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Investigating components of the host-pathogen interaction of *Septoria musiva* and *Populus*

by

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ABSTRACT

Components of host-pathogen interactions in the *Septoria musiva-Populus* disease system were investigated in an attempt to better understand resistance and susceptiblity to Septoria canker disease. A system for the production and detection of the extracellular proteolytic enzymes produced by *Septoria musiva* was established using the synthetic medium, casein culture medium (CCM). An isolate of *S. musiva* (MP1) showed a growth-limiting effect when grown in CCM that contained initial concentrations of <60 mM glucose. SDS PAGE activity gels revealed two putative proteinases, P1 and P2 (M_r 24 and 75 kd), in culture filtrates of MP1. Proteinase P1 was isolated and further characterized for optimum pH (8.0) and reaction temperature (60 C). Proteinase inhibitor II (PI II) and phenylmethanesulphonylfluoride (PMSF) reduced proteolytic activity by 55% and 95% respectively. Inclusion of PMSF in SDS PAGE activity gel samples, resulted in inhibition of P1 activity, but not P2 activity. *In vitro* and *in vivo* studies were conducted to determine the effect of PI II on the physiology of the fungal pathogen *Septoria musiva*. PI II reduced *in vitro* serine proteinase activity in culture filtrates of *S. musiva*, and reduced conidial germination, mycelial growth, and culture sporulation when the fungus was cultured in medium containing PI II. *In vivo* tests were conducted with *S. musiva* and leaf and stem tissues of a transgenic *Populus x euramericana* clone ('Ogy') that expresses PI II protein. A reduction in lesion size was observed, but there was no significant difference in infection

frequency of leaves. Stem inoculation results were inconclusive because of environmental conditions which may have favored host defenses. Expression studies of PI II in transgenic 'Ogy' have revealed an ~24 kd native proteinase inhibitor of *Populus* that has immunoreactivity to antibodies against PI II. The native inhibitor protein in 'Ogy' has approximately the same biochemical properties, and similar migration patterns, as PI II. A rating system for evaluation of canker disease in *Populus* clones was developed to standardize ratings of Septoria canker disease. The rating system evaluates both disease incidence and severity and has permitted identification of some possible sources of resistance in hybrid clones of *Populus*.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Increased concerns over the environmental toxicity and cost of fossil fuels, and the shrinking reserves of forested lands, have stimulated growing interest in short rotation intensive culture of woody plants as a viable fuel and fiber resource (Phelps 1983). Toward this alternative, biomass researchers in the United States and Europe have focused on fast growing tree species such as those found in the genus *Populus*. This genus is capable of phenomenal growth rates, but is limited by serious pest problems. Two of the major insect pests of North American *Populus* species are the cottonwood leaf beetle *Chrysomela scripta* F. (Coleoptera: Chrysomelidae) and the imported willow leaf beetle *Plagiodera versicolora* (Laicharting) (Coleoptera: Chrysomelidae). The major fungal pathogens are *Septoria musiva* Peck (teleomorph *Mycosphaerella populorum* Thompson), *Melampsora medusa* Thuem., and *Marssonina brunnea* (Ell. & Ev.) Sacc. All can cause considerable damage to their *Populus* host, but *S. musiva* has the potential to cause outright mortality in (some) susceptible clones.

Little information is available about the effects of fungal proteolytic enzymes on the disease process in woody plants, and what factors *S. musiva* uses to cause

canker disease. Field studies have been conducted to screen poplar clones for resistance to the fungus, but disease incidence and severity has varied between test sites (Ostry and McNabb 1983, 1985) making it hard to determine characteristics that may be related to resistance.

The *Populus* host in this study is 'Ogy', a clonal selection from the progeny of a cross of a North American *Populus deltoides* Bartr. and the European *Populus nigra* L. Hybrids from crosses of these species are known as *Populus* X *euramericana* (Dode) Guinier. Transgenic 'Ogy' containing a chimeric fusion of the proteinase inhibitor II gene (*pin2*) with a CaMV 35S promoter, and showing expression of the proteinase inhibitor II protein (PI II), have been engineered to test the importance of proteinases in host-parasite interactions (Heuchelin et al. 1997).

Determining the possible role of proteases in the pathogenesis of leaf and stem tissue, and observing the host-parasite interaction in clones with a range of resistance, has provided our laboratory a unique look at the disease process in the interactions of *Septoria musiva* and *Populus* X *euramericana*. Pathogen proteinases may be able to be inhibited sufficiently by strong expression of proteinase inhibitors so that a stable form of resistance may be realized. Proteinase inhibitors have the potential to interfere in the degradation of cell components during infection and limit the effective nutrient value of host tissues. The goals of this project were: 1) to identify the types of proteases used by *S. musiva*, 2) to determine the effect these proteases have in the disease process, 3) to determine the effect of proteinase inhibitor II on the physiology

and proteases of *S. musiva*, 4) to identify any endogenous proteinase inhibitors that may exist in the fungus's *Populus* host, and 5) determine possible resistance mechanisms to Septoria canker disease in *Populus* clones.

Dissertation Organization

This dissertation consists of six chapters which contain: a general introduction and literature review (Ch. 1), three papers for publication (Ch. 2-4), studies in field disease screening (Ch. 5), general conclusions for the dissertation (Ch. 6), and an acknowledgments and literature cited section. References cited in the general introduction, literature review, chapter 5, and general conclusions follow the general conclusions section (Ch. 6). The doctoral student will be the senior author on the publications derived from the manuscripts. The three manuscripts, co-authored with Dr. H. S. McNabb, Jr., will be submitted to the journals: Phytopathology, Molecular Plant-Microbe Interactions, and the Canadian Journal of Forest Research, respectively.

Literature review

Septoria musiva

History

Septoria musiva Peck. (teleomorph *Mycosphaerella populorum* Thompson) is an endemic fungal pathogen in native stands of eastern North American *Populus* (Dinus 1974). In 1884, Peck first described the fungus *S. musiva* while observing its signs and symptoms on living leaves of *Populus deltoides*. Other evidence of woody plant Septoria pathogens in northcentral North America were recorded as early as 1925 by Tehon and Daniels, and later definitive work was conducted by Thompson in 1937. In 1941, Thompson's description of *M. populorum* provided the missing link in the disease cycle of Septoria leaf spot and canker by elucidating the sexual stage (teleomorph) of *S. musiva*. The loculoascomycete *Mycosphaerella populorum* is associated with the overwintering leaf litter of *Populus* species in North America (Barr 1972; Thompson 1941). *M. populorum* is cosmopolitan in most all forest communities east of the continental divide, with only *Septoria populicula* present in the west coast. The indigenous North American species *Populus deltoides*, is susceptible to foliar infection (Bier 1939; Waterman 1954), but resistant to the canker disease (McNabb and Ostry, personal communication). *S. musiva* spores, which are only capable of infecting the leaves of the native *P. deltoides*, infect susceptible hybrid clones and introduced *Populus* species and can result in serious foliar (Septoria

leaf spot) and stem (Septoria canker) disease (Bier 1939; Ostry and McNabb 1985, 1989; Waterman 1954; Zalasky 1978). With the large-scale introduction of inter- and intraspecific hybrids, *S. musiva* became a serious pathogen causing Septoria leaf spot and Septoria canker in hybrid poplar in North America (Bier 1939; Ostry and McNabb 1983; 1985; 1989; Spielman et al. 1986; Thompson 1941; Waterman 1954).

Impact

Septoria leaf spot and canker have minimal impact in the natural forest ecosystems of eastern North America. The indigenous species *P. deltoides* has been found to be susceptible to foliar infection, but does not incur significant loss (Waterman 1954). The native *Populus* species of eastern North America provide an immense reservoir of inoculum for infections by *M. populorum* (Bier 1939; Thompson 1941; Waterman 1954) on susceptible genotypes. Poplars or hybrids introduced to North America with parentage from the Tacamahaca or Aigeiros sections of the genus *Populus*, have been moderately to highly susceptible (Ostry and McNabb 1983, 1985, 1989; Waterman 1954). Leaf spot and canker formation has been reported in some *P. deltoides* selections (Bier 1939; Filer et al. 1971; Zalasky 1978), but this information has been challenged by recent research (Ostry and McNabb, personal communication) that supports the premise that pure *P. deltoides* selections are resistant to canker development. The selections observed in the previous studies may possibly be hybrids, the progeny of an outcross between a native *P. deltoides* and a separate

Populus species or hybrid. In susceptible clones, the fungus can drastically reduce photosynthetic area on the leaves of poplars, and more importantly can cause cankers that can girdle the main stems. Both types of disease can cause serious loss of biomass accumulation (McNabb et al. 1982; Ostry 1987) and the canker can also cause death of the main stem. *M. populorum* reduced the production of biomass by 37% in a susceptible clone when compared to a resistant clone in a 3-year growth study in Iowa (McNabb et al. 1982). Stem infection and girdling resulted in a 30% reduction in stand 1 year after the same clone was coppiced. There is a large degree of variability in resistance to the leaf spot and canker disease among hybrids of common parentage and clones grown in different locations (Ostry and McNabb 1985; Cooper and Filer 1976). These types of losses seem to occur regardless of latitude, as evidenced by reports from the southern United States (Weiss et al. 1976) to Canada (Zalasky et al. 1968). Considerable variability in fungal aggressiveness (Krupinsky 1987, 1989) has been observed and may be explained by different strains of the pathogen (Hubbes and Wang 1990). Changes in strain populations may help explain breakdowns in clones previously thought to be resistant (Vanderplank 1984). Selection of genotypes for utilization in intensive culture systems, without regard to disease resistance, has increased the importance of both the foliar and stem disease in *Populus* and *Populus* hybrids dramatically. Because of this, damage to foliage and

stems resulting from infection by *M. populorum* has greatly limited the use of numerous fast-growing *Populus* hybrids in short rotation, intensive culture plantations (Waterman 1954; Ostry and McNabb 1985).

The asexual stage of *S. musiva*

Most of the published accounts of foliar and stem infection are attributed to the asexual stage (anamorph) of *Septoria musiva* Peck. The asexual reproductive structure, or fruiting body, of *S. musiva* is the pycnidium (Alexopoulos et al. 1996). The pycnidium produces asexual spores (conidia) that are exuded in long spore tendrils or in pinkish masses (Thompson 1941; Waterman 1954). The conidia are described as hyaline, cylindrical, straight or slightly curved, and septate (Thompson 1941; Palmer et al. 1980). As many as six septations have been reported (Waterman 1954; Davis 1915) with the conidia. The most commonly observed range of septa is one to four (Thompson 1941; Palmer et al. 1980). The conidia can range in size from 17-56 um x 3.5-4.0 um (Bier 1939; Thompson 1941; Waterman 1954; Palmer et al. 1980). The range in size is effected by the species or clone on which they are produced (Thompson 1941; Waterman 1954).

The sexual stage of *S. musiva*

The vast majority of the primary inoculum (ascospores) for *M. populorum* infections in the spring is disseminated by ascocarps that develop in the overwintering

leaves of *Populus* (Bier 1939; Thompson 1941; Waterman 1954; Filer et al. 1971). The sexual ascospores of this loculoascomycete are produced in pseudothecia (Alexopoulos et al. 1996) and are ejected into wind currents with the objective of infecting both stems and leaves of susceptible *Populus* (Lulley 1986). The pseudothecia also have been found in cankered branch and stem tissues (Bier 1939; Waterman 1954), though they are not believed to be a significant source of primary inoculum (Waterman 1954).

At maturity, the ascospores are hyaline, straight or slightly curved, and have a septum which separates the two nearly equal cells. They are slightly constricted at the septum, and range from 13-24 X 4-6 um (Niyo 1986).

Disease cycle

The disease cycle begins with last year's leaf liter as the infected substrate. This substrate is the primary inoculum for the system. The substrate contains pseudothecia from the previous year's disease cycle. The psuedothecia begin to develop in autumn, as evidenced by production of spermagonia prior to winter (Thompson 1941), and continue to slowly develop over winter and early spring based on degree days (Lulley 1986). This developmental process has not been well studied in the field, though Niyo et al. (1986) conducted a laboratory study of psuedothecial development of *M. populorum* and found ascocarp development was typical of that described for the Dothideales (Luttrell 1973).

Maturity and subsequent release of ascospores can occur from early spring to late summer, depending on weather conditions (Lulley 1986). With the appropriate environmental stimuli of light, temperature, and moisture, the pseudothecia will produce and eject ascospores. Lulley (1986) observed a distinct seasonal periodicity in the ascospore production of *M. populorum*. Dissemination of ascospores began in April, at the time of bud swell on *P. deltoides*, and continued for an extended period of 5-6 months. Peak ascospore production occurred in May. Moisture is also an important component of ascospore discharge. Thompson (1941) and Gerstenberger (1983) both reported the release of ascospores of *M. populorum* after wetting of leaves by rain. Lulley (1986), in a quantitative study, also found that ascospores were released primarily after rains. He also concluded from his observations that dew was unimportant in the induction of ascospore release, and that the majority of ascospores were released in daylight hours. Seasonal environmental factors such as degree days, light, and moisture can all affect ascospore production and dissemination.

The airborne ascospores may travel in air currents to a new *Populus* stand or re-establish in the present stand at an appropriate infection court. The spores may arrive at the infection court via settling, rain deposition, or wind impaction with a wet or adhesive surface. The infection court for Septoria leaf spot is the stomates of the leaf lamella (Thompson 1937). The infection courts for stem canker are wounds, lenticels, leaf petioles, and stipules (Bier 1939; Waterman 1946). Long et al. (1986) showed that, of these, wounds and leaf scars are the most successful sites of infection.

Once in juxtaposition with an infection court, germination may occur if environmental conditions are favorable. Free water and a temperature of approximately 27° C will provide optimal germination and germ tube elongation (Thompson 1937). Once the germ tube has penetrated the stomate or bark and is in living tissue, infection is established.

Stem infections are initiated during the current year's growth (Bier 1939) before rhytidome formation takes place in the development of bark (Zalasky 1978). Infected stem tissues are typically dark brown to black, with areas of lighter color near the center of the canker where pycnidia can sometimes be observed (Bier 1939). These ephemeral pycnidia are typically buried in the host stem tissues or develop in black stroma-like fungal tissues within the lenticels (Bier 1939; Waterman 1954). This type of pycnidial production is rarely observed on cankered stems that are 2 years of age or older. *M. populorum* can completely girdle first-year stems, or if less aggressive on its host, may continue colonizing stem tissues and result in a perennial canker (Bier 1939; Filer 1976). The perennial cankers typically have depressed to flat centers, and the canker margin often has a profusion of callus tissue (Bier 1939; Waterman 1954).

The symptoms of Septoria leaf spot are visible approximately 10 - 20 days after infection (Thompson 1941; Long et al. 1983). Leaf spots are typically circular, irregular, or angular, 1 to 15 mm in diameter, reddish brown to dark brown, and have grey to white centers (Thompson 1941). The species of *Populus* and the age of the

leaf tissues infected can affect symptom development (Thompson 1941). Lulley (1986) found the abaxial surfaces of the leaves were more susceptible to infection than the adaxial surface. This is probably because of the greater number of stomates found on the abaxial side of the leaf. Infected leaf tissues give rise to pycnidia that can develop on both leaf surfaces (Thompson 1941). Production of conidia generally occurs soon after symptoms appear in late spring (Bier 1939); in central Iowa, these typically appear in early June (Young et al. 1980). The conidia are exuded in long spore tendrils or pinkish masses, which when dry appear white to cream colored (Thompson 1941; Waterman 1954). Conidia are primarily released during periods of rain, with as little as 1.3 mm of rain being a sufficient factor in induction of spore dispersal (Gerstenberger 1983). Trapping of conidia during the growing season revealed that wet growing seasons result in increased production and release of conidia compared to dry seasons. The trapping and subsequent analysis of the dispersal gradients for *S. musiva* conidia in *Populus* plantations showed steep dispersal gradients, which suggested that the conidia were likely dispersed in droplets of rain (Gerstenberger 1983). Field populations of conidia in Iowa increased on a weekly basis thoughout the growing season, from early May or June until leaf drop (Lulley 1986). Intensification of host disease is caused by repeating generations of the asexual conidia during the growing season. This repeating stage of conidial release and infection greatly amplifies disease in the host and is characterized by a series of concatenated processes that make up the secondary disease cycle. Leaves that are

infected at leaf drop are the substrate and repository for the development of sexual ascocarps from late fall to spring (Lulley 1986). With the production of psuedothecia and the release of ascospores in the spring, the disease cycle begins for a new season.

Fungal nutrition

Each fungal pathogen is unique in its enzyme systems and nutritional needs. Though many standardized culture media are utilized for a variety of fungi, there may be essential organic or inorganic nutrients that are not present in sufficient quantity for optimial growth and reproduction of the fungus. Fungal nutrition is critical for mycelial growth and differentiation, as well as the production of enzymes (Garraway and Evans 1984). The initial source of nutrition for the germinating fungus are its intracellular oil droplets and protein reserves. The stored reserves provide enough nutrition to allow establishment of the germ tube and the enzymatic processes contained in its cells (Alexopoulos et al. 1996). The subsequent nutrients must be acquired from the environment by absorption or enzymatic processes. The amount of carbon and nitrogen in the environment can greatly affect growth and the type and quantity of enzymes that are produced. This carbon to nitrogen ratio (C/N) can result in dramatic differences in growth, even when both substrates are at higher than essential concentrations (Aube and Gagnon 1969). The amounts of these nutrients also can affect fungal colony differentiation and reproductive development (Ng et al. 1972).

