



Biological and chemical characterization of active metabolites produced by *Pyrenophora teres*
by Sami Satouri

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology

Montana State University

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Abstract:

The ethyl acetate fraction of the culture fluid of *Pyrenophora teres* showed a phytotoxic activity on an excised leaf of a susceptible cultivar of barley. The purification of the active metabolites involved three chromatographic methods, namely sieve size chromatography, thin layer chromatography, and high performance liquid chromatography. Three active compounds were purified. The two less active metabolites were crystallized and their structures were determined using x-ray crystallography.

Both compounds are related and differ by a methoxy group on carbon #7. Pyrenoline A and Pyrenoline B are the trivial names given to these two metabolites.

The third and most active compound was chemically characterized using conventional spectroscopy and was found to have the same chemical properties as Pyrenolide A, a compound that was identified by Nukina in 1980. This thesis describes the purification steps of the three compounds and their biological activities on the host plant (*Hordeum vulgare* sp) as well as non-host plant species. Attention was paid to Pyrenolide A. This included its production in different isolates of *P. teres*, its quantification, and the effect of different leaf extracts on its production.

Pyrenolide A is active on a wide range of plant species including weeds. Leaf extracts from host and nonhost plants were effective in increasing toxin production in culture. Four isolates including net and spot forms, were tested for toxin production. Pyrenolide A was produced in the liquid culture of all four fungal isolates.

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This thesis has been read by each member of the author's committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

The ethyl acetate fraction of the culture fluid of Pyrenophora teres showed a phytotoxic activity on an excised leaf of a susceptible cultivar of barley. The purification of the active metabolites involved three chromatographic methods, namely sieve size chromatography, thin layer chromatography, and high performance liquid chromatography. Three active compounds were purified. The two less active metabolites were crystallized and their structures were determined using x-ray crystallography. Both compounds are related and differ by a methoxy group on carbon #7. Pyrenoline A and Pyrenoline B are the trivial names given to these two metabolites.

The third and most active compound was chemically characterized using conventional spectroscopy and was found to have the same chemical properties as Pyrenolide A, a compound that was identified by Nukina in 1980. This thesis describes the purification steps of the three compounds and their biological activities on the host plant (Hordeum vulgare sp) as well as non-host plant species. Attention was paid to Pyrenolide A. This included its production in different isolates of P. teres, its quantification, and the effect of different leaf extracts on its production.

Pyrenolide A is active on a wide range of plant species including weeds. Leaf extracts from host and non-host plants were effective in increasing toxin production in culture. Four isolates including net and spot forms, were tested for toxin production. Pyrenolide A was produced in the liquid culture of all four fungal isolates.

INTRODUCTION

Man has been intrigued by plant diseases for many centuries. The Romans were concerned about the periodic plagues of rust that appeared on their crops. In their attempt to control this disease, they held an annual festival where a red dog was sacrificed in appeasement of the god Robigus. Since Roman times the advent of microbiology has allowed the identification of thousands of pathogens. Many of these pathogens are organisms which produce a variety of biochemical compounds deleterious to their host plants. These compounds, as well as the pathogens, may vary in their degree of biological activity and their host specificity (60). A dramatic example is the southern leaf blight of maize. In the summer of 1970, this disease devastated much of the corn crop in the United States. The epidemic caused the greatest crop loss in the shortest time span of any plant disease ever reported. The maize epidemic was similar to an earlier plant disease disaster that affected the oat crop in North America during the summer of 1946. In both cases, toxins produced by the causal fungi were the major factors in the destructive process (46,68).

There is no universal agreement upon the definition of the term phytotoxin (17,78). A widely accepted definition describes a phytotoxin as a toxic secondary metabolite produced and released by a pathogen, lacking enzyme, hormone or nucleic acid properties. An alternative definition was given by Luke and Gracen (41) who described a phytotoxin as a metabolite of microbial origin which is toxic to plants but does not play a major role in pathogenesis. Phytotoxins may be classified based on different criteria: host specificity, biological activity, general role in disease development or chemical structure. The term Vivotoxin describes a class of phytotoxins that can be recovered from infected tissue (17). Vivotoxins are not regarded as primary factors in disease development. A Pathotoxin is another class of phytotoxins defined as a host-specific toxin which, in reasonable concentrations, induces all the typical disease symptoms and the production of which is correlated with pathogenicity (78).

Anton de Barry (12) is credited with the idea that toxins are involved in plant disease. De Barry's work was the precursor of research on extracellular enzymes rather than toxin research (56). Rosen in 1926 questioned the meaning attributed to toxic components in culture filtrates (54). According to him, it is easy to prove that filtrates contain toxic substances, but very difficult to relate this finding to the etiology of disease. Gottlieb in 1943

extracted toxic fluids from tomato plants infected with *Fusarium wilt* in an attempt to relate the toxin production to the disease occurrence (29). This work was an important contribution to toxin research in that it is the first time that such steps had been taken. The isolation of a biologically active compound from infected tissue is important evidence for the involvement of the toxic metabolite in pathogenesis. In 1954 Gaumann (26) was the first to proclaim that microorganisms are pathogenic only if they produce toxins. To date, there is no evidence to support this claim. The postulates of Dimmond and Waggoner (1953) still have an influence on toxin research (17). They call for the separation of the toxin in pure form from the diseased plant, and reproduction of the characteristic disease symptoms following re-injection into healthy tissue.

Phytotoxins may have different useful applications. They can be utilized as plant disease models, gene markers, herbicide models, tools for determining the normal physiology of a plant, or for taxonomic, commercial and selection purposes (56). Phytotoxins have been used as a taxonomic tool in distinguishing between closely related bacterial species. Tabtoxin has been used to distinguish between *P. syringae* pv *tabaci* and *P. angulata* (7), the former being a toxin producer while the latter is not. These microorganisms are not distinguishable by morphological or

serological criteria (7). Although an attempt could be made to determine if toxins produced by Pyrenophora teres can distinguish P. teres from Pyrenophora graminea, such experiments have never been reported. Further, tentoxin, a phytotoxin produced by Alternaria tenuis has been used to determine the genealogy of some higher plant species. Its inhibitory effect on the chloroplast coupling factor (CF1) of a specific plant species has allowed the determination of species ancestry (8). PC-toxin is a host-specific toxin isolated from Periconia circinata (mangin) Sacc. Wolpert and coworkers in 1980 showed that toxin treatment of root tips of sorghum plants from susceptible cultivars selectively enhanced the synthesis of 14 Kd proteins (79). Traylor and coworkers in 1988 suggested the use of PC-toxin as a marker for the PC-gene (73).

The potential of phytotoxins as herbicides or as a model for new herbicides has been suggested recently (15, 20, 67, 68). Higher plants and their pathogens have, in all likelihood, evolved together and in the process may have had considerable biochemical interchange. An understanding of this relationship may help focus approaches to new herbicidal investigations (37). In the recent past, considerable effort has been expended in finding novel and selective phytotoxins with potential use for the control of weeds (37). As a result, several new compounds have been structurally defined using x-ray crystallography.

Exserohilone was isolated from Exserohilum holmii a pathogen of Dactyloctenium aegyptium (crow foot grass) (67). Drechslera gigantea, a pathogen of Bermudagrass (Cynodon dactylon) and quackgrass (Agropyron repens), produces several bioactive terpenoids, among them gigantane, a phytotoxin that mimics growth hormone properties and produces green island effects on the host leaf (25,38). Monocerin is a phytotoxin produced by Exserohilum turcicum, a pathogen of Johnsongrass (Sorghum halepense). It is also active against tissues of tomato and Canada thistle (37).

The use of plant phytotoxins in controlling weeds may have several advantages over the use of weed pathogens (15). The use of a living pathogen as a biocontrol agent is not without risks. For instance, some weed pathogens are equally capable of infecting economic crops as well as weeds. Further, these pathogens may be impossible to contain within a localized area because of the unpredictable nature of dispersal mechanisms. More importantly successful infection of target weeds may be difficult to accomplish. Several factors play important roles in successful weed infection: temperature, humidity, amount of inoculum and genetic differences among target species affecting their susceptibility. These variables must be optimized to allow efficient infection of target weeds and their subsequent control. A given pathogen may be efficacious in controlling a target weed in one geographic

location because of the existence of near optimal conditions in that area, but in a different locality the same pathogen may be found to be unsuitable as a control agent. These limitations merit consideration of the use of phytotoxins as more convenient control agents. Because bioproducts such as phytotoxins may be easier to degrade, phytotoxins have the additional advantage of ecological safety. The specificity of some phytotoxins towards a given species is another advantage over using the pathogen itself. Once synthesized, the toxins may be less expensive to apply in high concentrations. However, some obstacles have yet to be overcome. Phytotoxins have never been shown to kill any weed when they are sprayed on the plant.

