weidenhamer et al. (*69*) demonstrated that monoterpenes are soluble enough in water to act as potent biological inhibitors with solubilities ranges of 10^4-7x10^7 ppb and levels of activities under 10^5 ppb.

The normal tested range of activity for allelochemical is 10^{-4}-10^{-9}M c.a 0.1-10^4 ppb whilst we can consider good candidates for natural herbicide models when the range is between 10-10^3 ppb ca. 10^{-5}-10^{-7}M consequently phenolic compounds as benzoic derivatives, coumarins, flavonoids, alkaloids and quinones that has been demonstrated to be active at ranges between 10^{-2}-10^{-5}M ca. 10^3-10^6 ppb are very poor candidates as natural herbicides.

A clear idea about the future for natural herbicide structural models can be obtained from the analysis of Figure 6 where range of activity shown by different classes of allelochemicals isolated from higher plants are presented. From those data we can conclude that the most potential natural allelochemicals are terpenoids: monoterpenes, sesquiterpenes, sesquiterpene lactones and triterpenes, and fatty acids with activity range of 0.25-10^5 ppb, rather than the traditionally considered phenolics, quinones or alkaloids.

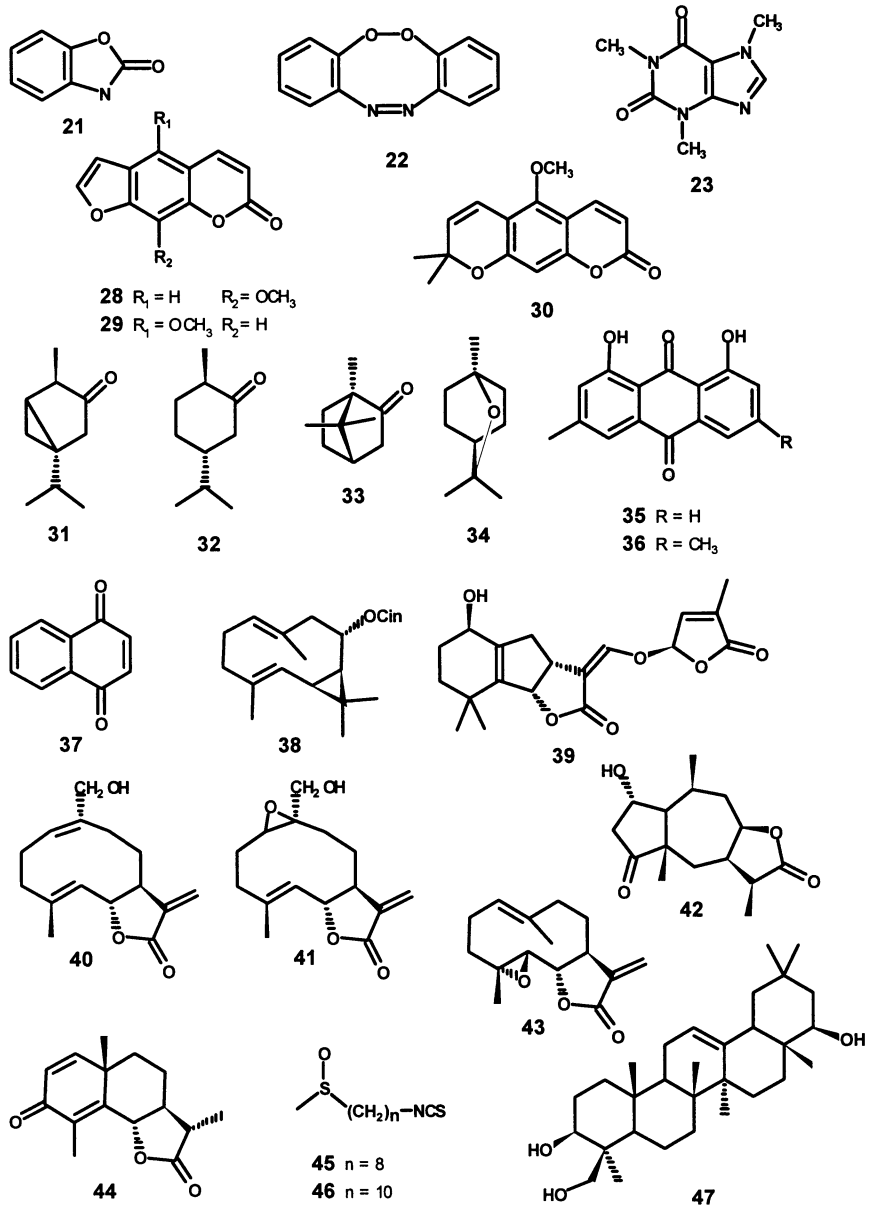
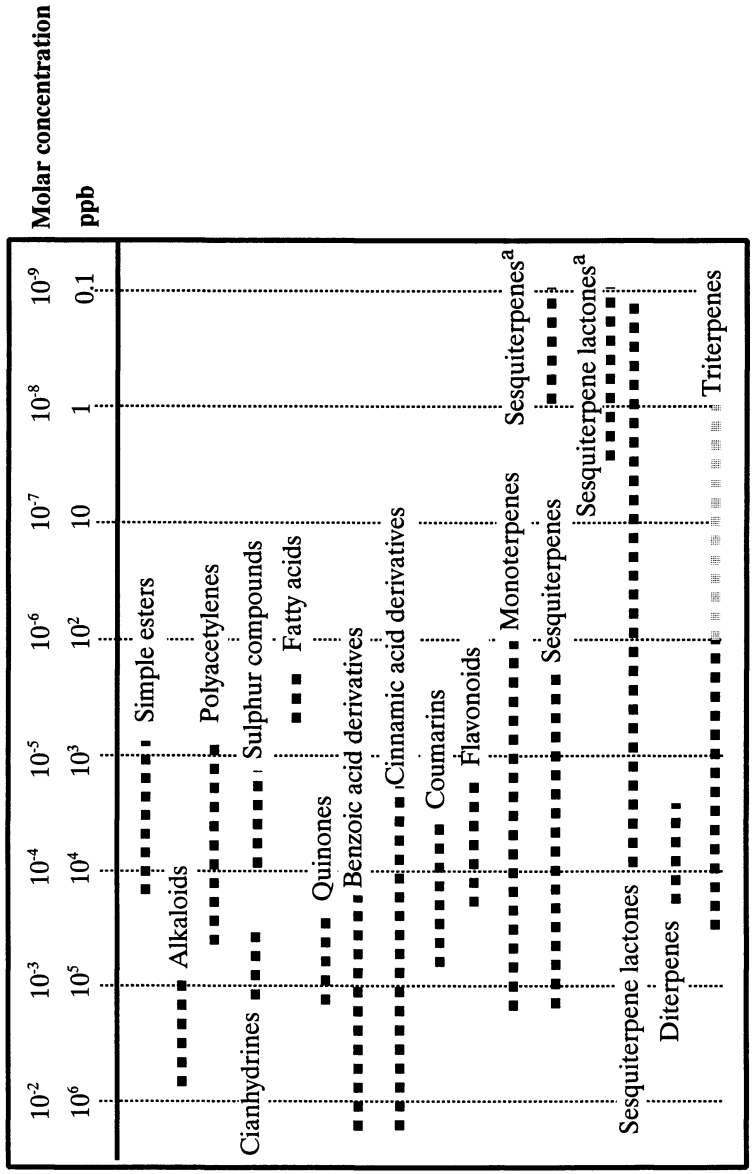


Figure 5. Structures of Allelopathic Agents from Table I.



[a] Bioactivity range over *Striga lutea* Lour.

Figure 6. Bioactivity range of allelopathic agent families from higher plants.

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Chapter 24

Role of Secondary Metabolites in Root Disease Suppression

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Bacterial secondary metabolites active in suppression of root diseases include antibiotics, siderophores and volatile compounds. With recombinant DNA technology it is possible to elucidate the regulatory mechanisms and structural genes involved in the production of these metabolites and to transfer genes for specific beneficial traits among bacterial strains. Some "transgenic" rhizobacteria that express such genes have provided greater protection against fungal pathogens of plants in growth chamber studies, indicating that genetic manipulation is a viable approach to the development of microbial biocontrol agents that are safe, reliable and effective.

Interest in biological control and the use of microbial products to suppress soilborne plant pathogens has been stimulated in recent years by trends in agriculture towards greater sustainability and growing public concern for hazards associated with the use of synthetic pesticides. Research strategies combining molecular technology with more traditional approaches have significantly advanced our understanding of the mechanisms involved in disease suppression by beneficial, plant growth-promoting rhizobacteria (PGPR) introduced on seed or planting material, and the results of these studies have been reviewed extensively (*1-8*). Here, we discuss recent evidence that microbial metabolites, including antibiotics, siderophores and volatiles, have a key role in the control of certain soilborne pathogens. These compounds may function not only directly against target pathogens through the classical mechanism of antibiosis, but also to increase the competitiveness of PGPR by inhibiting other, nontarget microorganisms. In addition, some of the metabolites produced are known to have broad-spectrum activity that extends to the tissues of host plants, where their effects may be either beneficial or deleterious to plant germination, growth, development or yield.