Excessive amounts of carbon, sulfur, or nitrogen sources such as amino acids and ammonium ions, can result in catabolite repression of extracellular enzymes (Klapper et al. 1973; Cohen et al. 1975; Ivanitsa et al. 1978; Shinmyo et al. 1978; Cohen 1981; Rollins and Gaucher 1994). A typical example of catabolite repression is high concentrations of a nitrogen source resulting in reduction of extracellular proteases. The response lowers protease production which provides free amino acids for nitrogen nutrition, thus conserving energy and substrate resources. Carbon source and concentration have been shown to affect the utilization of amino acids greatly in higher fungi (Weinhold and Garraway 1966). This evidence from the literature demonstrates the importance of proper fungal nutrition in any study involving fungal growth, differentiation, or sporulation.

Proteinases

Proteases degrade proteins into smaller polypeptides and their constituent amino acid residues by hydrolyzing peptide bonds between amino acids. Two different types of proteases are utilized in the degradation of a typical protein. The "endopeptidases" are specific for peptide bonds between distinct amino acid groups (residues), and are responsible for the initial stages of protein breakdown and subsequent degradation of polypeptides. The "exopeptidases" are not specific for the internal bonds which hold together an amino acid chain, but rather the bonds of the

terminal amino or terminal carboxyl residues. The cleavage of terminal residues by exopeptidases results in the generation of free amino acids from peptides. The currently accepted nomenclature for proteases assigns the term "proteinase" to proteolytic enzymes possessing endopeptidase activity (Barrett 1985).

Proteinases and their inhibitors are classified according to the mechanism used by the proteinase to hydrolyse a polypeptide bond. Six families of proteinases have been identified. They are named according to the specific amino acid residues which are active in their catalytic mechanisms, or according to compounds which are crucial to the catalytic activity (Neurath 1984). The six families can be grouped into four main functional groups; the aspartic, serine, cysteine, and metallo-proteinases. Reviews by Beynon and Bond (1989), Dalling (1986), and North (1982) are very informative, and together provide a comprehensive look at the proteinases used in biological systems and their mechanisms.

Aspartic proteinases are active in acidic environments and typically have optimal enzyme activity in the range of pH 3.5 to 5.5. The acidic aspartic proteinases are produced in most eukaryotic organisms and have been found in virtually every genus of the fungi (North 1982).

Serine proteinases are active in neutral to alkaline environments and typically have optimal activity in the pH range of 7.5 to 9.0. The alkaline serine proteinases are found in nearly all prokaryotic and eukaryotic organisms, including pathogenic fungi and bacteria, as well as phytophagous insects (North 1982).

Cysteine proteinases are a diverse group and are active in environments with a wide range of pH values. Optimal activity has been observed from pH 4.0 to 8.0, depending on the organism and its environment. Cysteine proteinase activity is typically activated or enhanced in the presence of cysteine, low molecular weight thiol groups, and other reducing agents. This group of proteinases has been found in nearly all organisms, including phytophagous insects. Reports of cysteine proteinases in fungi are few to date (North 1982).

Metallo-proteinases are typically active in near-neutral pH environments. Some metallo-proteinases can have optimal activity in environments with pH conditions as low as pH 5. Most metallo-proteinases contain a crucial zinc atom within their catalytic site. These proteinases are widespread in both prokaryotic and eukaryotic organisms, including some pathogenic fungi (North 1982).

Proteinase Inhibitors

Proteinase inhibitors have recently been the focus of considerable research and speculation as to their potential role as plant defense chemicals in host-pest and host-pathogen interactions. In general, proteinase inhibitors interfere with the active sites of proteolytic enzymes, thus slowing or halting the rate of hydrolysis by a protease on its protein substrate. These inhibitor proteins, and the genes that code for them, show promise as a source of resistance to insect and pathogen pests for tree improvement

programs. Detailed information on proteinase inhibitors and their origins can be obtained from the reviews of Ryan (1981, 1990) and Barrett and Salvesen (1986).

The inhibitors of aspartic proteinases are few; they include the protein pepstatin, epoxy propane, and diazoacetyl-compounds such as the diazoacetyl-norleucine methyl ester-copper ion complex (Beynon and Bond 1989; Dalling 1986). Specific inhibitors of serine proteinases include the PI I and PI II inhibitor proteins of the Solanaceae, the soybean trypsin inhibitor and its relatives in the Leguminosae, the protein aprotinin, and irreversible chemical inhibitors such as phenylmethanesulphonylfluoride (PMSF) and di-isopropylfluorophosphate (DIFP) (Beynon and Bond 1989; Dalling 1986). Specific inhibitors of cysteine proteinases include the oryzacystatin inhibitor (OC I) protein of rice (Abe et al. 1987), iodoacetamide, iodoacetate, N-ethyl maleimide, and heavy metals (Beynon and Bond 1989; Dalling 1986). Inhibitors of metallo-proteinases include phosphoramidon, hydroxamic acid dervitives, mercaptoacetyldipeptides, EDTA, and other metal-chelating agents (Beynon and Bond 1989; Dalling 1986).

Of these inhibitors, the small molecular weight proteins are of particular interest to researchers. These inhibitor proteins are, for the most part, tight-binding reversible inhibitors of their proteinase class and have very little reactivity with other types of proteinases. Because they are reversible inhibitors and thus follow an equilibrium dissociation constant, their activity is dependent of their concentration in the system studied. They typically have little to no toxicity to non-target organisms,

and if present, their effect on the target organism is rarely acute. These properties make them ideal for use in biological systems. Of even greater interest is the potential to create transgenic trees which express the inhibitor proteins in their leaf, root, and/or cambial tissues. Once the gene for the inhibitor protein has been cloned, it can be incorporated into a transformation vector and used in a transformation program to create transgenic trees that may possess a higher level of resistance to a particular pest or pathogen (Klopfenstein and Hart 1997). Research of this type has been conducted in recent years using *Populus* as a model transformation system for woody plants. The majority of the research has focused on two inhibitor proteins; proteinase inhibitor II (PI II), a serine proteinase inhibitor from potato (Heuchelin et al. 1997; Klopfenstein et al. 1991, 1993, 1997), and oryzacystatin inhibitor (OC I) (Cornu et al. 1996; Leplé et al. 1995), the cysteine proteinase inhibitor from rice.

Proteinase Inhibitor II

The protein

Proteinase Inhibitor II is a low molecular weight protein [monomer M_r 12,300 (Plunkett et al. 1982)] that inhibits the proteolytic activity of trypsin and chymotrypsin (Ryan 1981, 1990, Ryan and An 1988). It is the gene product of the proteinase inhibitor II gene (*pin2*).

The PI II dimer is composed of two protomers (monomers) that can vary in their molecular weight and isoelectric point (pI). Four different protomers; A, B, C, and D have been observed (Bryant et al. 1976). Protomer A has not been characterized because of problems in obtaining pure fractions of this protomer. The remaining three have pIs of 5.7 (B), 7.2 (C), and 8.2 (D). Any two of these protomers may combine to form one of 16 functional PI II dimers, though some combinations have slightly better inhibitory activity. The PI II dimer is capable of inhibiting two moles of chymotrypsin per mole of PI II. The inhibition of trypsin is not as strong and has been observed at approximately one mole of trypsin inhibited per mole dimer of PI II.

PI II is a heat stable and acid soluble protein. The protein resists disassociation and precipitation at pH as low as 2.2, or as high as 10.6. PI II can be heated to 70 C for 30 min at the pH extremes of 2.2 or 7.8, with only 6% of its activity being lost. PI II has been found in plants belonging to the Solanaceae, such as potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Miller) (Green and Ryan 1972; Ryan 1981, 1990).

The gene

The proteinase inhibitor II gene (*pin2*) was first cloned from tomato and potato. From mRNAs expressed during wounding, cDNAs were synthesized and their nucleotide sequences elucidated (Graham et al. 1985; Sanchez-Serrano et al. 1986). The genes responsible for the coding of the proteinase inhibitors in both potato and tomato

have been isolated and analyzed for nucleotide sequence (Fox 1986; Keil et al. 1986; Thornburg et al. 1987a). Through characterization and deletion analysis, the functions of the three major components of *pin2* were identified (Thornburg et al. 1987a) These regions are the wound-inducible promoter (Thornburg et al. 1987b) that allows transcription to proceed when signal molecules from wounding are present, the protein coding region that codes for PI II, and the terminator that both polyadenylates the transcript and seems to increase stability of the mRNA (An et al. 1989).

Effect of PI II on fungi

PI II is believed to reduce the vigor of fungi effectively by depriving them of necessary amino acids (Ryan 1990). A proteinase inhibitor of unknown structure from *Phaseolus vulgaris* L. seeds has reduced the activity of a proteinase in *Colletotrichum lindemuthianum* (Sacc. & Magnus) (Mosolov et al. 1979). Based on its pH properties, further investigation suggested the proteinase was of the serine type. *Fusarium solani* (Mart.) Sacc. (Mosolov et al. 1976) produces trypsin-like extracellular proteases when grown in liquid culture medium. Proteinase inhibitors were isolated from soybean, lima bean, wheat, and potato, and used to inhibit the *F. solani* protease. In the results, potato inhibitors provided the greatest inhibition of the protease. Preliminary studies from our laboratory show that *Mycosphaerella populorum* also uses serine proteinases to obtain nutrition from protein substrates in liquid culture. Media filtrate contained proteolytic enzymes that are inhibited by proteinase inhibitor II (Sillick et al. 1989). Proteinases

have been shown to have a significant role in the pathogenesis of host tissues (Movahedi and Heale 1990). Cells were treated with cell wall degrading enzymes (CWDE) as well as an aspartic protease. Cell death could be caused by CWDEs alone, but the addition of the protease caused an increase in cell death. The investigators concluded that the protease may be active with CWDEs to produce a synergistic effect in pathogenesis.

Transformation of *Populus*

Populus was first transformed by Parsons et al. (1986) using *Agrobacterium tumefaciens*. They were not able to regenerate the resulting callus tissue into plantlets, so they proceeded to confirm the transformation with callus tissue. Fillatti et al. (1987) were the first to transform and regenerate *Populus*. The poplar was transformed with a herbicide resistance gene, and resistance was confirmed in the regenerated transformants. Pythoud et al. (1987) transformed poplar with a different species of *Agrobacterium*, *A. rhizogenes*. Poplars were transformed with a kanamycin resistance gene by Chun et al. (1988) using a binary vector system. DeBlock (1990) transformed *P. trichocarpa* X *P. deltoides* and *P. alba* X *P. tremula* with the phosphinotricin herbicide resistance gene. *Populus* has been transformed via electric particle acceleration method by McCown et al. (1991) using a NOS-NPT, a CaMV 35S-GUS, and a CaMV 35S-BT construct. Klopfenstein et al. (1991) transformed *Populus* with an *Agrobacterium* binary vector system containing the wound-inducible promoter of *pin2*

and the CAT reporter gene. The construct pRT45 also contained the *npt* gene for kanamycin resistance. Transformants showed wound-inducible expression of CAT mRNA when the trees were artificially wounded. Hybrid poplar clones 'Ogy' (*Populus x euramericana*) and 'Hansen' (*P. alba x P. grandidentata*) were transformed with a chimeric construct of the pin2 gene containing either the cauliflower mosaic virus 35S or bacterial nopaline synthase (NOS) promoter (Heuchelin et al. 1997; Klopfenstein et al. 1993, 1997). An *Agrobacterium*-mediated transformation of a *P. tremula x P. alba* hybrid was accomplished using two different procedures, co-inoculation (Brasileiro et al. 1991) and co-cultivation (Leplé et al. 1992). An adaptation of these procedures lead to the transformation of a Leuce hybrid poplar (*P. tremula x P. tremuloides*) clone 'INRA 353-38' with the cDNA of oryzacystatin inhibitor (*OCI*), a cysteine proteinase inhibitor from rice (Leplé et al. 1995).

Agrobacterium Mediated Transformation

Wild type *Agrobacterium* transformation

Though first mentioned by Aristotle, the first true definitive work on the disease crown gall was by Smith and Townsend (1907). The investigators found that the disease was caused by *Agrobacterium tumefaciens*, a gram-negative, soil-borne pathogen. The bacterium was found to infect dicotyledonous plants in more than 61 plant families. The disease condition, tumour or gall formation, was shown to persist even after the infected

tissue was rid of the bacterium (White and Braun 1941). This distinguished crown gall from other galls caused by insects or fungi, and suggested that perhaps something was being transferred from the disease organism to the host tissues. Braun (1947) hypothesized that there was some tumour-inducing principle (TIP), possessed by the bacterium, that was responsible for the tumour formation. The work of Watson et al. (1975) and Van Larebeke et al. (1975) identified circular DNA plasmids in virulent strains of *A. tumefaciens* and showed that avirulent strains did not possess the plasmids. They also were able to cure the bacteria of their plasmids by growing them at 36 C, then restore their virulence by reintroducing the plasmid. This plasmid is called the tumour-induction (Ti) plasmid. Chilton et al. (1977) proved that a small portion of the Ti plasmid DNA was inserted into the tumour tissue genomic DNA. This portion of DNA is called the transfer DNA (T-DNA). The Ti plasmid contains, in addition to the T-DNA segment, a set of genes for virulence. The virulence genes recognize chemical signals, such as acetosyringone, that are produced by wounded plant cells (Stachel et al. 1985). Acetosyringone activates the vir genes that are responsible for T-DNA transfer. The vir genes (*virA*, *virB*, *virC*, *virD*, *virE*, and *virG*) work together efficiently to produce a single stranded T-DNA intermediate (Stachel et al. 1986). This single strand of T-DNA is incorporated into the plant genomic DNA seemingly at random. The T-DNA contains genes for auxin and cytokinin production (oncogenes), as well as opine synthesis genes (Zambryski et al. 1989). Once the T-DNA is stably integrated in the host plant it is transcribed to produce plant hormones and opines without control by the

plant's regulatory processes. The opines produced are used as a carbon and nitrogen source for the bacteria. The hormone imbalance causes the formation of the tumour tissue that in turn provides a protected environment for the bacteria, as well as a large number of opine-producing cells.

The *Agrobacterium* binary vector system

In the *Agrobacterium* binary vector system, the T-DNA is excised with its border sequences and placed in a plasmid vector that contains antibiotic resistance. The oncogenes and opine genes are removed and replaced with a foreign gene of interest as well as a selectable marker gene (An 1987). The vir genes are moved to a separate plasmid called the helper plasmid. The vir genes on the helper plasmid allow proper excision, and incorporation of the T-DNA construct to proceed as it would in the natural system. With the binary vector system, there is insertion of the foreign gene T-DNA construct without tumour induction or opine synthesis.

The *Agrobacterium* binary vector used in this dissertation was provided by co-investigator R. W. Thornburg. The bacterium contained a disarmed Ti plasmid (EHA 101) as the helper plasmid. The vector was plasmid pRT104 (Ebert and Thornburg, unpublished) containing *pin2* regulated by a 35S promoter of the CaMV virus (Odell 1985), and a selectable gene encoding neomycin phospho-transferase II (NPT II) regulated by a nopaline synthase promoter (An. et al. 1986). The vector also contained both tetracycline and ampicillin resistance genes to aid in selection of the bacterium.

Transformation vector plasmids containing *pin2*

Transformation vectors containing the *pin2 gene* have been created for use in *Agrobacterium* binary vector systems. The wound inducibility of the *pin2* promoter has been shown in transgenic tobacco (Thornburg et al. 1987b; Sanchez-Serrano et al. 1987) and transgenic poplar (Klopfenstein et al. 1991) using a chimeric gene composed of the *pin2* promoter fused with the chloramphenicol acetyltransferase (CAT) gene. The plasmid vector, pRT45 (Thornburg 1987b), also contained the selectable marker gene NPT II with a nopaline synthase promoter (An et al. 1986). The CAT gene protein or mRNA was expressed in foliage subjected to wounding, but was not detected in unwounded leaves.

Other chimeric plasmid vectors containing the PI II protein coding region and terminator, and a foreign (constitutive) promoter were created (Ebert and Thornburg, unpublished) and used in studies mentioned in this dissertation. Plasmid pRT102 has a nopaline synthase promoter from *Agrobacterium*, and in pRT104 the CaMV 35S promoter from the Cauliflower Mosaic Virus drives the coding region. Both pRT 102 and pRT 104 contain the NPT II selectable marker gene.

Hybrid and Native Clones of *Populus*

Clone of Study: *Populus* X *euramericana* 'Ogy'

'Ogy' is a clonal selection from the progeny of a cross of a North American *Populus deltoides* Bartr. and the European *Populus nigra* L. Hybrids from crosses of these species are known as *Populus X euramericana* (Dode) Guinier. 'Ogy' was selected from the breeding program of Vic Steenackers at the Poplar Research Center in Geraardsbergen, Belgium. Progenitor trees were chosen on the basis of bole form and disease resistance. The maternal tree of 'Ogy' was from a cross of a southeast Iowa *P. deltoides* (maternal) and a *P. deltoides* from Ontario (paternal). The tree resulting from this cross was pollinated by a *P. nigra* from Belgium. 'Ogy' was selected from the resulting seedlot (d'Oultremont and Steenackers 1973).

Of the many Euramericana hybrids created by the Belgian breeding program, 'Ogy' is one of the better clones (Steenackers 1984). 'Ogy' is known for its fast growth rate and good wood quality, especially for match veneer. In Belgium, 15-year-old 'Ogy' clones grew to an average circumference of 120 cm, a basal area of 1,156 cm^2, a height of 22.4 m, and a stem volume of 708 cm^3 (Steenackers 1987). Under favorable conditions, these growth properties make 'Ogy' an excellent tree for biomass production. Of the Euramericana hybrids, 'Ogy' is one of the easiest to propagate in tissue culture. It also produces shoots from leaf vascular tissues when stimulated with exogenous hormones.

