THESIS

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METABOLITES OF SOIL ACTINOMYCETES AS A SOURCE OF HERBICIDE CHEMISTRY

By

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ABSTRACT

METABOLITES OF SOIL ACTINOMYCETES AS A SOURCE OF HERBICIDE CHEMISTRY

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Three antibiotics previously identified as inhibitory to germination and growth were tested for their effect on barnyardgrass (Echinochloa crus-galli L.) and cress (Lepidium sativum c.v. Curly cress). Antibiotics ranked most to least inhibitory were: cycloheximide >oxytetracycline >streptomycin. An isolation and screening procedure was developed to identify soil antinomycetes that are able to produce germination and growth inhibitors on agar plates. Nine isolates of 120 demonstrated severe toxicity on cress and barnyardgrass indicators. Several isolates were observed to stimulate seedling growth. Repeatability of the agar screening method was demonstrated, but the appearance of toxins in agar media was not always correlated to their production during submerged culture.

Streptomyces sp. 101, was isolated from a Houghton muck soil within a dense quackgrass (Agropyron repens (L.) Beauv.) rhizosphere. This isolate produced herbicidal compounds in an aerobic fermentation. Active compounds appeared in the fermentation broth after 2 days, peaked at 6 days and appeared slightly reduced by 8 days. A methanol extract oflyophilized broth was sprayed on several species of 10-day-old weed seedlings. Selective foliar toxicity was recorded with dicotyledonous species that were generally more severely injured than monocots.

Herbicidal compounds were separated and purified using thin layer, column and high performance liquid chromatographic techniques. The compounds were identified by mass spectroscopy and in the case of cycloheximide, co-cchromatographed with known material.

Greenhouse and field experiments were initiated to determine if plant growth could be adversely affected by soil inoculations with a toxin producing Streptomycete. In the greenhouse experiments, barnyardgrass (Echinochloa crus-galli L.) was significantly inhibited by soil inoculation, whereas cucumbers (Cucumis sativus L.) were unaffected. In a field experiment, a band of inoculum was spread over indicator rows of oat (Avena sativa L.) and sorghum (Sorghum bicolor) which were planted with a no-tillage method into desiccated cover crops of oat, rye (Secale cereale L.), sorghum x sudangrass (S.bicolor x S.vulgare) and no cover crop. Oats were significantly inhibited (21 % reduction in dry weight) by soil inoculations with the 101 isolate.

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There are some very special people in Michigan that have made my time here both enjoyable and unforgettable. You all have a place in my heart and friend when you need me. Thank you, all.

As always my parents Beatrice and Steve DeFrank have been a constant source of support, love and Italian food. This thesis is dedicated to you.

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INTRODUCTION

The antibiotic era began with the discovery of a penicillin producing microorganism by Fleming in 1929 (8). Since that time many compounds have been identified with a wide diversity of structures and applications (28). The current age of biotechnology is firmly based on the concept of synthesis of useful chemicals by microorganisms. A recent issue of SCIENCE (1) has been entirely devoted to the scope and application of this area of technology and it's impact in many aspects of human endeavor.

Pesticides have played an ever increasing role in the production of agricultural commodities and subsequently the search for new, safe and more effective compounds has been an ongoing research objective. The use of antibiotics as agricultural pesticides has heretofore been limited to the control of pathogenic diseases (19). Demain (7) offers several new applications of antibiotics among these being insecticides, herbicides and growth regulators. It appears that expanded use of antibiotics in all areas of pesticide application will depend on several factors: recognition of the diverse biological action of microbial metabolites, rapid and reliable methods of screening microbial metabolites with pesticidal activity and the collaboration of industrial biologists, soil microbiologists and agricultural pesticide specialists to develop control strategies that make use of not only purified compounds as pesticides but also producing organisms as inoculum to soils or living plants.

Rapid advances in biotechnology and large scale bioreactors can provide the agricultural researcher with new tools to control weeds, insects and diseases. The advantages of natural products would include chemical and production techniques that are less polluting, and less reliant on fossil fuel and other chemical inputs. This thesis will focus on the use of microbial metabolites as herbicides.

CHAPTER 1

LITERATURE REVIEW

Effects of microbial products on plant growth.

Numerous chemical companies devote a substantial amount of capital every year to search for, develop and market herbicides. Empirical screening of synthetic chemicals has been the major method of herbicide discovery. In 1983 the author is aware of only 2 herbicides of microbial origin being commercially evaluated (19). The diversity of metabolites produced by soil microorganisms is well documented (16,28) and will not be elucidated here. The focus of this review is on microbial metabolites which have growth-altering effects on plants.

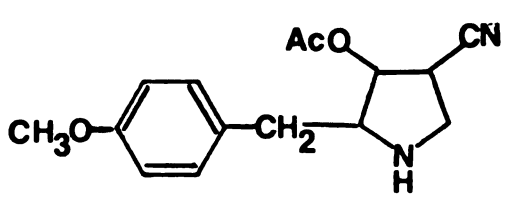
Early research with antibiotics investigated both their occurrence in natural soils and techniques for laboratory culture. The presence of antibiotics in the vicinity of soil organic matter and as protective treatment against disease attack naturally lead to investigation of their effects on plant growth and development. An early review (4) described the variety of responses obtained by exposing germinating seeds and living plants to antibiotic solutions. Thirty-eight antibiotics were discussed which provided plant responses ranging from inhibition of germination and growth, foliage desiccation and wilting. Consistent inhibitors of seed germination and root growth at concentrations of 5 ug/ml or less were: cycloheximide, azserine, alternaric acid and polymyxin. Nickel (20) examined the growth stimulating properties of twenty antibiotics using

concentrations of bacitracin, penicillin G, streptomycin and oxytetracycline had produced significant increases in fresh weight. At that time, severely toxic antibiotics were considered unusable as plant protective agents and their use as herbicides was not advanced.

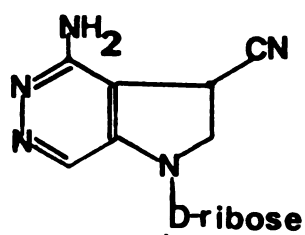
McCalla and Haskins (18) reviewed the topic of phytotoxic substances from soil microorganisms and crop residues. They examined a wide variety of research which involves the interaction of decomposing crop residues and associated soil microorganisms. Predominant factors in many cases of soil toxicity are the soil microorganisms which secrete toxins or alter plant compounds into toxic substances. Outlined are several studies in which soil microorganisms were isolated and shown to produce plant toxins in aqueous fermentation cultures. The authors suggested that the apparent potency of microbial metabolites may be at least as effective as currently used herbicides and other pesticides.

Lynch (16) reviewed the topic of soil microbial products and their effect on plant growth. Microbial metabolites are grouped as; growth regulators (gibberellic acid, ethylene and vitamins), phytotoxins (organic acids and antibiotics), soil stabilizers and enzymes. The potential to affect plant growth by manipulating microbial activity in the soil, using a crop rotation are suggested by the author. An undetermined aspect of previous work is whether rotational crop residues provide specific metabolites for the production of phytotoxins or if the extra carbon in the soil provides energy substrate for the growth of phytotoxin producing microorganisms.

These reviews provide substantial evidence that soil microorganisms, if properly cultured, could provide a vast array of metabolites with a broad spectrum of plant responses. Preliminary work which seriously considered the use of antibiotics as herbicides was recently initiated by several pesticide research groups in Japan (2,19,23,34). Yamada et al. (34) investigated the growth regulating activity of two antibiotics isolated from the fermentation broth of a steptomycete. Their objective was to discover a model substance of microbial origin which could then be chemically modified for use as a herbicide. Two active substances were identified as anisomycin and toyocamycin.



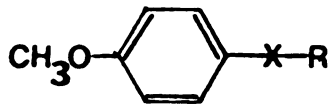
Anisomycin



Toyocamycin

The growth-inhibitory activity of these two antibiotics and two synthetic derivatives of anisomycin (n-acetyl anisomycin and deacetyl anisomycin) were evaluated on three monocotyledons and three dicotyledons. Anisomycin showed selective inhibitory activity to root growth at fairly low concentrations (6-12 ppm). The inhibitory activity of toyocamycin was less selective between shoot and root growth than that of anisomycin. The reduced activity of N-acetyl anisomycin demonstrated the necessity of the basic group for the inhibitory action of anisomycin. Two subsequent reports by Yamada (35,36) investigated the herbicidal activity as related to chemical substitution upon the base molecule, 4-methoxy benzophenones (34) and

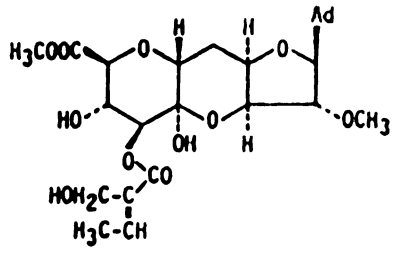
4-methoxydiphenylmethanes (36). Chemical substitution upon the ansiomycin base molecule are illustrated in the figure below:



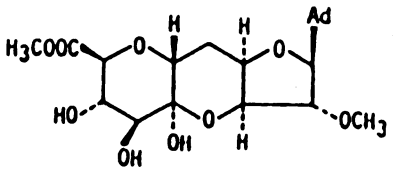
X=CH_2, 4, methoxydiphenylmethane
X= C=O, 4, methoxybenzophenones
R= acidic, basic or neutral groups.

These papers demonstrated that when searching for groups of compounds with herbicidal activity, synthetic alteration can improve or alter that molecules performance. Fujii et al. (9,10) continued this line of herbicide research with detailed studies of the mode of action of 3,3'dimethyl-4-methoxybenzophenone (9) and its herbicidal activity based on absorbtion, translocation and metabolism (9).

Arai et al. (2) reported on two new nucleoside antibiotics with potent herbicidal activity. Herbicidins A & B were isolated from the fermentation broth of Steptomyces saganonensis.



Herbicidin A



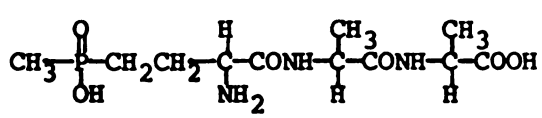
Herbicidin B

Ad=adenine

Both (A & B) antibiotics were tested as spray treatments on two-week old plants, five monocotyledons, eight dicotyledons and as seed germination inhibitors. These groups were composed of crop and weed species. Spray treatments of herbicidins showed an important selective activity in which the dicotyledonous plants were especially injured. Herbicidin A had a more selective effect between rice and other plants than Herbicidin B. These results indicate that antibiotics can be useful as contact herbicides with the ability to remove plant species selectively, a valuble characteristic of a herbicidal compound.

Takigachi et al. (27) discovered two additional herbicidins in the fermentation broth of Streptomyces saganonensis, herbicidins C & E. Detailed herbicidal activity was not described in this report. Omura et al.(23) isolated herbimycin from the fermentation broth of Streptomyces hygroscopicus. The antibiotic was tested on germinating seeds (preemergence system) and on two-week-old plants (postemergence system). Four monocotyledons and four dicotyledon species were treated. Potent herbicidal activity was recorded on both classes of plants. Rice, Oryza sativa, showed strong resistance and preemergence activity was greater than foliar activity.

Ogawa et al. (24) reported on the isolation of bialaphos from fermentation broth of Streptomyces hygroscopicus.



Bialaphos

This compound was reported to have wide spectrum herbicidal properties analogous to currently used herbicides paraquat (ICI) and glyphosate (Monsanto) (19). The herbicidal effects were discovered when it did not pass the screening test as a fungicide due to extreme phytotoxicity. Another phosphonated amino acid (glufosinate) is currently under development by Hoechst Co. It was discovered in batch culture, but is now being synthesized chemically. These reports illustrate the potential of antibiotics as herbicides. Currently in the U.S. there is no commercially available herbicide of microbial origin. It is clear that microbial metabolites and their derivatives may soon be appearing as commercial agricultural pesticides.

Misato (19) considers the reasons why the use of antibiotics as pesticides are expected to find increased usage in Japan. Several advantages were listed as follows: 1) antibiotics can be very potent and specific in their activity; reduced usage rates and easy decomposition within the environment could reduce non-point source pollution usually associated with agricultural practices of the past. 2) With regards to their manufacture, chemical plants with large scale fermentors can produce a variety of antibiotics by changing the producing organism and/or the culture conditions. This would allow the expansion of marketable products without a significant increase or change in capital investment. Also, microorganisms often produce rather complex structures easily while synthesis of such compounds involves many sophisticated manufacturing processes. 3) Agricultural antibiotics are produced by the consumption of plant products (corn

steep, sugar beet pulp and molasses (38) and thus the industry is fueled by a renewable resource.

Limitations for the use of antibiotics in agriculture also exist: 1) difficulty in microanalysis and identification of active compounds. 2) development of pest resistance. 3) regulatory constraints based on antibiotics traditionally viewed for medical purposes. As pointed out in a recent issue of Science (1) the dominance of biotechnology in the development and manufacture of new drugs and pharmaceuticals will become increasing obvious as advances in this rapidly changing field of applied biology come into being. Since progress in the area of antibiotic herbicides has received limited attention in the U.S. and since earlier allelopathic studies implicated a contribution of microbial products this research project was initiated. The diversity of soil microorganisms seems a promising reservoir of herbicidal metabolites.

Factors affecting antibiotic production

All of the herbicidal antibiotics discovered to date were from fermentation broths of various members of the order Actinomycetales. An agar screening technique for the detection of microorganisms that can produce herbicidal metabolites has not been previously described.

Several reports have indicated that certain streptomycetes which produced antibiotics on agar plates failed to produce them when cultured submerged conditions. The antibiotic, fumaramidmycin produced by Streptomyces kursanovii (17) was produced on agar plates but not in aerobic fermentation. Inactivation was caused by the

antibiotic contacting the vegetative mycelium. Physical separation of the antibiotic and vegetative mycelium by the agar preserved activity in that system. Shomura et al. (26) found that of all isolates producing antibiotics on agar plates, 1.9% could not produce an active compound in submerged culture. These examples indicate that the initial search for herbicidal antibiotics should be conducted with the producing organisms growing on agar plates. Even without the ability to produce an active compound in submerged culture, the discovery of active producers may allow elucidation of unique chemical structures and for the option for genetic manipulation to obtain a desired organism.