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Although much less is known about the metabolites produced by the so-called deleterious rhizobacteria (DRB) (*9*) or deleterious rhizosphere microorganisms, (DRMO) (*10*), we include them in our discussion from the standpoint of their potential to control weeds.

Antibiotics

Antibiotics are small organic compounds of microbial origin that, at low concentrations, are deleterious to the growth or other metabolic activities of other microorganisms (*11*). Although implicated in the suppression of soilborne pathogens for over 60 years, their importance over most of that time was questioned on the basis of the indirect nature of the evidence and on actual and perceived physical and biological constraints to antibiotic production, activity and detection in soil. More recently, definitive evidence that antibiotics have a critical role in microbial interactions has come from improved assay systems with enhanced specificity and sensitivity both in vitro and in situ, in the context of natural ecosystems. Many genera of bacteria and fungi are known to produce antibiotics, but current research is focused mainly on rhizosphere pseudomonads that produce compounds of known structure and broad-spectrum activity including pyrrolnitrin (*12*) and pyoluteorin (*13*), phenazine derivatives (*14-23*) and 2,4-diacetylphloroglucinol (*24-33*).

Phenazines. Phenazine compounds are pigmented, nitrogen-containing heterocyclic antibiotics produced by bacteria via the shikimic acid pathway (*34*). Phenazine-1-carboxylic acid (PCA) is a primary determinant in the ability of *Pseudomonas fluorescens* 2-79 to suppress take-all, a serious root and crown rot of wheat and barley caused by *Gaeumannomyces graminis* var. *tritici*. Strain 2-79 was isolated from the roots of wheat grown in a soil naturally suppressive of take-all near Lind, WA. Rhizosphere populations of fluorescent *Pseudomonas* spp. on wheat roots from soils in which take-all has spontaneously declined are typically more antagonistic toward *G. g. tritici* than populations on roots from nonsuppressive soils, a fact thought to reflect a natural enrichment that occurs with consecutive cropping of wheat (*35*). *P. aureofaciens* 30-84, another suppressive strain, was similarly isolated from wheat grown in a field in Kansas where take-all had declined. Strain 30-84 produces PCA, but also lesser quantities of 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine (*21*).

Phenazine-nonproducing (Phz^-) Tn*5* mutants of strains 2-79 and 30-84 failed to inhibit *G. g. tritici* in vitro and were significantly less suppressive of take-all on wheat than were the parental strains. Inhibition of *G. g. tritici* and suppression of take-all were restored in mutants complemented for phenazine production (Phz^+) with homologous DNA from genomic libraries of the respective parental strains (*14, 21*). Subsequent isolation of PCA from the roots and rhizosphere of wheat treated with 30-84, 2-79, or its Phz^+ complemented derivative and grown in raw soil provided the first direct evidence for the specific role of the antibiotic in disease suppression in situ (*15*). PCA was not recovered from control roots or roots colonized by Phz^- mutants, and take-all was reduced only on roots

from which PCA was isolated. Moreover, an inverse linear relationship was demonstrated on wheat roots in *G. g. tritici*-infested soil between the number of take-all primary lesions and the population size of Phz^+ wild type strain 2-79 or a complemented derivative applied to the seed. No such relationship was observed with a Phz^- mutant, which failed to reduce lesion number at any dose (*16*). Ability to produce the antibiotic was not a determinant of ecological competence in these studies, which lasted no longer than three weeks. In studies of up to 100 days, however, the ability to produce phenazines contributed positively to the persistence of 2-79 and 30-84 in soil habitats (*19*). Populations of Phz^- mutants of both strains declined more rapidly than did those of the parental or Phz^+ complemented mutants in raw rhizosphere and bulk soil but not in steam-pasteurized soil, suggesting that the antibiotic contributes to competitiveness against indigenous microorganisms.

From 60 to 90% of the biocontrol activity of strain 2-79 can be attributed to production of PCA, depending on the soil type. A pyoverdin siderophore and anthranilic acid made only minor contributions to suppressiveness (*17*). Competition for nutrients and niche exclusion probably account for the remainder of suppressive activity (*17, 20*), although the possibility that the bacteria or their products induce a host plant defense response cannot yet be ruled out. Included among the soil edaphic factors found by linear regression and principal component factor analysis to be positively interrelated with disease suppression by 2-79 were sulfate-sulfur, % sand, pH, sodium, zinc and ammonium-nitrogen. Cation exchange capacity, exchangeable acidity, manganese, iron, % silt, % clay, % organic matter, and total carbon were negatively interrelated (*18*). These correlations are the product of complex interactions among the environment, the host, the pathogen and the biocontrol agent, and should not be construed as indicating that a particular variable is related directly to PCA production or activity. However, the negative effect of clay, silt and organic matter may be associated with binding and inactivation of the antibiotic on the surface of charged soil particles. In vitro, both PCA and cell mass accumulation increased in response to the addition of boric acid and sulfates of iron and magnesium, whereas PCA accumulation alone was improved by addition of zinc sulfate, ammonium molybdate and cytosine. Interactive effects between the sulfates of zinc, iron and magnesium also were observed on biomass and PCA accumulation (*22*). Iron, but not zinc, was directly correlated with phenazine biosynthetic activity using reporter gene fusion constructs to the phenazine biosynthetic locus (Fujimoto, D. K., USDA-ARS, Washington State University, unpublished data).

The Phenazine Biosynthetic Locus. The phenazine biosynthetic locus initially was identified in clones from a genomic library of *P. aureofaciens* 30-84 by their ability to restore Phz^- mutants to Phz^+ and disease suppressiveness. Expression of this locus in *Escherichia coli* required the introduction of appropriate gene promoters and yielded all three antibiotics normally produced by strain 30-84. Sequences essential for phenazine production were localized to a segment of approximately 2.8 kb (*21*) that contains at least two genes: *phzB*, encoding a 55-kD protein involved in PCA production, and *phzC*, encoding a 19-kD protein needed for production of 2-hydroxyphenazine-1-carboxylic acid (*36*).

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An adjacent regulatory gene, *phzA*, also has been characterized; it is divergently transcribed from *phzB* and *phzC* and functions in trans to activate their expression (*37*; see below).

The phenazine biosynthetic locus from strain 2-79 has been cloned on a 12-kb fragment that hybridized strongly with the biosynthetic locus from 30-84. Sequences required for PCA production were clustered within divergently transcribed units of ca 5.0 kb and 0.75 kb that were strongly and weakly expressed, respectively, under conditions favorable to the production of PCA (Thomashow, L. S., Fujimoto, D. K., USDA-ARS, Washington State University, unpublished data). These sequences are thought to correspond physically and functionally to *phzB* and *phzA*, respectively. The 12-kb fragment from strain 2-79 transferred PCA biosynthetic capability to all of 27 other strains of *Pseudomonas* into which it was introduced. In growth chamber experiments, the incidence (but not the severity) of take-all was significantly reduced on the lower root segments of seedlings treated with the PCA-producing transgenic derivatives of strains Q69c-80 and Q65c-80, as compared with those treated with the unmodified parental strains (Hara, H., Weller, D. M., Thomashow, L. S., USDA-ARS, Washington State University, unpublished data). These results support the feasibility of genetic modification as one approach to the improvement of existing biocontrol agents.

Phenazines and Phytotoxicity. U. S. Patent 3, 367,765 for the use of *P. aureofaciens* cultures or extracts to control the growth of algae and noxious weeds was awarded to C. D. Nelson and J. I. Toohey in 1968 (*38*). The strain of *P. aureofaciens* presumably was isolated from pasture soils exhibiting a rare condition described as "barren ring", in which herbaceous vegetation was completely suppressed within an annually expanding ring-shaped area (*39*). Neither pure cultures of *P. aureofaciens* isolated from a barren ring and applied to plots seeded with cultivated plants, nor sods from infected soils transferred to areas of normal vegetation, incited disease symptoms, and the condition was attributed to an association of microorganisms including fungi.