The possible use of phytotoxins to create novel models for more effective herbicides is a very promising area of research and may result in herbicides that may enhance environmental safety. For these reasons, phytotoxins may constitute an efficient method of weed control. Although several metabolites from weed pathogens have been characterized, very few specific weed phytotoxins are known. One of the first host-selective weed phytotoxins was isolated from Bipolaris cynodontis and characterized by Sugawara and Strobel (70). Stierle recently described a phytotoxin found in the liquid culture of Alternaria alternata Lam (65). It is the first host-specific dipeptide to be isolated from a weed pathogen. The pathotoxin was named

maculosin and its structure has been determined and chemically synthesized (65). Alternaria alternata also produces other non-specific phytotoxins that have been characterized chemically and biologically (64).

Phytotoxins can be useful tools for plant breeders since their use may facilitate plant selection. Cochliobolus victoria (Nelson) is the causal agent of victoria blight in oats, which was described about 40 years ago. In 1947 Murphy and Meehan reported that C. victoria produces a specific phytotoxin to which they assigned the trivial name victorin. Forty years elapsed before the structure was elucidated (43). Victorin was utilized for selection of resistant cultivars of oats (32, 33). Similarly, eye spot disease on sugarcane seedlings caused by Drechslera sacharii is one of the most destructive diseases of sugarcane under favorable environmental conditions. Steiner and Byther correlated cultivar resistance of sugarcane plants and the symptoms caused by the application of the toxin on seedling plants under greenhouse conditions (66). Studies on the mechanism of action and the toxin specificity revealed a membrane-binding effect (69).

The oldest application of a phytotoxin is the one employed by Wheeler and Luke in 1955 in which a relatively crude toxin preparation was utilized to screen large populations of oat plants for resistant individuals (77). The practice soon extended to the selection of novel useful

plant genotypes as a step in the in vitro breeding programs, before the regeneration of plants (32, 33). In vitro selection of barley and wheat for resistance has been carried out using a crude toxin preparation from Cochliobolus sativus (10). According to Chawla and coworkers, barley and wheat lines that have been regenerated from callus lines surviving the toxin treatment were less sensitive to the pathogen when inoculated with the living organism.

The potential contribution of a given toxin to disease development can be determined by re-isolation of the toxin from infected tissue and the demonstration of its disease-producing potential. Yet there may be severe constraints in attempting to isolate toxins from diseased plants due to the instability of the phytotoxins, their presence in very low concentration or their irreversible binding to host components. Furthermore, the specific stage relative to the establishment of disease at which the toxin is isolated may be an important factor. Toxin isolated after the pathogen is well established in the host plant may have little or no relevance to pathogenesis. The question of the involvement of phytotoxins in disease etiology may be further compounded by the production of multiple metabolites with toxigenic potential. In these instances, disease induction by a single phytotoxin or several phytotoxins acting in concert may be difficult to demonstrate.

Drechslera maydis, D. oryzae, D. sorghicola and D. gigantea produce more than one active phytotoxin (25, 68, 80). Some of them belong to the same family. The relevance of each of these compounds to the occurrence of the disease is not known.

Pyrenophora teres is an important pathogen of barley that causes the disease known as net blotch. The economic losses caused by this pathogen during the last few years have initiated concern among the international community. Net blotch disease occurs wherever barley is grown in temperate and humid regions of the world (16). In recent years, with intensive management and increasing popularity of barley, diseases such as net blotch have assumed greater significance (61). The 1979 epidemic in the United Kingdom was particularly notable (35). During this epidemic, foliar application of fungicides was recommended to limit the spread of infection. Seed treatments were also used but they were only effective in reducing the level of the primary inoculum (59). The use of resistant cultivar remains as the most effective means of control.

The symptoms produced by P. teres occur on the blades and sheaths of the leaves and may extend to the flowers and grain. The initial lesions appear as minute spots or streaks which increase in size to form narrow, dark brown, longitudinal and transverse streaks producing a net-like pattern. This netting symptom is typical of Pyrenophora

teres Drechs. f. teres. Other isolates of P. teres produce spot-type rather than net-type symptoms. Isolates causing these spot-type symptoms are referred to as Pyrenophora teres Drechs. f. maculata Smedg (45, 62). Pyrenophora teres has been reported by several authors to produce more than one active metabolite (28, 63), some of which are host specific and some may have relevance to disease development.

Several people have studied biochemical aspects of P. teres (28, 44, 63). Smedegard-Peterson isolated and characterized two phytotoxins (63). The structure of these compounds has been elucidated (1) and their involvement in disease development was reported (63). They were found to be identical to aspergillomarasmine A and B.

Aspergillomarasmine A and B (AM-A and B) were described in 1965 by A. L. Haenni and coworkers (30). They caused wilting, necrosis and leaf fall of plants such as tomato, willow, olive, melon and barley. AM-A and B have been isolated from a number of other fungi besides P. teres, including Fusarium oxysporum f. sp. melonis (9), Aspergillus oryzae and Aspergillus flavus (30), and Colletotrichum gloeosporioides (2, 6). Most interesting is the fact that AM-A and B have been reported as new microbial hypotensive agents regulating the renin angiotensin system and producing an anti-inflammatory effect on burned skin (47).

In 1980 Nukina and coworkers in Japan isolated and chemically characterized a new metabolite from P. teres in liquid culture (41). The compound was found to be a ten-membered lactone and it was given the trivial name of Pyrenolide A. No biological activity was reported for this compound.

Many pathogens have been shown to produce more than one phytotoxin which had different levels of activities (64, 68, 80). A good example is the study on Alternaria alternata from knapweed (Centaurea spp). Isolates from A. alternata usually produce an abundance of known phytotoxins such as tenuazoic acid and perylene quinones. Alternaria alternata also produces similar compounds (64). Because the researcher pursued necrotic activity present in fractions not containing these compounds, the maculosin group was discovered, one member of which is a very host-specific dipeptide (65).

A number of the metabolites produced by P. teres were described by Smedegard-Peterson and Manabu (44, 63). Because of their proteinaceous nature, aspergillomarasmine A and B isolated from P. teres cannot be found in the lipophilic fraction of the culture filtrate of this fungus. The first objective of this study was to test the ethyl acetate fraction of the liquid culture of P.teres for phytotoxic metabolites. If a phytotoxic activity was found in this fraction, then purification would ensue using the

standard chromatographic techniques such as sieve size chromatography (sephadex LH20), thin layer chromatography (TLC), and high performance liquid chromatography (HPLC).

The establishment of a purification procedure for the active metabolites was the second goal in this study. The purified compounds were subjected to chemical characterization and biological analysis. The bioassays provide information about host specificity, minimum active concentrations required to produce a symptom, and the host range of the isolated compounds. The chemical characterization and the biological activity of the active metabolites constitute the ultimate goal of this study.

MATERIALS AND METHODS

Fungal Isolates

Pyrenophora teres, isolated from barley plants growing in Montana fields, was kindly provided by A. L. Scharen and B. Baltazar of Montana State University. The fungus was maintained on V-8 juice medium. Five isolates consisting of both Net and Spot forms were utilized throughout this study. The isolate Pt-WPb is a net-type isolate that was used for the toxin purification. This isolate was obtained in single spore culture and used to inoculate the broth medium.

Fungal Culture

Filner, in 1965, described an artificial medium, called M-1-D medium, that has been used successfully to grow different fungi (23). A modification of the M-1-D medium was described by Karr and coworkers in 1974 (36). In addition to the fact that it is a basic defined medium, the modified M-1-D medium has the additional advantage of being relatively easy to make and allows the addition of several supplements. For these reasons this medium was used throughout this study (see Appendix A for medium composition).

One of the problems that is often encountered in toxin isolation is the attenuation of toxin production by the fungus (50). To avoid the occurrence of such a phenomenon, a fresh transfer was utilized to inoculate the liquid culture. After every three transfers on V-8 juice agar plates, a primary culture was re-established from infected tissue. The liquid medium was supplemented with barley leaf extract following the method of Robeson and Strobel (52, 53). The leaf extract from barley plants was added at the rate of 0.1 g/l to the M-1-D modified medium prior to autoclaving. This method was effective in increasing toxin production.

Culture Conditions

One-liter Erlenmeyer flasks containing 500 ml of M-1-D medium were inoculated with agar plugs containing fungal mycelium. The flasks were shaken at 200 rpm for 2 to 3 weeks at 26°C under fluorescent lamps.