Hybrid and native *Populus* clones from the northcentral regional *Populus* programs

Clones with pure *Populus deltoides* parentage, as well as hybrid clones of species belonging to the Aigeiros and Tacamahaca sections, were used in this dissertation to study Septoria canker disease (Table 1). The hybrids and *P. deltoides* clonal selections represent a range of resistance to cankers caused by *Septoria musiva*.

Major disease and insect problems of the *Populus* clones

'Ogy' and other hybrids are susceptible to both leaf spot disease and cankering by *Septoria musiva* Peck (teleomorph *Mycosphaerella populorum* Thompson). This pathogen is considered an endemic organism in native stands of North American *Populus*. Evidence of woody plant pathogens belonging to the genus *Septoria* was recorded in northcentral North America in the early 20th Century (Tehon and Daniels 1924), and later studied definitively (Thompson 1937). With the introduction of inter- and intraspecific *Populus* hybrids, *S. musiva* became a serious pathogen causing Septoria leaf spot and Septoria canker in hybrid poplar in North America (Bier 1939; Ostry and McNabb 1983, 1985; Spielman et al. 1986; Thompson 1941; Waterman 1954). The fungus can drastically reduce photosynthetic area on leaves of poplars, and more importantly can cause cankers that can girdle main stems. Leaf spot, but not canker disease, is present on native *P. deltoides* selections. Both manifestations of the disease condition can cause serious loss of biomass accumulation (McNabb et al. 1982; Ostry 1987) and the canker also can cause breakage and death of the main stem.

Table 1. Selections of native and hybrid *Populus* used in the artificial-inoculation Septoria canker disease screening test

Parentage of cross or species of selection	Resistance to *S. musiva* [a,b]	Identification numbers or trade names	Source of original cross or selection
Populus deltoides	HR	'8000105'	Illinois (clonal selection)
Populus deltoides	HR	'8000113'	Illinois (open pollinated)
Populus deltoides	HR	'51-5'	North Central Regional Project
Populus X *euramericana* [c]	MR	'DN 34' ('NC 5326') 'Eugenei'	France (natural hybrid)
P. nigra x *P. maximowiczii*	MR	'NM 6'	Canada
Populus X *euramericana* [c]	MR	'DN 2'	Canada
P. nigra 'Charkowiensis' x *P. nigra* 'Caudina'	R/S	'NE 19'	Oxford Paper Company
P. deltoides x *P. maximowiczii*	MS	'313.55'	USFS Univ. of Minn., St. Paul.
P. deltoides x *P. maximowiczii*	MS	'502.37'	USFS Univ. of Minn., St. Paul.
P. nigra x *P. trichocarpa*	HS	'NE 285'	Oxford Paper Company
P. trichocarpa x *P. deltoides*	HS	'57-276'	Univ. of Washington

[a] Resistance values: HR = highly resistant, MR = moderately resistant, R/S = low resistance/susceptible, MS = moderately susceptible, HS = highly susceptible

[b] Resistance values represent the observed resistance to Septoria canker disease, as determined by researchers (Dickman and Stuart 1983; McNabb and Ostry, personal communication).

[c] *Populus* X *euramericana* hybrids are crosses of *P. deltoides* and *P. nigra*.

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Considerable variability in pathogen aggressiveness has been observed (Krupinsky 1987, 1989) and may be explained by the presence of different strains of the pathogen (Hubbes and Wang 1990). Strain differences may help explain resistance breakdowns in clones previously thought to be resistant (Vanderplank 1984). Field observations of the Euramericana hybrids suggest that there is some low level resistance (McNabb et al. 1982).

The hybrid and native clones are susceptible to *Melampsora medusa* Thuem., the common poplar leaf rust. In Europe, 'Ogy' is susceptible to various races of *Melampsora larici-populina* Kleb and is currently used as a differential clone for determining European rust races (Steenackers 1987).

The other foliar disease that can have a significant impact on the clones is leaf spot caused by *Marssonina brunnea* (Ell. & Ev.) Sacc. (Ostry and McNabb 1983; Ostry 1987). The necrotic spots on the leaves can reduce photosynthetic potential, and petiole infections can lead to leaf abscission that results in premature defoliation. 'Ogy' has been observed to possess moderate resistance to this pathogen (Steenackers 1985, 1986).

In North America, the major defoliating insect pest of *Populus* with *P. deltoides* parentage is the cottonwood leaf beetle, *Chrysomela scripta* F. (Coleoptera: Chrysomelidae), (Ostry et al. 1989). 'Ogy' is a preferred food source for both the larval and adult stages of *C. scripta* (Hart 1992, personal communication).

CHAPTER 2. DETECTION OF TWO *SEPTORIA MUSIVA* PROTEINASES PRODUCED *IN VITRO*

A paper to be submitted to the journal Phytopathology

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Abstract

A system for the production and detection of the extracellular proteolytic enzymes produced by *Septoria musiva* was established. A synthetic medium, casein culture medium (CCM), containing essential macro and micronutrients, glucose, and long chain polypeptides of casein as the sole source of amino acid and nitrogen nutrition, was developed. The medium proved to be a very good growth medium for both agar plate culture and liquid shaker cultures. Four isolates of *S. musiva* were screened for growth and reproductive potential, their ability to grow on a minimal long-chain polypeptide substrate (casein), and their ability to adapt to liquid culturing. The isolates responded differently in the screening tests and several did not exhibit

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vigorous growth in liquid culture. Growth data from the isolates showed a growth-limiting effect in CCM cultures that contained initial concentrations of <60 mM glucose. An isolate from Iowa (MP1) was chosen for the protease production studies. Culture filtrates of MP1 were concentrated and run on SDS PAGE activity gels. Two putative proteinases, P1 and P2, were visualized as clearings in the activity gels that resulted from proteolytic digestion of an incorporated casein substrate. Estimates of their molecular weights, 24 kDa (P1) and 75 kDa (P2), were determined by comparing the location of the clearing in relation to molecular weight standards on the gel. Proteinase P1 was isolated by ammonium sulfate fractionation and further investigated with a spectrophotometric gelatin assay for proteolytic activity. The optimum pH and temperature for P1 proteolytic activity were approximately pH 8 and 60° C. Preincubation with the serine proteinase inhibitors, proteinase inhibitor II (PI II) and phenylmethanesulphonyl fluoride (PMSF), resulted in reduction of proteolytic activity by 55% and 95% respectively. Inclusion of PMSF in SDS PAGE activity gel samples containing whole culture filtrate resulted in complete inhibition of P1 activity, but did not visibly affect the activity of P2. Studies to further define the P1 serine proteinase and to determine the nature of the P2 activity are in progress.

Introduction

Septoria musiva Peck. (teleomorph *Mycosphaerella populorum* Thompson) is a fungal pathogen of *Populus* and is considered endemic in the native stands of eastern North America. *S. musiva* is the causal organism in the Septoria leaf spot and Septoria canker diseases of hybrid poplar in eastern North America. It is one of the most serious pathogens of *Populus* in North America. With the introduction of more susceptible inter- and intraspecific hybrids, the disease has become a serious problem (Bier 1939; Ostry and McNabb 1983, 1985; Spielman et al. 1986; Thompson 1941; Waterman 1954). Severe Septoria leaf spot can result in greatly reduced photosynthetic area or even premature defoliation (Thompson 1941). Septoria canker can weaken or even girdle the main stem, predisposing the stem to wind damage (Moore 1984) or dieback of the distal portion of the infected stem (Waterman 1946). Biomass losses as high as 63% have been reported in plantations with severe Septoria disease outbreaks (McNabb et al. 1982). Primarily infections are mainly the result of ascospores released in the spring. Subsequent infections result from asexual conidia released in secondary disease cycles that greatly amplify the number of infections (Lulley 1987).

Proteinases have been shown to have a synergistic effect in cell necrosis when combined with cell wall degrading enzymes (Movahedi and Heale 1990). Therefore, fungal proteinases may play an important role in pathogenesis. There are four major

groups of proteinases, the serine, cysteine, aspartic, and metallo-proteinases. A review by North (1982) confirmed that other researchers have found serine proteinases, as well as proteinases belonging to the other three classes, in higher fungi. It has been proposed by Ryan (1990) that proteinase inhibitors could possibly reduce the vigor of fungi by depriving them of necessary amino acids. The activity of fungal proteinases in the pathogens *Colletotrichum lindemuthianum* (Sacc. & Magnus) and *Fusarium solani* (Mart.) Sacc. have been reduced by proteinase inhibitors (Mosolov et al. 1976; 1979). Preliminary studies from our laboratory have shown that filtrates from liquid cultures of *S. musiva* exhibit proteolytic activity that is partially inhibited by proteinase inhibitor II (Sillick et al. 1989; Heuchelin and McNabb 1995).

Proteinase Inhibitor II protein (PI II) is an inhibitor of the serine proteinases trypsin and chymotrypsin (Ryan 1981, 1990; Ryan and An 1988). PI II is a heat stable, acid soluble, low molecular weight protein [monomer M_r 12,300 (Plunkett et al. 1982)] and is the gene product of the proteinase inhibitor II gene (*pin2*) (Green and Ryan 1972; Ryan 1981, 1990). To further determine the potential of PI II and other proteinase inhibitors as antifungal agents of *S. musiva*, our laboratory is investigating the proteinases produced by the pathogen.

The main objectives of this study were to establish an artificial medium for cultures of *S. musiva* that could provide optimal nutrition and a defined nitrogen (protein) source for proteolytic enzyme analysis, and to identify proteinases that may be responsible for the proteolytic activity previously observed in *S. musiva* culture filtrates.

Materials and Methods

Septoria media and nutrition

V-8 PPA. Isolates of *S. musiva* were maintained on the V-8 Phytone Peptone Agar (V-8 PPA) developed by Krupinsky (1989). The V-8 PPA medium contains protein hydrolysates from soybean (BBL Phytone Peptone, Becton-Dickinson & Co. Cockeysville, MD) and a slurry of vegetable homogenate (V-8 juice) rich in vitamins, minerals, carbohydrates, and amino acids. The medium allowed better growth and sporulation (Krupinsky 1989) than all previously developed Septoria media.

CCM. A synthetic liquid medium, casein culture medium (CCM), was developed for the analysis of *S. musiva* protease production. The medium contains essential macro and micronutrients, glucose, and long chain polypeptides of casein (Vitamin free, Bovine Casein, Calbiochem San Diego, CA) as the sole source of amino acid and nitrogen nutrition. Casein (10 g/l) dissolved in distilled water at pH 11, was dialyzed exhaustively so that it contained only long chain polypeptides. Dialysis was conducted at room temperature using standard dialysis tubing (Spectrapor) in 3 liters of pH 11.0 ddH_2O with two changes per day for 3 days. The pH of the ddH_20 was adjusted to pH 11.0 with NaOH to maintain the structural integrity of the dialysis tubing. KOH was used to adjust the pH in the last change of

dialysis buffer to diffuse the possibly toxic sodium ions. Exclusion of low molecular weight peptides was confirmed with electrophoresis. The dialyzed casein was quantified using the Bradford assay (Bradford 1976), diluted with ddH_2O to a concentration of 5 g/l, and adjusted to pH 11.0 with KOH. A stock micronutrient solution [1000 X] consisting of 0.001 g/l of $CuSO_4$, $FeSO_4$, $MnSO_4$, $ZnSO_4$, H_3BO_3, $CoCl_2$, and NaCl was mixed and stored at 4 C. One milliliter of the stock micronutrient solution was added per liter to a phosphate buffered (22 mM, pH 5.0) nutrient solution [2X] containing $CaCO_3$ (0.25 g/l), KH_2PO_4 (3g/l), and $MgSO_4$ (0.001 g/l). Equal volume aliquots of the buffered nutrient solution and the casein solution were autoclaved separately and combined when cooled. This process was necessary to prevent denaturation and precipitation of the casein protein source. Final pH of the combined medium was pH 6.8.

Septoria isolates

S. musiva isolate MP1 was obtained from a local *Populus* plantation by our laboratory (Lulley 1986), and isolates MP2, MP3, and MP4 were collected from other plantations in the northcentral states by cooperators in the USDA Forest Service, St. Paul, MN. The asexual pathogen propagules (conidia) of *Septoria musiva* were collected from hybrid poplar field plots in Iowa (MP1), Wisconsin (MP2), North Dakota (MP3), and Minnesota (MP4). Pure cultures of the *S. musiva* isolates were

established on V-8 PPA and allowed to asexually sporulate The resulting conidia were frozen in glycerol at -70° C and used as stock propagules for fresh cultures.

Screening of isolates

Growth and sporulation on V8-PPA. The four isolates of *S. musiva* were grown on V-8 PPA plates for 14 days at 25° C. A 200 µl conidial suspension (1,000 conidia/ml) was applied to the surface of the media. Radial growth area (cm^2) and evidence of sporulation were monitored daily. There were 10 plates prepared per isolate. A separate germination assay using a thin film of V-8 PPA on microscope slides was run along with the petri plate cultures. Germination efficiency was assessed using low power magnification with a standard microscope.

Growth on solid CCM. Radial growth of *S. musiva* isolates on CCM containing agar (2% w/v), but without glucose, was observed for 30 days. Ten plates per isolate were stab-injected with a sterile needle coated with conidial exudate from freshly sporulating cultures of the isolates. Protease-dependent growth was measured as radial area (cm^2) occupied by mycelium. In a later experiment, MP1 was grown on the same medium amended with a range of glucose concentrations to determine the effect of carbon (glucose) on radial growth. Glucose was filter-sterilized and added in 10 mM increments from 0 to 100 mM.

Growth in liquid CCM. Shaker cultures of *S. musiva* isolates in liquid CCM were used to obtain proteinases produced by the pathogen in the culture medium. Inoculum consisted of 200 ul of a conidial suspension (10,000 conidia/ml sterile ddH_2O) applied to 20 ml of CCM in a 50 ml Erlenmeyer flask. An optimal concentration of the carbon source (glucose) was determined for highest levels of growth (mycelial mass). Glucose was filter-sterilized and added in 10 mM increments from 0 to 100 mM. The culture flasks were agitated at 100 rpm on an orbital shaker platform at 25° C. After 30 days, cultures were vacuum filtered through pre-weighed filter paper. The filter paper with the mycelium was dried in a 50 C oven for 48 hr. and weighed to determine dry mycelial mass. The filtered supernatant from the cultures was frozen at -70° C until analysis could be conducted.

Culture filtrates for subsequent experiments were derived from 100 ml Erlenmeyer flasks containing 40 ml CCM amended with 50 mM glucose and 500 µl of a conidial suspension (10,000 conidia/ml sterile ddH_2O) from isolate MP1.

Monitoring of glucose concentration in the liquid medium was accomplished using a spectrophotometric assay that uses Trinder reagent (Sigma Diagnostics, St. Louis). The reagent oxidizes available glucose and produces a chromophore that is measured at 505 nm (Trinder 1969).

PAGE assay for proteolytic activity

Protease activity was visualized using a modification of the substrate-electrophoresis (S-PAGE) method of Garcia-Carreno and Haard (1994). The non-denaturing sodium dodecyl sulfate (SDS) activity gels were electrophoresed in a Bio-Rad Protean II dual slab gel apparatus (Bio-Rad Laboratories, Richmond, CA). Samples were not heat denatured and contained no beta-mercaptoethanol. Lyophilized culture filtrate fractions were resuspended in ddH_20 (20 ul), diluted (1:1) in sample buffer (12mM Tris pH 6.8, 4% (v/v) glycerol, 4% (w/v) SDS, and 0.00025% (w/v) bromophenol blue), and loaded along with molecular weight standards into a SDS-PAGE gradient gel. The gradient gel consisted of a 5-20 % acrylamide resolving gel with a 4% stacking gel. The gel was electrophoresed, with cooling, at 60 V for 24 h.

After electrophoresis, the gel was rinsed with ddH_2O and immersed in casein substrate (2% casein, boiled in 50 mM Tris-HCl buffer, pH 7.5) for 45 min at 4° C. The gel was then rinsed in ddH_2O, drained, covered, and incubated at 25° C for 2 h to allow digestion of the substrate. The incubated gel was washed with ddH_2O and immersed in the staining solution (40 % EtOH, 10 % acetic acid, and 0.1 % Coomassie Brilliant Blue R250) for 2 h. The gel was destained for 90 - 120 min in a 40 % ethanol-10 % acetic acid solution with gentle agitation.

Ammonium sulfate precipitation

For the characterization studies described, a 51-70% ammonium sulfate [$(NH_4)_2SO_4$] fraction was used to segregate the proteolytic activities of two putative proteases. The 51-70 % fraction was selected from a trial of five different fractions, with the objective of excluding a high molecular weight protease from the fraction. The trial fractions were obtained by adding ammonium sulfate to five 40 ml aliquots of a pooled 200 ml culture filtrate solution and collecting the resulting precipitate. The salt was added to four of the aliquots until one of each contained a 30, 40, 50, and 60 % (w/v) concentration of the salt respectively. The solutions were mixed until all salt dissolved, incubated at 4° C for 4 h, and centrifuged at 12,000 x g for 20 min. The fifth aliquot contained no salt, but was incubated and centrifuged as the others. Supernatants were carefully decanted, in order to not disturb any resulting precipitate. Additional ammonium sulfate was added to the five collected supernatants until each contained 70% (w/v) of the salt. The solutions were mixed, incubated, and centrifuged as before. The resulting precipitates represented 0-70, 31-70, 41-70, 51-70, and 61-70 % ammonium sulfate fractions of the culture filtrate proteins. The precipitates were resuspended in 5 ml Tris-HCl buffer (50 mM, pH 7.5) and dialyzed 20 h at 4° C with three changes of the same Tris buffer. A final dialysis step was conducted in ddH_2O for 2 h at 4° C to remove Tris salt before lyophilization. Approximately 25 aliquots (200 ul/microfuge tube) of each fraction, were lyophilized

in a spin-vac, and stored at -20° C. Protein content of the fractions was determined using the Bradford assay (Bradford 1976).