PCA and 2-hydroxyphenazine-1-carboxylic acid prepared from cultures of *P. aureofaciens* and applied as a pre- or postemergence treatment at 10 lb/acre selectively killed pigweed and mustard. Application of the hydroxyphenazine to water at 2.7 lb/acre-ft. controlled the blue-green algae *Microcystis aeruginosa* and *Anabaena flos-aquae*, and PCA at 6.75 lb/acre-ft controlled the water weed *Lemna minor*. In vitro, purified 2-hydroxyphenazine-1-carboxylic acid markedly inhibited blue-green and green algae at concentrations of 2 μg/ml or lower, whereas PCA was effective only at much higher concentrations. Both *Phleum pratense* L. (timothy) and *Nasturnium officinale* R. Br. (garden cress) were more sensitive to PCA than to its hydroxy derivative. Either compound applied to an underlying filter paper at only 0.1 $\mu g/cm^2$ reduced root elongation of timothy to less than half the normal rate, and PCA at 2 $\mu g/cm^2$ completely inhibited timothy seed germination. No toxic effects on fish, insects or mammals were noted (*40*). In a more recent study (*15*) in which wheat seed was treated with *P. fluorescens* 2-79 or *P. aureofaciens* 30-84 and sown in natural soils, emergence and root growth were not reduced on seedlings from which PCA was recovered at concentrations

ranging from 30 to 130 ng/g fresh weight of root plus closely adhering soil. However, stunting was observed on seedlings colonized by strain 30-84 in a steamed soil. PCA was isolated at concentrations of up to 580 ng PCA/g root from such seedlings, but it is unclear whether the growth reduction was due to the higher PCA concentration, to undetermined amounts of 2-hydroxyphenazine-1-carboxylic acid, or to other unidentified metabolites that may have been present. The latter hypothesis is supported by the observation that formulations of washed cells of *P. fluorescens* 2-79 supplemented with purified PCA and applied to wheat seeds did not result in germination losses, but losses of 15 to 80% were observed when the treatment consisted of unwashed cells formulated in spent culture fluid. This finding suggests that the phytotoxicity of the spent medium is due to metabolite(s) other than PCA (Slininger, P. J., NCAUR USDA-ARS-MWA, personal communication, 1993).

Phloroglucinols. Phloroglucinol antibiotics are phenolic bacterial and plant metabolites with antiviral, antibacterial, antifungal, antihelminthic, and phytotoxic properties (*25,27,41-50*). Of particular interest is 2,4-diacetylphloroglucinol (Phl), which is thought to be synthesized via the polyketide pathway (*31*) and is produced by fluorescent pseudomonads isolated from soil or plant material worldwide, e.g., England, Ireland, Switzerland, Ukraine, and the United States. One such strain, *P. fluorescens* CHA0, isolated at Morens, Switzerland, is suppressive to soilborne diseases including black root rot of tobacco caused by *Thielaviopsis basicola*, take-all of wheat, and damping-off of cucumber caused by *Pythium ultimum* (*24,26,28,51*). Strain CHA0 produces secondary metabolites including not only Phl, but also monoacetylphloroglucinol, pyoluteorin, salicylic acid, indoleactic acid, hydrogen cyanide and a pyoverdine siderophore. Genetic analyses and gnotobiotic plant assay systems have been indispensable in defining the roles of these metabolites.

Phl is the major determinant in the suppression of take-all by strain CHA0. In combination with hydrogen cyanide, it also was responsible for control of black root rot of tobacco (*24-26*; see also below), but did not contribute significantly to protection of cucumber against *P. ultimum*, which was mediated mainly by pyoluteorin (*28*). The Phl$^-$ Tn*5* mutant CHA625 was less inhibitory in vitro against *G. g. tritici* and *T. basicola*, and less suppressive of take-all and black root rot, than was CHAO. Complementation of CHA625 with an 11-kb fragment from a CHA0 genomic library restored Phl production, fungal inhibition and disease suppression. Phl was isolated from the roots of wheat colonized by CHA0 (0.94-1.36 μg/g root) but not from roots colonized by CHA625 or control roots (*26*). In contrast, mutants deficient in siderophore or indoleacetic acid production were as suppressive of disease on both wheat and tobacco as was strain CHA0, indicating that these metabolites do not contribute to biological control (*24,25*).

In other systems, Phl production has been correlated with control of take-all by *P. aureofaciens* Q2-87 (*32,33*) and suppression of damping-off of sugar beet by *Pseudomonas* sp. F113 (*29,30*). *P. aureofaciens* Q2-87, isolated from wheat grown in a suppressive soil near Quincy, WA, produces HCN in addition to Phl. At least 20% of fluorescent pseudomonads from the Quincy site shared this phenotype (Keel, C.; Weller, D. M.; Thomashow, L. S., USDA-ARS, unpublished

data), suggesting that such strains may be naturally enriched in certain suppressive soils. *Pseudomonas* sp. F113, isolated in Ireland from the root hairs of a sugar beet plant, inhibited several fungi and bacteria in vitro, and a Tn*5* mutant was less inhibitory than the parental strain. Production of the antibiotic was optimal at 12C in cultures with a high surface to volume ratio and in media containing fructose, sucrose or mannitol as carbon source (*29*), with ammonium ion as nitrogen source (*31*).

Genetics of Phloroglucinol Biosynthesis. Three loci involved in the production of Phl have been identified in strain CHAO. The first, *gacA* (*g*lobal *a*ntibiotic and *c*yanide control), functions as a global regulator of secondary metabolism and is required for production of not only Phl, but also pyoluteorin, HCN (*27*), and an extracellular protease and phospholipase (*52*). A similar locus has been cloned from *P. fluorescens* BL915, which produces pyrrolnitrin, chitinase and HCN and protects seedlings from damping-off caused by *Rhizoctonia solani* (*53*). The *gacA* gene encodes a 24 kD protein similar to UvrY from *E. coli* and other response regulators in the FixJ family of two-component regulatory systems (*27*). In BL915, both *gacA* and a gene similar to *lemA* from *P. syringae* and other known bacterial sensor components, were necessary for production of antifungal metabolites. The products of these two genes may interact as a two-component system to coordinately regulate the expression of antifungal activities (*53*).

Plasmids pME3128 and pME3090 contain additional Phl-related loci from strain CHA0. The 11-kb fragment in pME3128 complemented the Tn*5* Phl^- mutant CHA625 to Phl^+ (*26*) and is distinct from the 22-kb insert in pME3090. The latter was selected initially for its ability to cause overproduction of pyoluteorin when reintroduced into wildtype CHA0; it subsequently was found to also increase Phl production by about 50% on malt agar (*28*). Overproduction of the two antibiotics is thought to be due to the increased copy number of the cloned gene(s), which may function as structural or regulatory elements of antibiotic synthesis or to increase the supply of biosynthetic precursor molecules.

The locus in pME3128 also differs structurally and genetically from that present in a 7-kb insert in pMON5122, described by Vincent *et al.* (*32*) as encoding gene(s) for Phl synthesis in *P. aureofaciens* Q2-87 (Keel, C.; Thomashow, L. S., USDA-ARS, unpublished data). All of 15 *Pseudomonas* recipients of pMON5122 produced Phl in vitro, indicating that this biosynthetic capability, like that for PCA, can be transferred readily among strains. In the growth chamber, Phl-producing transgenic derivatives of strains Q69c-80 and Q65c-80, like the PCA-producing derivatives described above, were more suppressive of take-all than the parental strains. However, Phl-producing transconjugants reduced disease severity whereas PCA-producing strains lessened disease incidence (Hara, H.; Weller, D. M.; Thomashow, L. S.; USDA-ARS, unpublished data), suggesting that the two kinds of derivatives may interact differently in situ with the take-all pathogen. Sequences in pMON5122 required for production of Phl have been localized to approximately 5 kb and shown to contain at least two transcriptional units by mutagenesis with the transposon Tn*3*HoHo1 and complementation analysis (*54*).

Two loci from *P. fluorescens* F113 involved in Phl synthesis have been cloned on 6-kb and 25-kb genomic fragments (*30, 55*). The 6-kb fragment in pCU203 partially restored Phl production to the Phl⁻ Tn*5* mutant F113G22 and transferred Phl biosynthetic capability to M114, one of eight nonproducer strains into which it was introduced. Strains F113(pCU203) and M114(pCU203) were more inhibitory to *P. ultimum* in vitro and increased sugarbeet seedling emergence in soil relative to the parental strains (*30*). Cell-free extracts from strain F113 have monoacetylphloroglucinol acetylase activity, which is thought to catalyze the final enzymatic step in a pathway proposed for the biosynthesis of Phl (*31*). This enzymatic activity has been shown to be encoded on pCU203 (*55*). Another Phl- nonproducing strain, E1/7, was able to produce Phl when transformed with both pCU203 and pCU204, but not with either plasmid alone. The 25-kb insert in pCU204 encodes a positive regulator for synthesis of Phl, HCN and protease (*55*).