Influence of Different Leaf Extracts on Toxin Production

Leaf extracts from the following plant species were used to supplement the culture medium: Fescue (Festuca eliator.), bermudagrass (Cynodon dactylon), barley (Hordeum vulgare, cv. Compana and cv. CI 9819). Leaf extracts were obtained using the method of Robeson and Strobel (53) with

the following modifications: freshly harvested leaves were minced. Double distilled water was added up to 20 ml per gram of fresh weight. The flasks were placed in an oven at 35°C overnight. The suspension was filtered through 6 layers of cheese cloth to remove leaf residues. The filtrate was then reduced to a final concentration of 1 g of leaf tissue per 10 ml of distilled water using rotary evaporation in vacuo at 35°C. The residue was filter-sterilized using a 0.2 μ analytical filter unit (type A, Nalgene, VWR) and the solution stored at 4°C. Flasks of 125 ml containing 50 ml of M-1-D modified medium were supplemented with 50 μ l of leaf extract (1 ml of leaf extract/1 l of M-1-D) prior to autoclaving. Each treatment was conducted in triplicate. All flasks were inoculated from the same petri dish culture of P. teres using the isolate Pt-WPb. The flasks were shaken for 10 days before extraction and dry weight quantification.

Isolation and Purification

The culture broth was filtered through 4 layers of cheese cloth and reduced to half of its original volume by rotary evaporation in vacuo at 35°C. The fluid was then extracted five times with half the volume of ethyl acetate. The combined ethyl acetate fractions were washed with an equal volume of distilled water and evaporated to dryness at

25°C under reduced pressure. Isolation involved the following chromatographic methods:

Sieve Sizing Chromatography

The residue was permeated through a sephadex LH-20 (65 g) using methanol as a mobile phase.

Thin Layer Chromatography (TLC)

TLC was performed on Merck silica gel 60 F 254 (0.5 mm) precoated plates. The following solvent systems were used:

Methanol : chloroform (7:100); ethanol : chloroform (7:93); pyridine : chloroform (6:1); chloroform : methanol (12:1); benzene : ethyl acetate (6:4); ethyl acetate : methanol : water (80:5:5); benzene : ethanol (9:1); acetonitrile : methanol (1:2) and butanol : pyridine : water (6:4:3).

Two solvent systems were selected for their effectiveness:

(A) ethyl acetate : methanol : water (80:5:5); (B) chloroform : methanol (12:1). The thin layer chromatography was repeated twice in the phytotoxin purification, using both solvent systems A and B. UV light (254 and 380 nm) was used for the detection of the compounds of interest. None of the universal reagents including anisaldehyde, ninhydrin, and sulphuric acid reagents were found to be useful in visualizing any of these compounds.

High Performance Liquid Chromatography (HPLC)

The most active fraction was subjected to reverse phase HPLC. The HPLC used was a Waters M6000A solvent delivery pump, fitted with a Waters model UK6 injection valve. This

was connected to a 6 x 150 mm C18 column (Senchu pak) which in turn was coupled to a Waters Model 440 UV detector set at 254 nm. Different solvent systems were used:

Methanol : water (7:3); methanol : acetonitrile (35:65); methanol : acetonitrile (65:35); acetonitrile : water (65:35); acetonitrile : water (1:1) and acetonitrile : methanol : water (7:3:2).

Two solvent systems were selected for their effectiveness, solvent system A: acetonitrile : water (1:1), and solvent system B: acetonitrile : water (65:35). These two solvents were used for purification and quantification, respectively.

Toxin Production and Quantification

Analytical HPLC was used for the identification and quantification of different metabolites (27, 70, 75). This method was quick, easy and reliable. In a defined set of conditions (column, flow rate, solvent mixture, sensitivity of the detector), a given compound can be partially characterized by its retention time. Analytical HPLC was used to quantify the most active compound. The peak height or the peak area was calculated and a standard curve was used to determine the amount of compound in the injected sample.

Standard Curve

A standard curve was generated using a pure toxin solution, acetonitrile:water (65:35) as a mobile phase, a flow rate of 1.0 ml/min, and a detector set at 0.1 AUFS.

Toxin samples of known concentrations were injected. Each concentration was made in triplicate. The peak height was calculated and a regression curve was generated using the MSU-STAT program (R.D.I, MS, 1987) (Figure 1).

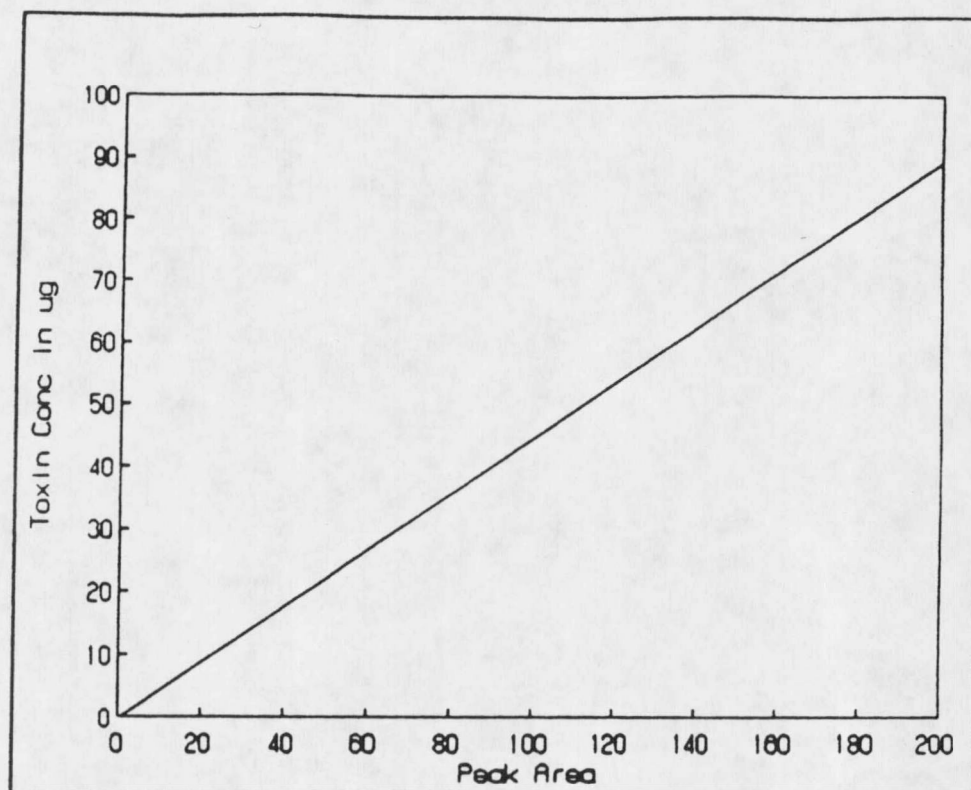


Figure 1. Standard curve for the quantification of Pyrenolide A produced by P. teres. The curve was generated using a pure toxin solution, acetonitrile : water (65:35) (v:v) as a mobile phase, a flow rate of 1.0 ml/min, and a detector set at 0.1 AUFS. MSU STAT was used to obtain the regression curve.

Bioassays

Plants were grown under controlled environmental conditions of 8 hrs of darkness at 28°C and 16 hrs of light (25 μ einstein/m²/s) at 32°C. The leaf puncture assay consisted of puncturing a detached leaf. The wound was then overlaid with 5-10 μ l of the test solution (70). Ethanol (5%) was used to dissolve the compounds. The treated leaf was incubated in a moist chamber for 42 hrs and the lesion size was measured. The three active compounds were assayed on barley cultivars and other plant species to determine their host specificity and their minimum active concentrations. The control in all bioassays was run using 5% ethanol.

Screening of Different Isolates for Toxin Production

Four isolates of P. teres, two net form and two spot form isolates, were tested for toxin production using analytical HPLC. The isolates were grown in 150 ml flasks containing 50 ml of basic M-1-D medium. The experiment consisted of four replicates for each isolate. The fungus was grown for 10 days. The filtrate was filtered through Whatman #1 filter paper. The liquid culture was reduced to 1/3 of its original volume and extracted three times with 50 ml ethyl acetate. The extracts were combined and washed with distilled water, then dried under reduced pressure.

Every sample was eluted in 1 ml of acetonitrile : water (65:35) prior to injection into the HPLC. The presence of the phytotoxin was tested using the HPLC analytical method.

Chemical Analyses

Nuclear Magnetic Resonance

The proton nuclear magnetic resonance (NMR) spectrum of Pyrenolide A, Pyrenoline A and Pyrenoline B was recorded on a 500 MHz Bruker spectrometer. Chemical shifts were recorded in ppm units relative to trimethylsilane (0 ppm) with CDCL₃. The NMR interpretation was provided by Steve Coval, Department of Chemistry, at Cornell University.

Mass Spectroscopy

High resolution mass spectroscopy was provided by J. Sears, Chemistry Department, Montana State University. The interpretation was provided by Steve Coval at Cornell University.