The occurrence of many antibiotic producing strains in the soil actinomycetes (28) provided the primary reason for screening these organisms for antibiotic production. Other groups of soil organisms have also demonstrated the ability to produce herbicidal metabolites (18). A procedure for isolating actinomycetes from soil has been developed (13). A brief description follows: Soils collected from natural environments are air dried to reduce the viable number of bacterial cells. Incubation of dried soils mixed with $CaCO_3$ under humid conditions reduces the growth of fast growing fungi. Soil thus treated and plated out from serial dilutions to an agar of minimal nutrient composition results in easy identification and isolation of actinomycete colonies. The details of the agar screening method (ASM) for the detection of microorganisms producing herbicidal metabolites will be presented in the materials and methods section (Chapter 2).

Plant pathologists were among the early researchers who attempted to alter the microbial equilibrium in natural soils through the incorporation of cover crop residues. Hildebrand and West (14) evaluated strawberry (Fragaria ananassa Duchesne) growth in naturally infested root-rot soils into which consecutive crops of several agricultural plants had been incorporated. Crop residue treatments were compared with steam sterilized controls. The crops used to alter root rot incidence and severity were; tomato (Lycopersicon esculentum Mill.), corn (Zea mays L.), red clover (Trifolium pratense L.), soybean (Glycine max (L) Merr.) oats (Avena sativa L.), rye (Secale cerale L.) timothy (Phleum pratense L.) and rape (Brassica campestris L.). Significant changes in representative groups of fungi, nematodes and bacteria were recorded in the various soil treatments. The incidence of strawberry root-rot in soils with manure, corn, red clover, timothy or no organic matter input increased in this respective series. Incorporated soybean residues and sterilized soil produced strawberry plants which were free of disease. West and Hildebrand (30) continued work in this area and described the contributing factors of soybean decomposition which reduced disease severity of the root-rot complex. Glucose and acetic acid could be substituted for soybean decomposition in reducing disease severity. Additional aspects of these reports serve to indicate the dynamic nature of biological activity in soil and the ability of decomposing organic substrate to shift relative levels of microbial populations. Rouatt and Atkinson (25) recorded the effects of three plant materials (rye,soybean and sweet clover(Melilotus sp.) on the microbial balance

of potato (Solanum tuberosum) scab (Streptomyces scabes) in natural soils. Soybean residues reduced the incidence of potato scab and increased the levels of bacteria requiring simple nutrients on an agar plate. Rye and clover caused little to no reduction in scab and increased bacterial populations of those with more complex growth requirements. Sustained changes in soil pH were also developed with various plant residues.

Alterations in microbial populations to improve the "health" of the soil may have been the basis for early recommendations of specific crop rotations. In addition to studying shifts in microbial populations, many articles discussed the ability of various organic substrates to support the growth and antibiotic production of soil borne fungi and actinomycetes. Grossbard (12) evaluated the production of patulin by Penicillium urticae Bainer on sterilized organic substrate and sterilized soil amended with wheat straw, sugar beet pulp and glucose. The substrates studied for the support of fungal growth and antibiotic production were; bracken (Pteridium aquilinum Kuhn.), red fescue (Festuca rubra L.), white mustard (Brassica hirta Moench.), red clover, Italian ryegrass (Lolium multiflorum Cam.), lawn mowings, lucerne (Medicago sativa L.), saniform (Onobrychis viciaefolia Scop.), timothy grass, wheat straw, manure and sugar beet pulp. Materials which consistently supported antibiotic production were wheat straw, timothy grass and sugar beet pulp. Substrates which varied in their adequacy for antibiotic production were red clover, red fescue, white mustard and rye grass. The district in which the

crops were grown and the time of year of plant harvest appeared to affect the substrates ability to support antibiotic production. The addition of glucose increased the antibiotic titre in every case. The duration of antibiotic production also varied with different plant materials. The leguminous crop materials were generally unsuitable for the formation of stable antibiotics. Increasing amounts of antibiotic were formed in sterile soils amended with wheat straw, sugar beet pulp and glucose, respectively. Unsterilized soils could not produce the antibiotic of the inoculating organism as measured by the techniques (inhibition of bacterial growth) employed in this report. All results suggest that available carbohydrate is the prime requirement for antibiotic production on all substrates tested.

Gregory et al. (11) studied the required soil conditions for antibiotic production and the control of several damping-off fungi. Amendments which allowed for antibiotic production in non-sterile soils included, 0.5% soybean meal, 0.5% glucose, 0.2% corn steep and the addition of $CaCO_3$ to give a pH of 6.7. Activity peaked at 4 days after inoculation and decreased rapidly upon subsequent incubation. Only P.urticae and a Bacillus sp. could produce an antibiotic in a non-sterile soil. Antibiotic titre in non-sterile soils was always lower than the titre in sterile soils. The data indicates that antibiotic production in the soil occurs only when readily available food substrates are present at the proper pH. When comparing reports of antibiotic production in non-sterile soils it is important to note the growth form of the organism when applied to the soil. In some cases spore suspensions are used (12), in others vegetative mycelia are

used (11). What has not been critically compared is the form of the microbial inoculum (spores, fresh mycelia or dormant mycelia on an organic carrier) and its importance in establishing a desired population in the soil. Another aspect which has not been clarified is the preconditioning effects of organic substrates on microbial populations before the addition of an antibiotic producing organism.

Wright (31) studied the production of gliotoxin by a strain of Trichoderma viride, under various soil conditions. The effect of autoclaving soil was studied in detail to determine its ability to enhance antibiotic production. Increased antibiotic titre of autoclaved soils was not totally due to the removal of competing organisms. Beneficial effects of steam sterilization became marked after a period of heat treatment in excess of that required to kill the microflora. Increased nitrogen levels could not account for enhanced gliotoxin production. Results suggested an increase in available energy yielding carbon compounds as a primary stimulator. pH was again found to have a significant effect on antibiotic production but results varied with different soil types. Unautoclaved soils, amended with clover and wheat straw supported gliotoxin production. Wright (32) continued studies of gliotoxin formation in the soil and found sites of activity confined to the immediate area of the soil amendment. The pH of the amendment was found to be more important for gliotoxin production than the soil itself. Spores of T. viride remained viable and able to produce antibiotic for up to eight weeks. The production of antibiotics on seed coats of three

plant species (white mustard, corn and pea (Pisum arvense L.) was demonstrated by Wright (33). Seeds inoculated with spores of T. viride and Penicillium frequentas resulted in antibiotic production peaking at 4-6 days after incubation in non-sterile soils. Natural colonization of uninoculated peas by T. viride resulted in seed coat production of gliotoxin. It is clear that seed coats can provide an adequate environment for the growth and antibiotic production of soil microorganisms. If weed seeds could be selectively colonized by organisms secreting an antibiotic that is phytotoxic, soil inoculation with such an organism could provide a form of preemergent weed control.

Dawson et al.(6) describes the distribution of soil microorganisms under two soil management systems; plowing (soil well mixed in the 0-12 cm soil layer) and subtilling (plant residue primarily on the surface). Populations of soil fungi, bacteria and actinomycetes were determined at two soil depths and within three cropping schemes. Generally, the evidence for the various rotations indicates that when crop residues are allowed to decompose on the soil surface, greater numbers of anaerobic microorganisms develop in the top 2.5 cm layer than when residues are plowed under. Plowing dilutes the effect of residues with regards to population concentrations in the top 2.5 cm soil layer. The stimulating effects of the plant resiidues peaked in the first month after application and had disappeared within nine months. This paper clearly and convincingly demonstrates that microorganism populations can be stimulated by placement of plant residues on the soil surface . Large amounts of plant residues can be retained on the

soil surface with a no-tillage method of crop production (37) This developing cultural practice can establish a dynamic soil environment in which manipulation of microbial populations can be attempted through the selection of certain plant residues as a mulch coupled with microbial inoculations, whose metabolites are of a pesticidal nature. The size, timing and form of the microbial inoculations to develop significant and repeatable field results will require a substantial research effort. An initiation of this effort will be reported later in this thesis.

Norstadt and McCalla (22) reported on the environmental prerequisites for the induction of phytotoxicity in non-sterile soils. Soils mixed with wheat straw showed two specific peaks in phytotoxicity on corn plants were used to follow decomposition over a 35 day time period. In a related experiment, soil and straw were mixed on a staggered schedule to obtain incubations of 7, 11, 14 and 18 days before the addition of spores of the patulin producing fungus Penicillium uriticae B.. Patulin is a potent phytotoxic substance (21). Results indicated that incubations of 11 to 14 days provided suitable substrate for the proliferation of P. uriticae B. and subsequent patulin formation. In field applications of microbes for pest control, their dormant inocula could remain viable in the soil until the proper environmental cues for metabolite production occurred.

Krasilnikov (15) reviews Russian research in the area of plant nutrition as it relates to metabolites of associated soil microorganisms. Many instances of plant uptake of microbially produced amino acids, vitamins and antibiotics from natural soils are reported. Antibiotics either added directly to soils or produced by actinomycetes were found to accumulate in roots and leaves of corn, peas and wheat. Current methods in chromatography and immunoassay of very small amounts of compounds (29) would make the detection of microbial metabolites, absorbed from the soil by plant roots, a routine procedure. Living plants could then function as biological traps of microbial metabolites. The absorption and detection of microbial metabolites within plant tissue could provide a unique method of studying factors affecting the production of these compounds in undisturbed natural soils at biologically significant levels. Berestetskii et al. (3) studied phytotoxic substances produced by Penicillium granulatum in liquid culture and non-sterile soils. Results indicated that the toxin produced in liquid culture was similar to that produced in spore inoculated soils after a 10-13 day incubation period. The fungal metabolite was toxic to most of the plant species evaluated.

To use soil inoculations to affect a level of weed control in no-tillage cropping systems a substantial research effort must be completed. A prerequisite for developing a marketable inoculum is the mass production of a viable biological unit which can be evenly distributed across a crop field. A recent book chapter by Churchill (5) discusses the considerations and successes in the mass production

of fungal spores of Colletotrichum gleosporioides for use as a microbial herbicide. This fungus has been shown to be effective in the control of northern joint vetch Aeschynomene virginica, a serious weed in rice and soybeans in certain areas. Churchill explains the application of modern fermentation technology methods and equipment in the production of large quantities of viable fungal spores. The introduction of spores or other forms of inocula into agricultural fields, predisposed for the proliferation of that organism and the production of its desired metabolite, may lead to new strategies in the biological control of diseases, insects and weeds.

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CHAPTER 2

SCREENING PROCEDURES TO IDENTIFY SOIL-BORNE ACTINOMYCETES WHICH CAN PRODUCE HERBICIDAL COMPOUNDS

ABSTRACT

Three antibiotics previously identified as inhibitory to germination and growth were tested for their effect on barnyardgrass (Echinochloa crus-galli L.) and cress (Lepidium sativum L. c.v. Curly cress). Barnyardgrass germination and growth was more sensitive to the inhibitory effects of the antibiotics than cress. Antibiotics ranked most to least inhibitory were: cycloheximide> oxytetracycline >streptomycin. An isolation and screening procedure was developed to identify soil antinomycetes that can to produce germination and growth inhibitors on agar plates. Nine isolates of 120 demonstrated severe toxicity on cress and barnyardgrass. Several isolates were observed to stimulate seedling growth. The repeatability of the agar screening method was demonstrated. Organisms shown to produce toxins in agar media did not always produce inhibition when grown in submerged culture.

INTRODUCTION

Fleming's (6) account in 1929of the antibiotic activity of penicillin (produced by Penicillin notatum) began a new era in the discovery and use of compounds derived from microorganisms. Since that time, many compounds have been identified with a wide diversity of structure and application (18). The current age of biotechnology is firmly based on the concept of chemical synthesis by micro-organisms. A recent issue of SCIENCE (1) was entirely devoted to the scope and application of this area of technology and its impact in many aspects of human endeavor.

Soon after their discovery, antibiotics were considered for use as protective agents for plants against pathogenic attack. Wright (21) evaluated 12 antibiotics at various pH's to determine germination and root growth inhibition of three indicator species, wheat (Triticum aestivum L.), white mustard (Brassica hirta Moench.) and red clover (Trifolium pratense). In Wright's study, alternaric acid was the most toxic antibiotic to wheat and mustard but had much less effect on clover. Brian (3) reviewed the effects of antibiotics on plants, providing many examples of phytotoxicity expressed as germination, shoot/root growth inhibition, foliage toxicity and wilting. Norman (13) demonstrated the inhibition of root growth and cation uptake by antibiotics. L-cycloserine was found to be the most potent inhibitor of cucumber (Cucumis sativus L.) root elongation as well as root dry weight accumulation in barley (Hordeum vulgare L.). Suppression of K^+ uptake was also demonstrated but only at concentrations many times

greater than that needed to inhibit root elongation. Norman also pointed out the possibility of root zone colonization by antibiotic-producing organisms with a subsequent inhibition of host plant growth. In a recent article, Schroth and Hancock (15) proposed the exploitation of beneficial root colonizing bacteria to exclude toxin-secreting bacteria as an untapped resource for improvement of crop growth.

Lynch (9) extensively reviewed the topic of soil microorganisms and their products which can affect plant growth. He described a large variety of compounds and their plant responses that range from growth inhibition to stimulation. Kaminsky (8) convincingly demonstrated the role of soil microorganisms in the allelopathic potential of a desert shrub, (Adenostoma fasciculatum H & A). He discovered that the shrub supported a toxin producing microbial population. These organisms responded to soil environmental cues of drought and rewetting with a release of compounds toxic to oats(Avena sativa L.) and Bromus diandrus. He concluded that with a thorough examination of soil toxicity problems, the role of microorganisms will become increasingly dominant.

In the Peoples' Republic of China, Mr. Chen Yong-Zheng (personal communication, 1982) was involved in a group project (1971-1977) to find actinomycetes which could produce compounds useful as herbicides. His group evaluated the culture filtrates of 11,000 isolates of which 400 produced compounds inhibitory to root growth of rice (Oryza sativa L.), rape (Brassica napobrassica), oats (Avena sativa L.) and

barnyardgrass. Many of the isolates were identified as producing cycloheximide and the project was terminated.