Phloroglucinols and Phytotoxicity. Phloroglucinol derivatives are potent photosystem II inhibitors, with the two acyl and three hydroxyl groups on the phenolic nucleus all seen as structural features important for activity (*48*). Herbicidal activity, which resembles that of 2,4-dichlorophenoxyacetate (*42,44*), was reported initially in 1969 and more recently by Keel *et al.* (*26,56*). The minimal amount of Phl causing 50% inhibition of plant growth in vitro ranged from 8 μg/ml for cress and tobacco to 32-64 μg/ml for wheat, and monocots (corn, wheat) were more resistant than dicots (cress, cucumber, flax, tobacco, tomato) (*26*). Similarly, Phl at 16 μg/ml or less inhibited seed germination of cress, flax, tobacco and tomato by 50%; cucumber (128-256 μg/ml) and cotton (256-512 μg/ml) were more resistant; and wheat required in excess of 1,024 μg/ml for 50% inhibition. In a gnotobiotic system, the addition of 40 μg synthetic Phl/g soil reduced tobacco plant and root weight in the absence of the pathogen *T. basicola*; roots were stunted and deformed and showed increased root hair proliferation. In the presence of *T. basicola*, plant weight was drastically reduced regardless of the presence or absence of the antibiotic (*56*). The amounts of Phl and pyoluteorin (which also is phytotoxic) produced by wild type strain CHA0 in the rhizosphere were not sufficient to affect growth of tobacco, wheat (*26*), cucumber, sweet corn or cress (*28*) in the absence of pathogens. However, CHA0(pME3090), an overproducer of Phl and pyoluteorin, reduced the growth of cress and sweet corn by 51 and 32%, respectively, compared to plants colonized by the wild type strain. Cucumber was more resistant, and growth was unaffected by CHA0(pME3010). Both Phl and pyoluteorin probably contributed to the overall herbicidal effect, as pyoluteorin itself at 128μM reduced the fresh weight of cucumber, cress and corn by 72, 90 and 95%, respectively. In contrast, complete inhibition of *P. ultimum*, the target pathogen in these studies, occurred at 128 μM of pyoluteorin or 640 μM of Phl (*28*). These data clearly show how the balance between plant disease suppression and phytotoxicity depends on the kinds and amounts of metabolites produced by introduced strains and the relative sensitivities of the host plant and its pathogens to those metabolites.

Regulation of Antibiotic Production. Antibiotic synthesis by *Pseudomonas* spp. is highly responsive to the environment and is modulated by a complex hierarchy

of genes, as summarized in a scheme proposed in 1990 (*3*) for the regulation of synthesis of oomycin A. This structurally uncharacterized antibiotic is largely responsible for control by *P. fluorescens* Hv37a of pre-emergence damping-off of cotton caused by *P. ultimum* (*3,57*). Oomycin A synthesis is induced by glucose and requires the activity of glucose dehydrogenase (*58*), an enzyme representative of the many that function in catabolism and intermediary metabolism, and thereby influence antibiotic production without direct involvement in biosynthetic processes *per se*. Activation of glucose dehydrogenase and glucose sensing in HV37a requires the expression of *afuA* and *afuB*. These uncharacterized genes, as well as other positive and negative effectors including *cin* and *afuP*, comprise a second level of regulation that is functionally, and probably also structurally, conserved among antibiotic-producing pseudomonads. Mutations in *afuA, afuB* and *afuP* had pleiotropic effects on Hv37a function (*3*), suggesting that their products act globally, either as two-component sensor-response regulators (*27,52,53*) or as elements of the multigene systems and regulons that maintain cellular homeostasis (*59*). The *gacA* and *lemA* homologues (see above) that control secondary metabolism in strains CHA0 and BL915 are active at this level, and a similar pleiotropic locus, *phzP*, has been described (*23*) that is required for synthesis of PCA in *P. fluorescens* 2-79.

In the oomycin A model, the third and fourth levels of regulation are transcriptional and autoregulatory and affect *afuE*, a key structural gene in the biosynthetic operon. Expression of *afuE* is transcriptionally activated by *afuR*, an adjacent but divergently transcribed regulatory gene activated in turn by the product of *afuP* and by oomycin A itself. Synthesis of this antibiotic is therefore both positively autoregulated and cell cycle- or cell density-dependent (*3*). With limiting substrate the pathway is uninduced, conserving valuable resources but preventing accumulation of oomycin A to the threshold required for autoinduction. With nutrients to support growth, the cells can produce oomycin A to the level needed for maximal pathway expression. Such regulation is intuitively apppropriate in environments like the rhizosphere where nutrients may be transiently available.

The clustered, divergent arrangement of regulatory and structural genes at the oomycin A locus is conserved in the phenazine biosynthetic loci of *P. aureofaciens* 30-84 and *P. fluorescens* 2-79 (*36,37*; see above), and may prove to be a general feature among antibiotic biosynthesis loci in *Pseudomonas*. A second common aspect may be the dependence on cell density and autoinduction. In studies with isogenic wild type and Phz$^-$ mutants of strain 30-84, expression of the biosynthetic locus required accumulation of an autoinducer in the culture medium (*37*). The regulatory gene *phzA* in the biosynthetic locus encodes a protein (PhzA) of 27 kD similar to the transcriptional activators LasR of *P. aeruginosa*, LuxR of *Vibrio fischeri* and the 28-kD UvrC protein of *E. coli* (*36*). Significantly, LuxR and LasR have been implicated in intercellular communication and cell density-dependent expression of bioluminescense genes in *V. fischeri* (*60*) and of virulence genes in *P. aeruginosa* (*61*); in both cases, activity required the presence of a freely diffusible, homoserine lactone-containing autoinducer. Thus, a similar mechanism of regulation may apply to all of these pathways.

Siderophores

Siderophores are low molecular weight compounds that are produced under iron-limiting conditions, chelate the ferric ion (Fe^{3+}) with high specific activity, and serve as vehicles for the transport of Fe(III) into microbial cells (*62*). The yellow-green fluorescent siderophores produced by fluorescent pseudomonads also are known as pseudobactins or pyoverdines and are responsible for the characteristic pigmentation observed in cultures grown under iron limitation. The hypothesis that siderophore-mediated competition for iron is a mechanism by which introduced fluorescent pseudomonads antagonize soilborne pathogens assumes that conditions exist under which iron is a growth-limiting nutrient in the rhizosphere. This question has been examined extensively over the past 15 years and is the subject of many comprehensive reviews (*3,10,65*). Results of these studies, summarized briefly below, have sometimes substantiated and in other instances refuted the importance of siderophores in biological control. However, as the techniques used to study microbial activity in soil environments have become more sensitive and specific, it has become increasingly apparent that siderophore-mediated iron deprivation has a role in the control of only certain plant diseases, and then only when the chemical and physical factors that influence iron availability in soil are favorable to siderophore production and activity. Further, because iron influences the production of antagonistic metabolites other than pyoverdines, and its effects on growth and development differ among phytopathogens, mechanisms in addition to simple competition for this essential element also must be considered.

Early evidence for the effectiveness of siderophores came from studies demonstrating iron-regulated antagonism by fluorescent pseudomonads or purified pyoverdines against a wide variety of phytopathogens in vitro and in soil (*65-69*). Additional support came from work with nonfluorescent mutants of *P. fluorescens* and *P. putida*; such strains were less effective than the corresponding wild types in plant growth promotion (*70*) and protection of wheat against *P. ultimum* (*71*) and *G. g. tritici* (*72*). Recent studies have benefitted from the use of better-defined Tn*5* mutants with single-site transposon insertions that inactivate both pyoverdine production and biocontrol activity (*10,73-75*). One of the best-characterized strains is *P. putida* WC358, which suppresses minor pathogens of potato (*10,73,74*) and, when co-inoculated with the nonpathogenic *Fusarium oxysporum* strain Fo47b10, contributes to control of fusarium wilt of carnation caused by *F. oxysporum* f.sp. *dianthi* (*76*). The structure of the siderophore pseudobactin 358 has been elucidated (*77*), and genes for its synthesis (*78,79*) and uptake (*80,81*) have been identified and characterized. This pyoverdine is critical to the ability of WCS358 to intensify the antagonism of *F. oxysporum* Fo47b10 against pathogenic *F. oxysporum*. Siderophore-mediated iron limitation reduces the efficiency of glucose metabolism in both strains of fungi but the effect is more pronounced in the pathogen, rendering it less competitive for carbon substrates than the nonpathogenic antagonist (*82*).

Numerous reports based on comparisons of biocontrol activity of wild type fluorescent pseudomonads and their pyoverdine-deficient Tn*5* mutant derivatives have documented the *absence* of a role for pyoverdines in the control of certain

plant diseases. Examples include the suppression of take-all by *P. fluorescens* strains 2-79, M4-80R (*17*) and CHA0 (*25*), control of septoria tritici blotch of wheat by *P. aeruginosa* LEC1 (*83*), and protection by *P. putida* N1R and *P. fluorescens* Pf-5 against damping-off caused *P. ultimum* on cucumber (*84,85*). The pyoverdine siderophore produced by *P. fluorescens* CHA0 had no effect on germination of endoconidia and chlamydospores or mycelial growth of *T. basicola*, whereas the ferric pyoverdine was inhibitory, and in fact more toxic than free iron, perhaps because the concentration of available iron was increased to a toxic level (*86*).

Because iron so profoundly influences microbial metabolism in general and siderophore production and activity in particular, Loper *et al.* (*87*) have developed a novel biological sensor in order to determine iron availability to microbes in microhabitats on plant surfaces. The sensor, a reporter gene fusion termed *pvd-inaZ*, consists of an iron-regulated promoter isolated from a pyoverdine gene (*pvd*) and fused upstream of a promoterless ice-nucleation-activity gene (*inaZ*) from *P. syringae*. Ice nucleation activity is proportional to the amount of InaZ protein in the bacterial outer membrane, and in theory a single cell with an active ice nucleus can be detected on plant surfaces or in culture, making the reporter both quantitative and exceptionally sensitive. Cells of *P. fluorescens* harboring *pvd-inaZ* expressed ice nucleation activity differentially in response to the levels of iron available both in culture and in the plant rhizosphere, illustrating the potential value of this approach to clarifying the role of siderophores in microbial interactions *in situ*.