Protease activity in the fractions was visualized by resuspending the lyophilized samples in ddH_2O (20 ul), adding activity gel loading buffer, and running the samples on SDS activity gels as described above.

Protease activity assays

Extracellular proteinase activity in the culture filtrate was determined by hydrolyzing gelatin with a modification of the Tseng and Mount (1974) method, and quantifying the hydrolysate using a Perkin-Elmer Lambda 5 UV-VIS spectrophotometer. Attempts to use casein as the substrate were unsuccessful because of solubility problems, so the proven gelatin substrate was used. Reaction mixtures consisted of 0.25 ml of 1% (w/v) gelatin (Sigma Chemical Co. St. Louis, MO) in 0.05 M Tris-HCl buffer (pH 8.0) and 0.25 ml of the resuspended lyophilized protease sample in a microfuge tube. The mixture was incubated for 3 h at 30° C in a heat block. At the end of the incubation period, 0.75 ml of 20% trichloroacetic acid (TCA) was added to the reaction and mixed well. The solution was allowed to stand at room temperature for 10 min and centrifuged at 10,000 rpm for 10 min. The absorbance of the supernatant at 280 nm was recorded. The control blank was prepared by incubating the buffer-gelatin solution and enzyme sample separately and combining them for TCA treatment. In experiments where enzyme inhibitors were

used, the inhibitor was added to the enzyme sample and pre-incubated for 10 min at 30 C prior to incorporation with the reaction mixture. The enzyme sample for the control blank in the inhibitor assay also was pre-incubated. The blank was used to zero the spectrophotometer before reading the samples. An increase in absorbance of 0.01 was defined as one unit of protease activity.

Serine protease assay. The gelatin spectrophotometric assay was used to determine the effect of proteinase inhibitors on enzyme activity in the protease sample. The protease sample consisted of a resuspended lyophilized 51-70 % ammonium sulfate fraction of culture filtrate. Purified PI II (CalBiochem-Novabiochem Corp. San Diego, CA), an inhibitor protein of serine proteases, was added to assess its ability to inhibit protease activity. The crystalline PI II was dissolved in ddH_2O (10ug/ul). Ten microliters of the PI II solution were added to the enzyme fraction. Phenylmethanesulphonyl fluoride (PMSF), an irreversible inhibitor of serine proteinases, also was added (1 mM) to a resuspended lyophilized sample. Inhibitors were incubated with the enzyme samples for 10 min at room temperature prior to incorporation in the gelatin spectrophotometric assay.

PMSF also was applied to a SDS-PAGE activity gel sample at an equivalent concentration and electrophoresed as previously described for SDS activity gels.

Protease activity optimum pH. The gelatin assay was used to determine the effect of pH on enzyme activity of the protease sample. The sample was a lyophilized 51-70 % ammonium sulfate fraction, resuspended as before. One percent gelatin solutions were prepared in each of the following buffers: 0.1 M acetic acid-sodium acetate buffer (pH 5), 0.1 M monobasic sodium phosphate-dibasic sodium phosphate buffer (pH 6 and 7), 0.1 M Tris-HCl buffer (pH 8 and 9), and 0.1 M glycine-sodium glycinate buffer (pH 10). The gelatin assay was performed as described above. There were four replications per treatment and the experiment was performed twice.

Protease activity optimum temperature. The gelatin assay was used to determine the effect of temperature on enzyme activity in the protease sample. The sample was prepared as described in the pH optima assay. Reaction mixtures were incubated at 10, 20, 30, 40, 50, 60, 70, and 80 C for 3 h, and treated as previously described. There were four replications per treatment and the experiment was performed twice.

Results

Screening of isolates

Growth and sporulation on V8-PPA. All isolates grew and sporulated on the standard V-8 PPA Septoria medium. Radial growth was confined to the area of the inoculation solution, and isolates produced mycelia at similar rates. Differences were noted among the isolates in the length of time required for sporulation (Table 1). Isolate MP1 sporulated most rapidly at 5 days, MP3 and MP2 required more time (6 and 7 days respectively), and MP4 was slow to sporulate, with conidia being produced at 13 days. Germination testing revealed that the conida produced on the V-8 PPA media were viable and germinated well. All isolates had conidial germination efficiencies of approximately 90% or greater (data not shown).

Growth on solid CCM. The radial growth of *S. musiva* isolates on CCM agar without glucose was determined (Table 1). The growth exhibited (cm^2) showed the potential of each isolate to utilize proteases to obtain nutrition for mycelial growth. MP4 grew the best on the carbon deficient medium (8.2 cm^2), with MP2 and MP1 exhibiting good growth (6.0 and 5.9 cm^2 respectively) and MP3 faring the worst (3.1 cm^2). In the later experiment with MP1 grown on glucose amended CCM (data not shown), radial growth ceased or was greatly limited from approximately day 10 to

Table 1. Growth, sporulation, and polypeptide utilization potential of the four *Septoria musiva* isolates

Isolate	MP1	MP2	MP3	MP4
Isolate source	IA	WI	ND	MN
Reproductive maturation period on V8-PPA medium (Days from germination to sporulation)	5	7	6	13
Radial growth on CCM agar containing no glucose (30 day growth period, radial growth in cm^2)	5.9	6.0	3.1	8.2
Growth in liquid CCM amended with glucose (growth = mean mycelial dry weight [mg] of treatments)	24.6	9.0	10.8	5.3

day 30. With glucose in the medium, radial growth began at a higher rate, but was severely slowed or halted when sporulation of the fungus was visible. Sporulation was initiated within 10 days on all glucose concentrations (10-100 mM). No sporulation was observed on the control CCM (0 mM glucose), even after 30 days growth. Amount of sporulation increased with glucose concentration until approximately 50 mM, where greater glucose concentrations did not visibly enhance sporulation. The 10mM glucose CCM medium allowed light sporulation, 20-30 mM provided moderate sporulation, and 40 mM or greater resulted in heavy sporulation by day 10.

Growth in liquid CCM. Thirty-day cultures of *S. musiva* in liquid CCM showed a growth limiting effect by CCM glucose concentrations of 50 mM or less (Fig. 1). The optimal concentration of glucose for the liquid medium was a minimum of 60 mM. Higher levels of glucose provided no significant advantage in production of mycelial dry weight. No maximum concentration or possible limiting effect from excess glucose concentration was investigated. The average mycelial mass produced over the range of glucose concentrations varied by isolate (Table 1). MP1 was superior for growth in liquid culture. MP1 exhibited good results in all the selection areas (Table 1) and was selected as the experimental isolate for the following biochemical analyses.

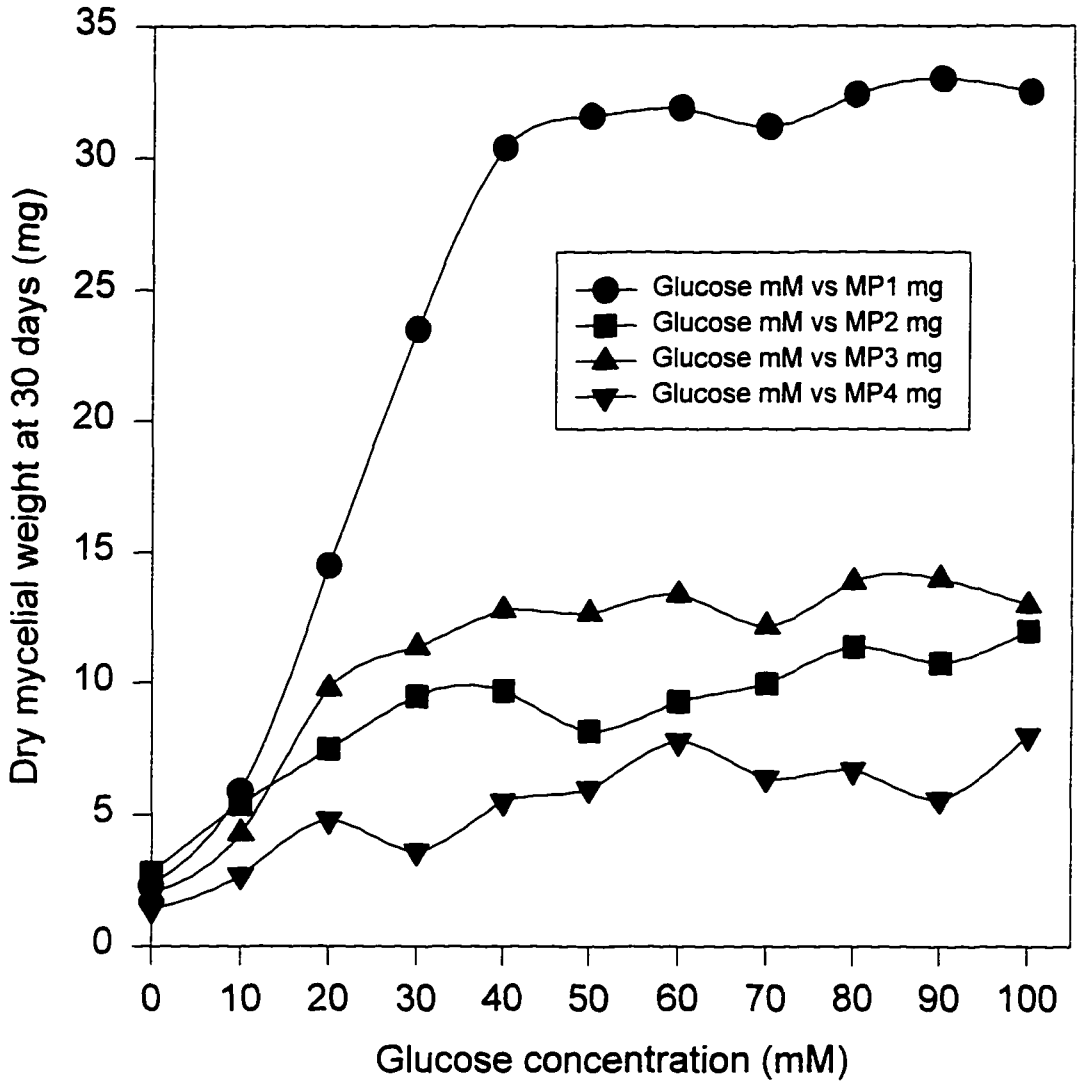

Fig. 1. The effect of the initial glucose concentration in CCM medium on the growth of *S. musiva* isolates MP1-4 after 30 days of liquid culture. Growth = mycelial dry weight (mg). Initial glucose concentrations (treatments) = 0 mM (control) and 10-100 mM glucose/L.

Trinder assay. Analysis of glucose content in the 30 day-old MP1 culture filtrates, provided data on the concentration of residual glucose (mM) in the culture medium (Fig. 2). Further analysis revealed the amount of glucose consumed by the fungus in each of the treatments and, by comparing the amount of mycelial mass produced, the efficiency of glucose utilization in the production of biomass (Fig. 2). The initial glucose concentration of the medium had an effect on the efficiency of glucose consumption in the production of mycelial mass. The breakpoint for low efficiency vs. high efficiency metabolism was at approximately 60 mM initial glucose concentration.

PAGE assays for proteolytic activity

Proteolytic activity was observed on the SDS-PAGE activity gels. Whole culture filtrate from isolate MP1 showed proteolytic activity in two separate regions of the gel. The location of the putative proteases on the gels was revealed as clear bands where the gelatin had been degraded (Fig. 3). Activity was present at approximately 24 and 75 kd and will be referred to as protease P1 and P2 respectively.

The banding intensity varied slightly depending how long the culture medium was in contact with an established mass of mycelium. Fresh medium introduced to an actively growing mycelial mass showed slightly greater activity by P1 after 2 days.

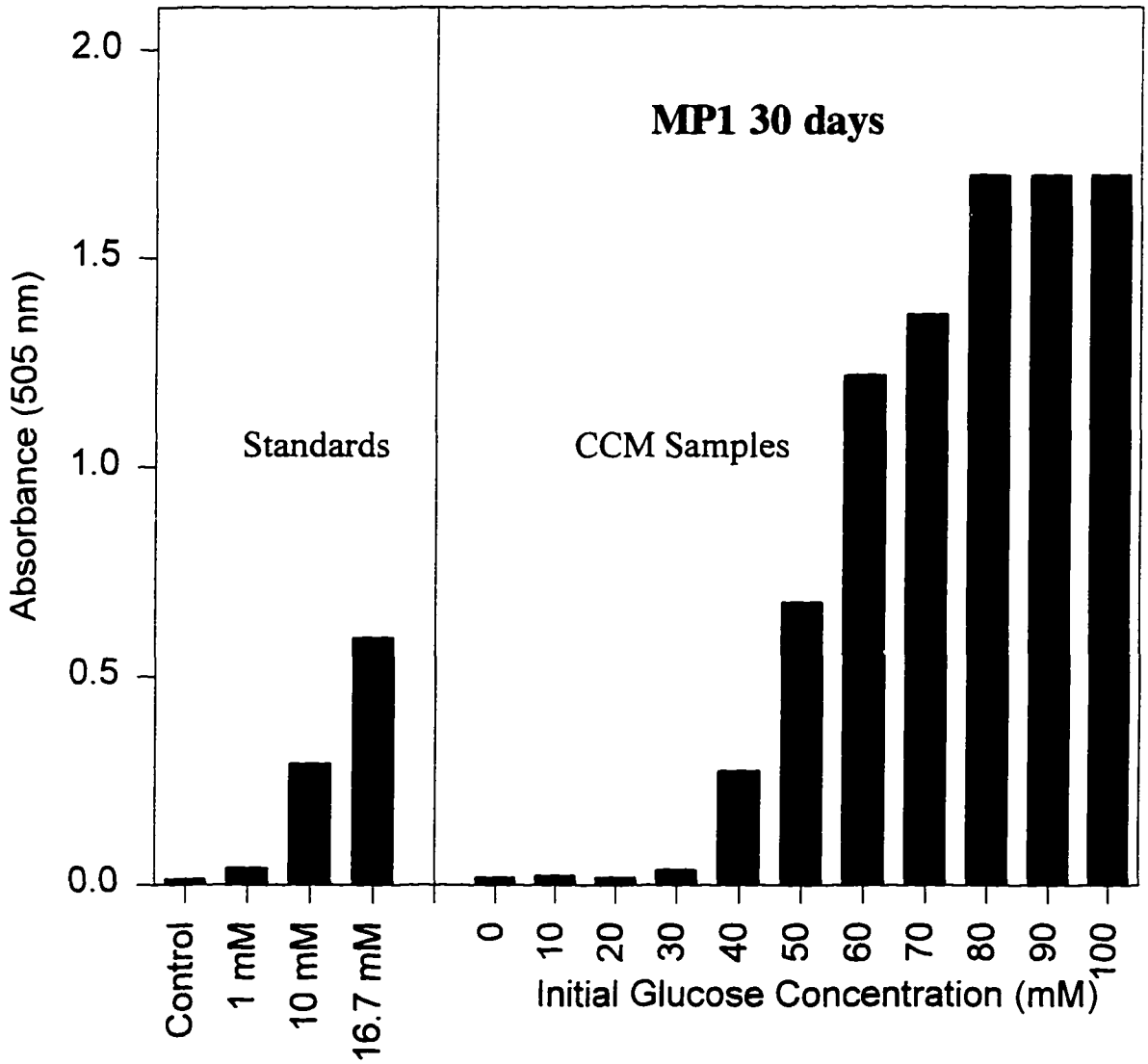

Fig. 2. The effect of the initial glucose concentration of CCM medium on the amount of glucose available (residual) to *S. musiva* isolates MP1-4 after 30 days of liquid culture. Residual glucose = absorbance at 505 nm, as determined by the Trinder assay for glucose concentration. Standards are CCM medium containing designated amounts of glucose (mM). Control standard = CCM without glucose.

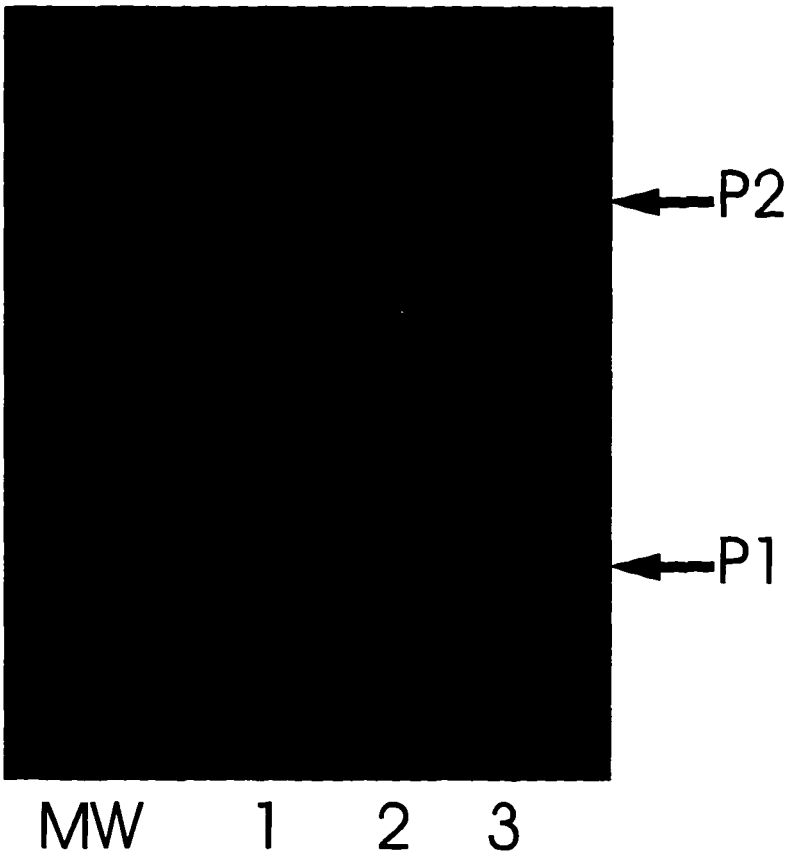

Fig. 3. The appearance of two proteases, designated P1 and P2, (indicated by arrows) on a SDS activity gel in samples from *S. musiva* isolate MP1 grown in liquid CCM containing 50 mM glucose. Clearings in the stained gel indicate proteolytic activity. P1 is approximately 24 kDa, and P2 is approximately 75 kDa. Lane 1 = proteolytic activity after 2 days growth by an established mycelial mass of *S. musiva* in fresh medium., Lane 2 = activity at day 10, Lane 3 = activity at day 30. Protein content was 100 μg per lane.