The literature contains many examples of soil microorganisms which can produce compounds toxic to higher plants. The phosphonated amino acid herbicide bialaphos (2-amino-4-methylphinoyl butyl alanylalanine) is an antibiotic with glyphosate-like activity (11). Other chemical groups with herbicidal activity are the herbicidins and herbimycins (2,14) also discovered in Japan. A simple means of screening soil microorganisms, specifically for plant toxins could be a useful approach in the search for new herbicide chemistry. The order Actinomycetales was chosen as a starting point in the search for toxin producers because of the known diversity of chemical compounds produced by members of this group (18). The objective of this research was to develop a screening procedure for soil microorganism, specifically the actionmycetes, which could produce compounds with potential use as herbicides.

MATERIALS AND METHODS

Effect of known antibiotics on seed germination and seedling growth.

Three antibiotics known to inhibit plant growth were obtained from Sigma Chemical Co. St Louis, MO. Aqueous solutions of cycloheximide 19 oxytetracycline (=terramycin (13) and streptomycin (21) were prepared in concentrations of 1, 10, and 100 ug/ml. Test solutions were composed using 0.01 M potassium phosphate buffer at pH 6.5. Cress and barnyardgrass were used as indicator species . Fifteen seeds (non-sterile) of each species were placed in separate 15 x 60 mm plastic petri dishes (Becton Dickinson and Co., Oxnard, CA.) containing 1.5 ml of test solution on a 5.5 cm Whatman No. 2 filter disc. The factorial experiment included, 2 species, 3 antibiotics and 3 concentrations. Each treatment was replicated 4 times with data (% germination, shoot and root length) analyzed as a completely randomized design. The incubation environment was a humidified plastic box at 28^{o}C for six days in the dark. Data for root length were used in trend analysis for the factors, antibiotic and concentration. All seed germination assays were carried out in a similar manner, except the days for germination and growth varied.

Actinomycete Isolation and toxin screening procedure.

Nine soils were used for actinomycete isolation. The areas sampled are described below:

Site code	Site description
100	Houghton muck soil (Histosol) below pine needle cover.
200	Houghton muck soil, within a dense quackgrass (Agropyrens repens L.) sod
300	Mineral soil (Hillsdale sandy loam) Baker woodlot Michigan State University, below rotting log.
400	Mineral Soil (Conover loam) within 10 cm discing layer in old cherry orchard.
500	Organic matter, livestock manure heap.
600	Mineral soil (Brookston loam) garden site.
700	Mineral soil (Brookston loam) within living oat (Avena fatua L.) rhizosphere.
800	Mineral soil (Spinks sandy loam) within sorghum (Sorghum bicolor Pers.) rhizosphere.
900	Mineral soil, (Spinks sandy loam) within close proximity of incorporated sorghum residue.

The methods used for soil enrichment of actinomycetes were described by Tsao et al. (17). Soils thus prepared were serially diluted and plated out on arginine-glycerol-salt agar (AGS) (5). Soil dilutions of 10^{-4} to 10^{-6} usually provided a sufficient number of isolates for clean removal from agar plates. Johnson and Curl (7) described the morphological features of actinomycetes for use in identifying isolates on soil dilution plates. Based on their descriptions, all isolates which appeared different within each soil sample type were removed from plates and streaked-out several times to remove bacterial contaminants. Isolates which appeared free of bacterial contaminants were processed through the toxin screening procedure (TSP). Warren et al. (20) compared 10 liquid media for use in aerobic fermentation of actinomycetes. They ranked media by the number of cultures which produced antibiotics and the titre of the filtered broths. Based on their optimum medium and availability of medium components, medium A-9 was used to make the agar for the TSP. This agar (TSA) is described below:

10g	glucose
20g	blackstrap molasses (unsulfured)
5g	peptone (Bacto peptone Detroit MI.)
15g	Bacto agar
1000 ml	double distilled water, adjust to pH 7.0 with 1N NaOH before autoclaving.

The TSA selected for this procedure provided for rapid growth of the test organisms and was non-inhibitory to indicator plant species. Thus, 40 ml of TSA was poured into 100 x 15 mm square petri dishes (Lab-Tek. Products Naperville Ill.). The test organism was streaked on the TSA in a 1.0 cm band at one side of the plate and incubated at

28°C for 14 days in a dark humidified environment. Control (uninoculated) plates were also incubated in a similar way to compensate for any changes in the TSA which may have occured during incubation. Since actinomycetes grow very slowly, indicator species could germinate and grow without physically contacting the test organism. Thus, seeds of two indicator plant species were partially inserted into the agar following incubation . Seeds completely immersed within the agar failed to germinate properly in control plates. Twenty barnyardgrass and 10 cucumber seeds (cv. Greenstar) were surface sterlized with a 15 min. soak in .1% $HgCl_2$ and rinsed with sterile distilled water before being placed into the agar with the test organism. TSA with no test organism was used as the control plate.

Plates with plant indicator seeds were placed in a humidified growth chamber in the dark for 6 days at 28°C. Initial screening of a large number of test organisms did not allow for detailed measurements of germination inhibition or subsequent growth. Instead visual ratings were used to record plant growth inhibition on the agar plates. Since indicators were planted in 3 rows of 5 seed each, gradients in growth inhibition and stimulation could be easily observed. Generally, test organisms which elicited a 60% or more inhibition of emerged radical growth of both indicators compared to control plates were considered useful as producers of toxic metabolites. Of 120 isolates evaluated in this manner, nine were considered useful producers as described above.

Replicated TSP with barnyardgrass.

The repeatability of the TSP was determined with isolates which had been designated as useful producers of toxic metabolites. Seven organisms were selected from the TSP for this replicated test. The TSA was used as the growth medium with a 1.5 cm band of the test organism on both sides of the plate. Two bands of the test organism provided a greater concentration of toxins in the agar. This experiment was repeated twice. Data presented are representative of both runs.

Twenty sterilized barnyardgrass seeds were placed onto the TSA after test organisms had incubated for 19 days at 28°C. Control plates were subjected to the same incubation environment. Indicator seeds germinated and grew for 8 days at 28°C in the dark . At the end of 8 days, seed germination and length of shoots and roots were recorded. The data were subjected to an analysis of variance. Each treatment was replicated four times and date were analyzed as a completely randomized design.

Production of germination inhibitors during aerobic fermentation

Two isolates were selected from the TSP (101 and 903) to evaluate their ability to produce phytotoxic compounds in submerged culture. The isolates were grown on agar for 5 days at 28° C in the dark. Growth from the plate, containing spores, was transferred with a wire loop to a 250 ml erlenmyer flask containing 50 ml of the following medium, toxin producing medium (TPM):

1.0 g corn steep solids (Corn Products, Cook County Illinois)
2.5 g peptone
5.0 g blackstrap molasses, unsulfered
5.0 g glucose
1000 ml deionized and double distilled water.
Adjust to pH 7.0 before autoclaving.

The 250 ml flasks were placed on a rotary shaker (Lab-Line Instruments, Melrose Park Ill.) at 110 rpm for 2 days at 28° C. From this flask, 2.0 ml were transferred to several other, 250 ml flasks and shaken for another 2 days. These flasks were stored at 4° C and used as seed stock cultures. Organisms remained viable for up to two years under these conditions. Two ml of the stored stock cultures were transferred to two liter flasks with 500 ml of TPM. This start-up procedure provided a rapidly growing vegetative inoculum that was used in an 8-day fermentation at 28° C. The two liter flasks contained four 1.5 cm x 6.5 cm tapered bevels equally spaced around the bottom. These bevels facilitated mixing and aeration of the fermentation broth and prevented clumping of the producing organism.

At the end of 8 days, the broth cultures were centrifuged at 2,300 g for 15 minutes in 250 ml plastic bottles, followed by filtration through a Whatman no. 42 filter disc. Uninoculated TPM was subjected to the same conditions and used as a control treatment in a seed germination assay.

Fifteen seeds of cress and barnyardgrass were used as indicators. Treatments were composed of diluted (1:5) broths of isolates of 101, 903 and uninoculated TPM and were replicated 4 times. Full strength uninoulated TPM showed some inhibition of germination. Evaluation of broth components revealed corn steep as responsible for the inhibitory nature of unused TPM. At a dilution of 1:5, the TPM effects on germination and growth were not significantly different than distilled water (data not presented).

RESULTS AND DISCUSSION

Effect of known antibiotics on seed germination and seedling growth.

Root length data were converted to % of control (distilled water) before the analysis of variance was performed. The AOV indicated a highly significant (1%) F test for main effects of species, antibiotics and concentration. Only the species x antibiotic interaction produced a significant F test. This indicated that the two species did not respond the same to all antibiotic solutions. Cress roots were less inhibited by streptomycin and oxtetracycline than barnyardgrass roots. Cycloheximide had a similar effect on both species (Table 1).

Table 1. The effect of three antibiotics on root growth of barnyardgrass and cress.

	Root Growth[a] (% of Control)	
Antibiotic	Cress	Barnyardgrass
Streptomycin	109 d	82 c
Oxytetracycline	88 c	52 b
Cycloheximide	13 a	10 a

[a]Means separated by Duncans' multiple range test at the 5% level. Means followed by the same letter are not significantly different.

Trend analysis revealed a significant negative linear relationship between root length and the log of the concentration (Figure 1). Antibiotics can be ranked from most to least inhibitory to root length as follows: cycloheximide> oxytetracycline> streptomycin. These data complement previous accounts of plant toxicity and selectivity among species in response to antibiotic solutions (3). The injury symptoms of barnyardgrass and cress were the same in response to cycloheximide. This antibiotic at concentrations of 10 and 100 mg/ml completely prevented the emergence of a root radical and shoot tip. At 1 mg/ml, cycloheximide allows for emergence of a root tip which only grows for about 3 mm. Inhibitory concentrations of oxytetracycline and streptomycin caused a stunting of shoot and root growth without any necrosis of tissue. These antibiotics were used in this test to observe injury symptoms and to determine inhibitory concentrations for comparison with any new compounds identified in this screening trial.

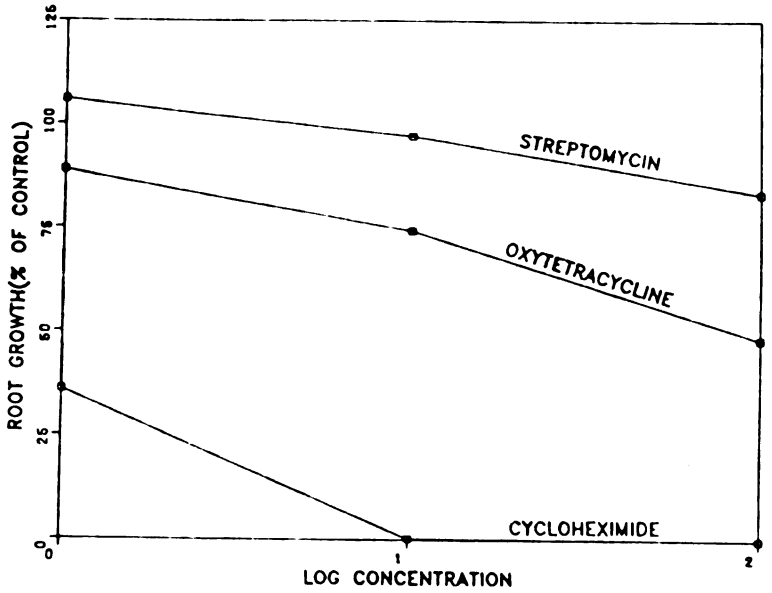

Figure 1. The relative toxicity (root growth as % of control) of three concentrations. Each point represents the mean response of both indicators (barnyardgrass and cress), eight observations.

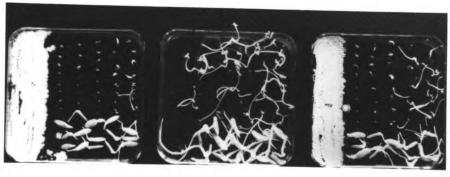

Figure 2. TSP as modified by R. Heisey. Top row of agar plates with cress, middle row with barnyardgrass and bottom row with cucumber. Plates to the far right and left showing inhibiton of germination and growth of indicator plants. Middle plate is a control.

Low concentrations of streptomycin (10 mg/ml) showed stimulatory effects on cress root growth. Nickel and Finlay (12) demonstrated growth stimulation of Lemna minor with 10 ppm solutions of bacitracin, isomicotinic hydrazide and penicillin G. As demonstrated in this experiment, concentration-dependent inhibition and stimulation of plant growth is possible with antibiotics.

Actinomycete Isolation and Toxin Screening Procedure

Of 120 isolates evaluated, nine inhibited cucumber and barnyardgrass germination and growth (Photo 1, Table 2). Soil suspensions of the manure heap contained the greatest diversity of actinomycete morphological forms. However, no toxin producing strains were isolated from this site. Five of eight toxin producing strains were isolated from Histosols (4 from site No.200 and 1 from site No.100). Sites No. 700, 800 and 900 contained one toxin producing isolate each. In general, root growth inhibition was manifested as blackened roots tips where contact with the agar was made. A negative response to geotropism was also a common response of indicator roots. Inhibition of shoot growth (shortened internodes and unexpanded true leaves) was always accompanied by root growth inhibition. Twenty-one isolates provided 5 to 20% stimulation in growth of at least one indicator species over control (% estimates by visual rating only). Further investigations with isolates stimulating plant growth will not be reported here.

These findings appear to be consistent with the wide range of plant responses to soil microbial products as reviewed by Lynch (9). The high percentage of toxin producing organisms in the root rhizosphere of quackgrass (site No. 200) allows for limited speculation as to the ability of this plant to grow in relatively pure stands. Perhaps the ability of quackgrass to sustain a population of toxin producing organisms within its rhizosphere contributes to the plants' ability to aggressively interfere with the growth of neighboring plants.

Buchholtz (4) observed severe mineral deficiency of corn plants growing in areas infested with quackgrass. Heavy fertilization with nitrogen and potassium had little impact on corn yield. He felt that nutrients were made unavailable to the corn in some other way than direct competition. He also suggested that the absorptive capacity of the corn may have been impaired by close association with the quackgrass roots. Norman (13) showed inhibition of cation uptake in barley roots by antibiotics. Although his measurements were under laboratory conditions, locally high concentrations of antibiotics within a living plants rhizosphere might exert similar effects.