Volatile Compounds

Interest in the role of volatile compounds in the rhizosphere has centered on the production of hydrogen cyanide and the consumption by bacteria of metabolizable seed volatiles that may trigger the development and growth of fungal pathogens. Depending on the system under investigation, bacterial cyanogenesis in the rhizosphere may be either beneficial, resulting in pathogen suppression, or deleterious, causing plant growth inhibition and yield reduction. Studies have focused mainly on the suppression by *P. fluorescens* CHA0 of black root rot of tobacco caused by *T. basicola* (*25,86,88,90*), and the control by *P. putida* WCS358 of deleterious rhizosphere microorganisms (DRMO) associated with growth reduction of potatoes planted into high cropping frequency soils (*10,73,74,91*).

P. fluorescens CHA0 grown in vitro produced cyanide in quantities sufficient to inhibit *T. basicola* (*86,88*), and Hcn^- mutants constructed by gene replacement or deletion were less protective against black root rot than was the parental strain (*25,88*). Further, introduction of a clone containing hcn^+ genes from CHA0 to the weakly protective, noncyanogenic *P. fluorescens* P3 rendered the recipients Hcn^+ and increased their suppressiveness (*88*). The structural genes for cyanide synthase have been sequenced and shown to be under transcriptional control by anaerobic regulation (*52*). An early hypothesis that cyanide stress might stimulate the host plant's defense responses (*88*) now appears untenable; a *gacA* mutant of CHA0 defective in the production of secondary metabolites including cyanide was as effective on tobacco as CHA0 in inducing pathogenesis-

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related proteins and systemic resistance to tobacco necrosis virus (*89*). This suggests that cyanide controls black root rot by direct antagonism of *T. basicola.* The observation that an Hcn^- deletion mutant was as suppressive of *G. g. tritici* on wheat as CHA0 itself may indicate either that this pathogen is less sensitive to cyanide toxicity or that the wheat rhizosphere is less supportive of cyanide production, which requires a suitable precursor such as glycine and an adequate supply of Fe(III) (*88,90*).

Iron availability also may be central to the ability of *P. putida* WCS358 to protect potato from yield reductions associated with growth in short-rotation soils. It is postulated (*10*) that growth inhibition is due to the phytotoxicity of cyanide produced by DRMO in the rhizosphere, and that WCS358 reduces cyanide levels by making Fe(III) biologically unavailable for its production. Approximately half of *Pseudomonas* spp. isolated from the rhizosphere of potato produced cyanide, and in a gnotobiotic system supplemented with ferric siderophore, the Hcn^+ derivative of *P. fluorescens* P3 described above decreased potato plant growth relative to cyanide-nonproducing P3 (*91*). The cyanogenic P3 mutant was not deleterious to tobacco (*88,90*), suggesting that sensitivity differs among plant species. WCS358 appears uniquely suited to the role of iron scavenger, as it can not only produce and take up its own iron-complexed pseudobactin, but also those of other pseudomonads (*74*).

Bacterial antagonists may control certain seed rot and damping-off diseases through the consumption of metabolizable volatiles that are released by germinating seeds. Treatment of cotton seed or seed exudates with strains of *Enterobacter cloacae* greatly diminished their ability to stimulate germination of sporangia of *P. ultimum*, apparently by reducing the level of ethanol present (*92*), and *P. putida* N1R lowered the concentration of ethanol and acetaldehyde produced by germinating seeds of pea and soybean (*93*). Seeds treated with N1R had increased emergence and stimulated less hyphal growth of *P. ultimum* than did untreated seeds.

Induced Resistance

Induced disease resistance is an active plant defense process that depends on the host's physical or chemical barriers and is activated by biotic or abiotic inducing agents (*94*). Early studies (reviewed in *1,94,95*) had suggested that bacteria could induce systemic physiological changes in plants, but direct evidence that such changes might contribute to protection against spatially distant pathogens has only recently been reported. However, Fusarium wilt of carnation, induced by inoculation of the stems with *Fusarium oxysporum* f. sp. *dianthi*, was significantly reduced if the roots were bacterized previously with *Pseudomonas* sp. WCS417r. Stems of bacterized and infected plants accumulated phytoalexins more than did stems of nonbacterized, uninoculated plants, and the bacteria could not be isolated from stem tissue (*95,96*). Similarly, treatment of cucumber seeds with any of 6 PGPR strains protected emerging leaves that were subsequently challenge-inoculated with *Colletotrichum orbiculare* (*97*), and seed treatment with a strain of *P. fluorescens* reduced the number of foliar lesions caused by *P. syringae* pv. *phaseolicola* (*98*). In all three cases the PGPR strains and the pathogen were

spatially separated, eliminating the possibility that direct antagonism and competition were involved in the interactions. The mechanisms underlying bacterially-induced systemic resistance are only beginning to be investigated, but they appear to differ from those of classical induced resistance, at least in the cucumber/*Colletotrichum* model. Whereas the classical response required leaf treatment with any of a number of necrosis-causing pathogens, bacterial induction produced no root necrosis and was strain-specific (*94,97*). This specificity may be due to differences among strains in the production of metabolites translocated within plant tissues, as suggested in a report (*99*) that herbicolin A, a cyclic peptide antibiotic produced by *Erwinia herbicola* B247 on roots of wheat, was present in washed root and crown tissue, but it could not be detected at the root surface. Inducer metabolites need not be antibiotics, however (*89*; see above), as global inactivation of secondary metabolism in a mutant of *P. fluorescens* CHA0 did not diminish its ability to induce resistance to tobacco necrosis virus. Indeed, cell surface lipopolysaccharides (LPS), which are important determinants of antigenic specificity in gram-negative bacteria, have been implicated in the induction of resistance in carnation. Treatment of the roots with live cells of *Pseudomonas* WCS417, or with heat-killed cells or partially purified LPS, increased phytoalexin accumulation in the stems of plants inoculated with *Fusarium* and reduced the incidence of wilt (*95*).

Deleterious Rhizobacteria for Biocontrol of Weed Species

Although the concepts and strategies applicable to the development of bacterial agents for control of weeds are fundamentally similar to those for the suppression of plant pathogens, and intense efforts are underway to develop biocontrol agents for soilborne fungi, the potential for bacterial biocontrol of weeds remains virtually unexplored. Nevertheless, the limited evidence available supports the feasibility of such investigations. Deleterious rhizobacteria (DRB) are a major component of the rhizosphere populations of important crop plants including sugar beet (*9*), wheat (*100*) and potato (*10*); they have been isolated from the roots of important weed seedlings (*101,102*); and they probably are ubiquitous and common to all plant root systems (*9,101*). Such DRB may exhibit considerable target plant specificity; of over 1000 isolates from soils of winter wheat cropping systems, 81 were active against downy brome but did not reduce growth and development of winter wheat (*102*). A particularly promising isolate, *P. fluorescens* D7, reduced shoot growth and seed production in downy brome by as much as 53% and 64%, respectively, in field trials in Washington state; weed control was accompanied by winter wheat yield increases of up to 35% (*102*). Phytotoxic activity was present in an extracellular aggregate from cultures of D7 that contained mainly lipopolysaccharide and at least three different polypeptides of four to ten amino acids each (*103*).

Future Prospects

Challenges to the exploitation of rhizobacteria as biocontrol agents of agricultural pests, whether fungal pathogens or weeds, are largely the same and include all

aspects of strain identification and improvement, risk assessment, and the development of cost-effective production and formulation technologies. For the current generation of fungal biocontrol agents, elucidation of the identity, structure and regulation of the secondary metabolites involved in pest control are expected to provide a rational basis for adaptation or enhancement of activity of the existing strains. Thus, it may be possible to achieve more uniform performance under field conditions either by tailoring strains to perform in diverse soil types or by modifying the rhizosphere environment, e.g. by the addition of micronutrients, to enhance bacterial viability and competitiveness or to increase the production of biologically active metabolites. Advances in genetic manipulation will facilitate creation of biocontrol agents that are less dependent on environmental triggers for the production of active metabolites, that produce metabolites in larger quantities, or that produce metabolites in combinations that act synergistically or against a wider range of target pathogens. Ultimately, the ease with which genes for at least some biosynthetic pathways can be mobilized supports the feasibility of expressing them conditionally in a tissue-specific, wound-inducible manner in plants, assuring the production of metabolites in the sites where they can function most effectively.