RESULTS AND DISCUSSION

Introduction

Following purification of the active components of the ethyl acetate fraction, chemical and biological analyses were performed. Three pure active compounds were recovered. The structure of two of these was elucidated using x-ray crystallography and found to be related. After a thorough on-line computurized chemical search both metabolites were classified as novel compounds. They were given the trivial names of pyrenoline A and pyrenoline B. Both compounds were tested for their biological activities on barley and other plant species. The third and most active compound did not crystalize. When the chemical analysis was performed, it became clear that the same compound had been described in previous studies and was given the trivial name of Pyrenolide A (44). Nukina in 1980 reported that this compound had a fungistatic effect and also caused hyphal abnormalities in different phytopathogenic fungi including Fusarium oxysporum and P. teres. However, the purification procedure of this compound and its biological activity on plants is still not known. The biological activity on different plant species, the effect of plant supplements on toxin production, and the presence of this compound in the liquid

culture of different P. teres isolates will be presented and discussed in this chapter.

Purification of the Three Active Compounds

The crude ethyl acetate extract of P. teres culture was permeated through sephadex LH20 (65 g). Fractions of 5 ml were collected and the elution was monitored at 289 nm. Four peaks were recovered and assayed for their biological activities on an excised barley leaf. The highest activity was found in peak #2 (F: 73-83) and smaller amounts of activity were found in peak #3 and peak #4 (F: 85-100) (Figure 2). The fractions collected under peak #2, #3, and #4 were subjected to two cycles of thin layer chromatography. Three active compounds were recovered. These three metabolites had different R_f values in a number of solvent systems (Table 1). Two TLC cycles allowed the purification of the less active compounds (Figure 3). Crystallization followed and the compounds were structurally characterized using x-ray crystallography. An on-line computer chemical search on these metabolites was done, the results of which revealed that these compounds are novel. They were given the trivial names of Pyrenoline A and Pyrenoline B.

The third and the most active compound required one more chromatographic step for purification. Thus, HPLC was used. Pyrenolide A had different retention times in a number of solvent systems (Table 2).

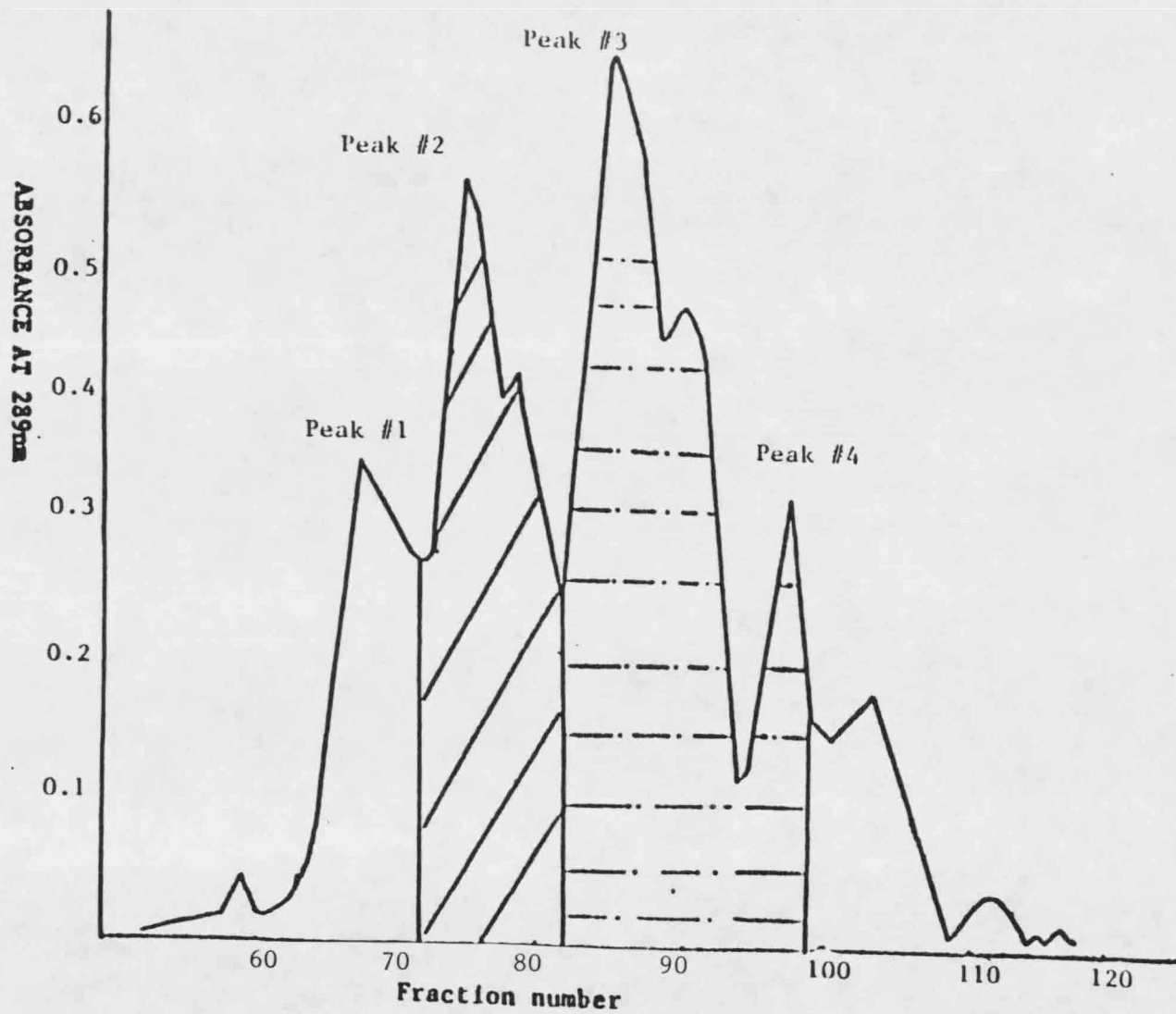


Figure 2. Sieve size chromatogram from a sephadex LH20. The mobile phase used was MeOH and the fractions were read at 289 nm. The bioassays performed indicated that the active fractions were under peaks #2, #3, and #4.

Table 1. The Rf values of the three phytotoxins Pyrenolide A (Pyrde-A), Pyrenoline A (Pyrne-A), and Pyrenoline B (Pyrne-B) in different solvent systems.

Solvent	Ratio (v/v)	Pyrde-A	Pyrne-A	Pyrne-B
CHCL3 : MeOH	9:2	-	0.59	0.53
CHCL3 : MeOH	12:1	-	0.52	0.48
CHCL3 : EtOH	93:7	0.55	0.36	-
CHCL3 : Pyridine	1:6	0.87	0.81	0.78
benzene : EtOAC	6:4	0.27	0.10	-
benzene : EtOH	9:1	0.44	0.19	-
ButOH : pyridine : water	6:4:3	0.83	0.65	0.53
toluene : EtOH	9:1	0.18	0.14	-
acetone : EtOH	9:1	0.80	0.82	-
butOH : CHCL3	10:2	0.65	0.53	-
EtOAC : MeOH : water	0:5:5	0.7	0.58	0.46

Table 2. Retention times of Pyrenolide A (PyrdeA) in different solvent systems in the HPLC purification step.

Solvent	Ratio (v/v)	PyrdeA retention time (min)
MeOH : AcCN	35:65	3.75
MeOH : AcCN	65:35	3.1
AcCN : water	65:35	4.3
AcCN : water	1:1	5.3
AcCN : MeOH : water	7:3:2	4.06