The data collected in the agar screen in no way allows for valid conclusions of the role that microorganisms play in allelopathic expression. However, with adequate sampling procedures it may provide a useful tool in comparing the relative numbers of toxin producing organisms between two distinct soil environments (eg. rhizosphere vs. non-rhizosphere soils). These data suggest that root rhizospheres may be a good source for the isolation of toxin producing actinomycetes.

Replicated TSP with barnyardgrass.

All isolates significantly reduced the germination of barnyardgrass seeds. However, root growth was the most sensitive measure of toxin production (Table 2.).

Table 2. The response of germinating barnyardgrass seeds to actinomycetes on agar plates.

		LENGTH (mm)	
Isolate	% Germination	Shoots	Roots
Control	98	36	10.0
101	24	3	.1
206	74	15	.9
210	74	15	.0
211	75	22	3.5
216	70	13	.3
715	85	25	5.3
903	73	16	.2
LSD 5%	12	12	3.3

These data indicate that toxin production on agar is repeatable and that the TSP was a reliable method of selecting toxin producing actinomycetes from soil suspension plates. The double streaking of test organisms probably provided a greater concentration of toxins in the agar used to germinate and grow the indicator seeds. However, the double streaking probably is not desirable in the initial TSP where gradients of toxicity appear on the agar and thus allow for a more rigorous screening for highly potent toxin producers.

Production of germination inhibitors during aerobic fermentation

Indicator seeds germinated and grew for 6 days at 28° C, after which % germination, shoot and root length data were taken. The data (Table 3) indicated that the 101 isolate added potent germination and growth inhibitor(s) to the fermentation broth whereas the response to 903 broth was not significantly different from control (uninoculated TPM)

Table 3. The response of cress and barnyardgrass (BYGR) to 7-day fermentation broths (1:5 dilution) of isolates 101 and 903.

	% Germ.		Length (mm)			
			Shoot		Root	
Broth	cress	BYGR	cress	BYGR	cress	BYGR
101	25 a	10 a	2 a	1 a	.5 a	.3 a
Control	90 b	95 b	18 b	12 b	21.0 b	15.5 b
903	90 b	95 b	20 b	11 b	19.0 b	14.3 b

Means separated by Duncans multiple range test at the 5 % level. Means within a column followed by the same letter are not significantly different.

The data indicated that production of germination inhibitors on agar does not always correlate with similar performance in aerobic fermentation.

An agar screening technique for the detection of microorganisms that can produce herbicidal metabolites has not been previously described. The antibiotic, fumaramidmycin produced by Streptomyces kursanovii was produced on agar plates but not in aerobic fermentation(10). Shomura et al. (16) found that of all isolates producing antibiotic on agar plates, 1.9 % could not produce an active compound in submerged culture. These examples indicate that the initial search for herbicidal antibiotics should be conducted with organisms growing on agar plates. Even without the ability to produce an active compound in submerged culture, the discovery of active producers allows for the options of (1) genetic manipulation to obtain organisms which are more efficient producers and (2) identification of unique chemicals for chemical synthesis.

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CHAPTER 3

THE PRODUCTION OF HERBICIDAL MICROBIAL METABOLITES BY *STREPTOMYCES* SP

ABSTRACT

An actinomycete, *Streptomyces* sp. 101, was isolated from a Houghton muck soil within a dense quackgrass (*Agropyron repens* (L.) Beauv.) rhizosphere. This isolate produced herbicidal compounds in an aerobic fermentation. Active compounds appeared in the fermentation broth after 2 days, peaked at 6 days and appeared slightly reduced by 8 days. A methanol extract of lyophilized broth was sprayed on several species of 10-day-old weed seedlings. Selective foliar toxicity was recorded with dicotyledonous species that were generally more severely injured than monocots. Greenhouse and field experiments were initiated to determine if plant growth could be adversely affected by soil inoculations with a toxin producing *Streptomycete*. In the greenhouse experiment, barnyardgrass (*Echinochloa crus-galli* L.) was significantly inhibited by soil inoculation, whereas cucumbers (*Cucumis sativus* L.) were unaffected. In a field experiment, a band of inoculum was spread over indicator rows of oat (*Avena sativa* L.) and sorghum (*Sorghum bicolor*) which were planted with a no-tillage method into desiccated cover crops of oat, rye (*Secale cereale* L.), sorghum x sudangrass (*S.bicolor* x *S.vulgare*) and no cover crop. Oats were significantly inhibited (21 % reduction in dry weight) by soil inoculations with the 101 isolate.

INTRODUCTION

The ability of soil borne microorganisms to produce compounds which affect plant growth is well documented (4,10,11,19) Yamada et al. (21) investigated the growth regulating activity of two antibiotics isolated from the fermentation broth of a Streptomycete sp. Their objective was to discover a model substance of microbial origin which could then be chemically altered for use as a herbicide. The two active substances were identified as anisomycin and toyocamycin. Two subsequent reports by Yamada et al. (22,23) investigated the herbicidal activity as related to chemical substitution upon base molecules of microbial origin. These papers illustrate that when evaluating microbial compounds with herbicidal activity, synthetic alterations may alter their effectiveness.

Arai et al. (1) reported on two new nucleotide antibiotics with potent selective herbicidal activity. Herbicidins A and B were isolated from the fermentation broths of Streptomyces saganonensis. Herbicidin A showed greater selective postemergence activity between rice (Oryza sativa L.) and other weed species than herbicidin B. Omura et al. (14) isolated herbimycin from the fermentation broth of Streptomyces hygroscopicus. Potent herbicidal activity was observed on both mono and dicotyledonous species. Bialaphos, produced by Streptomyces hygroscopicus SF 1293 (13) and S. viridochromogenes (12) is reported to have wide spectrum herbicidial properties analogous to currently used herbicides paraquat and glyphosate (12). Another phosphonated amino acid, glufosinate is currently under development by

the Hoechst Co.(Putnam personal communication,1983). This compound was discovered in a batch fermentation culture but is now being synthesized chemically. Currently in the U.S., there is no commercially available herbicide of microbial origin, although microbial pathogens are being used as biological control agents. It is clear that microbial pesticides will be an important addition to the arsenal of compounds needed to enhance crop productivity.

Plant pathologists were among the early researchers investigating antibiotic production in natural soils. Their objectives were to develop soil treatments to protect plants against pathogenic attack (5,6,19). Krasilinikov (8) reviews Russian literature in the area of plant nutrition as affected by the metabolites of soil microorganisms. He reports that antibiotics added directly to the soil or produced by actinomycetes were found to accumulate in roots and leaves of corn (Zea mays L.), wheat (Triticum aestivum L.) and peas (Pisum sativum).

Berestetskii et al. (3) studied phytotoxic substances produced by Penicillium granulatum in liquid culure and non-sterile soils. The toxic compounds (not identified) produced in liquid culture were similar to that produced in soils after a 10-13 day incubation period. The detection of toxins in the soil by living plants was not attempted. The accumulation of herbicidal microbial metabolites in natural soils appears possible. Soil requirements appear to be a readily available carbohydrate source and high populations of the producer organism. The coordination of these and perhaps several other variables could provide a form of biological preemergence weed

control. The objective of this research was to produce herbicidal microbial metabolites in aerobic fermentation broths and assess their postemergence activity as well as preemergence activity in inoculated soils under greenhouse and field conditions.

MATERIALS AND METHODS

Time course of toxin production of isolate 101

The organism, 101 (Streptomycete sp. as characterized by Dow Chemical Co., personal communication with Dr.J. Whalen) was selected for detailed studies of toxin production in aerobic fermentations. This selection was based on results of an agar screening technique previously described (Chapter 2). This isolate was used to develop a protocol for determining the usefulness of compounds produced during fermentation as herbicides. The procedure for inoculation of shake flasks used for fermentation is the same for all fermentation runs reported. The 101 isolate was grown on agar for 5 days at 28°C in the dark. Growth , containing gray spores, from the agar plate was transferred with a wire loop to a 250 ml erlenmyer flask containing 50 ml of the following medium, toxin producing medium (TPM):

1 g	corn steep solids (Corn Products, Cook County Illinois)
2.5 g	peptone
5.0 g	blackstrap molasses unsulfered
5.0 g	glucose
1000 ml	of deionized and double distilled water. Adjusted to pH 7.0 before autoclaving.

The 250 ml flasks were placed on a rotary shaker (Lab-Line instruments, Melrose Park Ill.) at 110 rpm for 2 days at 28°C. From these flasks, 2.0 ml were transferred to 10 other, 250 ml flasks and shaken for another 2 days. These 10 flasks were stored at 4°C and used as seed stock cultures. Organisms remained viable for up to two years under these conditions. Two ml of the stored stock cultures

were transferred to the TPM in 250 ml flask and shaken for two days after which 2.0 ml were transferred to a two liter flask with 500 ml of TPM. The two liter flasks contained four 1.5 x 6.5 cm tapered bevels equally spaced around the bottom. These bevels facilitated mixing and aeration of the fermentation broth and prevented clumping of the producing organism. This start-up procedure provided for an inoculum that averaged 4.5×10^7 counts/ml, as measured by counts on serial dilution plates.

A time course study was initiated to determine a profile of broth toxicity to seed germination over time. Five 2 liter flasks were inoculated with the 101 organism as previously described. Thirty ml of fermentation broth were aseptically removed from the different flasks 2,4,6 and 8 days after inoculation. Fermentation broths were centrifuged immediately upon removal from the flasks in 30 ml high-speed centrifuge tubes at 2,300g for 15 min. Centrifuged samples were frozen until cress seed bioassays were performed. Fermentation broths from each day were evaluated at full strength and 1:5, 1:25 and 1:125 dilutions. A control broth was uninoculated TPM which was not subjected to fermentation conditions. The pH of the full strength test solutions were: 0-day, 7.3; 2-day, 7.1; 4-day, 6.6; 6-day, 7.2; 8-day, 7.5.

Ten cress seeds, selected for uniform size were used as an experimental unit for each treatment. At the end of 5 days, % germination and length of shoots and roots were measured. The experimental design was a randomized complete block with treatments replicated four times.

Centrifuged fermentation broths were filtered through Whatman no. 42 filter disc and the solubility of the toxin(s) determined. Filtered broth (150 ml) was lyophilized, producing .65 g of dried material. Twenty-three mg of the dried residue were placed in seven 5 ml test tubes with screw top caps. To each test tube was added 2 ml of one of the following solvents:

water
methanol
acetonitrile
acetone
dichloromethane
toluene
petroleum ether

Solvents and lyophilized broth were mixed for 2 min. with a vortex mixer. Undissolved particles were removed by filtration with an AP-20 prefilter (Millipore Corp. Bedford Mass.) Filtered solvents were taken to dryness under N_2 at 40°C. The residues from the dried extracting solvents were reconstituted in 1.0 ml of the same solvent after which 0.1 ml aliquots of the reconstituted extract were added to 5.5 cm sheets of Whatman's no. 2 filter paper and allowed to dry for 24 hours. Dried filter paper sheets were placed in a plastic petri dish and hydrated with 1.5 ml of distilled water for the cress seed bioassay.

Postemergence activity of fermentation broth extract

Postemergence toxicity on six weeds was evaluated twice with extracts of the broth culture of the 101 isolate in greenhouse experiments. Based on the data of toxin solubility from lyophilized culture broths, methanol was used as an extracting solvent of dried residues.

Experiment 1:

Three hundred ml of filtered fermentation broth were lyophilized and reconstituted in 90 ml of methanol. A similar quantity of unused fermentation broth (TPM) was lyophilized and extracted with 90 ml of methanol to make-up the control spray. The lyophilized broths were extracted at 4°C for 2 weeks. This unusually long extraction period was due to an unavoidable delay and is not intended as a significant aspect of the procedure. The methanol extracts were filtered and taken to dryness under vacuum at 40°C.

Ten day-old seedlings of the following weed species were used as indicators of postemergence spray activity.

1. Velvetleaf (Abutilon theophrasti(Medic))
2. redroot pigweed (Amaranthus retroflexus L.)
3. Common purslane (Portulaca oleracea L.)
4. Green foxtail (Setaria viridis L.)
5. Red millet (Panicum miliaceum)
6. Barnyardgrass

Seeds of the six indicators were planted into greenhouse flats using a soil mixture of 3:1, Spinks sandy loam:Sunshine soil mix (Fisons-Western Peat Co. Vancouver, B.C.). Each weed species was thinned to 10 plants per row before spraying. Seedlings were grown with supplemental flourescent light from January 19-29, 1983. The mean day and night temperatures were 26° C and 18°C respectively. Light intensity was particularly low during this time of the year. The broadleaf seedlings were just past the cotyledon stage at the time of spraying where as the grass seedlings had 1-2 fully expanded true leaves.

The dried material remaining after removal of the methanol was reconstituted in 30 ml of distilled water. The 30 ml aqueous solutions were split into 15 ml portions to test spray activity with two surfactants: 0.1% (wt./wt.) X-77 (Chevron Chemical Co., San Francisco, CA.) and 0.1% Tween-20 (ICI America, Inc., Wilmington Del.). Test solutions were applied with a glass chromatographic atomizer (Supelco, Inc. Bellefonte, PA.) until droplets formed on the foliage. Treatments were replicated 3 times. Visual injury ratings were used to evaluate spray treatments 7 days after spraying. An injury scale of 0-10 was used (9). This rating system assigns 0 for no injury and 10 for complete kill. Dry weights for each indicator were taken 14 days after spraying.

Experiment 2:

Conditions in this experiment were slightly altered. The long broth extraction period (2 weeks) in experiment 1 raised the possibility of alteration in the chemical structure of compounds causing the observed foliar toxicity. Two aliquots (400 ml) of 10l broth were lyophilized and extracted for 6 hours (room temperature, using a wrist action shaker) and 7 days (4^{o} C, no agitation). Control treatments were composed of methanol extracts (7 days, 4^{o}C, no agitation) of lyophilized unused fermentation broth. Methanol extracts were filtered, taken to dryness (under vacuum, 40^{o} C) and reconstituted in 40 ml of distilled water and 0.1 % (wt./wt.) X-77. Spray treatments thus composed were applied to the same set of indicator species. Seedlings germinated and grew using supplemental flourescent lights from August 5-15, 1982. The mean day and night temperatures were 30^{o}C and 23^{o}C

respectively. Under these conditions broadleaf seedlings were at the 1-2 true leaf stage and grasses had 2-3 expanded true leaves at the time sprays were applied. Sprays were applied as previously described with treatments replicated 4 times. Spray ratings were made 7-days post application and plants harvested for dry weights 14 days post application. Experimental design was a randomized complete block.