For control agents of weeds, screening programs based directly on toxicity to target plants should to lead to the isolation of candidate strains that are effective and highly host-specific. Also of interest are strains that can act in conjunction with other agents to increase their activity, in a manner reminiscent of the combined effect of fluorescent pseudomonads and nonpathogenic *Fusarium* spp. against pathogenic *Fusarium*. In this regard, Fernando *et al.* (*104*) recently presented evidence that fluorescent pseudomonads on the phylloplane of velvetleaf increased leaf necrosis and disease severity caused by the bioherbicide *Colletotrichum coccodes*; the effect was correlated with increased appressoria formation by *C. coccodes* and apparently was due to iron sequestration by the bacteria. Finally, as we are reminded by Cutler (*105*), all biologically active secondary metabolites can be considered novel templates that may be altered by biotransformation or chemical modification, potentially enhancing properties such as activity or specificity while retaining desirable attributes such as biodegradability. A genetic approach to template modification also should be considered for metabolites produced by rhizosphere bacteria, many of which are amenable to genetic manipulation.

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Chapter 25

Natural Products with Potential Use as Herbicides

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There is great incentive to discover effective and economically feasible herbicides that are toxicologically and environmentally benign. Several hundred active ingredients of herbicides are now sold world-wide. Virtually all of these compounds were discovered by screening compounds from organic chemical synthesis programs. Leads for the structure of one group of patented herbicides came from 1,8-cineole, a well known plant allelochemical. However, plants have thus far not been as lucrative as microbial products as a source of phytotoxic compounds with the potential for direct use as herbicides or as templates for new synthetic herbicide classes. Microbial phytotoxins have provided valuable information on potential molecular target sites for herbicides. This information has also been useful in probing the biochemistry of plants. Phosphinothricin (glufosinate when synthetic), a product of *Streptomyces viridochromogenes,* is a successful herbicide that is environmentally and toxicologically benign. Bialaphos, a tripeptide from *S. hygroscopicus* which degrades to phosphinothricin in target plants, is the only commercial herbicide produced by biosynthesis. Many other microbial products have been patented as herbicides and some of these are under active development. Most of these compounds are from non-plant pathogens; however, there is growing interest in utilizing plant pathogens as sources of herbicide leads. Tentoxin, cornexistin, and AAL-toxin are discussed in detail as examples of microbial toxins with the potential for herbicide development. An overview of natural phytotoxins that have been considered for herbicides is provided, as well as considerations of the pesticide industry in using this approach.

In Allelopathy; Dakshini, K., et al.;
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Secondary compounds of plants and microorganisms have been known to be toxic to insect, microbial, and weed pests for many years. Such compounds have been used successfully, either in their natural or a modified form, as insecticides, fungicides, and herbicides. This chapter will deal only with their use as herbicides or leads for new herbicides, a topic that has been previously reviewed with emphasis on plant products (*1-4*), microbial products (*5-10*), or both (*11-16*).

There is increasing interest in this topic because of the perceived need to decrease our dependence on synthetic pesticides, of which two-thirds by volume of those used in agriculture are herbicides. Along with this perception is the view that naturally-produced compounds are inherently more toxicologically and environmentally benign than are synthetic chemicals. Pesticide manufacturers are interested in natural phytotoxins as sources of compounds that can: 1) be used directly as herbicides; 2) be used as leads for new chemical families of herbicides; and 3) provide clues to new molecular sites of herbicide action on which to base biorational design strategies.

Discovery Factors

Beginning with 2,4-D, synthetic herbicides have been used for approximately 50 years. Virtually all cropland used for agronomic crops is treated with at least one herbicide annually in developed countries. Thus, the herbicide market is largely a replacement market, with better and more desirable herbicides replacing older compounds. The replacement compounds may be more desirable because of improved performance, environmental and toxicological properties, and/or economics. Since this is an international replacement market, competition between herbicide-producing companies is intense.

Virtually all successful herbicide discovery efforts have centered around synthesis of organic compounds and testing them initially for biological activity in greenhouse-based whole plant bioassays. When interesting biological activity is discovered, structure-activity relationship studies are conducted to maximize desirable properties of the new chemical lead. This strategy has been highly successful in producing a diverse array of synthetic herbicides with generally increasing safety and target activity. Despite the success of this strategy, it is increasingly difficult to generate new, viable herbicide leads with this method. How much of the diminishing returns is due to increased performance criteria in the competitive replacement market and how much is due to limitations on the types of compounds produced by organic chemists is not clear. However, the need for new, more focused discovery strategies is obvious. Two other methods of herbicide discovery are being employed.

The first of these is biorational design; that is, designing a herbicide around a molecular site of action. For example, one might synthesize a range of substrate or transition-state analogs for an enzyme target site, with the objective of discovering a potent inhibitor with concomitant herbicidal activity. This strategy has been successful in drug discovery and design, but there is no public acknowledgment of success with this method for herbicide discovery.

The other alternative is to rely on nature to provide new compounds for use as herbicides or as leads for new herbicides or new herbicide sites of action. Natural products have been a very successful source of new insecticides and fungicides (*e.g., 17, 18*). However, this strategy has met with limited success for herbicides. There are three divergent approaches to natural product-based herbicide discovery efforts.

Naturally-occurring compounds can be randomly screened for their activity as phytotoxins, a biological rationale can be used to focus on fewer compounds for which there might be a higher probability of success, or known natural phytotoxins can lead to exploitation of new molecular sites of action for biorational design. All three methods have been used, but it is unclear which method is more effective. Herbicide discovery groups routinely obtain natural products for their screening programs, sometimes with suspected or reported activity and sometimes not. Fermentation broths of exotic microbes are screened for herbicidal activity, and phytotoxins are isolated if the culture broth has such activity. Less effort has been made to isolate toxins from plant pathogens suspected or known to produce phytotoxins or from plant species suspected to produce allelochemicals involved in interspecific competition. However, the majority of the discovery effort with natural products has been with microbial products, perhaps because plants are less likely to produce extremely potent phytotoxins.

New molecular sites of herbicide action are of growing importance for several reasons. Herbicides with new sites of action can be used to combat herbicide resistance. The evolution of weed resistance to many commercial herbicides has become a matter of concern to herbicide manufacturers, weed scientists, and farmers. Biotypes of many species have evolved resistance to numerous herbicides and herbicide classes (*19, 20*). The problem is exacerbated by weeds that are cross resistant to all or many herbicides with the same molecular target site and/or weeds that are multiply resistant to herbicides with different molecular sites. Another reason for interest in new sites of action is that a new site of action will almost certainly provide a company with a unique product which may be protected from competitors if the number of compounds active at the site is not so large that competitors can find loopholes in the patents. Lastly, development of a herbicide site of action lead provides the opportunity to manipulate the site of action in crop plants to produce herbicide-resistant crops with which the new herbicides will be selective (*21, 22*).

The molecular sites of action of most commercial herbicides are known, whereas those of relatively few natural phytotoxins have been determined. Nevertheless, the known molecular sites of action of natural phytotoxins are almost all different from those of commercial herbicides (Table I). This observation supports the view that natural products are particularly good sources of phytotoxins with unique molecular sites of action.

Discovery of natural products with potential for use as herbicides or herbicide leads is complicated by three factors: overly complicated chemical structures, extremely small amounts of compound available for bioassays, and rediscovery of known compounds.

Table I. Known Molecular Sites of Action of Commercial Herbicides and Natural Phytotoxins[a]

Site of Action	*Commercial Herbicides*	*Natural Phytotoxin*
Photosystem II	Many, including triazines substituted ureas, uracils, *etc.*	Cyanobacterin, aurachins
Protoporphyrinogen Oxidase	Diphenyl ethers and others	Not reported
Acetyl-CoA carboxylase	Aryloxyphenoxypropionates Cyclohexanediones	Not reported
Acetolactate synthase	Sulfonylureas, imidazolinones, sulfonanilides	Not reported
Tubulin	Dintroanilines, phosphoric amides	No potent ones
Phytoene desaturase	Many, including pyridazinones	Not reported
EPSP synthase	Glyphosate	Not reported
PS I	Bipyridiliums	Not reported
Dihydropterate synthase	Asulam	Not reported
Lycopene cyclase	Aminotriazole	Not reported
Glutamine synthetase	Glufosinate[b]	Many, including, phosphinothricin[b] bialaphos, tabtoxin
Cellulose synthase?	Dichlobenil	Phthoxazolin A?
CF_1 ATPase	Not reported	Tentoxin
Acyl transferase	Not reported	Fumonisins AAL-toxins
β-Cystathionase	Not reported	Rhizobitoxine
Ornithine carbamoyl transferase	Not reported	Phaseolotoxin
Aspartate amino transferase	Not reported	Gostatin, perhaps cornexistin
Acetyl-CoA transacylase	Not reported	Thiolactomycin
Plasma membrane ATPase	Not reported	Fusicoccin
3-Oxoacyl-ACP synthase	Not reported	Cerulenin
ALA synthase	Not reported	Gabaculine
Jasmonic acid receptors	Not reported	Coronatine

[a]Taken from refs. *6*, *15*, and *23-28*.
[b]Glufosinate is the synthetic version of phosphinothricin.

Phytotoxic natural product chemical structures can range from the very simple to the extremely complex. Determination of the structures can be a

formidable task; however, modern instrumentation for isolation and chemical structure determination has greatly reduced the effort required for this discovery step.