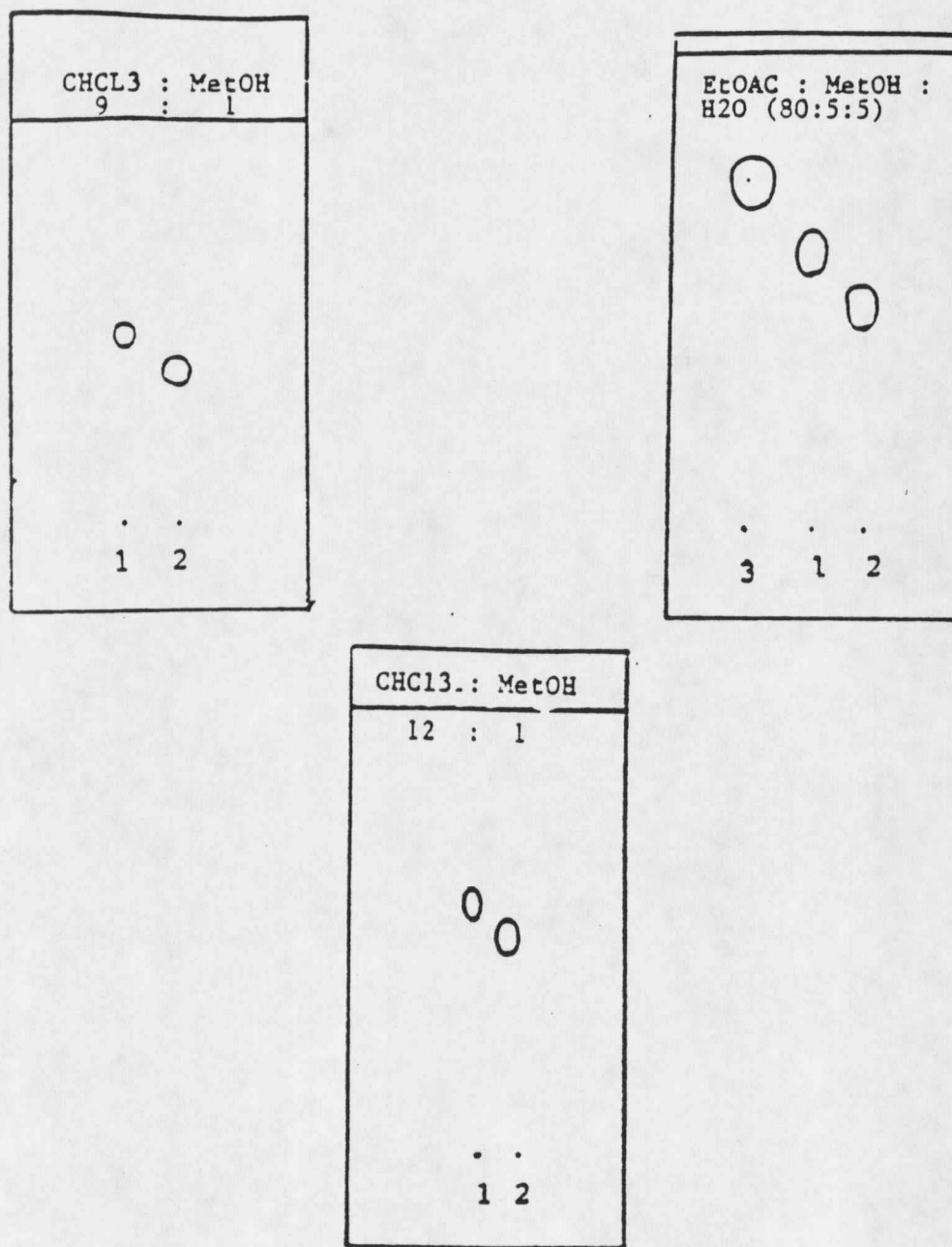


Figure 3. Mobility of Pyrenoline A, Pyrenoline B, and Pyrenolide A in different solvent systems. 1 = Pyrenoline A, 2 = Pyrenoline B, 3 = Pyrenolide A.

Confirmation of Purity

Purity is a prerequisite for any chemical characterization and biological analysis. A single peak upon HPLC elution and migration of the compound as a single spot on the TLC plate using more than one solvent system are important criteria for the confirmation of purity. On the other hand, the advent of GC-MS allows the detection of nanomolar quantities of a given compound, which makes it the technique of choice for testing the purity of different compounds. All three techniques were employed to test the purity of the three metabolites. A purified sample was injected into a reverse phase HPLC and a single peak was eluted (Appendix B, Figure 9). Furthermore, when spotted on a TLC plate and run in two solvent systems, all three compounds migrated as a single spot. Also when each compound was injected into a GC-MS system a single peak was recovered.

Chemical Analysis

Pyrenoline A

Crystals of Pyrenoline A were obtained by dissolution in methanol/chloromethane, and this solution was allowed to vapor diffuse with benzene. The crystals belonged to space group P2₁, (z=2) with a= 5.145 (6), b= 19.267, c= 6.626(8) Å. The structure of Pyrenoline A was analyzed by x-ray

diffraction, and a drawing of the final x-ray model showing relative stereochemistry only is shown in Figure 4.

The high resolution mass spectrum of Pyrenoline A indicated the molecular formula $C_{15}H_{15}N_1O_4$. The ^{13}C NMR spectrum showed the presence of nine aromatic carbons which, in conjunction with the presence of nitrogen, suggested a quinoline or isoquinoline aromatic nucleus. In the ^1H NMR spectrum there are only four proton signals which display any proton-proton coupling. Two geminally coupled protons at $\delta = 2.86$ (dd = 3.3, 17.8 Hz) and 3.17 (dd $J = 5.8, 17.8$ Hz) are each further coupled to a methine multiplet at $\delta = 3.99$ (h-7). This methine proton is further coupled by less than 1 Hz to a one proton broad singlet at 4.97 (H-8, br s).

Pyrenoline B

The high resolution mass spectrum of Pyrenoline B indicated the molecular formula $C_{14}H_{13}N_1O_3$, which suggests that Pyrenoline B is lacking the methoxy group relative to Pyrenoline A. This was confirmed by the proton ^1H NMR spectrum of Pyrenoline B which showed no methoxy signal in the vicinity of 3.35 as observed in the spectrum of Pyrenoline A. In place of the missing methoxy, and its adjacent methine proton, are signals for two additional methylene protons at $\delta = 2.34$ (m) and 2.24 (m). These new methylene protons both show coupling to geminally coupled methylene protons at $\delta = 2.71$ (ddd = $J = 4.9, 8.3, 18.1$ Hz) and

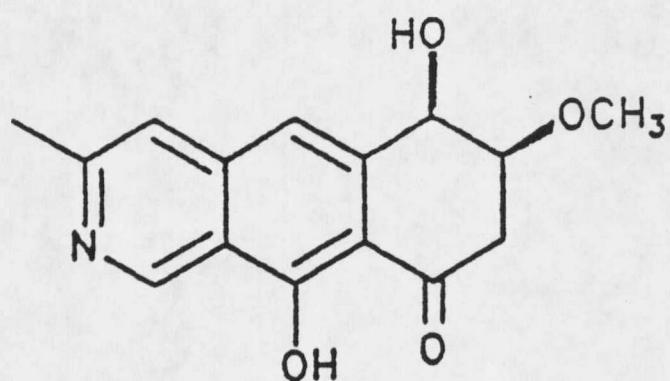
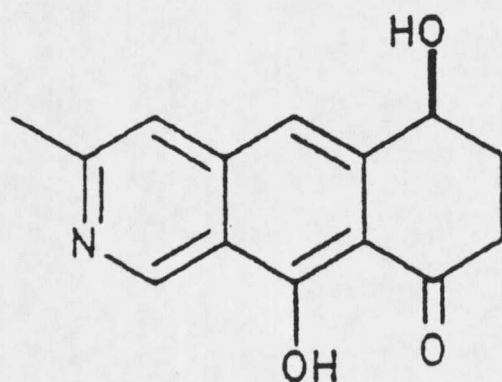
Pyrenoline APyrenoline B

Figure 4. Structure of Pyrenoline A and Pyrenoline B.

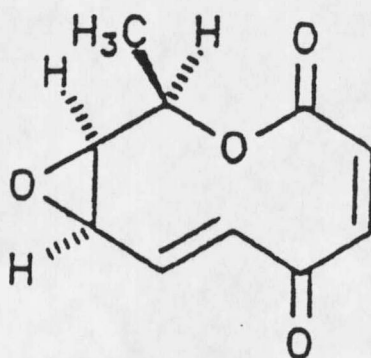
Pyrenolide A

Figure 5. Structure of Pyrenolide A.

3.04 (ddd= J= 4.9, 7.9, 18.1 Hz), and also to the hydroxymethine proton at δ = 4.99. Pyrenoline A thus lacks the C-7 methoxy present in A. Pyrenoline A and B were otherwise spectroscopically identical.

Pyrenolide A

The mass spectrum obtained from pure active metabolite showed exactly the same spectrum as Pyrenolide A described by Manabu (44) (Appendix C, Figure 11). The empirical formula indicated by the M.S. is $C_{10}H_{10}O_4$ (EI-MS, m/e 194.05). The wave lengths of the maximum absorbance were 222 nm and 245 nm, with an extinction coefficient of 6700 and 7200 respectively. The molecule is a ten-membered lactone (Figure 5).

Bioassays of Pyrenoline A

Pyrenoline A was assayed on both monocot and dicot plant species. Pyrenoline A was non-specifically active on host and non-host plant species (Table 3). The lowest active concentration on monocots Hordeum vulgare and Avena sativa and the dicot Hibiscus sabdariffa were 4 mM and 100 μ M respectively. Pyrenoline A was more active on the non-host plant Hibiscus sabdariffa than on barley. In all bioassays performed, 5% EtOH was used as a control and no discernable symptoms were observed.

Table 3. Effect of Pyrenoline A on different plant species, using the leaf puncture assay. Lesion measurements were taken 42 hrs after treatment. Toxin solution was applied in drops of 5 μ l on the wounded leaf.

<u>Plant species</u>	<u>Conc in mM</u>				
	8	4	0.4	0.1	0
<u>Hordeum</u> <u>vulgare</u> <u>cv Compana</u>	+	+	-	-	-
<u>Avena sativa</u>	+	+	-	-	-
<u>Esculenta</u> <u>esculentum</u>	++	+	-	-	-
<u>Euphorbia</u> <u>heterophila</u>	+	+	-	-	-
<u>Hibiscus</u> <u>sibderiffa</u>	+++	+++	++	+	-

Lesion size:	1 - 2.5 mm	2.5 - 5 mm	5 - 10 mm
Activity:	+	++	+++

-: No symptom at all, +: active, ++: Fairly active
 +++: Highly active.