Induction of soil phytotoxicity by 101 inoculation

Aluminum pans (20 x 14 cm) were filled with a greenhouse soil mix of 3:1 Spinks sandy loam:Sunshine soil mix. Each pan consisted of two layers, the top was amended in the following way: 10 g of $CaCO_3$ and 4.0 g dried finely ground sorghum residue (c.v. Bird-A-Boo, Taylor Evans Seed Co. Tulia Texas) were incorporated into 200 g of dried soil. When this soil was amended with 5, 10, 20 and 40 g of $CaCO_3$ the pH of the soil solution was adjusted to 7.0, 7.2, 7.4 and 7.6 respectively.

Soil, in pans, was sterilized in a 208 liter steel drum with 680 g of Dow-Fume MC-20 (98% methyl bromide and 2% chloropicrin, Dow Chemical Co, Midland MI.) for 24 hours. A diluted soil sample was streaked on a petri dish containing the TSA. The absence of any microbial growth indicated that complete sterilization was achieved with this procedure. The sterilant was removed by pumping it out of the drum with an electric pump for 12 hours. The absence of chloropicrin (odor) at the drum vents was assumed to be an indication of complete sterilant removal. Each pan received 200 ml of tap water before the addition of 101 inoculum. Inoculations were staggered to

obtain two soil incubation times of one and two weeks before the addition of indicator species. Counts for the two inoculum times were: 1.9 x 10^8/ml and 8.0 x 10^7/ml respectively.

Two 14 cm rows in each pan were inoculated with 10 ml of the 101 organism. Incubations were carried out in plastic bags in the greenhouse in bags to prevent drying of the soil. Twenty barnyardgrass and ten cucumber seeds were planted in rows where inoculum had been applied to the soil. The indicators grew in the soil pans for 19 days (2/4-23/83) in a greenhouse with supplemental flourescent lights. Day and night temperatures were 26° C and 18° C, respectively. The experiment was a 3 way factorial with 2 soil amendments, 2 inoculum levels and 2 indicator species. Data collected were plant densities and dry weight accumulation.

The field experiment was conducted on the Michigan State University, Horticulture Research Farm in East Lansing, MI. Three crop species were planted on June 14, 1982 on a Miami silt loam to provide surface mulches into which the 101-organism would be placed. The mulch treatments were: no-crop; sorghum x sudangrass (Sorghum bicolor Pers. x Sorghum vulgare c.v. Haygrazer), rye (Secale cereale c.v. Wheeler) and oats (c.v. Gary). Seed rates of these crops were: sorghum x sudan grass, 104 kg/Ha; rye and oats, 166 kg/Ha. Plots for mulch treatments were 1.8 x 12.2 m. A 'Moore Uni-Drill' (Moore Uni-Drill Ltd., Newhill House, 33 Kirk Rd. Balbymoney BT 53 6PP CO Antrin, N.Ireland) was used

to plant crops for mulch and indicator species under no-tillage (NT) field conditions. Treatment design was a 3 way factorial with 4 mulches, 2 inoculum levels and 2 indicator species. Each treatment was replicated 5 times.

Crops were grown for 39 days and sprayed with the systemic herbicide, glyphosate . This herbicide completely killed the crops and reportedly has no residual soil herbicidal activity. Twenty days after spraying, the 101 inoculum was added to mulch plots on the same day that indicator species of sorghum (c.v. Bird-A-Boo) and oats (c.v. Gary) were planted with a no-tillage method. Indicator seeding rates for sorghum and oat were 224 kg/Ha and 157 kg/Ha respectively. Five rows 11.4 cm apart of each indicator were planted directly into the mulches.

The 101 inoculum was prepared for delivery to the field in the following manner. Japanese millet (Echinochloa sp.) (Mangelsdorf Seed Co., St. Louis MO.) seed (400 g) were autoclaved three times for 1 hour in 32 x 24 cm pans covered with aluminum foil. Twenty pans of seeds were prepared in this way. A four day-old shake flask culture (500 ml) of 101 was added to each pan containing the millet. The 101 isolate grew on the millet for 19 days at 28^{o}C. The inoculum was then air dried in a greenhouse for 5 days before delivery to the field. The inoculum, thus prepared, contained 4.6 x 10^{7} counts/g. A standard gravity feed lawn spreader was used to place a 0.3 m x 3 m band directly over seeded indicator rows.

Solid rows (1.5 meters) of each indicator plant was harvested 25 days after planting. Data obtained were plant densities and dry weight accumulation.

RESULTS AND DISCUSSION

Time course of toxin production of isolate 101

The AOV for % cress germination and shoot and root growth indicated a significant F test (1% level) for the time (fermentation) x concentration interaction (Figure 1 a-c) All parameters measured indicated that toxic concentrations appeared at 2 days of fermentation and reach a peak at 6 days. Using root length as a representative response, the two day-old broth was inhibitory at every concentration as compared to 0 day broth. At a dilution of 1:25, 4 and 6 day broths were the only test solutions significantly more inhibitory than 2 day broth.

Figure 1 a-c. The response of cress to 0,2,4,6 and 8 day fermentation broths of isolate 101.

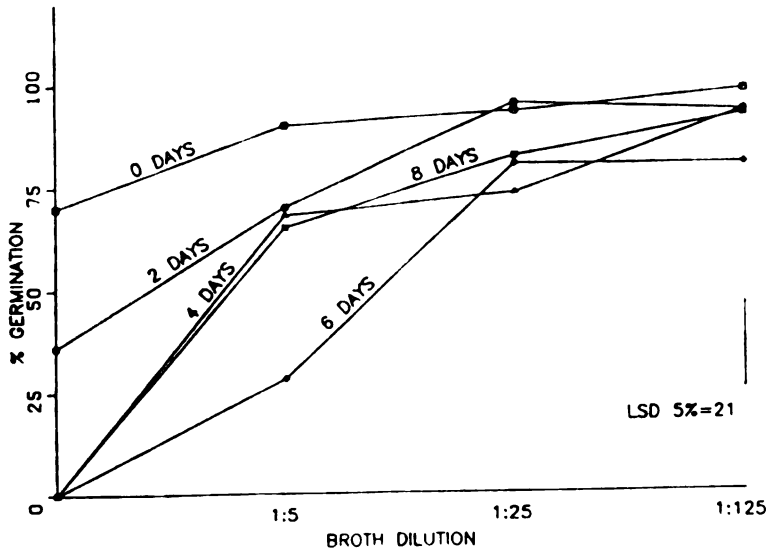

Figure A.

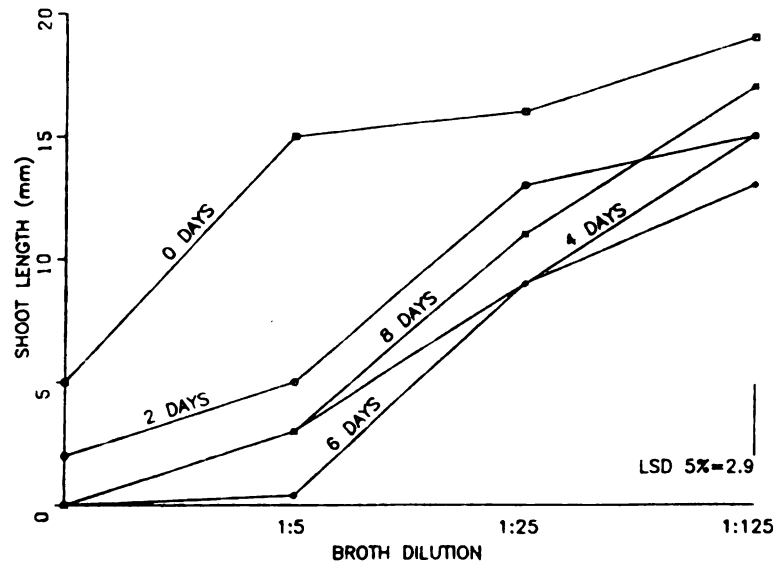

Figure B.

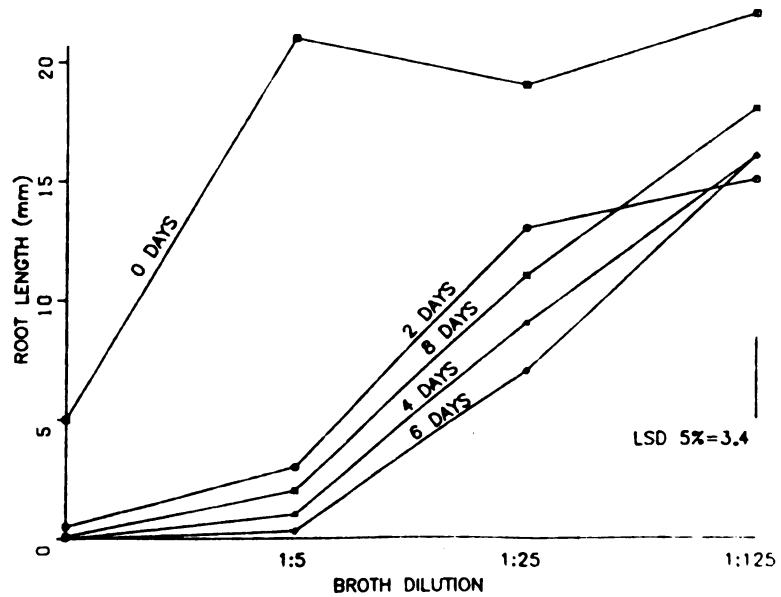

Figure C.

These results agree well with those of Warren et al. (17). They reported on the antibiotic production of 110 cultures in 10 fermentation media. Fermentation for 7-8 days at 28°C was considered optimum for reproducible assays as a basis for appraising antibiotic production. In all subsequent fermentations, 8 day-old broth was used for evaluation of toxin characterization and identification.

The solubilities of the toxic components of the lyophilized broth were determined and found to be soluble in water and methanol solvents only (Table 1).

Table 1. Solubility characterization of inhibitors in freeze dried 101 fermentation broth.

		LENGTH (mm)	
Solvent	% Germination	Shoots	Roots
water	88	8.1 a	7.9 a
methanol	88	11.1 a	8.4 a
acetonitrile	90	25.3 b	18.4 b
acetone	91	26.1 b	21.6 b
dichloromethane	91	26.0 b	23.1 b
toluene	93	26.9 b	23.3 b
petroleum ether	95	24.4 b	23.5 b

Means separated by Duncans multiple range test at the 5% level. Means within a column followed by the same letter are not significantly different.

Both root and shoot growth indicated that the toxin(s) present in the lyophilized broth were only extracted with water and methanol. These results indicated that relatively polar compounds are responsible for the observed activity from the lyopilized broth.

Postemergence activity of 101 fermentation broth extract

Experiment 1:

Spray treatments were rated on a scale of 0 to 10, 0 being no effect and 10 being complete kill of the indicator species. Visual ratings of spray treatments were made 7 days after application. Two weeks after spraying, plants were harvested and dried (Table 2). Velvetleaf was the least affected dicot species and also the only indicator that showed a significant response to the type of surfactant used. Tween 20 appeared to enhance foliar toxicity of the spray solutions on velvetleaf. Two days after spraying, necrosis of leaf margins was obvious but plants eventually grew out-of this initial injury. Redroot pigweed was the most sensitive dicot species. Two days after spraying, stem tissue below the cotyledons collapsed, followed by death of the entire seedling. The seedlings of common purslane showed some upward-cupping of true leaves in response to the spray treatment. At 4 days after spraying, these seedlings had bleached cotyledons a condition soon followed by wilting and death. All grasses showed some degree of leaf curling two days after spraying. Green foxtail was the most sensitive grass showing a high level of leaf curling and wilting, followed by death. Red millet and barnyardgrass both showed less leaf curling and some wilting, although they eventually grew normally.

Table 2. The response of six weed species to a methanol extract of freeze dried 101 fermentation broth and unused TPM.

	7-Day Rating				Dry Weight (g)			
	101		TPM		101		TPM	
Weed Species	X-77	T-20	X-77	T-20	X-77	T-20	X-77	T-20
Barnyardgrass	2.0 a	2.3 a	0.0 b	0.7 b	0.3 b	0.5 b	1.0 a	0.8 a
Red millet	3.3 b	2.6 b	0.0 a	0.0 a	0.3 a	0.3 a	0.4 a	0.4 a
Green Foxtail	9.3 a	9.0 a	0.0 b	0.0 b	0.0 a	0.0 a	0.4 b	0.4 b
Common purslane	8.6 a	8.6 a	0.0 b	0.0 b	0.0 a	0.0 a	0.3 b	0.2 b
Redroot pigweed	9.0 a	8.7 a	0.7 b	0.7 b	0.0 a	0.0 a	0.1 b	0.2 b
Velvetleaf	3.7 a	7.0 b	0.0 c	0.0 c	0.4 b	0.2 a	0.7 c	0.5 bc

Means separated by a Duncans multiple range test at the 5% level. Means within a row for either 7-day rating or dry weight accumulation followed by the same letter are not significantly different.

The order of species sensitivity (based on 7-day ratings) to the spray solution from most to least sensitive was: green foxtail redroot pigweed common purslane green foxtail red millet barnyardgrass velvetleaf.

Experiment 2:

Treatments in this experiment were designed to determine if the length and method of broth extraction could affect indicator plant response to spray solutions. The 7-day ratings for all grass species and velvet-leaf indicate that the 6 hour extraction was slightly more phytotoxic than the 7-day extraction (Table 3). The advanced physiological stage of the seedlings in this experiment resulted in a general reduction in foliar phytotoxicity in response to spray solutions. An additional source of variation between the two experiments could have been the nature of the broths extracted. Differences in

fermentations batches were not quantified and therefore not adjusted during the composition of the spray solutions. Redroot pigweed responded equally to spray solutions regardless of extraction method. Common purslane was unaffected by any spray treatment.