Plants and microbes usually produce biologically active compounds in very low yields. Traditional bioassays for synthetic herbicide tests require several grams of product. Isolated secondary products are rarely available in such amounts. Thus, microbioassays that are not as conclusive as standard screens are generally used in screening synthetic compounds. *Arabidopsis* and/or small-seeded monocotyledonous plants grown in microtiter plates on agar containing the test compounds are used by some. Small aquatic plants, such as duckweed, in microtiter plate wells have also been used. Other microbioassays are available. Most companies prefer microbioassays in which the compound is tested on a whole, photosynthesizing plant.

Rediscovery of already known phytotoxins can be costly. Compounds are generally tested before structure is determined. If a compound tests positive in a bioassay, the decision as to whether to determine the structure is made. If this sometimes costly effort is made and the compound is already known, considerable time, money, and effort will have been wasted. This has been reported to be a major problem and concern by at least one company (*29*). Bioassay and chemical profiles of known natural phytotoxins can help to eliminate such duplication.

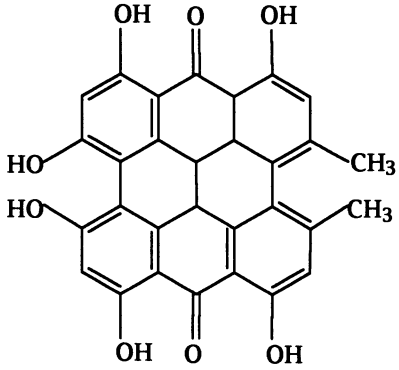
In this review, we will provide some of the more important examples of natural phytotoxins and some examples from our own work.

Phytotoxins from Plants

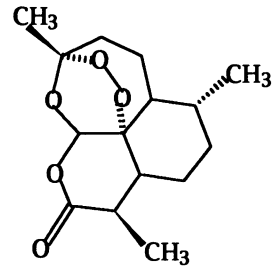
Many compounds produced by plants that inhibit the growth of other plants have been discovered. For most of these compounds, there is little or no evidence that they play an ecological role in plant-plant interactions. Thus, there has been no evolutionary selection for phytotoxicity for many of these compounds, and this is reflected in their relatively weak herbicidal properties, compared with those of many microbial phytotoxins. This has led to relatively little interest by the pesticide industry in plant-derived compounds as herbicides, despite a strong interest in them as insecticides and fungicides (*4*), biological activities for which there apparently has been much more natural selection.

Nevertheless, plants produce thousands of secondary compounds with novel structures. Regardless of function, the more phytotoxic of these compounds might be expected to be sequestered by the plant into plant parts that do not come into contact with plant cytoplasm in order to prevent autotoxicity. This is apparently the case with artemisinin (Figure 1), a sesquiterpenoid hydroperoxide produced by annual wormwood (*Artemisia annua* L.). For a plant-produced compound, artemisinin is highly phytotoxic and is autotoxic (*30-33*). Artemisinin and artemisitene, another phytotoxin, are entirely localized within the subcuticular space of capitate glands that cover the epidermis of this plant (*34, 35*). Other highly phytotoxic compounds, such hypericin (Figure 1) (*36, 37*), are also apparently localized in glandular or internal oil cavity locations, away from tissues where they could do harm. Thus, a biorational approach to

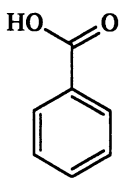
discovery of phytotoxic compounds of plants might be to concentrate on compounds that have been sequestered.



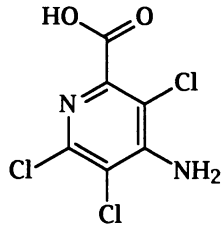
hypericin



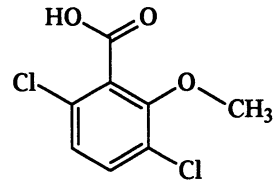
artemisinin



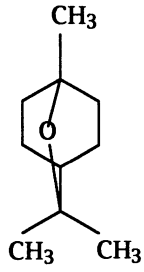
benzoic acid



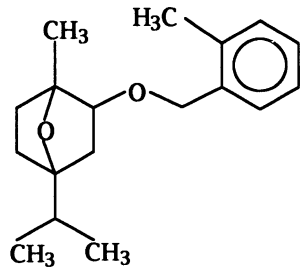
dicamba



picloram



1,8-cineole



cinmethylin

Figure 1. Some plant-derived phytotoxins and their derivatives that are mentioned in the text.

Hypericin is a red, photodynamic pigment that is toxic to all living organisms due to its photosensitizing nature. Its lack of selectivity between plants and animals would preclude its use as a herbicide.

With modification, some of the plant compounds with weak phytotoxicity might provide the basis for new herbicides that function

entirely differently than the unmodified plant-produced compounds. For example, one might consider the highly potent halogenated benzoic acid herbicides such a dicamba or picloram to be modifications of the plant product benzoic acid (Figure 1).

One of the first and most potent phytotoxins to be well studied in plants is 1,8-cineole (Figure 1). This compound is produced by many plant species and has been implicated in plant-plant allelopathy (*38*). It is quite phytotoxic, but it is also too volatile to be used effectively as a herbicide. Modification of 1,8-cineole led to cinmethylin (Figure 1), a synthetic herbicide that underwent some product development (*39, 40*), but was never marketed.

Camphene, a relatively weak phytotoxin from plants, when polyhalogenated to produce a witch's brew of almost 200 permutations called toxaphene, was sold as both an insecticide and a herbicide (*41*). Toxaphene was removed from the market because of toxicology concerns. However, the phytotoxic compound(s) in the mixture may have passed toxicological review if they could have been isolated and identified.

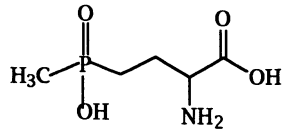
In summary, higher plants have not yielded many compounds that have generated interest in the herbicide industry. Algae, a diverse group of aquatic plants and microbes, might hold more promise. Algae produce a wide range of secondary products with biological activity (*42*), and many of these compounds from marine algae are halogenated. Few of these compounds have been tested for phytotoxicity. The only reported potent algal phytotoxins of which we are aware will be mentioned in the next section, as the algae producing them are microbial algae.

Phytotoxins from Microbes

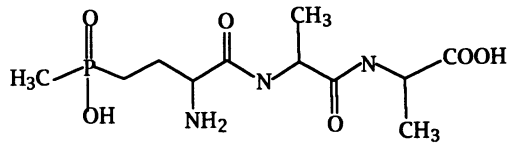
Microbes have been a lucrative source of phytotoxins with the potential to lead to new herbicides. Good candidates have come from soil microbes, saprophytic microbes, plant pathogens, and microbial algae. This topic has been the subject of numerous reviews (*e.g., 5-10, 43-46*). Despite the activity in this area, only two commercial herbicides, glufosinate and bialaphos, are known to have resulted from microbial phytotoxins. Glufosinate is the synthetic version of phosphinothricin (Figure 2), a potent inhibitor of glutamine synthetase produced by several soil *Streptomyces* species (*47, 48*). Bialaphos (Figure 2) is a tripeptide that is converted to phosphinothricin within the plant (*49*). Bialaphos is the only commercial herbicide produced by fermentation. Its cost of production has limited it to the Japanese market, whereas, the less costly synthetic version of phosphinothricin, glufosinate, is sold throughout the world. Other compounds from both non-pathogenic and pathogenic microbes are inhibitors of glutamine synthetase, however none are effective as phosphinothricin.

Many other microbial products have been patented as herbicides (*16, 50*), however, none have yet proved commercially successful. In this review we will only give some recent examples and developments with microbial phytotoxins with the potential for use as herbicides or as leads for development of new herbicides. Our emphasis will be novel

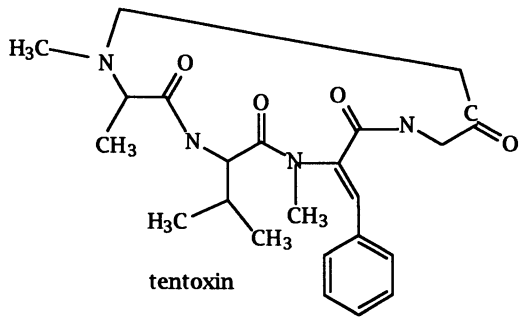
mechanisms of action and on structure-activity relationships, since these aspects are of great interest to those involved in herbicide development.



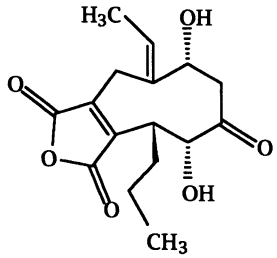
phosphinothricin or glufosinate



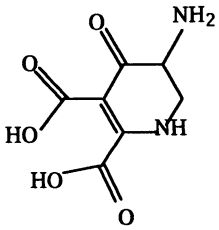
bialaphos



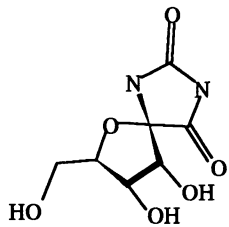
tentoxin



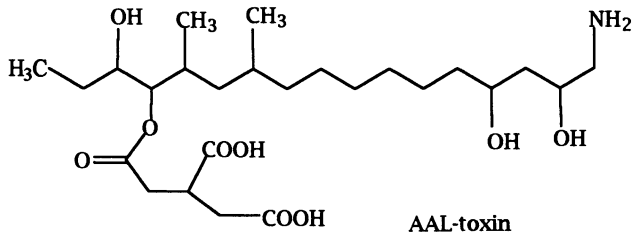
cornexistin



gostatin



hydantocidin



AAL-toxin

Figure 2. Some phytotoxins mentioned in the text that are produced by microbes.