At 10 and 30 days, P1 continued to provide more clearing on the gel than P2, but the amount of clearing by P2 had increased (Fig. 3). Addition of phenylmethanesulphonyl fluoride (PMSF) to the enzyme sample prior to gel loading resulted in inactivation of P1 but did not affect the activity of P2 (Fig. 4).

Protease activity assays

Assays for protease activity were solely run on a fraction containing P1 activity. Fractionation of the sample by ammonium sulfate precipitation allowed the exclusion of P2 from the sample. P2 activity was excluded when a 51-70 % fraction of the culture filtrate was used on activity gels (Fig. 5).

Serine protease assay. Protease activity of P1 was substantially reduced by both PI II and PMSF. The reversible protein inhibitor, PI II, provided significant inhibition of the protease (Fig. 6) resulting in an enzyme activity decrease of approximately 55%. The irreversible inhibitor, PMSF, provided almost complete inhibition of the proteolytic activity in the assay (~95%).

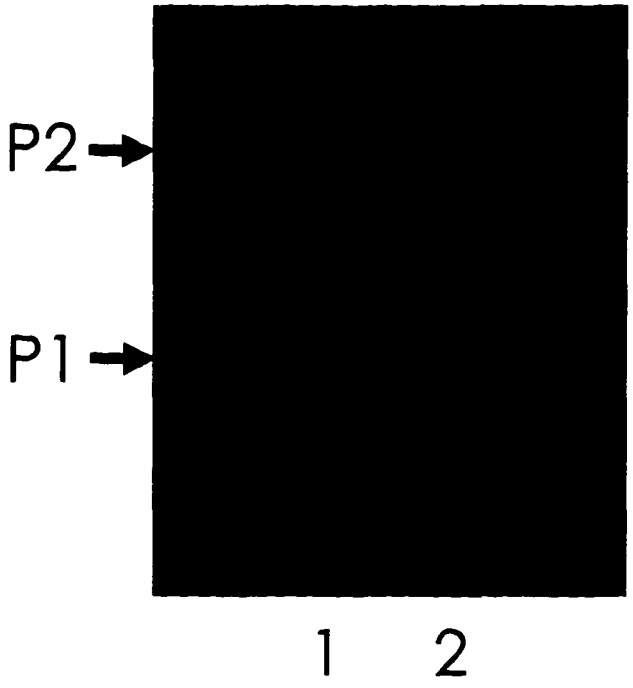

Fig. 4. The effect of PMSF treatment on the activities of P1 and P2 as visualized by a SDS activity gel. Samples were from a 30 day, *S. musiva* MP1 liquid CCM culture containing 50 mM glucose. Clearings in the stained gel indicate proteolytic activity. Lane 1 = proteolytic activity of sample (untreated), Lane 2 = proteolytic activity of sample treated with PMSF (10 mM). Sample protein content was 100 μg per lane.

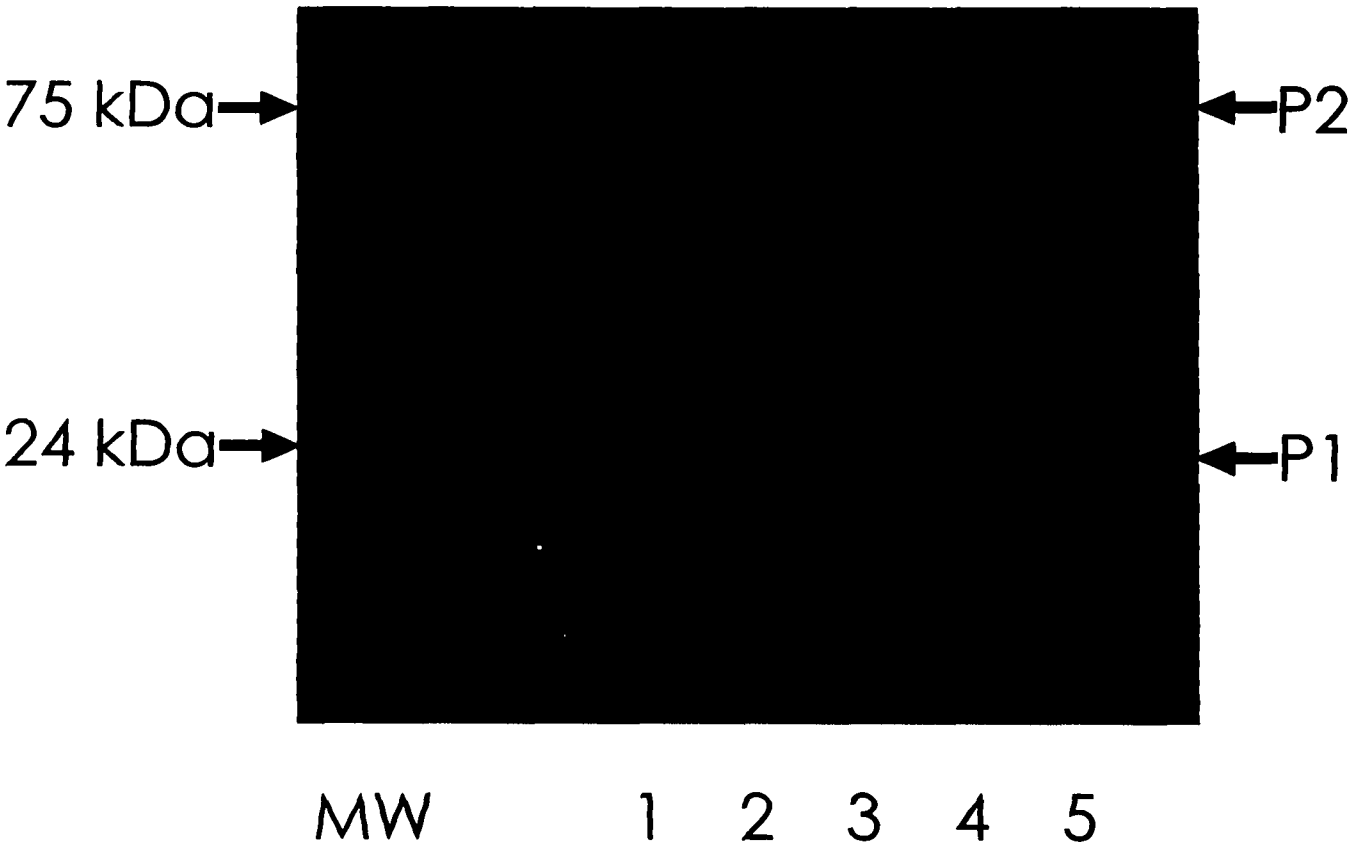

Fig. 5. Proteolytic activity of the five ammonium sulfate fractions derived from culture filtrates of 30 day, *S. musiva* MP1 liquid CCM cultures, containing 50 mM glucose. Clearings in the stained gel indicate proteolytic activity. Lanes: 1 = 61-70%, 2 = 51-70%, 3 = 41-70%, 4 = 31-70%, 5 = 0-70%.

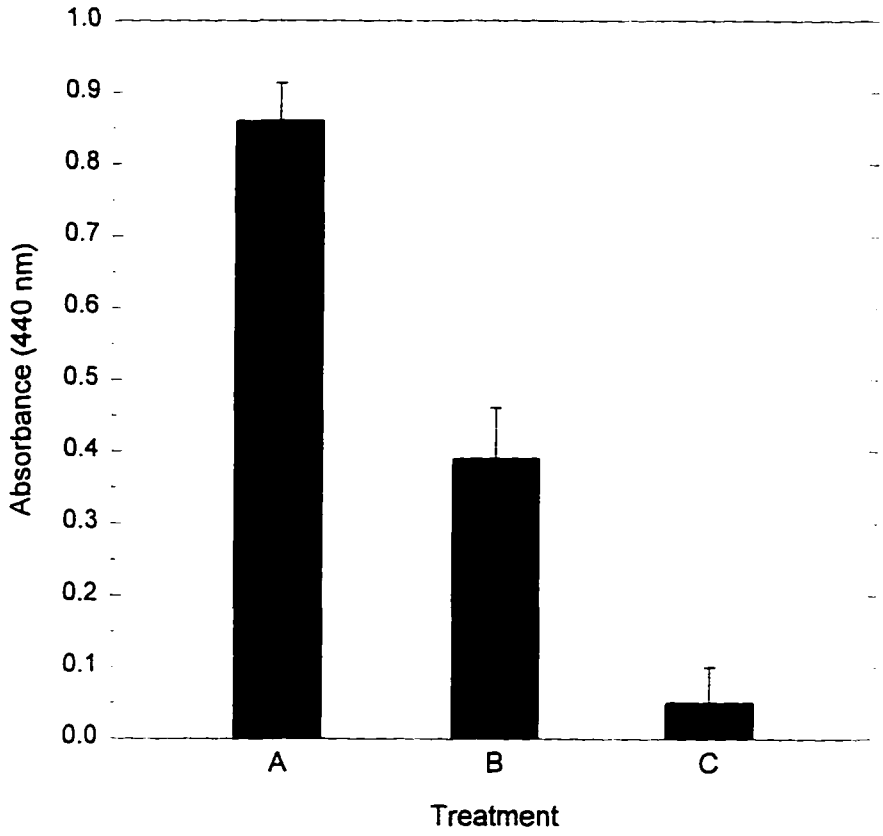

Fig. 6. The effect of proteinase inhibitor II (PI II) and phenylmethanesulphonyl fluoride (PMSF) treatments on the activity of a 51-70% ammonium sulfate fraction of MP1 culture filtrate which contained P1 proteolytic activity. Activity was measured as absorbance with the gelatin spectrophotometric assay. Treatment A = 51-70% culture filtrate fraction (control), Treatment B = 51-70% fraction preincubated with PI II (100 μg/ml), Treatment C = 51-70% fraction preincubated with PMSF (10 mM). One unit of proteolytic activity equals an increase in absorbance of 0.01 at 440 nm. Error bars represent standard deviation of the mean.

Protease activity optimum pH. The protease P1 possessed activity across a range of mostly alkaline pH (pH 6-10). Protease activity increased to an optimum at approximately pH 8.0, then declined slightly, while still maintaining good activity to pH 10 (Fig. 7).

Protease activity optimum temperature. Protease P1 was active across a wide range of temperatures (10-70° C). Protease activity increased to an optimum around 60° C, and then declined with a sharp decrease in activity from 70 to 80° C (Fig. 8).

Discussion

This study provides basic information toward the elucidation of the proteolytic enzyme profile of *S. musiva*. Each pathogen system is unique in its biochemical profile, environmental interactions, and nutritional needs. Though many media and procedures work for a variety of fungi, techniques and conditions need to be optimized and standardized to provide data that are efficacious and can be used by other researchers studying *S. musiva*. Fungal nutrition is critical in cell growth and differentiation, as well as in the production of enzymes (Garraway and Evans, 1984).

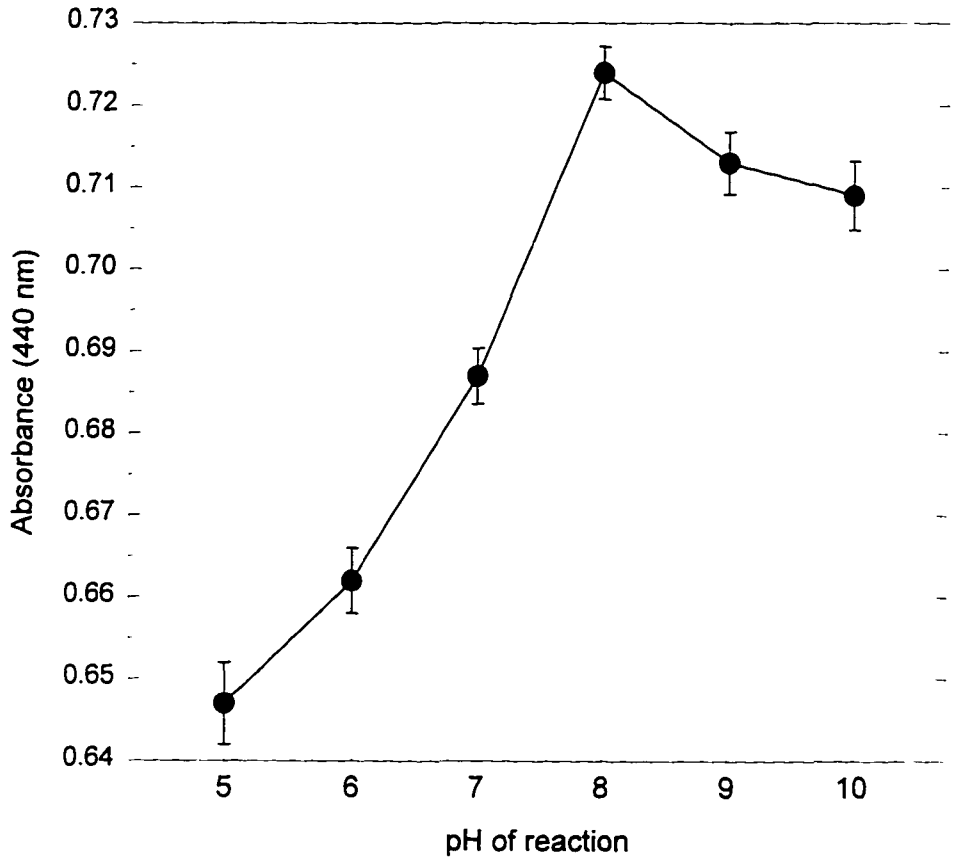

Fig 7. Effect of pH on proteolytic activity. Samples were a 51-70% ammonium sulfate fraction of MP1 culture filtrate that contained P1 proteolytic activity. Activity was measured with the gelatin spectrophotometric assay. Samples were mixed with 1% gelatin solutions made with: 0.1 M acetic acid-sodium acetate buffer (pH 5), 0.1 M monobasic sodium phosphate-dibasic sodium phosphate buffer (pH 6 and 7), 0.1 M Tris-HCl buffer (pH 8 and 9), and 0.1 M glycine-sodium glycinate buffer (pH 10). Error bars represent standard deviation.

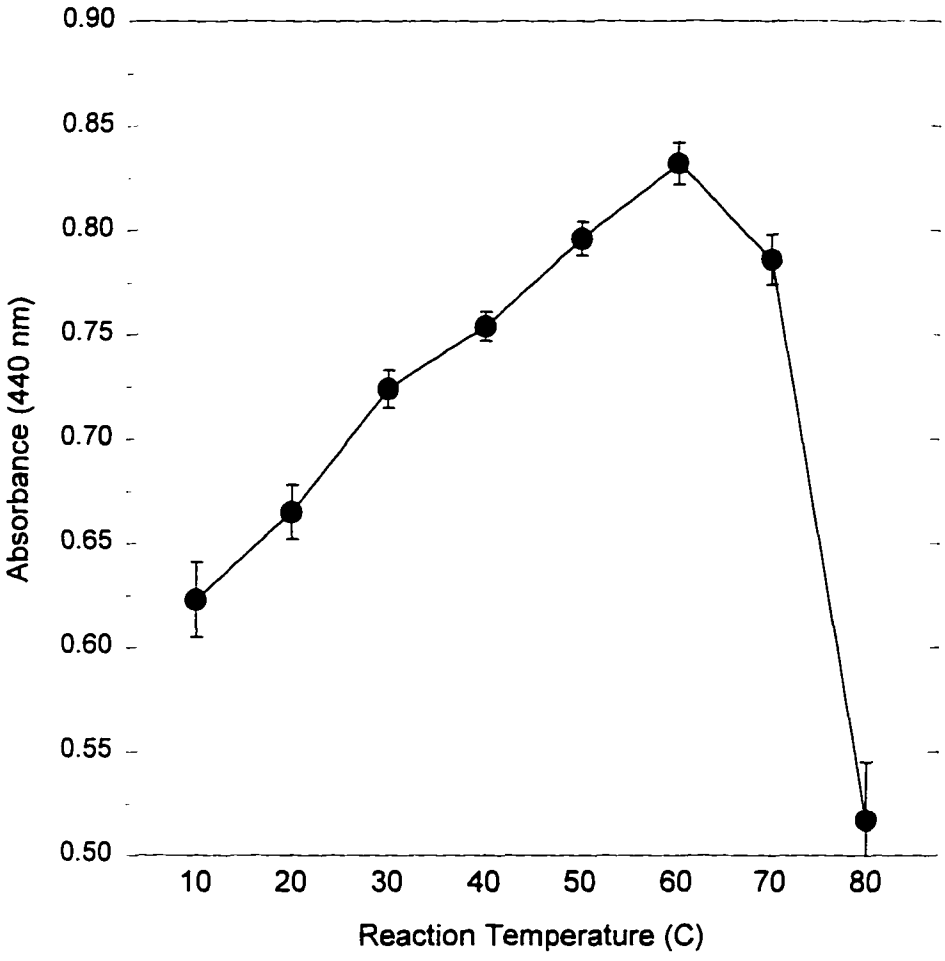

Fig 8. Effect of reaction temperature on proteolytic activity. Samples were a 51-70% ammonium sulfate fraction of MP1 culture filtrate which contained P1 proteolytic activity. Activity was measured with the gelatin spectrophotometric assay. Reaction mixtures were incubated at 10, 20, 30, 40, 50, 60, 70, and 80 C for 3 h. Error bars represent standard deviation.