Dry weights of velvet leaf, common purslane and red millet were not adversely affected by spray solutions. Barnyardgrass, green foxtail and redroot pigweed all showed significant reductions in dry weights in response to 101 spray solutions, regardless of extraction procedure.

Table 3. The response of six weed species to methanol extracts (6 hour, 7day) of freeze dried 101 fermentation broth and unused TPM (control).

	7-Day Rating			Dry Weight (g)		
	Spray Treatments					
Weed Species	Control	6 Hour	7-Day	Control	6-Hour	7-Day
Barnyardgrass	.8 a	5.8 c	3.3 b	3.8 b	2.3 a	2.9 a
Red millet	1.0 a	6.3 c	2.0 b	1.2	1.4	1.8
Green foxtail	0.8 a	7.0 c	5.0 b	1.0 b	0.5 a	0.7 a
C. purslane	0.8	2.0	1.8	1.1	0.8	1.0
R. pigweed	3.0 a	10.0 b	10.0 b	0.2 a	0.0 b	0.0 b
Velvet leaf	0.5 a	2.0 b	1.0 a	1.2	1.4	2.0

Means separated by Duncans multiple range test at the 5% level. Means followed by the same letter are not significantly different.

Another postemergence experiment was conducted in which spray solutions of 50 and 100 mg/ml cycloheximide were compared to methanol extracts of 101 broth. The 101 broth extract and 100 mg/ml cycloheximide sprays had similar effects on indicator species, i.e. leaf curling of grasses and bleaching of broadleaf foliage.

Several conclusions can be derived from the postemergence activity observed in these experiments. The phytotoxic effects of the 101 broth extract sprays appeared similar to a 100 mg/ml solution of cycloheximide. Thus the 101 broth extracts may contain cycloheximide. The activity observed in experiments 1 and 2 appeared inconsistent, perhaps due to differences in growth stage of indicator species and possibly the potency of broth used to compose the sprays. These inconsistancies do however indicate a narrow window of plant growth at which the 101 sprays are effective. Limitations such as this would have to be overcome before the broth components can be considered promising with respect to postemergence herbicidal activity. Variations in spray performance due to fermentation batch could be eliminated if facilities for large scale production were available and thus provide enough active material for several runs of postemergence experiments.

Induction of soil phytotoxicity by 101 inoculation

Cucumber and barnyardgrass plant numbers were unaffected by any treatment (Table 4), however, barnyardgrass dry weights were significantly affected by the treatment factors of sorghum residues and soil inoculation with the 101 isolate (F test significant at the 5% level for both factors). Incubation time did not have any effect on barnyardgrass weights. The dry weights increased 21 % in the presence of sorghum residues (data averaged for effects of inoculation and incubation) and reduced 18% with inoculation (data averaged for effects of sorghum residues and incubation time). In general, the data show that

a reduction in barnyardgrass dry weights occurred with one week of incubation, regardless of sorghum residues. However, with two weeks of incubation only the treatment with sorghum residues showed a reduction in dry weights.

Table 4. The response of barnyardgrass dry weights to sorghum residues and soil inoculation (101 isolate) at the end of a 1 and 2 week incubation period .

	Treatment Factors		Barnyardgrass dry wt. (g)
Incubation time	Sorghum residues	Inoculation	
1 week	-	-	.36
"	-	+	.25
"	+	-	.46
"	+	+	.35
2 week	-	-	.31
"	-	+	.33
"	+	-	.39
"	+	+	.32

F test for inoculation and sorghum residue significant at the 5%level.

In the field experiment, oat and sorghum were used as indicators to determine the effects of the treatment factors of cover crop (rye,oat and sorghum x sudangrass) mulches and inoculum (+ = present, - = absent). Densities of both species were unaffected by any treatment combination. The AOV for dry weight of indicators revealed that the species x inoculum interaction was significant at the 5% level (Table 5).

Table 5. Oat and sorghum dry weights in response to 101 soil inoculation.

Indicator	Inoculum level	Dry wt./1.3 m of row (g)
oats	+	65 a
oats	-	82 b
sorghum	+	69 a
sorghum	-	72 a

Means separated by Duncans multiple range test at the 5% level. Means followed by the same letter are not significantly different.

The means were averaged for 4 mulch treatments. The magnitude of the reduction in dry weight in the various mulch treatments was not significantly different. Oats were a more sensitive indicator to the treatment factor of inoculation than sorghum.

The main effect of mulches showed a significant F test at the 5% level (Table 6). Indicator species did not vary in their response to this factor.

Table 6. The response of both indicator species (oat and sorghum) to mulches. Means are averaged over species and inoculum levels.

Mulch	Dry wt./1.3 m of row (g)
no crop	78 b
oat	75 b
rye	72 ab
sorghum x sudangrass	63 a

Means separated by Duncans multiple range test at the 5% level. Means followed by the same letter are not significantly different.

The data for the field experiment can be summarized as follows: Inoculation with the 101 isolate resulted in an average 13% reduction in indicator plant dry weight, oats being more sensitive toinoculation than sorghum. Sorghum x sudangrass mulch significantly reduced

indicator plant dry weight over that of no crop and oat mulch. Putnam and DeFrank (15) previously reported on the ability of several cover crops, used as a surface mulch, to inhibit weeds. Populations of Portulaca oleracea L. and Digitaria ishaemum Schreb. Muhl. were reduced by 70% and 80%, respectively, by sorghum residues. The results of the field experiment support previous results where indicator plant dry weights were significantly reduced by sorghum x sudan grass mulch.

In designing this experiment, it was thought that soils covered with a mulch would provide a buffered environment and thus aid in the establishment of the applied inoculum. Mulches in no-tillage cropping systems can increase soil moisture levels and moderate temperatures (24) in addition to supplying a carbon source within close proximity to the inoculum and germinating weed seeds. In this experiment, the effects of surface mulches were not significantly different in their ability to enhance the toxic expression of the applied 101 inoculum. Although not shown, the effects of organic mulches may be crucial for the establishment of introduced microbial inoculums on other soils, with different organisms, other planting dates or soil types.

These data indicate that soil inoculation with an actinomycete can slightly affect plant growth and points out a need for a more sensitive method of quantifying herbicidial antibiotics produced in the soil. A precise and sensitive method will be crucial in determining the environmental cues for antibiotic formation in natural soils.

Weiler et al. (18) described a specific solid phase immunoassay for the detection of as little 3-4 pico-grams of indole-3-acetic acid (IAA)in oat coleoptiles. With this system very small samples of plant tissue are required for quanitative IAA analysis with little or no extract purification. A similar immunoassay could be devised for a herbicidal metabolites produced by a specific organism. With inoculations of such an organism, antibiotic production could then be measured under a variety of soil environmental conditions, over time, with a simple and sensitive immunoassay procedure. Plants growing in inoculated soils could also be assayed for antibiotic uptake (provided it occurred) and thus serve as biological accumulators. The uptake and accumulation of antibiotics by plants from soil has been reported (8).

Previous attempts to influence plant growth by inoculation of non-sterilized soil without prior incubation time failed to affect plant growth. This could be due to a variety of reasons. In these initial experiments, fresh vegetative growth from submerged cultures was used as an inoculum. The shift from sterile submerged culture to non-sterile soil may have imposed too great an environmental shock for successful establishment. Plant pathologists indicate a need for soil inoculums to be in a dormant state and preferably established on an organic substrate. These recommendations were followed for the development of the millet seed carrier as employed in this experiment with only limited success.

There have been several reports of antibiotic formation in non-sterile soils (5,6,20) Antibiotic formation was always less in non-sterile soils than in sterile ones. However, Wright (20) showed that the beneficial effects of autoclaving on antibiotic production (gliotoxin) were not totally due to the removal of competing organisms. She felt that the increased availability of carbon was partially responsible for the observed effect. In these and the current study, it can be concluded that establishment of an introduced organism in the soil is very difficult.

Rouatt and Atkinson (16) demonstrated the shift in microbial populations through the incorporation of various types of plant residues. The incidence of potato scab (Streptomyces scabies) was considerably reduced with soybean residues while rye and clover had no effect. Population shifts in bacteria, actinomycetes and fungi in response to plant tissue incorporation were recorded. This research and that of Kaminsky (7) indicated that soil microorganisms can play a significant role in the growth and development of plants and that shifts in soil populations may occur under certain circumstances. Although the 101 isolate was relatively ineffective, the use of microbial inoculations to induce selective soil phytotoxicity may still be a viable scheme for biological weed control. With more effective strains, a reliable method of inoculation and a predisposition of the soil for subsequent proliferation (by selection of cover crop residue) the induction of selective phytotoxicity upon weeds in certain crops may be possible.

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CHAPTER 4

CHROMATOGRAPHIC CHARACTERIZATION AND IDENTIFICATION OF HERBICIDIAL METABOLITES OF *STREPTOMYCES sp.* 101.

ABSTRACT

Streptomyces sp. 101 was cultured in an aerobic fermentation flask for 8 days. Dichloromethane extracted two compounds from the aqueous broth that were inhibitory to cress (*Lepidium satvium* L.), cycloheximide and an unknown. These compounds were separated and purified using thin layer, column and high performance liquid chromatographic techniques. The compounds were identified by mass spectroscopy and in the case of cycloheximide, cochromatographed with known material.

INTRODUCTION

The production of herbicidal antibiotics in aerobic fermentation broth has already been previously reported for several actinomycetes (1,4,5). These chemicals include phosphonated amino acids (5), herbimycins (4) and herbicidins (1).

In the Peoples Republic of China, Mr. Chen Young-Zheng (personal communication, 1982) was involved in a group project (1971-1977) to find Actinomycetes which could produce metabolites useful as herbicides. His group evaluated 11,000 isolates of which 400 produced metabolites in fermentation culture inhibitory to rice (Oryza sativa L.), rape (Brassica napobrassica) and barnyardgrass (Echinochloa crus-galli). This group used paper chromatography and a yeast bioassay to determine if culture filtrates contained cycloheximide. They believed that the presence of this compound in the fermentation broth rendered it unsuitable for continued evaluation of herbicidal components. What they failed to realize is that the production of additional metabolites in the broth could be useful as herbicides. Because of these observations, cycloheximide content was monitored in the fermentation broth of 101 and other isolates. Streptomyces sp. 101 was selected from the agar screening procedure (Chapter 2) for production and subsequent identification of herbicidal metabolites. The objective of this research was to develop a protocol to detect cycloheximide and identify other metabolites with herbicidal properties.

MATERIALS AND METHODS

Partitioning of 101 fermentation broth inhibitors into an organic solvent.

Fermentation conditions for the 101 isolate are previously described (Chapter 2). One hundred ml of raw 101 fermentation broth were successively extracted with two 100 ml volumes of each of the following

solvents, in the order given: hexane (HEX), toluene (TOL), and dichloromethane (DCM). Solvents were passed over a drying agent (Na_2SO_4 anh.) and taken to dryness under vacuum at 40°C. The extracted residue of each solvent was brought up into 1.0 ml of that solvent, then 0.2 ml aliquots were placed on a Whatman No.2 5.5 cm filter disc, dried and used in the cress bioassay. One ml of raw fermentation broth before and after extraction was loaded onto filter discs and dried. The treatments were replicated five times using 10 cress seed per treatment. Data was analyzed as a randomized complete block design.

Detection of cycloheximide in 10l fermentation broth.

The initial detection of cycloheximide employed the use of Whatman LKD (250 micron, thickness) silica gel plates (Whatman Chemical Separation Inc. Clifton N.J.). One liter of 8-day fermentation broth was partitioned against one liter of DCM two times. The DCM extract was passed over a drying agent and taken to dryness under vacuum at 45°C. The extract was transferred to a preweighed test tube and taken to dryness under a N_2 gas stream at 40°C. The air dried weight of the extracted residue was 1.6 mg. This material was reconstituted in 160 ul of DCM to obtain a final concentration of 10 ug/ul. A standard of cycloheximide of equal concentration was also prepared in DCM. The silica-gel TLC plates used in this experiment were channelized and contained a preabsorbent area (PAA) which facilitated rapid loading of test samples. Samples (10 ul) were loaded onto the plates which had been prewashed in the eluting solvent (9:1 acetonitrile:water).

Prewashed plates were placed in a 60°C drying oven for 24 hours before use. Five channels were loaded with 10 ul of the crude DCM extract of the 101 fermentation broth and 10 ul of the cycloheximide standard. The plates were eluted for 10 cm from the top of the PAA. After eluting and before complete drying had occurred, each channel was scraped in 1 cm widths starting at the top of the PAA. Each segment was bioassayed with cress seed for determination of the R_f of toxic components. The entire PAA constituted a single treatment in itself.

The plate scrapings for each channel were placed in 13 mm x 100 mm disposable test tubes. Above the TLC scrapings a Ap-20 prefilter was placed to provide a firm surface for cress seed germination. Distilled water (0.4 ml) was added to treatments containing the PAA, while 0.3 ml were added to all other treatments. Five cress seeds, selected for uniformity, were added to the test tubes and placed in a 28° C humidified box for 5 days in the dark. Treatments were replicated four times. At the end of 5 days, % germination, shoot and root length were recorded.

An eluted channel of each test solution, not used in bioassay, was sprayed with a detection reagent consisting of: 5 g KOH in 10 ml of water brought up to 100 ml with methanol. Sprayed plates were heated to 100° C for 5 min. In this system, cycloheximide, develops a yellow spot at R_f .7 (5) At 40 ug, cycloheximide produced a yellow spot is visible in white light. A lower limit of detection of 8 ug was possible with visualization using 366 nm light (transilluminator "Black-Ray", Ultraviolet Products, San Gabriel CA.).

Clean-up procedure for the DCM extract of the 101 fermentation broth.

The composition of the fermentation broth used for all experiments was previously described (Chapters 2 & 3) and was designated toxin producing medium (TPM). Unused TPM showed some inhibition of cress seed germination (Chapter 3, Figure 1 a-c). Therefore, during isolation of inhibitors from the 101-fermentation broths, toxicity from the broth components themselves had to be accounted for. This was accomplished by subjecting the unused broth to the same clean-up procedure as the 101 fermentation broth and determining where the inhibitory activity of the two broths differed.