Tentoxin (Figure 2) is a chlorosis-causing cyclic tetrapeptide produced by the plant pathogen *Alternaria alternata* and several other *Alternaria* species. Many important weeds are highly susceptible to tentoxin, whereas soybean and corn are resistant (*51*). Furthermore, it appears to have

unique molecular target sites. It prevents import of the nuclear-coded protein, polyphenol oxidase, into the chloroplast (*52*), and it inhibits chloroplast coupling factor ATPase activity (*53*). The two effects appear to be strongly related (*54*), perhaps because certain chloroplast envelope ATPase activities that are also inhibited by tentoxin (*55*) are involved in polyphenol oxidase import into the chloroplast.

Despite the attractiveness of tentoxin as a herbicide, the cost of producing tentoxin, either by biosynthesis or by chemical synthesis, prohibits its use as a herbicide. Considerable effort has been expended in attempts to produce simpler molecules with similar biological activity (*56, 57*). These structure-activity studies have been unsuccessful, resulting in the conclusion that only advances in production by fermentation have the potential to bring the cost of production to a level at which tentoxin might be competitive as a commercial herbicide. The genes for tentoxin production may exist on a virus that infects *A. alternata* (*55*), and attempts to isolate the genes for production in order to genetically engineer more amenable microbes for production, such as *Bacillus brevis,* are underway (A. R. Lax, personal communication).

Cornexistin (Figure 2) is a patented herbicide extracted from the saprophytic basidiomycete *Paecilomyces variotii* (*58, 59*). It is active against dicotyledonous weeds and some monocotyledonous weeds. However, corn is the only important crop in which it can be used selectively. It is much less toxic to mammals than the structurally-related rubratoxin B. It is relatively slow acting, like most herbicides that inhibit amino acid synthesis. Recent mechanism of action studies have suggested that it is metabolized to an active phytotoxin within the weed, where one or more isozymes of aspartate amino transferase are inhibited (*25*). A structurally related microbial phytotoxin, gostatin (Figure 2), has also been reported to be an inhibitor of this enzyme (*60*).

Hydantocidin (Figure 2) is a relatively new patented herbicide from *Streptomyces hygroscopicus* (*59, 61, 62*). Hydantocidin is highly effective as a herbicide against both monocotyledonous and dicotyledonous weeds. Its low fermentation yield has resulted in development of synthetic procedures that give good yields (*59, 62-64*). It is non-selective and, thus, could only be used selectively with crops if the crops can be genetically engineered to be resistant, as been done with other non-selective herbicides (*21*). The molecular site of action of hydantocidin is unknown, but it appears to be unique.

There has been renewed interest in AAL-toxin (Figure 2), a product of the plant pathogen *Alternaria alternata.* This relatively complex molecule was originally reported to be a host-selective phytotoxin to which only certain tomato varieties were sensitive (*65, 66*). However, most plant species are susceptible to this highly potent phytotoxin, with only certain tomato varieties being highly resistant (*24, 67-70*). Its molecular site of action was thought to be inhibition of plant nucleotide synthesis through inhibition of aspartate carbamoyltransferase (*71*). However, this mechanism of action could not be confirmed (*69, 72*), and newer evidence strongly indicates that the phytotoxicity of AAL-toxin is through the same mechanism as that of the *Fusarium* mycotoxins, the fumonisins, that cause damage to mammalian cells (*24, 73*).

Both fumonisins and AAL-toxins are analogs of the sphingoid base substrates, sphingosine (phytosphingosine in plants) and sphinganine, for the enzyme ceramide synthase. These fungal products apparently competitively inhibit this enzyme in animals (*74*) and plants (*75*), causing massive accumulation of sphingoid bases in both (*73, 76*). Sphingoid bases are cytotoxic to mammals (*77*) and phytotoxic to plant cells (*78*), apparently contributing to or causing the toxic effects. AAL-toxin is about ten-fold more toxic to plant cells than fumonisins (*78*), and has been patented as a herbicide (*79*). Only limited tests of AAL-toxin's toxicity to animals have been made (*80*). It is possible that AAL-toxin or an analog could be safe enough for use as a herbicide. Even if this fungal toxin or a compound with similar structure is never used as a herbicide, AAL-toxin has demonstrated that this molecular target (ceramide synthase) is a very effective one.

These are but a few of the microbial secondary products that are of interest because of their herbicidal activities. By chance, the compounds mentioned here reflect several of the developmental factors and concerns that the pesticide industry must consider and deal with in herbicide development.

Development Factors

Production costs are a critical factor in deciding whether to commercialize a natural product as a herbicide. Numerous promising natural herbicides have been abandoned because of the high cost of synthesis. Natural products can be produced by fermentation or synthesis. Modern molecular biology allows us to move the genes for biosynthesis of secondary products from one organism to another, so that plant-derived compounds can be produced by fermentation or microbial compounds can be produced by microbes more amenable to fermentative processes. Improved knowledge of the genetics of toxin production (*e. g., 81*) will allow genetic manipulation of producing strains to increase greatly toxin yield. As mentioned above, the herbicide bialaphos is produced by fermentation in Japan, and the less complex herbicidal derivative of bialaphos, glufosinate, is synthesized and sold throughout much of the world.

Biosynthesis by fermentation might be the only viable option for compounds with complex structures. However, this method of production may only be economically successful for specialized markets such as the rice herbicide market of Japan, where farmers can afford to spend up to US$200 per hectare on weed management chemicals. Furthermore, there are many technical problems in fermentation such as strain stability. Advances in biotechnology are improving the yield and quality of fermentation processes. Such advances will improve the prospects for production of natural compounds for use as herbicides.

Chemical synthesis is likely to be the only viable option for most compounds at this time. If the natural compound does not lend itself to economical synthesis, an active analogue may be amenable to synthesis. For example, the commercial herbicide methoxyphenone was the result of QSAR studies with anisomycin (*82, 83*). Nevertheless, the search for

simpler active analogs that might be economically synthesized has been unsuccessful for tentoxin (*57*), hydantocidin (*59*), and many other natural phytotoxins.

Natural compounds cannot be considered to be toxicologically benign, because some of the most potent mammalian toxins are natural compounds. Extensive toxicological studies have not been carried out on most of the natural products that have been proposed as potential herbicides. For the one compound discussed here for which data exists, AAL-toxin, we know that it may be a mammalian toxin (*79*). Ames (*84-86*) has estimated that many natural compounds, when tested in the same way that synthetic compounds are tested, would be classified as toxic on some level.

However, many natural compounds probably have a significantly shorter environmental half-life than most commercial herbicides. In some cases this could be problematic, because herbicides must persist long enough to kill the target weeds. One might generalize that significant contamination of food products, the soil, and water would be less likely with natural compounds than with most synthetic herbicides used at the same rates.

Summary and Prospectus

Natural products may be the most readily accessible source of novel compounds with biological activity toward plants. Tremendous effort has been expended in chemically characterizing thousands of natural compounds, yet comparatively little effort has been made to determine their herbicidal potential. Most of the information available on the phytotoxicity of natural products is not useful in evaluating their potential as herbicides. In only a few cases in which such compounds have been found to be phytotoxic has a mechanism of action been determined. In the few cases in which a molecular target site has been established, it has generally been one that has not yet been exploited by the herbicide industry. The potential for future discovery is enormous.

Some natural products will be useful without modification. Bialaphos and phosphinothricin (glufosinate) illustrate the potential of previously discovered compounds for development. Their success has increased the interest in natural products as herbicides. In other cases, modification of the structure of natural phytotoxins will improve their utility as herbicides. Those compounds with prohibitively complex structures may still provide valuable clues of unexploited molecular sites of action for those involved in biorational design of herbicides. Furthermore, structure-activity efforts might reduce the molecular complexity of natural compounds to produce more simple molecules that retain good herbicidal activity. A major advantage of these approaches is that they promise to produce new herbicides with previously unexploited molecular sites of action. The chances of finding new sites of action by exploitation of older chemical classes and by empirical screening are relatively low. With the rapidly increasing incidence of weeds evolving resistance to herbicides, new mechanisms of actions will be a highly desirable trait in herbicides of the future.

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There are, however, some formidable obstacles to success with the strategy of the use of natural compounds as herbicides or leads for new herbicides. They include the structural complexity of many natural toxins, the difficulties in working with very small amounts of materials, and the cost of rediscovering known compounds. The last two of these difficulties can be minimized with advances in technology.

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