Considerable nutrient variability can exist in media that consist of commercial food-products, such as V-8 juice, or vegetable produce. The CCM medium designed for this study contains moderate amounts of all the known essential macro and micronutrients needed by most fungi (Garraway and Evans, 1984). CCM is ideal for proteolytic studies because the sole nitrogen and amino acid sources are the long chain polypeptides of casein (~23.6 kd). Other established media contain protein hydrolysates that could provide individual amino acids or small peptides that could be absorbed by the fungus without the use of extracellular proteases. CCM can be amended with glucose to achieve a desired carbon/nitrogen (C/N) ratio, or used without glucose to require the fungus to obtain carbon from amino acid degradation.

S. musiva isolates selected for radial growth on CCM agar without glucose were assumed to be producing abundent extracellular proteolytic enzyme activity. Intracellular oil droplets and protein reserves in the conidia were likely the initial carbon and nitrogen sources for germination and mycelial growth. The subsequent C/N nutrition for the growth observed had to be derived from the CCM agar medium. It was not known in this experiment whether the additional carbon for growth was derived from amino acid degradation or from digestion of the agar matrix. The additional nitrogen needed for the substantial mycelial growth was undoubtedly obtained from proteolytic cleavage of the casein polypeptides. When isolate MP1 was grown on CCM agar containing glucose, mycelial growth flourished until sporulation was initiated 8-10 days after inoculation on all the glucose supplemented treatments

(10-100 mM). With increasing concentrations of glucose in the medium, radial growth prior to sporulation was diminished and the mycelial colony became more fecund. The cessation of radial growth was likely not a function of glucose inhibition, but of colony differentiation and reallocation of nutrient reserves to conidial development (Ng et al., 1972). We have observed growth of *S. musiva* on a variety of media and the results with CCM are typical of the fungus in culture. Reproductively fit *S. musiva* isolates grown on rich, nutritionally complete media will rarely produce large amounts of vegetative mycelium unless it is on minimal media, or has lost its ability to sporulate because of mutations (sectoring). Similar cessation of mycelial growth at the inception of sporulation has been observed *in vivo* (Zalasky 1978).

The greatly reduced growth by MP2-4 in liquid CCM may be the result of successive transferring of the isolates in culture. MP1 was collected in 1985, pure cultured, allowed to sporulate, and the resulting conidia frozen in glycerol at -70 C (Lulley, 1986). For this study, the conidia were thawed and inoculated onto V8-PPA. Conidia arising from the sporulating culture were frozen as conidial stock to generate fresh cultures for collection of conidia used in this study. The other isolates (MP2-4) were obtained as mycelial cultures from our cooperators at the U. S. Forest Service in St. Paul, MN. We later learned that the cultures had been transferred systematically to fresh media for many months prior to arriving in our laboratory. After arrival, spores derived from these cultures were used as conidial stock and treated the same as

MP1. The relative age of the isolates (time of somatic growth and differentiation) may have affected the ability of isolates MP2-4 to grow well in liquid culture.

The effect of glucose concentration on mycelial growth in the liquid CCM cultures was not linear. The lower initial concentrations of glucose in the medium, in relation to the uniform amount of initial protein, had a visible effect on growth (Fig. 1). Glucose concentrations below 50 mM seemed to limit growth potential. Cultures that contained greater than 50 mM glucose continued to grow in mycelial mass when cultured beyond 30 days. Glucose analysis with the Trinder assay showed minimal amounts of residual glucose in the 30-day limited growth (<50 mM) cultures (Fig. 2) and adequate supplies of residual glucose in the non-limited cultures (>50 mM). Initially, the sole limiting factor in the <50 mM glucose cultures seemed to be glucose depletion. The depletion of glucose was obviously a contributing factor, but not the only factor responsible for the differences in mycelial mass produced. Further analysis revealed that in some treatments where the initial glucose concentration was below 60 mM, the fungus used up to two times the amount of glucose to produce a unit of mass, as the >60 mM cultures (Fig. 2). Some of the limited growth cultures reached their near-starvation levels of glucose at 30 days, not only because they started with less, but because they also used the glucose at a much faster rate than the >60 mM non-limited cultures. Other researchers in fungal nutrition have found that the amount of carbon available to the fungus, in relation to the amount of nitrogen available, can have a major effect on growth and development. Carbon source and concentration have been shown

to greatly affect the utilization of amino acids in higher fungi (Weinhold and Garraway, 1966), and different C/N substrate ratios can result in dramatic differences in growth even when both substrates are at higher than essential concentrations (Aube and Gagnon, 1969). Catabolite repression of proteinases (Klapper et al. 1973; Cohen et al. 1975; Ivanitsa et al. 1978; Shinmyo et al. 1978; Cohen 1981; Rollins and Gaucher 1994) by high amino acid or ammonium concentration in the medium should not be contributing to the limiting factor in the <60 mM cultures, since the casein is in long polypeptide form. Repression by excess carbon or sulfur is also not likely because the >60 mM glucose cultures grew well, and sulfur was equally available to all glucose treatments. The limiting effect observed in this study is perhaps a combination of decreased availability of glucose and low carbon in the nutritional C/N ratio. The carbon/nitrogen substrate ratio for CCM with 60 mM glucose is 10.8 grams glucose per 2.5 grams casein.

The SDS-PAGE activity gels provided an efficient means of visualizing proteolytic enzyme activities that were favored by the gel conditions. Possible limitations of the activity gel assay, for detecting the representative proteinases of a sample, may relate to the pH of the separating gel and the substrate incubation step. These procedures place the gel-separated enzymes in neutral to alkaline environments ranging from pH 7 - 9. Thus, the conditions of this activity gel assay favors enzymes with optimal activity in the neutral-to-alkaline pH range. Proteinases with low isoelectric points and pK_a values, such as many of the cysteine proteases (Shipton et

al. 1975) and many aspartic proteinases (North 1982), are likely inhibited or even denatured in environs of pH 7 or greater (Barrett 1986). There are exceptions to the acidic optimal pH activities of cysteine and aspartic proteases in fungi (a cysteine elastase w/pH 8 and an aspartic w/pH 7.4), but they are few (Rippon and Varadi 1968; Tapia et al. 1981). Proteinase types that are likely to be favored by the activity gel conditions are serine and metallo-proteinases. Serine proteinases are optimally active in slightly alkaline pH environments, and the metallo-proteinases can be active at pH values ranging from slightly below to slightly above neutral pH.

The two visualized proteases, P1 and P2, showed good proteolytic activity in the neutral-to-alkaline conditions of the assay. Preliminary experiments by our laboratory, using a colorimeteric spectrophotometer assay, showed reduction of proteolytic activity by *S. musiva* culture filtrates when pre-incubated with the serine proteinase inhibitor PI II (Sillick et al. 1989; Heuchelin and McNabb 1995). Based on these data implicating serine proteinase activity, the irreversible serine proteinase inhibitor PMSF was added to enzyme samples prior to electrophoresis to determine if the gel-clearing activity of P1 and P2 could be inhibited. The resulting inhibition of gel-clearing activity by PMSF suggests that P1 is a serine protease.

Ammonium sulfate fractionation of the culture filtrates allowed exclusion of P2 activity and provided a fraction containing P1 activity for use in the enzyme activity assays. The fraction containing P1 was not a pure P1 extraction, and contained other compounds that co-fractionated with P1. This crude P1 fraction was used in the PI II

and PMSF inhibition, optimal temperature, and optimum pH assays. The same optimum assays were not run on P2 because of variability in P2 expression and difficulties in obtaining an exclusive fraction with good proteolytic activity. The optimal pH for proteolytic activity in the P1 fraction (~pH 8.0), the range of temperature conducive to proteolytic activity, and the approximate molecular weight (~23-25 kDa) of P1 are all typical data for a serine proteinase. The high temperature optimum may be explained by simple reaction kinetics. The following sharp drop at 80° C is likely because of denaturation. P2 is likely not a serine proteinase because PMSF had little to no effect on its activity. Because of occasional reports of non-serine proteinases possessing activity in neutral to alkaline conditions, P2 could be a cysteine, aspartic, or metallo-proteinase. Continued work will focus on isolating a stable fraction of P2 for characterization and inhibition assays. There may be other major proteinases produced in this *in vitro* system that are not detectable by this activity gel assay and its alkaline conditions. Acid activity gels, containing a substrate other than acid-insoluble casein, may allow visualization of other major proteases in the system. Additional biochemical analyses are needed to purify the enzymes and further define the characteristics of the *in vitro* extracellular proteinases P1 and P2 from *S. musiva*.

Acknowledgment

We would like to thank Mike Ostry of the USDA North Central Experiment Station for providing MP isolates 2-4, and Sean Murphy of Iowa State University for technical assistance.

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CHAPTER 3. INHIBITION OF GERMINATION, GERMTUBE ELONGATION, AND SPORULATION OF *SEPTORIA MUSIVA* BY PROTEINASE INHIBITOR II

A paper to be submitted to the journal Molecular Plant-Microbe Interactions

Scott A. Heuchelin[1] and Harold S. McNabb, Jr.[2,3]

Abstract

In vitro and *in vivo* studies were conducted to determine the effect of a serine proteinase inhibitor, Proteinase Inhibitor II (PI II), on the physiology of the fungal pathogen *Septoria musiva*. Enzyme inhibition assays, using PI II as the inhibitor, demonstrated that PI II was capable of reducing serine proteinase activity in the culture filtrates of *S. musiva*. *In vitro* tests to determine the effect of PI II on germination, growth, and sporulation of the fungus were conducted using microscope depression slides. The fungus was grown on cleared V-8 PPA containing a range of PI II

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concentrations (0-800 ug/ml). Conidial germination, mycelial growth, and culture sporulation were all reduced with increasing concentrations of PI II. Percentage reduction of germination ranged from 18-74%, mycelial growth was reduced 6-73%, and reduction in development of pycnidia ranged from 55-100% with concentrations of 100-800 ug/ml. *In vivo* tests with *S. musiva* and transgenic *Populus* expressing PI II protein were conducted with leaf and stem tissues. Leaf disk assays showed a reduction in lesion size when conidia were applied to transgenic leaves. There was no significant difference in the infection frequency. Results of the stem inoculations were inconclusive because of environmental conditions that may have favored the defenses of the host.

Introduction

The fungal pathogen *Septoria musiva* Peck. (teleomorph *Mycosphaerella populorum* Thompson) is considered an endemic pathogen in native *Populus* stands of eastern North America. With the introduction of inter and intraspecific hybrids, *S. musiva* has become a serious pathogen, causing Septoria leaf spot and Septoria canker in hybrid poplar in eastern North America (Bier 1939; Ostry and McNabb 1983, 1985; Spielman et al. 1986; Thompson 1941; Waterman 1954). Heavy foliar infection can result in greatly reduced photosynthetic area of the leaves of poplars, and stem

infections can cause lethal cankers that girdle the main stems and promote breakage by wind (Waterman 1954). Infection is primarily initiated by ascospores in the spring, and then greatly amplified by the asexual propagules called conidia (Lulley et al. 1989; Sinclair et al. 1987).

Proteinase Inhibitor II (PI II) is a low molecular weight protein [monomer M_r 12,300 (Plunkett et al. 1982)] that inhibits the proteolytic activity of the serine proteinases trypsin and chymotrypsin (Ryan 1981, 1990; Ryan and An 1988). The protein is the gene product of the proteinase inhibitor II gene (*pin2*). The *pin2* gene and PI II have been found in plants belonging to the Solanaceae, such as potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Miller) (Green and Ryan 1972; Ryan 1981, 1990).

PI II has been proposed to cause possible reduction in the vigor of fungi by depriving them of necessary amino acids (Ryan 1990). Proteinase inhibitors have reduced the activities of proteinases in *Colletotrichum lindemuthianum* (Sacc. & Magnus) (Mosolov et al. 1979), *Fusarium solani* (Mart.) Sacc. (Mosolov et al. 1976), and studies from our laboratory show that *Septoria musiva* Peck produces proteinases that are inhibited by proteinase inhibitor II (Heuchelin and McNabb 1995; Sillick et al. 1989). Proteinases have been shown to have a synergistic effect in cell necrosis when combined with cell wall degrading enzymes (Movahedi and Heale 1990). Therefore, fungal proteinases may play an important role in pathogenesis of host tissues.

This study was designed to elucidate whether inhibition of fungal serine proteinases by PI II may result in a reduction of the infection, colonization, and sporulation by the fungus. Reductions in any of these processes could result in less disease.

Materials and Methods

Septoria isolate

Asexual pathogen propagules (conidia) of *Septoria musiva* collected from four hybrid poplar field plots in Iowa, Wisconsin, North Dakota, and Minnesota were established in pure cultures and allowed to asexually sporulate. Conidia produced from the isolates were placed in spore-suspension storage in sterol-glycerol at -70 C. The four isolates (MP1-4) were screened for conidial germination efficiency, mycelial growth rate, sporulation potential, and protease production. Isolate MP1, from Iowa, was selected for further studies because of its consistantly good growth and sporulation in the screening tests.

Media

Isolates of *S. musiva* were grown and allowed to sporulate on V-8 Phytone Peptone Agar (V-8 PPA) to obtain maximum growth rate and sporulation (Krupinsky 1989). A broth medium was made using a variation of V-8 PPA for the serine protease assay. The agar was excluded, the remaining V-8 PP ingredients mixed, filtered with Whatman #1 filter paper, and autoclaved for use.

Another variation of V-8 PPA was used for the PI II inhibition assays. The V-8 phytone peptone solution was filtered by suction flask with #1 Whatman filter paper to remove particles that would make microscopic observations more difficult. The effluent was mixed with 1/10th the amount of agar (2 g/l) normally incorporated into V-8 PPA, autoclaved, and allowed to cool to 45 C in a warm water bath for incorporation of the PI II inhibitor.

PI II protein

Purified PI II (CalBiochem-Novabiochem Corp. San Diego, CA) was added to both liquid and solid culture media at various concentrations to assess its effect on *in vitro* fungal germination, development, sporulation, and protease activity. The crystalline PI II was dissolved in ddH_2O (10mg/ml), added by filter sterilization to the 45 C modified V-8 PPA medium in calculated aliquots, and mixed to obtain a range of PI II concentrations.

Serine proteinase activity in *Septoria musiva*

Shaker cultures of *S. musiva* in liquid V8-PP medium were used to elicit production of extracellular fungal proteinases. Cultures consisted of 200 ul of a conidial suspension (100,000 conidia/ml sterile ddH_2O), applied to 20 ml of autoclaved V-8 PPA filtrate in a 50 ml Erlenmeyer flask. The culture flasks were agitated at 100 rpm on an orbital shaker platform at 25 C. After one week of growth the broth was filtered, lyophilized, and resuspended at 1/10 original volume for the protease activity assay. Protease activity in the culture filtrate was determined spectrophotometrically, using an enzyme inhibition assay (EIA) which is a modification of the Kunitz assay for proteolytic activity (Kunitz 1947). The following were mixed in a glass test tube: 100 ul of culture filtrate, 1 ml of 3% azocasein, and 900 ul of phosphate buffer (0.1 M, pH 7.0) for determination of proteolytic activity in the filtrate. To assay inhibition of the proteolytic activity in the filtrate by PI II, buffer volume was reduced to 880 ul and 20 ul of PI II solution (10 ug/ul) was added to the culture filtrate and incubated for 10 min prior to incorporation in the reaction mixture. The negative control tube (blank) contained fresh culture medium (filtered 10X) in lieu of culture filtrate. The tubes were incubated at 37 C for 30 min and 1 ml of the solution was placed in a clean tube. One milliliter of 5% trichloroacetic acid was added, contents mixed, and incubated 20 min at 23 C. Contents were centrifuged for 10 min at 1,100 x *g*. One milliliter of the supernatant was placed in a

spectrophotometer tube that contained 1 ml of 2N NaOH, and 1 ml of ddH_2O. The contents were mixed and absorbance was read at 440 nm. One unit of proteolytic activity was defined as an increase in absorbance of 0.01 at 440 nm.

Inhibition of fungal development *in vitro*

In a sterile environmental hood, autoclaved microscopic depression slides were filled with sterile modified V-8 PPA media that contained Purified PI II at the concentrations of 0, 100, 200, 400, 600, and 800 ug PI II/ml medium. Upon media solidification, conidia of *S. musiva* isolate MP1 were applied (100 ul) at a concentration of 10,000 spores/ml ddH_2O. The conidia-coated slides were maintained at 23 C in a sterile high humidity environment to prevent desiccation of the medium and fungus.

A light microscope was used to view conidial germination at 12 h intervals for 84 h. A sample slide of each treatment was fixed with 10 % glacial acetic acid at each time interval to allow accurate measurements at the specific time increment. Percentage germination was determined by assessing the number of germinated conidia vs. the total number of conidia in the field of view using the 40X objective (400X magnification). Five "field of view" locations were arbitrarily chosen per slide. Slide position was adjusted to obtain an evenly distributed group of conidia.

Conidial germination was defined as a conidium having at least one germtube that was half as long as the conidium from which it was developing.

Germinated conidia on the fixed 12 h sample slides were assessed for length of germ tube elongation with a light microscope. Ten germtubes were systematically chosen for measurement from varied locations on the slide. If a conidium had more than one germinated cell, the longer germtube was chosen for measurement. Germtubes were measured at 400X using units from a micrometer scale in the eyepiece of the microscope. The scale units were converted to millimeters after viewing a standard millimeter scale in the microscope eyepiece at 400X. Length was measured as the distance from the generative cell to the apical tip of the germtube. Side branching of mycelia from the germtube was not measured.