Two liters of unused TPM (pH 7.0) were partitioned against an equal volume of DCM. The DCM, after extraction, was passed through a glass funnel containing Na_2SO_4 (anhydrous) held in place with a filter paper. Passage of the DCM through this salt broke-up all emulsions. $MgSO_4$ powder (40g/liter) was added to the DCM for 24 hours to remove any remaining moisture and was subsequently removed by filtration. The DCM was taken to dryness, under vacuum at 45^{o}C. The dried residue was transferred to a preweighed test tube to determine the dry weight of the DCM extractable residues of the unused TPM.

Twenty-four mg of the DCM extracted residues were fractionated using a preparative open-bed silica gel column. Column dimension and conditions were: I.D: 25 cm; Bed volume:50 ml; Bed resin: silica gel; 70-230 mesh (MC/B Manufacturing Co. Cincinnati OH.); Flow rate was not controlled, but determined by gravity flow. The silica gel resin was

slurry packed in DCM after soaking for 24 hrs and degassing.

The DCM extracted residue (22.7 mg) of the unused TPM was loaded onto the top of the prep-silica gel column with DCM. The loaded material was eluted with a step gradient of 500 ml increments. The first step was composed of 100% DCM, followed by steps increasing in methanol concentration by 0.25 % until a final step of 2.0% methanol was used to elute the column. The material eluted by each step of the gradient was taken to dryness under vacuum for dry weight determinations.

The dried residues from each step were brought-up with DCM to a concentration of 10 ug/ul. Twenty ul of each step solution were added to a filter paper disc, the solvent evaporated and cress assays performed.

Three and a half liters of 8-day 10l fermentation broth were extracted with DCM, yielding 58.6 mg of dry material. The extracting solvent was treated as previously described for unused TPM. The extracted residues were loaded onto the prep-silica gel column and eluted with a step gradient as previously described (100 % DCM to 2.0 % methanol:98.0 % DCM).

Absence of inhibitory activity in any portion of the step gradient elution of the unused TPM did not allow further identification. The (1.25MeOH) step of the 10l DCM extract elution was fractionated for identification of active components. A high pressure liquid chromatography (HPLC) carbohydrate analysis column (Waters Associates, Milford

conditions for the fractionation and subsequent collection of eluting samples were as follows: Mobil phase: 1.5:98.5 (MeOH:DCM); Flow: 3.0 ml/min; Detector: (SF 770 Spectroflow Monitor, Schoffel Ins. Corp. Westwood N.J.), u.v. 254 nm; Detector attenuation: 0.2; Fraction collection by time: 1 fraction/ 0.7 min. (1.9 ml/fraction). Four 20 ul injections of the 1.25 MeOH step at concentrations of 10 ug/ul were made, each injection being considered a replication of treatments. Sixteen fractions were collected with a ISCO model 1200 fraction collector (ISCO, Lincoln, Nebraska). Fractions were loaded onto filter paper discs for bioassay with cress. A trace of the HPLC run of these injections was recorded on chart paper with a speed of 40 cm/hr. Comparing peaks on the HPLC trace with the fractions bio-assayed with cress allowed for a determination of the retention volume(R_v) of the active components of this sample(Table 5). Four 20 ul injections of a standard solution of cycloheximide were also fractionated under these same conditions to determine its R_v.

Mass Spectra of active components of 1.25 MeOH step.

To prepare the 1.25 MeOH step for generation of a mass spectra (MS) of its active component, an additional clean-up was performed. An open bed silica gel column with an isocratic eluting solvent was used to enrich the 1.25 MEOH step for the active component. The open bed column conditions were: Bed resin: silica gel 70-230 mesh; Mobil phase: 2:98 (MeOH:DCM): I.D. of column: 1.5 cm; Height: 12.5 cm; Bed volume: 50 ml; Flow rate: 1.75 ml/min; Fraction collection by time: 1 fraction/4 min.. Twenty mg of the material from the MeOH step were

loaded onto the top of the column in a 0.5 ml volume of DCM. Fractions were collected and taken to dryness under an N_2 gas stream at 45°C. Fractions were reconstituted in 0.5 ml of DCM of which 5 ul were injected onto the carbohydrate analysis column. These injections onto the HPLC column were made to trace the elution of 1.25 MeoH step components on the open-bed silica gel column. In this system, fractions 32-40 were pooled, thus enriching for the active component as previously identified. The pooled fractions collectively contained 9.6 mg of sample material.

The pooled material was brought-up in DCM to a concentration of 10 ug/ul after which 20 ul were injected onto the the carbohydrate analysis column for fractionation and collection for cress bioassay. This assay was performed to determine if activity still remained after the open-bed silica gel column clean-up. Four injections were made and fractions collected by time (1 fraction/0.7 min) Fractions were collected for bioassay with cress and after visual evaluation only fractions 1,5,6,7,8,9,10,11, and 12 were statistically analyzed. Each injection was considered a replicate of the 12 fractions collected and used in the cress bioassay.

The fractionation of the 1.25 MeOH step and cycloheximide standard on the carbohydrate analysis column indicated the active component cochromatagraphed with cycloheximide. To determine if the active component was cycloheximide, several injections of the enriched 1.25 MeOH step were made on the HPLC carbohydrate analysis for sample collection and MS analysis. A standard solution of cycloheximide was

also injected onto the HPLC column for fraction collection and MS analysis. The spectra were run on a Hewlett Packard 5985 MS using a direct probe heated from 30°C to 150°C at a rate of 20° C/min.. Samples were ionized with a 70 eV of energy. The mass spectra of cycloheximide has been reported (3) and was used for comparison with the spectra generated.

Chromatographic characterization of other compounds with inhibitory activity.

MS data confirmed the presence of cycloheximide in the 1.25 MeOH step of the 101 DCM prep-silica gel elution. However the use of silica gel TLC plates to fractionate crude 101 DCM extracts indicated two R_fs with inhibitory activity. Whatman LKD silica gel plates with a PAA were used to fractionate 10 ug/ul solutions of 1.00, 1.25, 1.50 and 1.75 % MeOH step of the prep-silica gel elution series. Twenty ul of test solutions were added to the PAA and an eluting solvent of 9:1 acetonitrile:water was used to elute the plate for 10 cm. After elution, only R_f .55 was scraped and bioassayed with cress in test tubes as previously described. Control treatments were composed of a 1 cm width of silica-gel above the solvent front and distilled water. This test identified the fractions of the step gradient containing inhibitory compounds which do not cochromatograph with cycloheximide. Each channel of the the TLC plate was considered a replicate (five were used).

DCM was partitioned against 6.3 liters of 8-day fermentation broth, yielding 163 mg of extractable residue. This material was loaded onto a prep-silica-gel open-bed column and eluted with a 500 ml/step DCM:MeOH solvent series, as previously described. The weights of the material recovered from the 1.50, 1.75, 2.00 and 5.00 MeOH steps were 6.9,3.9,4.2 and 3.9 mg respectively. These residues and cycloheximide were brought-up in DCM to a concentration of 10 ug/ul. Ten ul of these solutions were injected onto an HPLC silica-gel column. Column conditions were: Column: Perkin-Elmer silica B/5; Mobil phase: 4:96 (MeOH:DCM); Flow: 1.5 ml/min; PSI: 1000; Fraction collection by time: 1 Fr./0.5 min. Fractionated samples were collected and loaded onto filter paper for cress bioassay for R_v determination of inhibitory components. Injections (two per sample) were used as replicates of treatments (composed of HPLC fractions).

RESULTS AND DISCUSSION

Partitioning of 101 fermentation broth inhibitors into an organic solvent.

The extraction of aqueous 101 fermentation broth with organic solvents indicated that hexane could not remove any compounds inhibitory to cress. Toluene and DCM both removed compounds inhibitory to cress. The fermentation broth after successive extraction with solvents remained inhibitory to cress, activity was however substantially reduced (Table 1).

Table 1. Response of cress to inhibitory components of 101 fermentation broth which are extractable with organic solvents immiscible with water.

Treatment	% Germination	LENGTH (mm) Shoots	Roots
101 before Ext.	22 b	.6 a	.5 a
101 after Ext.	94 c	21.8 b	8.6 b
Hex. extract	96 c	29.1 c	20.7 c
Tol. extract	12 b	.1 a	.1 a
DCM extract	0 a	.0 a	.0 a
water	96 c	24.2 bc	21.1 c

Means separated by Duncans multiple range test at the 5% level. Means within a column followed by the same letter are not significantly different.

These data indicate that inhibitory factors of the fermentation broth can be extracted with organic solvents. It appears contradictory that DCM and toluene could extract activity from the aqueous broth whereas only water and methanol could extract inhibitory compounds from lyophilized broth (Chapter 3, Table 1). The process of freeze drying may have altered solubility characteristics or caused volatility losses of inhibitors in some undetermined manner. For convenience in removal of solvent, DCM was used in all subsequent extractions. Studies with broth components revealed corn steep as the contributor of inhibitory compounds. These compounds are apparently very polar and not retained by a C_{18} Sep-Pak cartridge (Waters Associates, Milford MA), using water as a loading solvent.

Detection of cycloheximide in 101 fermentation broth

Cress germination and growth was used in all studies for inhibitor detection using chromatographic separation techniques. Cycloheximide, in this system, had an R_f on TLC plates of .67 as determined by cress response to the 6-7 cm portion of the plate and uv visualization of developed spot.

Table 2. The response of cress to cycloheximide and unknown inhibitors of the DCM extractable residues of the 101 fermentation broth. Inhibitory compounds fractionated on silica-gel TLC plate using a 10 cm run and 9:1 (acetonitrile:water) eluting solvent

	Cycloheximide			101 DCM Extract		
		Length (mm)			Length (mm)	
Treatment	% Germ.	Shoots	Roots	% Germ.	Shoots	Roots
PAA	96 b	30.9 b	15.6 b	96 b	30.0 b	21.0 bc
0-1 cm from PAA	92 b	25.8 b	9.4 b	96 b	28.9 b	13.2 b
1-2 "	92 b	30.8 b	9.1 b	92 b	28.6 b	19.1 b
2-3 "	100 b	24.2 b	13.2 b	96 b	31.5 b	18.7 b
3-4 "	96 b	24.8 b	10.6 b	100 b	33.0 b	16.0 b
4-5 "	100 b	22.6 b	9.6 b	92 b	32.1 b	13.6 b
5-6 "	80 b	28.0 b	8.4 b	15 a	1.8 a	.6 a
6-7 "	56 a	.1 a	.5 a	0 a	.0 a	.0 a
7-8 "	92 b	28.0 b	15.8 b	85 b	31.7 b	18.5 b
8-9 "	96 b	21.3 b	8.8 b	88 b	29.8 b	19.1 b
9-10 "	88 b	25.6 b	12.1 b	92 b	30.1 b	14.4 b
10-11 "(control)	96 b	29.3 b	11.6 b	100 b	39.1 b	15.5 b

Means separated by Duncans multiple range test at the 5% level. Means within a column followed by the same letter are not significantly different.

The DCM extract of the 101 fermentation broth showed two R_fs' with inhibitory activity (5-6 cm and 6-7 cm section of plates) (Table 2). Spray development and uv visualization showed two areas with bright spots at R_f .67 and .55. These data indicate that the DCM extract of the 101 broth contains at least two compounds that inhibit cress. One

of these compounds cochromatographs with cyclohexmide (R_f.67) and another is more polar. Since silica gel is a very polar material, smaller R_fs' can roughly be correlated to increased polarity.

Clean-up procedure for the DCM extract of the 101 fermentation broth

The bioassay results with the unused TPM indicated that no cress inhibitors were extracted with DCM (data not presented). Thus, no inhibitory activity was present in any step of the gradient used to elute the open-bed silica gel column. Therefore all inhibitory components of the 101 fermentation broth extractable with DCM were added by the 101 organism (Table 3). DCM extractable residues of unused TPM and 101- culture broth (22.7 mg and 58.6 mg respectively) were loaded onto the prep-silica-gel column. Data for % recovered material from this column indicated that a substantial quantity of material at fractions 5 and 6 was added to the broth by the organism during fermentation.

Table 3. Summary of step gradient elution of open-bed silica gel column. Fractionation of DCM extractable residues of 101 fermentation broth and unused TPM.

Step Gradient Composition						
	% MeOH	% DCM	101 broth		Unused TPM	
Fraction			mg eluted	% Recovered	mg eluted	% Recovered
1	0	100	4.4	8.3	5.7	27.0
2	.25	99.75	1.4	2.6	1.8	8.5
3	.50	99.50	.9	1.7	3.0	14.2
4	.75	99.25	5.3	10.1	1.7	8.1
5	1.00	99.00	8.6	16.3	1.0	4.7
6	1.25	98.75	17.3	34.8	2.5	11.8
7	1.50	98.50	3.4	6.5	2.1	10.0
8	1.75	98.25	1.6	3.0	1.4	6.6
9	2.00	98.00	2.1	3.9	1.9	9.0

Table 4. Cress response to DCM extractable residues of 101 fermentation broth.

		Length (mm)	
Treatment Description	% Germination[a]	Shoots[a]	Roots[a]
water control	93 b	22.6 c	25.0 c
DCM extract, before column	10 a	.1 a	.0 a
0-100 DCM step of gradient	93 b	20.9 c	23.0 c
.25-99.75 "	98 b	20.0 c	27.0 c
.50-99.75 "	85 b	20.9 c	29.6 c
.75-99.25 "	85 b	20.7 c	23.8 c
1.00-99.00 "	88 b	14.1 b	10.9 b
1.25-99.75 "	8 a	.1 a	.0 a
1.50-99.50 "	88 b	12.9 b	8.5 b
1.75-99.25 "	0 a	.0 a	.0 a
2.00-98.00 "	3 a	.0 a	.0 a

[a]Means separated by Duncans multiple range test at the 5% level.Means with in a column followed by the same letter are not significantly different.

The 1.25 MeOH step contained the greatest amount of material with inhibitory activity (Table 3,4). Therefore, fractionation of the components of this step were performed on an HPLC carbohydrate analysis column. Cress bioassay of the fractions collected allowed for the determination of retention volume (R_v) of active components (Table 5). The R_v of cycloheximide was also determined in this system. Identification of inhibitory compounds in the 1.75 and 2.00 MeOH steps was performed using M.S. and C^{13} NMR spectroanalysis (by Dow Co.).