Bioassays of Pyrenoline B

Pyrenoline B was non-specifically active on different plant species (Table 4). Sorghum halepense as well as Euphorbia heterophila showed symptoms at 400 μ M. Below 100 μ M no activity was observed on any of the plant species assayed. Pyrenoline B was non-specific and required a fairly high amount of compound to generate any discernable symptoms. In all bioassays the control did not show any symptoms.

Influence of Different Leaf Extracts on Toxin Production

The influence of leaf extracts on the ability of plant pathogenic fungi to stimulate toxin production has been described previously (50, 53). A leaf extract from a susceptible cultivar of sugarcane stimulated the production of helminthosporoside, a phytotoxin produced by D. saccharii. Serinol was isolated, identified and shown to be the cause of the increase in the toxin production and was called an activator in this particular study (50). In another study the production of deoxyradicin in the culture fluid of Alternaria alternata, a pathogen of sunflowers, increased when different leaf extracts from host as well as non-host plants were added to the liquid culture (53). In the present study leaf extracts from host and non-host plant species were added to a basic M-1-D medium.

Table 4. Effect of Pyrenoline B on different barley cultivars (Hordeum vulgare) as well as other plant species, using the leaf puncture assay. Observations were made 42 hrs after treatments. Toxin solutions were applied in drops of 5 μ l on the wounded leaf.

	Conc in mM				
	8	4	0.4	0.1	0
<u>Hordeum vulgare cv</u>					
<u>Compana</u>	+	+	+/-	-	-
<u>Gallatin</u>	+	+	+	-	-
<u>Clark</u>	+	+	-	-	-
<u>Wabet</u>	+	+	+/-	-	-
<u>Other plant species</u>					
<u>Agropyron repens</u>	+	+	+/-	-	-
<u>Dactylon cynodon</u>	++	+	+	+/-	-
<u>Fescuta elatior</u>	+	+	-	-	-
<u>Sorghum halepense</u>	++	+	+	-	-
<u>Avena sativa</u>	+	+	+/-	-	-
<u>Hibiscus sabdariffa</u>	-	-	-	-	-
<u>Euphorbia heterophila</u>	++	+	+/-	-	-

Lesion size: <1 mm 1 - 2.5 mm 2.5 - 5 mm
 Activity: +/- + ++

-: No symptom at all, +/-: moderate activity, +: active, ++: Fairly active.

Flasks were inoculated the same day and shaken for 10 days at 28°C. The basic M-1-D modified medium was used as a control. Culture fluid was tested for toxin presence and mycelium growth. The data collected shows that toxin production was enhanced upon addition of leaf extract material from both host and non-host plant species (Figure 6).

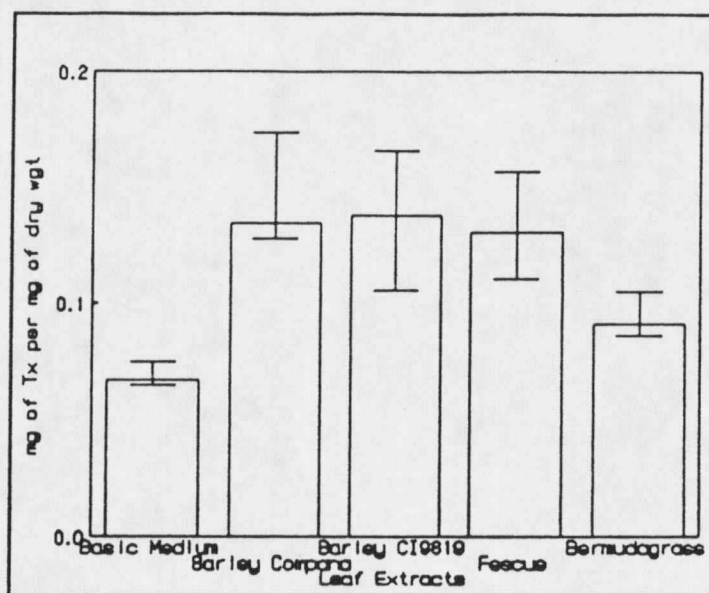


Figure 6. Effect of different leaf extracts on Pyrenolide A, a toxin produced by P. teres. Bars represent the average of three replications. The control consist of a basic -1-D medium. The leaf extracts were added at the level of 0.1 g of leaf extract per liter of basic medium. All flasks were shaken for 10 days.

A putative activator may be responsible for the increase in toxin production. This may involve several mechanisms such as the activation of an enzyme that is involved in the Pyrenolide A synthesis pathway. The activator may be contained in the plant extract of both host and non-host plant species. The leaf extracts of barley plants and fescue increased the toxin production in a significant manner when compared to the control. However, the effect of leaf extract from bermudagrass was not statistically significant from the control or the other treatments.

Bioassays of Pyrenolide A on Different Plant Species

When a solution of Pyrenolide A was placed on the leaves of both monocots and dicots of different plant species various symptoms were observed. Necrotic lesions, bleached spots, and green islands (15) were observed. However, the control did not show any symptoms. The lowest amount of toxin that caused a green island effect was 352 μM on Sorghum halepense. However, 70 μM of Pyrenolide A caused necrotic lesions on Convolvulus arvensis and Saccharum officinarum (Table 5). Bleached spots were formed on Zea mays at 700 μM . The data collected indicates that Pyrenolide A is a non-specific phytotoxin. It caused different symptoms on different plant species. Further investigations should be performed to assess the biological

Table 5. Effect of Pyrenolide A on monocots and dicots.

	Conc in mM				
	8	4	0.4	0.1	0
<u>Monocots</u>					
<u>Sorghum</u> <u>halepense</u>	GI +++	GI ++	GI +	-	-
<u>Zea mays</u> <u>"W64 N"</u>	BC IV+	BC +++	-	-	-
<u>Zea mays</u> <u>"W64 T"</u>	BC +++	BC ++	-	-	-
<u>Digitaria</u> <u>ischaenum</u>	NL +++	NL ++	-	-	-
<u>Convulvulus</u> <u>arvensis</u>	NL +++	NL +	NL +	NL +	NL -
<u>Agroperon</u> <u>repens</u>	NL ++	NL +	-	-	-
<u>Phloem</u> <u>pratens</u>	NL ++	NL +	-	-	-
<u>Saccharum</u> <u>officinatum</u>	?	NL ++	NL ++	NL +	-
<u>Dicots</u>					
<u>Portulaca</u> <u>oleacea</u>	NL +++	NL +	-	-	-
<u>Cucumis</u> <u>pepo L.</u>	GI +	GI +	-	-	-

NL: Necrotic lesion, BC: Bleached spot, GI: Green island.
The level of activity was evaluated according to the following scale.

Lesion size: 1 - 2.5 mm 2.5 - 5 mm 5 - 10 mm
Activity: + ++ +++

-: No symptom at all, +: active, ++: Fairly active, +++:
Highly active.

activities of Pyrenolide A, Pyrenoline A and Pyrenoline B on other biological systems. A number of phytotoxins have been reported to possess useful biological properties in mammalian systems as well as on plants. For example, aspergillomarasmine A and B are two phytotoxins isolated from Pyrenophora teres and were found to have an anti-inflammatory effect on the injured human skin (47).

Bioassays of Pyrenolide A on Different Barley Cultivars

Necrotic lesions were observed on all barley cultivars at 14.1 mM and 2.94 mM. However, at 700 μ M only Compana displayed a necrotic symptom. Below this concentration none of the cultivars tested showed any symptoms (Table 6). The cultivar Compana is a very susceptible cultivar to the isolate Pt-WPb which was used in the purification of Pyrenolide A. The symptom displayed by this cultivar may or may not be related to its susceptibility. Resistant cultivars as well as susceptible cultivars displayed the same lesion sizes (Clark and Gallatin). Therefore, the symptoms caused by the phytotoxin and cultivar resistance or susceptibility are not related.

Summary of the Bioassays

Pyrenolide A is a non-specific phytotoxin active on both dicots and monocots. Different symptoms were observed

Table 6. Effect of Pyrenolide A on different barley cultivars (Hordeum vulgare), using the leaf puncture assay. Lesion measurements were taken 42 hrs after treatment. Toxin solution was applied in drops of 5 μ l on the wounded leaves. The reaction of the cultivars used in this experiment towards P. teres was not assessed.

