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

Abstract:
The ethyl acetate fraction of the culture fluid of Pvrenophora 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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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.

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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 aegiptium (crow foot grass) (67). Drechslera gigantea, a pathogen of Bermudagrass (Cynodon dactylon) and quackgrass (Agropyron repens), produces several bioactive terpenoids, among them gigantanone, a phytotoxin that mimics growth hormone properties and produces green island effects on the host leaf (25,38). Monocerin is a phytotoxin produced by Excerohilum 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 μl 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.