A dissecting microscope was used to observe formation of pycnidia on the PI II treatment slides. Developing mycelial masses with dense locules were counted as pycnidia. Total number of pycnidia, within the area of the slide's circular depression (3 cm^2), was recorded.

Each treatment (PI II concentration) was represented by 10 replicate slides. The experiment was run twice. Data referred to in text and figures on germtube length are means of the two experiments combined.

Transgenic *Populus* clone expressing PI II protein

The transformed hybrid poplar used in this study is *Populus* X *euramericana* (Dode) Guinier 'Ogy'. 'Ogy' is the progeny of a cross of North American *Populus deltiodes* Bartr. (a cross-pollinated hybrid between individuals from Iowa and Ontario) and the European *Populus nigra* L. (a Belgian selection). Euramericana hybrids are susceptible to both leaf spot and cankering by *S. musiva*, but have low level resistance to cankering (McNabb et al. 1982). We have observed 'Ogy' with both leaf spot and cankers in the field (McNabb and Heuchelin, unpublished). The transgenic 'Ogy' clone, TR5, contains a chimeric fusion of the proteinase inhibitor II gene (*pin2*) with a CaMV 35S promoter. Clone TR5 expresses the proteinase inhibitor II (PI II) protein in its leaf (Heuchelin et al. 1997) and stem tissues (Heuchelin and Yung, unpublished), and is being used as a tool to test the importance of proteinases in host-pathogen and host-insect interactions (Kang et al. 1997; Klopfenstein et al. 1997).

In vivo* tests with *Septoria musiva* and transgenic *Populus

Transgenic *P.* x *euramericana* Ogy TR5 and untransformed checks were grown in the BL-1P confinement area in the Forestry Greenhouse. The TR5 clone represents transformation with pRT104 (Heuchelin et al. 1997).

Leaf disk assay. Leaf disease on the transformant and control were evaluated by assays using excised leaves in a petri-plate method that has been modified from the

leaf-disc method (Spiers, 1978; Ostry, 1987). Leaves of the same Leaf Plastochron Index (LPI) were obtained from transgenic trees housed in the ISU Forestry greenhouse. Each plate contained five leaf disks (2.5 cm diameter) set in equal sized cutouts of 2% water agar. The abaxial surface of the leaf was inoculated with a conidial suspension (10,000 conidia/ml) in order to maintain uniform infection and colonization levels among the tests. Experiments were conducted in a constant temperature (23 C), lighted incubator (16L:8D photoperiod).

Stem canker assay. Assays to determine PI II inhibition of *S. musiva* canker establishment and colonization were conducted in the greenhouse in a transgenic approved bay. Transgenic test material and untransformed controls were inoculated with either conidia, ascospores, or ddH_20. Fresh leaf scars were the site of inoculation. Three-month-old stems (1 cm diameter) of hybrid poplar with a range of susceptibilities were inoculated with conidial and ascospore suspensions of *M. populorum* at a concentration of 10,000 spores/ml. Conidial suspensions were from a single highly aggressive isolate, MP1. Ascospore suspensions were pooled populations of ascospores from the field, representing the natural variation in pathotypes found. The 20 ul droplets of inoculum were placed on the leaf scar of a freshly removed petiole and the area wrapped with paraffin film to maintain high humidity at the infection court. There were three inoculation sites per replication. One site was inoculated with conidia, one with ascospores and one with distilled water

(control). Inoculation sites were at every other node and at approximately 120 degree angles to one another. Inoculum type was alternated among the three possible sites of each replication to minimize interference. Canker growth and pathogen sporulation were monitored externally at bi-weekly intervals for 10 replicates of each clone. Axial and lateral measurements of canker size were recorded to estimate the area (cm^2) of tissue infected.

Results

In vitro inhibition assays

Serine Protease Activity. Enzyme inhibition assays (EIAs), using PI II and the modified spectrophotometric method of Kunitz described above, suggested serine proteases are present in the culture filtrates of *S. musiva*. Addition of PI II to the culture filtrates reduced proteolysis of the azocasein substrate by an average of 33% (Fig. 1).

Inhibition of fungal development. Germination of conidia, elongation of germ tubes and subsequent hyphal growth, and pycnidia formation and thereby subsequent sporulation, were all inhibited by increasing concentrations of PI II in the

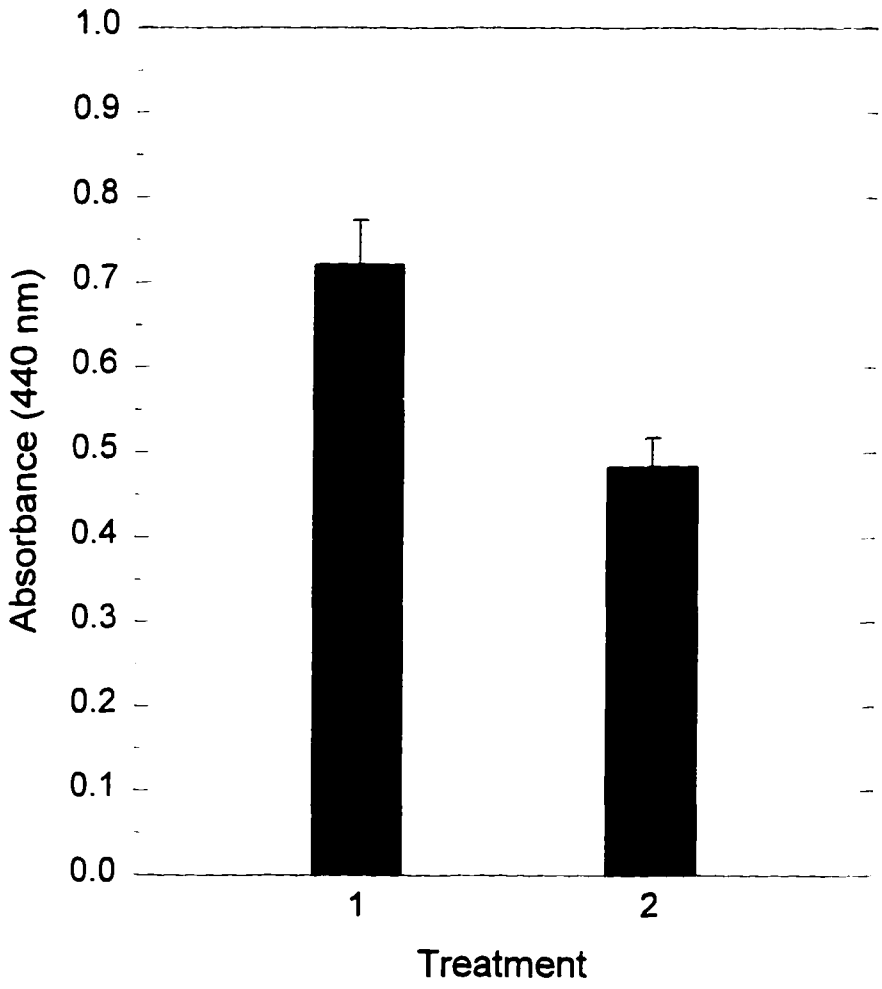

Fig. 1. Effect of PI II on proteolytic activity of culture filtrates of *Septoria musiva*. Treatment 1 (control) = culture filtrate with 7 days fungal growth. Treatment 2 = 7 day culture filtrate with 200 ug PI II. One unit of proteolytic activity equals an increase in absorbance of 0.01 at 440 nm. Error bars represent standard deviation of the mean.

media. Incorporation of PI II in the medium provided inhibition of germination at levels that ranged from 18% (100 ug/ml) to 74% (800 ug/ml) of the germination efficiency observed in the control (Fig. 2).

PI II demonstrated inhibition of germ tube elongation at levels ranging from 6% (100 ug/ml) to 73% (800 ug/ml) of the hyphal elongation observed in control medium without PI II (Fig. 3).

PI II provided significant inhibition of pycnidial development at levels ranging from 55% (100 ug/ml) to 100% (800 ug/ml) of the pycnidia produced in the control (Fig. 4). These data indicate the potential for at least this much reduction in subsequent sporulation.

In vivo tests with *Septoria musiva* and transgenic *Populus*

Leaf disk assay. Results from leaf-disc assays for Septoria leaf spot on transgenic leaves expressing PI II protein have shown a reduction in lesion frequency and size. Reduction in the frequency of lesions was not significant (TR5 mean = 4.56 / disk, control mean = 6.28 / disk, $p > 0.05$) but the lesions were considerably smaller (visual assessment).

Stem canker assay. Results from greenhouse stem canker inoculations do not show significant reduction of canker development by the transgenics. Canker formation was minimal and growth was extremely slow. Many of the inoculation sites failed to produce cankers on both control and transgenic materials.

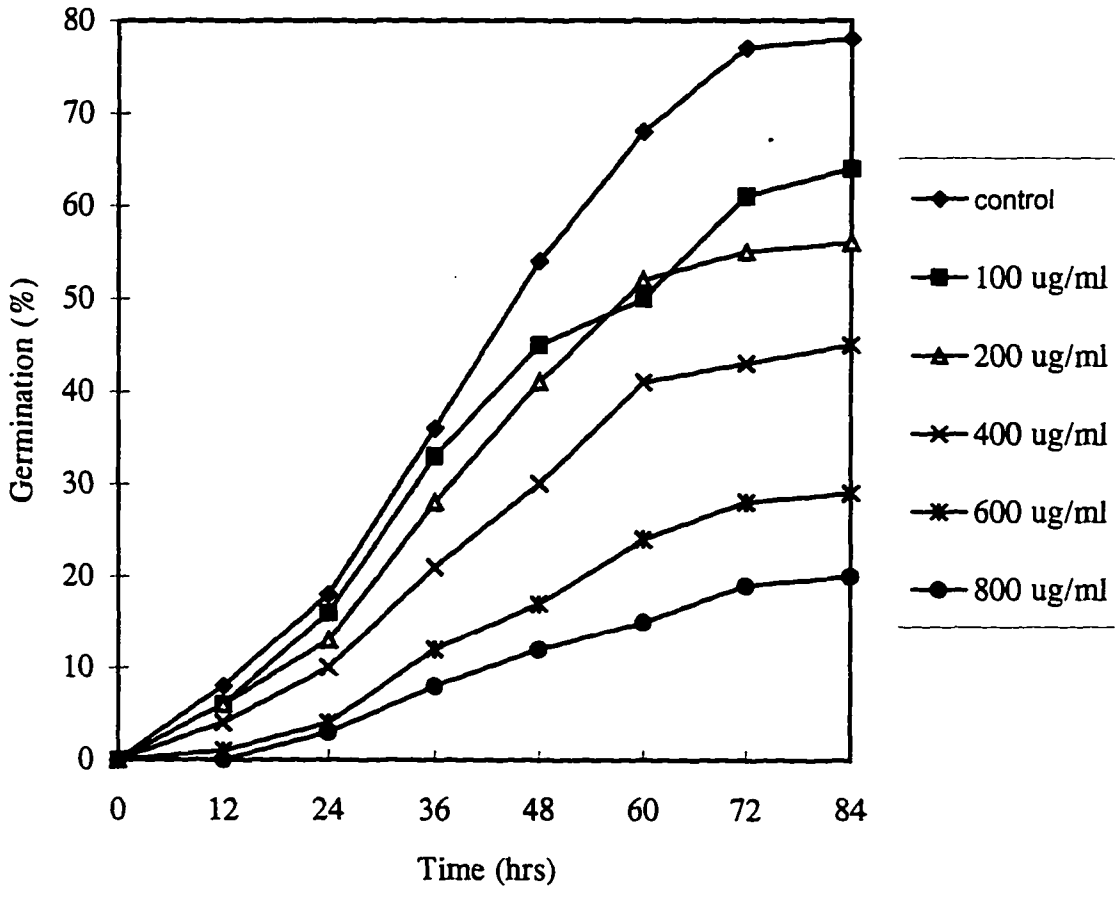

Fig. 2. Effect of proteinase inhibitor II (PI II) concentration on the germination of *Septoria musiva* conidia. Concentrations are μg PI II/ ml agar medium. Percentage germination was determined by counts of germinated conidia (approximately 10-15 conidia per viewing area) at 12 h intervals. Points shown are means of five counts from each of two experiments (n= 10).

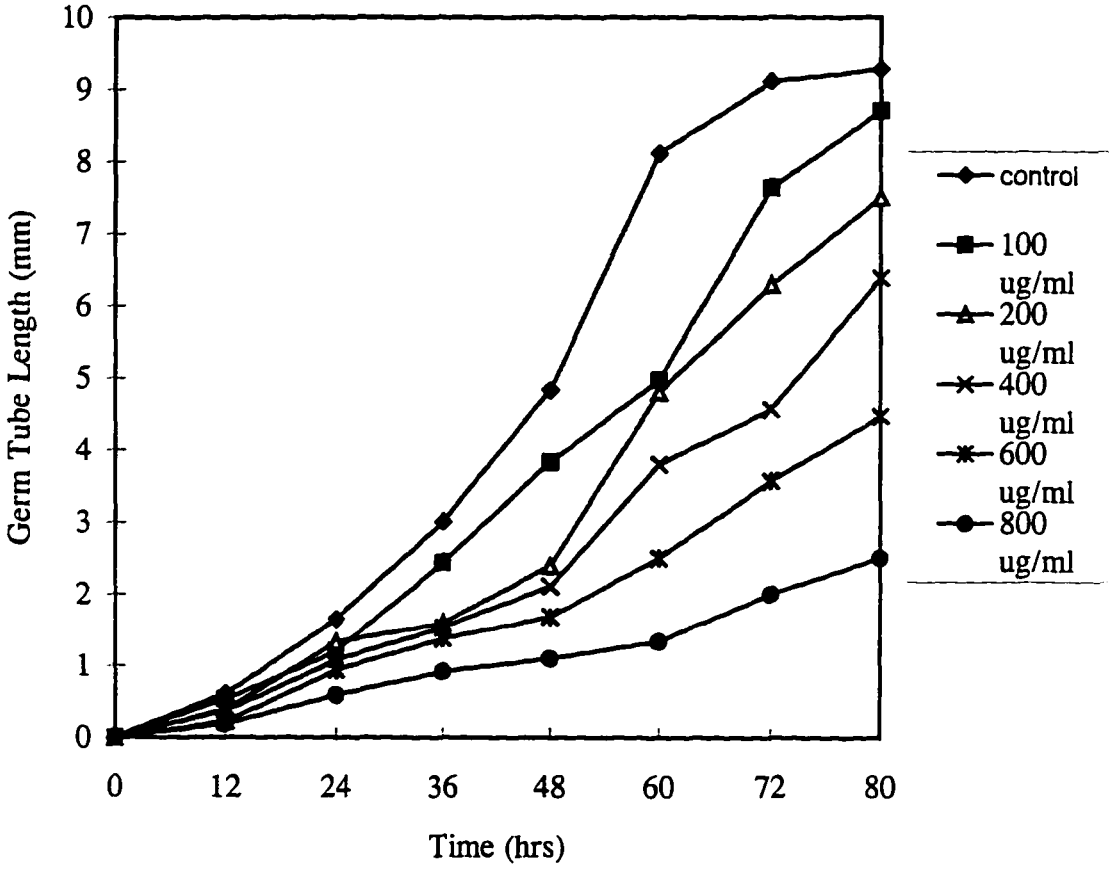

Fig. 3. Effect of proteinase inhibitor II (PI II) concentration on germtube elongation of *Septoria musiva* conidia. Concentrations are μg PI II/ ml agar medium. Germtube length was determined by arbitrary counts of germinated conidia at 12 h intervals. Points shown are means of 10 measurements from each of two experiments (n= 20).

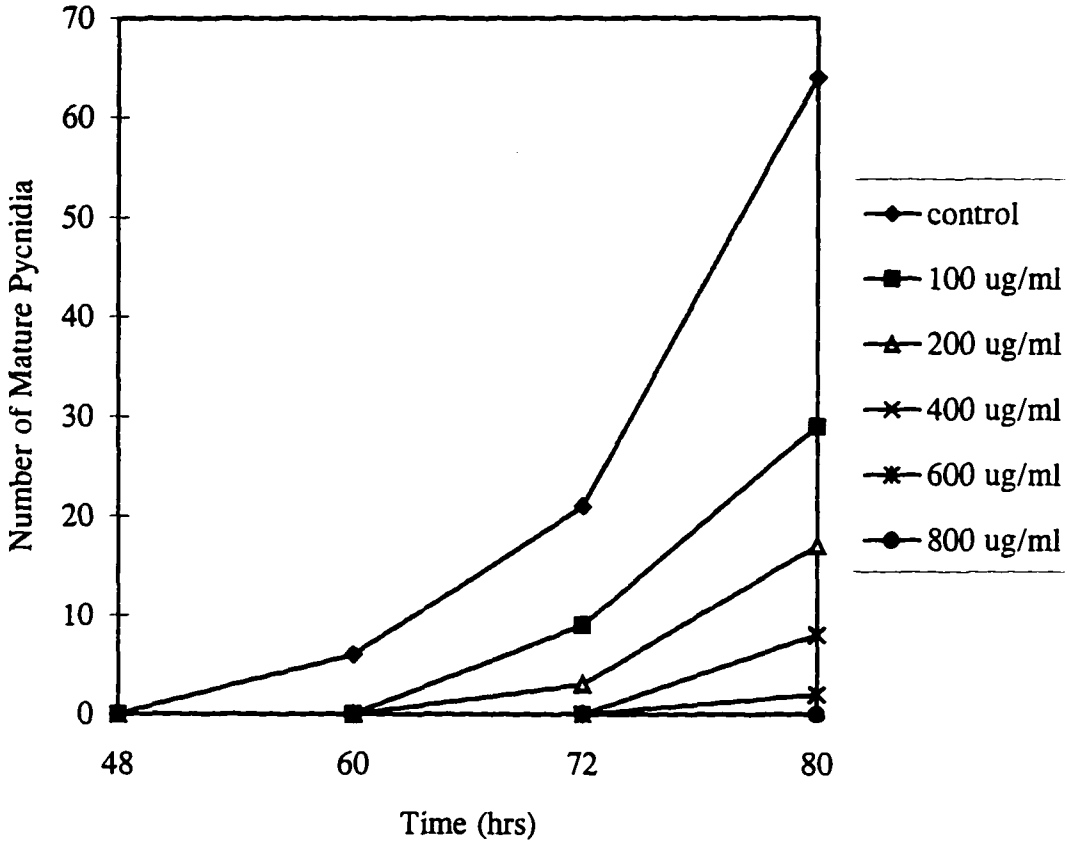

Fig. 4. Effect of proteinase inhibitor II (PI II) concentration on *Septoria musiva* pycnidia formation. Concentrations are μg PI II/ ml agar medium. Number of mature pycnidia were determined by counts of total mature pycnidia / slide at 12 h intervals. Points shown are means of total counts from each of two experiments (n= 2).