<u>Hordeum</u> <u>vulgare</u> cv	Conc in mM				
	14.1	2.94	0.70	0.007	0.00
Wapana	+	+	+/-	-	-
Compana	++	+	+	-	-
Betzes	++	+	+/-	-	-
Lewis	++	+	+/-	-	-
Pirolina	+	+	+/-	-	-
Hector	++	+/-	+/-	-	-
Wabet	+	-	-	-	-
Gallatin	++	+	+/-	-	-
Clark	++	+	+/-	-	-

Lesion size : <1 mm 1 - 2.5 mm 2.5 - 5 mm
Activity +/- + ++

-: No symptom at all, +/-: moderate activity, +: active, ++: fairly active.

on different plant species including green islands, necrotic lesions, and bleached spots. The least active concentration on the non-host plant species was 70 μ M on crabgrass (Digitaria ischaemum) and sugarcane (Saccharum officinarum) and 700 μ M on barley cultivars. When placed on different barley cultivars, Pyrenolide A elicited similar size lesions

on resistant cultivars as on susceptible cultivars. The control consisted of a solution of 5% EtOH and did not display any symptoms. Pyrenoline A was more active than Pyrenoline B.

Recovery of Pyrenolide A from the Culture Fluid of Different Pyrenophora teres Isolates

Five isolates of P. teres were tested for Pyrenolide A production. The liquid culture was shaken for 10 days and then extracted. Samples of 1 μ l of ethyl acetate extract were injected into a RP HPLC to test for the presence of Pyrenolide A. All five isolates produced a compound with similar chromatographic properties as Pyrenolide A (Figure 7). The virulence of the isolate Pt WPb is the only one known.

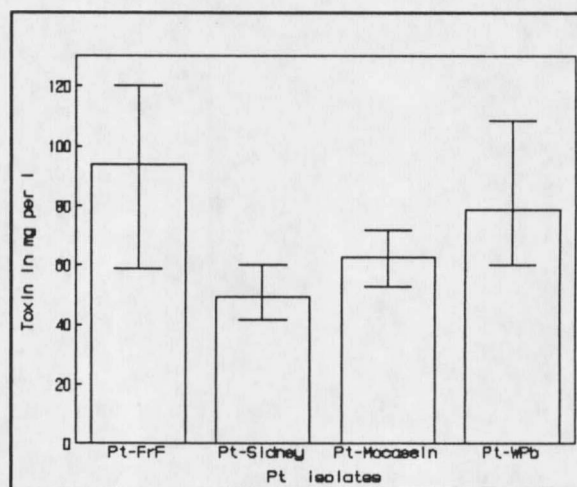


Figure 7. Recovery of Pyrenolide A from the culture fluid of different P. teres isolates. Pt WPb is highly virulent isolate. The virulence of the other isolates is not known.

SUMMARY

Three new phytotoxins were found to be produced by Pyrenophora teres. Purification, chemical characterization and biological activity on a wide host range of these phytotoxins were performed. These compounds are different from aspergillomarasmies A and B which were described by Smedegard-Peterson, in that they are not proteinaceous species and they have never been shown to be produced by other fungi. Two of these phytotoxins are chemically related and differ by a methoxy group on carbon #7. They were assigned the names Pyrenoline A and Pyrenoline B. The third active compound was found to be identical to Pyrenolide A, a ten-membered lactone described by Manubu in 1980.

The lowest active concentration of Pyrenoline A was 100 μM on Avena sativa and the lowest active concentration of Pyrenoline B was 400 μM on barley. Pyrenolide A caused various symptoms on different plant species and required as low as 70 μM to cause a lesion on kenaf (Hibiscus subderiffa) and 352 μM on barley (Hordeum vulgare).

Pyrenolide A was found to be produced by different isolates of P. teres collected in Montana. When leaf extracts from barley cultivars as well as fescue were added to the medium, Pyrenolide A production increased in a statistically significant manner.

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APPENDICES

APPENDIX A
LIQUID CULTURE

Modified M-1-D Medium

Stock solutions

Each stock solution was made by dissolving the compound in 100 ml of double distilled water and 1 ml of each stock solution is added to one liter of medium.

Compound	Weight in (mg)
Ca(NO ₃) ₂	28
KNO ₃	8
KCL	6
MgSO ₄	36
Na ₂ HPO ₄ H ₂ O	2
FeCL ₃ 6H ₂ O	0.2
MnSO ₄	0.5
ZnSO ₄ 7H ₂ O	0.25
H ₃ BO ₃	0.14
KI	0.07

Medium

The following compounds are added to 1 l of double distilled water.

Compound	Weight (g)
Sucrose	30
Ammonium tartrate	5
Yeast extract	0.25

After all the above compounds were added the pH of the liquid medium is then adjusted to 5.5 with 0.1 M HCL and autoclaved for 20 mn at 120°C.

APPENDIX B

Purification of Pyrenolide A

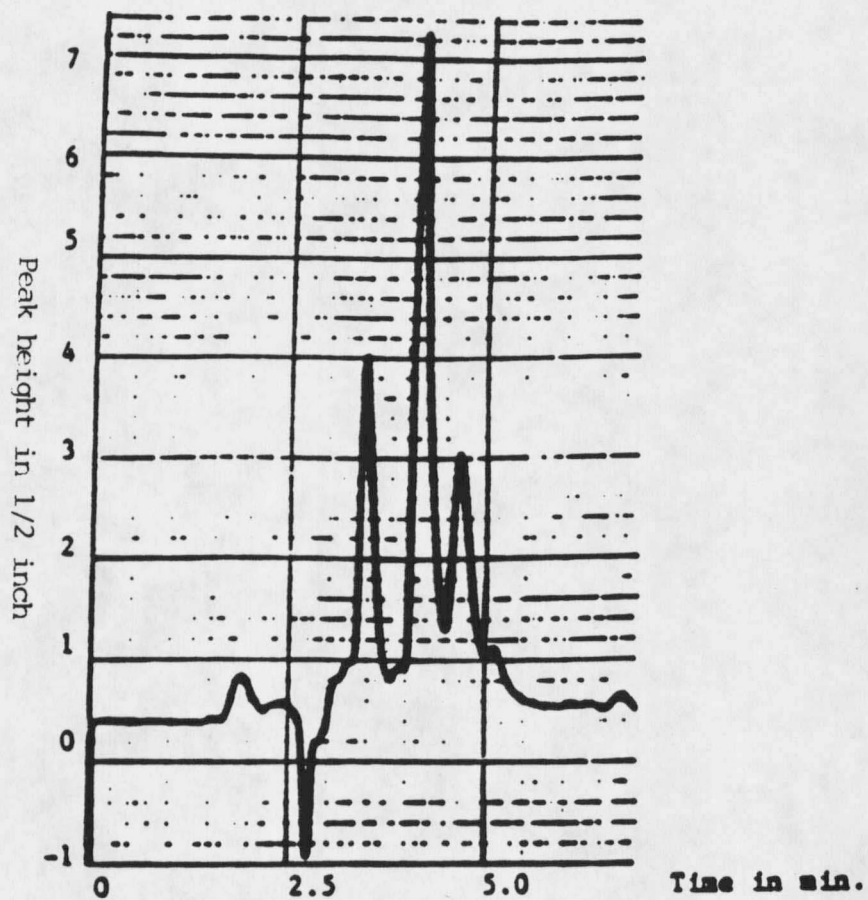


Figure 8. RP-HPLC of the active fraction obtained from thin layer chromatography. The mobile phase used was AcCN:H₂O (65:34) and the detector was set at 254 nm. The active peak was peak #2 on this chromatogram.

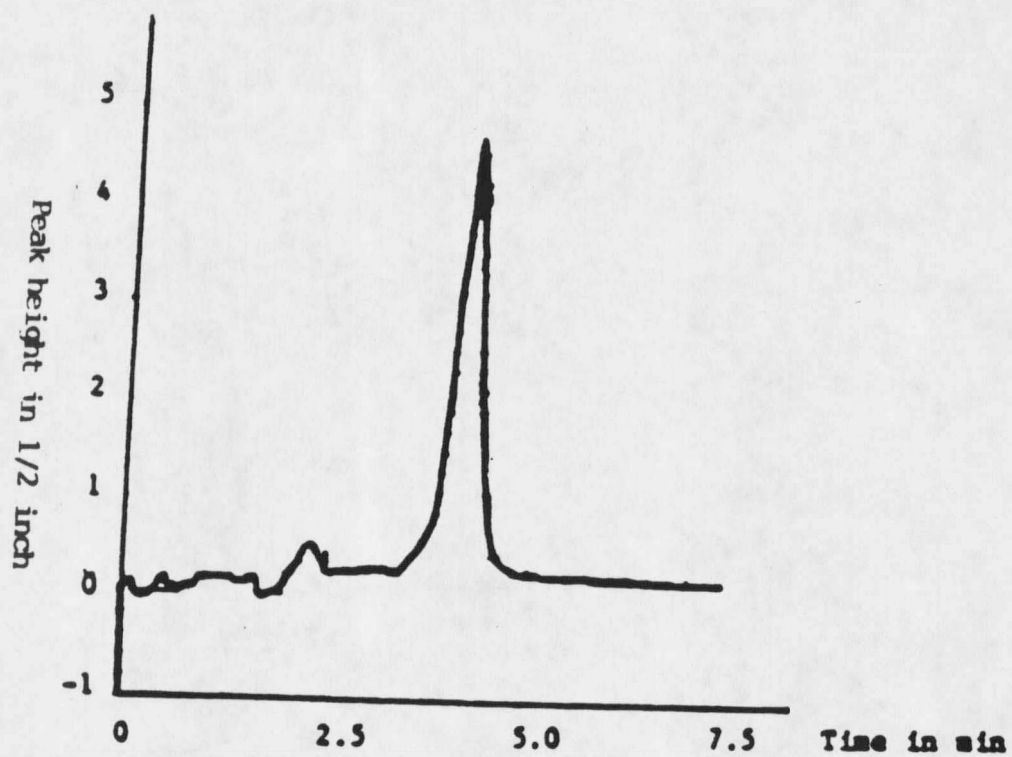


Figure 9. RP-HPLC elution profile of pure Pyrenolide A.