Table 5. The response of cress to components of a standard cycloheximide sample and 1.25 MeOH gradient elution step of 101 DCM extract. Components were fractionated on a HPLC carbohydrate analysis column.

		Cycloheximide			101 1.25 MeOH Step		
			Length (mm)			Length (mm)	
Fraction	#/Rv	% Germ.	Shoots	Roots	% Germ.	Shoots	Roots
1	.0- 1.9	90 bc	21 bc	24 b	98 bc	20 c	23 c
2	1.9- 3.8	100 c	32 c	45 c	90 bc	12 b	18 b
3	3.8- 5.7	90 bc	27 bc	31 bc	98 bc	17 bc	22 c
4	5.7- 7.6	90 bc	27 bc	31 bc	85 b	14 bc	18 b
5	7.6- 9.5	100 c	31 c	32 bc	90 bc	17 bc	19 bc
6	9.5- 11.4	100 c	30 c	35 bc	98 bc	18 c	22 c
7	11.4- 13.3	15 a	1 a	1 a	18 a	2 a	1 a
8	13.3- 15.2	80 b	17 b	19 b	93 bc	14 bc	18 b
9	15.2- 17.1	100 c	26 bc	24 b	90 bc	17 bc	20 c
10	17.1- 19.0	90 bc	26 bc	29 b	93 bc	19 c	22 c
11	19.0- 20.9	95 c	23 bc	25 b	100 c	17 bc	22 c
12	20.9- 22.8	98 c	31 c	30 bc	90 bc	18 c	20 c
13	22.8- 24.7	90 bc	27 bc	29 bc	98 bc	18 c	23 c
14	24.7- 26.6	100 c	25 bc	28 b	85 b	15 bc	18 b
15	26.6- 28.5	100 c	26 bc	29 bc	95 bc	15 bc	21 c
16	28.5- 30.4	90 bc	24 bc	27 b	88 b	19 c	19 bc

Means separated by Duncans multiple range test at the 5% level. Means within a column followed by the same letter are not significantly different.

Cress bioassay of the various fractions identified a R_v for cycloheximide of 11.4-13.3 ml and an inhibitory component of the 1.25 step with the same value.

Mass spectra of active components of 1.25 MeOH step

The 1.25 MeOH step was enriched for the active R_v as identified with cress on the carbohydrate analysis column using an open-bed silica gel column with a isocratic mobile phase. As fractions were eluted from this column, they were taken to dryness with N_2 gas, brought up in 20 ul of DCM of which 5 ul were injected onto the carbohydrate analysis column. This technique allowed for the tracing of compounds from the silica gel open-bed column as they were eluted. The pooling of fractions from the open-bed column was based on the HPLC traces of the various fractions containing material which appeared at the inhibitory R_v. Material pooled from the open-bed column was injected onto the HPLC carbohydrate analysis column and the compound eluted at the R_v of 11.4-13.3 ml was collected over successive runs for generation of a mass spectra. A cycloheximide standard solution was injected and collected from the HPLC column for generation of a mass spectra in a similar way. A mass spectra of a cycloheximide standard and the inhibitory component of the 1.25 step are presented in Figure 1a & b.

Figure 1 a & b. Mass spectra of a cycloheximide standard (a) and inhibitor from isolate 101 (b) as collected from HPLC column (Table 5).

Figure 1 A.

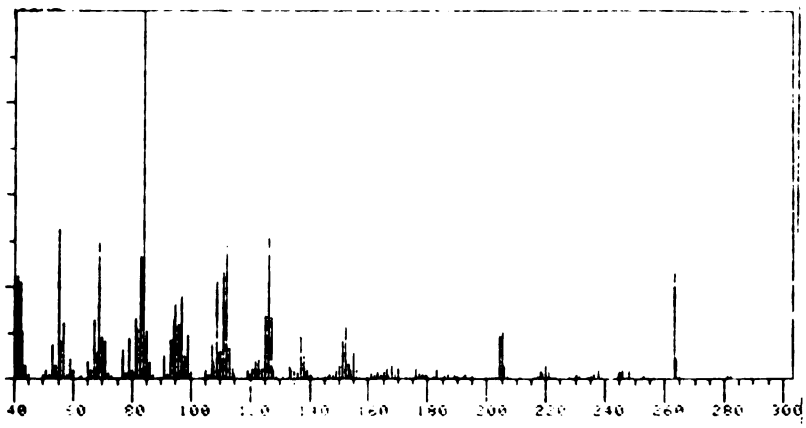
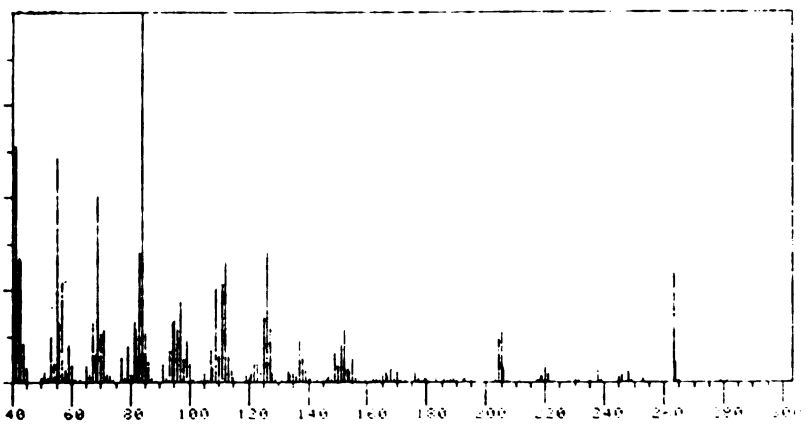


Figure 1 B.



A comparison of the mass spectra confirmed the identity of the active component of the 1.25 MeOH step as cycloheximide.

Chromatographic characterization of other compounds with inhibitory activity

A silica-gel TLC plate was used to fractionate the 1.0, 1.25, 1.50 and the 1.75 MeOH steps from the silica gel elution series. Previous TLC studies had identified two R_fs' (.67 and .55, Table 2) from the 101 DCM crude extract with inhibition against cress. The material at R_f .67 was identified as cycloheximide.

The material from R_f .55 was subjected to continued analysis to derive its identity. Bioassays of steps of the silica-gel open-bed column elution series containing material with inhibitory activity were conducted (Table 6). The 1.75 MeOH step contained the greatest amount of inhibitory material.

Table 6. The response of cress to an unknown with an R_f of .55 on TLC silica gel plates. Solvent system was 9:1 (Acetonitrile:water) with a solvent run of 10 cm.

		Length (mm)	
Sample fractionated	% Germ.	Shoots	Roots
1.00 MeOH step	93 b	21 c	11 b
1.25 "	80 b	20 c	7 b
1.50 "	30 a	5 b	1 a
1.75	0 a	0 a	0 a
10-11 cm (control)	100 b	23 c	12 b
water	95 b	24 c	13 b

Means separated by Duncans multiple range test at the 5% level. Means within a column followed by the same letter are not significantly different.

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An HPLC silica-gel column was used to fractionate a standard solution of cycloheximide and the 1.50, 1.75, 2.00 and 5.00 MeOH steps for determination of R_v as identified using the cress bioassay. On this column, cycloheximide was found to have a R_v of 4.25-5.00 ml (data not shown). The data for the R_v of the active components of the four silica-gel elution indicated consistent toxicity in fraction 12 (Table 7).

Table 7. The response of cress to the components of 1.5, 1.75, 2.00 and 5.00 MeOH steps of a open-bed silica-gel column elution series. Samples fractionated on an HPLC silica-gel column.

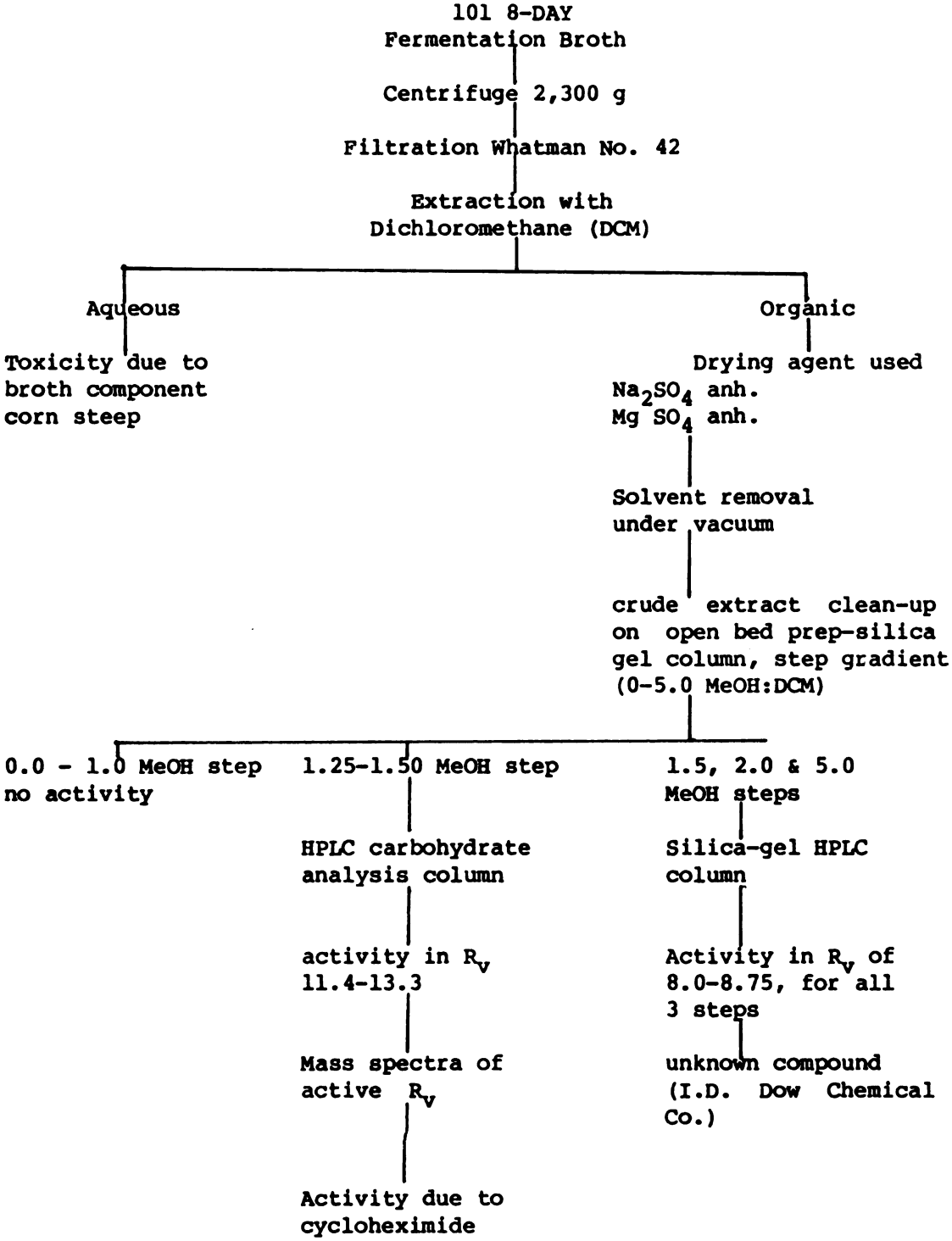
		Length (mm)							
		1.5 STEP		1.75 STEP		2.00 STEP		5.00 STEP	
Fr.no	Rv (ml)	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
6	3.5- 4.25	23.9	25.5	24.2	22.7	25.4	25.5	23.1	28.8
7	4.25- 5.00	.0	.5	21.6	19.9	28.9	29.3	22.4	25.8
8	5.00-5.75	16.5	13.2	23.3	32.4	20.9	15.9	28.7	30.7
9	5.75- 6.50	22.5	23.7	27.1	30.2	27.0	27.0	23.8	25.3
10	6.50- 7.25	23.2	26.5	24.0	34.1	26.2	32.9	27.8	35.7
11	7.25- 8.00	24.6	27.5	27.3	28.3	17.8	16.0	14.2	24.9
12	8.00- 8.75	20.6	21.4	17.9	10.1	.1	.8	.0	.6
13	8.75- 9.50	27.8	29.1	18.6	20.9	24.5	24.4	21.7	21.5
14	9.50- 10.25	23.9	24.8	21.8	20.7	21.5	25.9	25.0	31.1

Each step sample was replicated two times, data were not subjected to an AOV. Each mean is the average of 20 cress seed, 10 per replicate.

Fractions not listed showed no obvious signs of inhibition. It is clear from these data that much of the inhibitory activity of the 1.50 MeOH step can be attributed to cycloheximide (R_v4.25-5.00). Steps 1.75,2.00 and 5.00 all show inhibitory activity in R_v 8.00-8.75. Material at this R_v was used to generate a mass spectra and C_{13} NMR for structure elucidation (by Dow Chemical Co.). A flow diagram of

the analytical procedure is provided (Figure 2):

Figure 2. Analytical protocol for isolation and identification of inhibitors in 101 fermentation broth.



These data indicate that the presence of cycloheximide in fermentation broths should not remove them from consideration as producers of other herbicidal metabolites. In a commercial scale fermentation, batch conditions can be adjusted to favor the production of a specific antibiotic even though the producing organism can form several others (Churchill, personnel communication, 1983). Iwai and Omura (2) provide an extensive review on the conditions that affect antibiotic production in commercial scale fermentations. They point out that the cultivation method for a specific isolate can vary fermentation products quantititively and qualitatively. The production of trace antibiotics can be increased by adjustment of culture conditions such as pH, temperature, agitation rate or nutrient composition of the fermentation broth. The data presented here indicated the need for precise and accurate isolation of all culture batch components with inhibitory activity. If common antibiotics, such as cycloheximide, are produced by a large number of isolates, then it is important to identify it in fermentation broths. However, analysis must continue to enrich for trace compounds which may also possess herbicidal activity. Techniques which can efficiently isolate and accumulate trace compounds will aid in the discovery and identification of new herbicidal antibiotics.

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