APPENDIX C
CHEMICAL CHARACTERIZATION

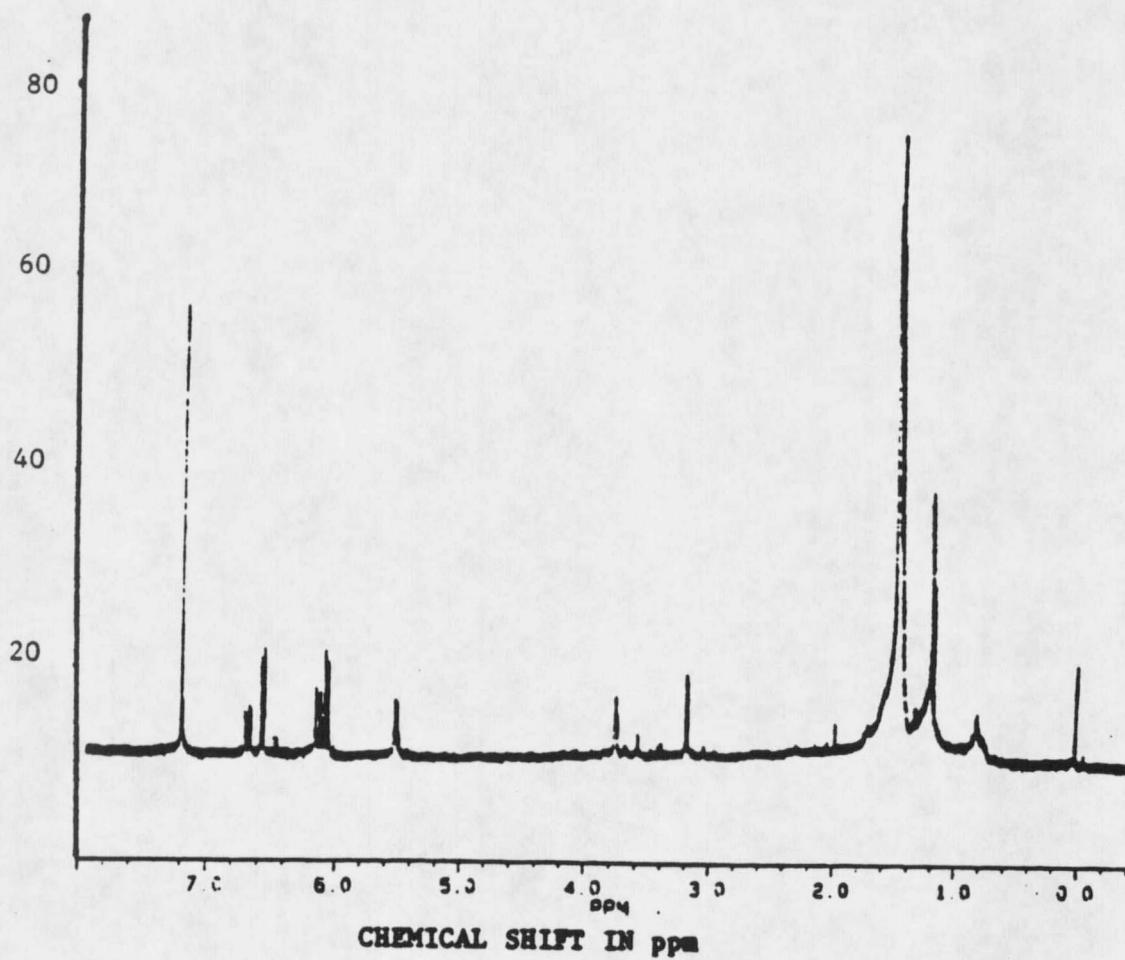


Figure 10. NMR of Pyrenolide A in deuterated chloroform.

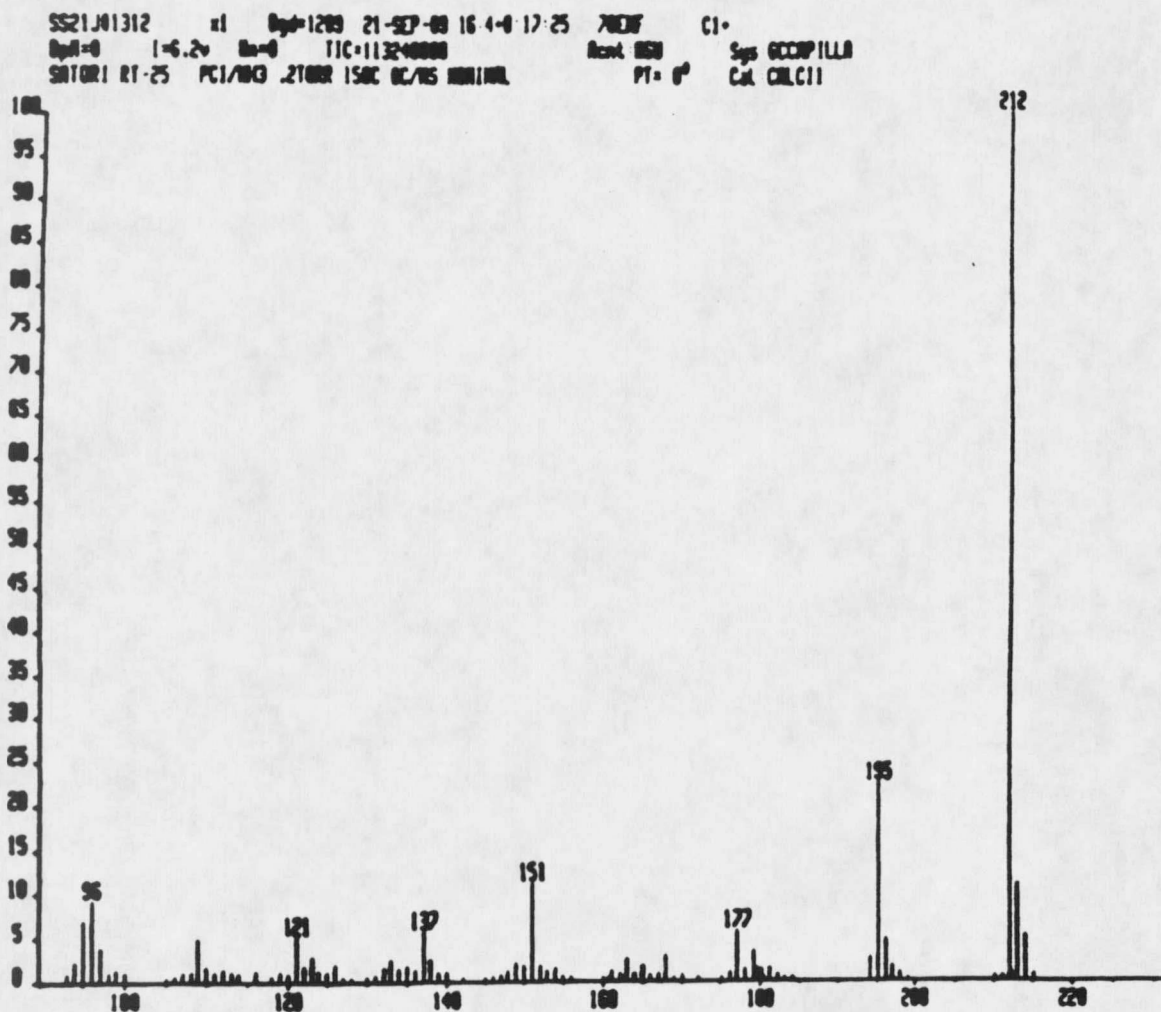


Figure 11. Mass spectrum of Pyrenolide A using ammonia as reagent gas. The molecular weight is 194 (212 - 18 = 194) corresponding to the empirical formula of $C_{10}H_{10}O_4$.

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