Discussion

PI II had a marked effect on conidial germination, and the subsequent hyphal growth and pycnidial development of *S. musiva*. Relatively high concentrations of PI II were required to greatly reduce germination efficiency. This may be because of the nutient reserves present in the conidia. Moderate levels of PI II provided substantial stunting of mycelial growth and even lower concentrations were able to delay pycnidial development. Perhaps as the spore uses its reserves of nutrients it is increasingly challenged by the PI II. Reduced nutrient uptake in the form of polypeptide-derived essential amino acids and their omnipresent nitrogen group, may result in slower germ tube formation and mycelial growth, reproductive differentiation, and subsequent reproductive success (Evans and Black, 1981; Ng et al., 1972). Lorito et al. (1994) detected cytoplasmic constituents issuing from hyphae in the presence of proteinase inhibitors. They proposed that some types of proteinase inhibitors may cause leakage in cell membranes. If cell leakage is occurring in this study, it could perhaps be the result of proteinase inhibitor induced amino acid deficiencies, possibly leading to protein deficient plasma membranes. The membrane leakage would further exacerbate any nutrient deficiencies in the cytosol that were induced by PI II's antinutrient properties.

The results of *in vivo* PI II inhibition with the transgenic TR5 clone were not as dramatic as the *in vitro* inhibition study. Our observations of leaf tissue infection in the leaf-disc assay, and canker development in the greenhouse, do not well represent the infection processes seen in susceptible genotypes of *Populus* in the field. Maxwell (1997) has shown the water potential of sample trees, at the time of leaf excision or canker inoculation, significantly affects fungal infection and colonization. Pre-harvest water potential and other environmental variables may possibly explain why even the moderately susceptible control leaves had such low infection frequencies. Though the reduction in lesion frequency was inconsequential, the reduction in lesion size was of note. Lesion size represents a host-pathogen interaction occurring within the leaf, where transgenically expressed PI II proteins are able to interact with the fungus. The failure of the greenhouse canker inhibition test may possibly be explained by Maxwell's recent findings. Maxwell found that drought stress significantly enhanced canker formation in his water-potential-controlled, Septoria canker susceptible clones. Some typically susceptible clones even failed to develop cankers when their water potentials were ideally maintained. Recent anatomical studies in our laboratory suggest that propensity for meristematic activity in the phellogen and parenchyma tissues of bark is a characteristic of canker resistance (Biela et al. 1996). Earlier studies by Bier (1961) and Haywood (1979) suggest a relationship between such activity and moisture levels. The canker inhibition assay in this study had to be run in transgenic approved bays of the greenhouse, because

APHIS approval for outplanting had not been obtained. The trees grew in near optimal conditions with their controlled environment and a regimented watering and fertilization schedule, which probably shifted disease interactions in favor of the host tree. Pending APHIS approval, the canker inhibition test will be re-run under natural field conditions.

The results of this study suggest that PI II has potential for limiting disease progression. Expression of PI II in transgenic poplar has been quantified at a level of 50 ug/g leaf tissue (Heuchelin et al. 1997). This is roughly equivalent to the 200 ug/ml concentration in the *in vitro* study. At this concentration, PI II allowed only 27% as many perithecia to develop as the control. This reduction in fruiting bodies combined with the increased time for substantial mycelial growth should result in increased latent periods of infection and reduction in the number of infective propagules. Considering that there are many secondary disease cycles in a growing season, PI II expression at this level could have a significant effect on disease build-up in the field over time.

Acknowledgement

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CHAPTER 4. AN ENDOGENOUS SERINE PROTEINASE INHIBITOR IN *POPULUS X EURAMERICANA* 'OGY'

A paper to be submitted to the Canadian Journal of Forest Research

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Abstract

Studies in expression of the serine proteinase inhibitor, Proteinase Inhibitor II in transgenic *Populus x euramericana* 'Ogy', revealed a native proteinase inhibitor in *Populus*. Western analysis revealed a ~24 kd protein that had strong immunoreactivity to antibodies against PI II. The native 'Ogy' protein has approximately the same molecular weight, and similar migration patterns as PI II. As with PI II, the newly discovered protein is also heat stable and acid-soluble. Cross-reactivity with the PI II antibody remained even after extensive purification via affinity chromatography. The 'Ogy' protein, or some of its components, can interact with protomers of PI II and form hybrids of the two inhibitors. Further study is under way to determine the amount of sequence homology that exists between the two proteins.

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Introduction

Transgenic hybrid poplars containing a chimeric fusion of the proteinase inhibitor II gene (*pin2*) with a CaMV 35S promoter, and expressing the proteinase inhibitor II (PI II) protein, were engineered to test the importance of proteinases in host-pathogen and host-insect interactions (Heuchelin et al. 1990; Klopfenstein et al. 1991; McNabb et al. 1990). The *Populus* transformation discussed in this paper involved the hybrid clone 'Ogy'. This clone is a second generation progeny containing germplasm from the North American, *Populus deltiodes* Bartr. and the European, *Populus nigra* L.

Proteinase Inhibitor II is a low molecular weight protein [monomer M_r 12,300 (Plunkett et al. 1982)] that inhibits the proteolytic activity of trypsin and chymotrypsin (Ryan 1981, 1990, Ryan and An 1988). The PI II dimer is composed of two protomers (monomers) that can vary in their molecular weight and isoelectric point (pI). The PI II dimer is capable of inhibiting 2 moles of chymotrypsin per mole of PI II. The inhibition of trypsin is not as strong and has been observed at approximately one mole of trypsin inhibited per mole dimer of PI II.

PI II is a heat stable and acid soluble protein. The protein resists disassociation and precipitation at pH as low as 2.2, or as high as 10.6. PI II can be heated to 70 C for 30 min at pH 2.2 or 7.8 with only 6% of its activity being lost

(Bryant et al. 1976). PI II has been found in plants belonging to the Solanaceae, such as potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Miller) (Green and Ryan 1972; Ryan 1981, 1990).

The objectives of this research were to obtain stable transformants using the CaMV 35S-*pin2* gene construct pRT 104, and develop a reliable protocol for detection of PI II in poplar tissues. While probing for PI II protein with Western analysis, a cross-reacting protein was observed in the control 'Ogy' material. The protein, which is native to 'Ogy', cross reacted strongly with the anti-PI II antibody and shares many of the biochemical properties of PI II. This paper describes the discovery of this protein, the PI II-like properties it possesses, and the possible role of the protein in the physiology of the clone 'Ogy'.

Materials and Methods

'Ogy'

'Ogy' is a clonal hybrid selection made by Vic Steenackers at the Poplar Research Center in Geraardsbergen, Belgium. This hybrid also carries the Belgian number Unal 5 and the Dutch #4154. Its grandparents are an Iowa *Populus deltoides* (#V.5) from the mouth of the Skunk River south of Burlington, Iowa, and a *P.*

deltiodes (#V.1) from Ontario, Canada. An individual (#S. 4-38, Dutch #1625) from this F1 progeny was then crossed with a *P. nigra* Ogy clone 17 to produce the 'Ogy' hybrid selection. This hybrid selection is now known as *P. x euramericana* 'Ogy' in the European trade. Apical shoots of 'Ogy' were surface sterilized with 10% ethanol and 10% chlorine bleach. These shoots were then placed in sterile tissue culture boxes. The boxes contained MS salts (Murashige and Skoog 1962) medium (Hazleton Biological Supply, Lexena KS.) supplemented with B5 vitamin complex and with exogenous hormones for shoot multiplication and rooting. A clonal population for transformation and greenhouse experimentation was established on MSB5 shooting medium [0.2 mg/L Naphthalene acetic acid (NAA) - 0.1 mg/L 6-Benzylaminopurine (BAP)]. Approximately 400 shoots were rooted in MSB5 rooting medium [0.03 mg/L Indole-3-butyric acid (IBA)]. These rooted shoots were grown as a sterile source of leaf material for transformation. Material was grown at 25 C under fluorescent lighting (50 uE/m^2/s) with a 16:8 (L:D) photoperiod.

Proteinase Inhibitor II

A preparation of commercially purified PI II, "Chymotrypsin Inhibitor II", (Calbiochem, San Diego, CA.) was used for antibody production, and as positive controls and standards in ELISA, Western, and EIA (enzyme inhibition assay) analyses. The commercially obtained PI II was prepared from potato according to the

technique described by Bryant et al. (1976). The inhibitor protein is the gene product of the proteinase inhibitor II gene (*pin2*) of potato (*Solanum tuberosum* L.).

The *pin2* gene of transformation vector pRT 104

The proteinase inhibitor II gene (*pin2*) was cloned from potato by Sanchez-Serrano et al. (1986). The genes responsible for the coding of the proteinase inhibitor in potato were isolated and analyzed for nucleotide sequence (Keil et al. 1986; Thornburg et al. 1987). Through characterization and deletion analysis, the function of the promoter region, the protein coding region that codes for PI II (Thornburg et al. 1987), as well as the terminator that both polyadenylates the transcript and seems to increase stability of the mRNA (An et al. 1989) were elucidated. The vector construct pRT 104, used in the transformations, does not contain the wound-inducible promoter which is native to the *pin2* gene. The vector gene-construct is a chimeric fusion of the proteinase inhibitor II gene (*pin2*) with a CaMV 35S promoter, and was created and provided by Robert Thornburg of Iowa State University's Biochemistry and Biophysics Department.

Transgenic *Populus* expressing PI II

The hybrid poplar clone, *Populus* x *euramericana* 'Ogy', was transformed with a transformation vector containing the *pin2* gene (Heuchelin et al. 1990; Klopfenstein et al. 1991; McNabb et al. 1990). Transformants were obtained using an

Agrobacterium binary vector system with a disarmed Ti plasmid and plasmid pRT 104 containing *pin2* regulated by a CaMV 35S promoter, and a selectable gene encoding neomycin phospho-transferase II (NPT II). Putative transformed shoots from co-cultured leaves were selected using three different kanamycin selection assays. Ten plantlets, derived from co-culture, showed strong NPT II expression and were tested by DNA analysis and protein immunoassays (Southern and Western blots). Confirmation of *pin2* expression was achieved for transformant numbers TR5 and TR10 (Heuchelin et al. submitted).

Protein purification

Protein extracts from the leaves of 'Ogy' were partially purified to obtain a group of proteins that had similar characteristics to PI II (acid solubility, heat stability, and small molecular weight). The partial purification was a modification of the PI II purification technique (Bryant et al. 1976), providing a crude inhibitor fraction. Leaves were collected from control (untransformed) 'Ogy' and the transformed trees, petiole and midrib removed, and ground with a mortar and pestle in liquid nitrogen. The frozen leaf powder was incubated in extraction buffer (0.1 M Tris HCl, 0.5 M sucrose, 0.1% ascorbic acid, 0.1% cysteine HCl, 1.0% w/v polyvinylpyrrolidone-10 (PVP-10), 0.01 M EDTA) overnight at 4° C. The suspensions were centrifuged at 1000 x *g* for 15 min to remove unsuspended material. The supernatant was acidified with 10 N HCl to pH 3, incubated 1 h at 23° C, and

centrifuged at 12,000 x *g* for 20 min to remove the acid-insoluble proteins. The acid-soluble proteins in the supernatant were then precipitated by adding $(NH_4)_2SO_4$ until the solution contained 70% of the salt. After centrifugation at 12,000 x *g* for 20 min, the pellet was resuspended in distilled water and heated to 80° C for 10 min to denature the heat sensitive proteins in the mixture. The solution was centrifuged at 12,000 x *g* for 20 min and filtered to remove the denatured proteins. The solution was dialyzed against ddH_2O for 48 h to remove any remaining salts. The solution was lyophilized to obtain a partially purified protein mixture (crude inhibitor) containing PI II and any other proteins that were heat stable and acid soluble. This protein mixture was hydrated, quantified (Bradford 1976) and used in Westerns, ELISAs and enzyme inhibition assays (EIA).

Western Blot Analysis

Partially purified PI II protein (crude inhibitor) extracts from 'Ogy' and the transgenics were prepared according to the Laemmli protocol (denatured at 100° C in SDS loading buffer containing beta-mercaptoethanol), as well as two modifications of the protocol. One preparation was not heat denatured, and the other contained no beta-mercaptoethanol in the loading buffer. All samples were run on a 5-22% SDS PAGE gradient gel for 30 h at 40 volts. The gel was destained in 50% methanol and 10% acetic acid to remove the tracking dye, stained with Coomassie brilliant blue R to

stain the protein bands, destained, and electroblotted onto a nitrocellulose membrane (MSI, Westboro, MA).

Immunodetection of the PI II proteins was accomplished by blocking the membrane with blotto containing ovalbumin (5% nonfat dry milk and 1% ovalbumin in PBS containing 0.5% Tween 20) overnight at 4° C, and warming to 37° C for 30 min. After rinsing in PBS Tween, the membrane was incubated with polyclonal anti-PI II rabbit antibodies for 2 h at 37° C. The membrane was rinsed extensively, blocked with goat normal serum for 1 h, rinsed, and incubated with alkaline phosphatase labeled anti-rabbit goat antibodies. After 1 h of incubation at 37° C, the membrane was rinsed exhaustively and placed in PBS.

PI II protein on the membrane was visualized using a NBT/BCIP (Sigma Chemical Co.) chemical detection system for the presence of alkaline phosphatase-labeled antibodies (Blake et al. 1984).

Anti-PI II antibody

New Zealand White rabbits were injected intramuscularly with PI II to elicit antibody production. Both the native and denatured forms of PI II were used as immunigens. After 4 weeks, serum was obtained and purified using a Protein A column (Bio-Rad Laboratories, Richmond, CA) to isolate the IgG fraction. Precipitation tests and Ochterlony double diffusion tests were run with PI II to

determine the quality and cross-reactivity of the antibodies. The polyclonal IgG fraction was further purified by affinity chromatography. The antibody preparation was run through an Affi-gel 10 column (Bio-Rad Laboratories, Richmond, CA) containing proteins isolated from leaves of 'Ogy'. Antibody that did not bind to the column was concentrated and run though an Affi-gel column containing purified PI II. Antibody that bound to the column was eluted, concentrated, and quantified.

Serine proteinase inhibition assay

A colorimeteric enzyme inhibition assay (EIA) was developed based on the assay for protease activity on a casein substrate (Kunitz 1947). The EIA assay was designed to detect inhibition of proteinase activity by proteinase inhibitors such as PI II. The enzymes in the study were trypsin and chymotrypsin, and azocasein was used as the substrate (Sigma Chemical Co., St. Louis, MO). The serine proteinase inhibitor, PI II, was supplied from extracts of the transgenic clone 'Ogy TR5'.

The following were mixed in a glass test tube: 100 ul of leaf protein extract, 1 ml of 3% azocasein, 800 ul of phosphate buffer (0.1 M, pH 7.0), and 100 ul of 0.01% (w/v) chymotrypsin or trypsin. The treatments contained control 'Ogy' leaf extract or transgenic TR5 leaf extract in combination with either trypsin or chymotrypsin. The negative control tube (blank) contained ddH_2O in lieu of plant extract. The tubes were incubated at 37° C for 30 min and 1 ml of the solution was

placed in a clean tube. One ml of 5% trichloroacetic acid was added, contents mixed, and incubated 20 min at 23° C. Contents were centrifuged for 10 min at 1,100 x *g*. One ml of the supernatant was placed in a spectrophotometer tube that contained 1 ml of 2N NaOH, and 1 ml of ddH_2O. The contents were mixed and absorbance was read at 440 nm. One unit of proteolytic activity was defined as an increase in absorbance of 0.01 at 440 nm.

Results

Antibody Production

Initially, the presence of PI II protein was difficult to confirm in extracts of TR5 vs. control 'Ogy'. The polyclonal IgG antibodies reacted strongly to the PI II dimer as well as its protomers, but there was also considerable cross reactivity with the antibody preparation to the control 'Ogy' proteins. Test Western Blots showed consistently high background signal in the lanes that contained extracts of control and transgenic 'Ogy'. Affinity chromatography of the antibodies, against 'Ogy' leaf proteins bound to an Affi-gel 10 column, provided a preparation that resulted in much lower background signal in test Westerns. Further affinity chromatography, using purified PI II, resulted in a concentrated antibody preparation that did not bind to most

native 'Ogy' proteins, but was specific in its binding of native and denatured PI II as well as protomers of the inhibitor.

Western Blot Analysis

The presence of PI II protein was confirmed in extracts of TR5 and TR10 (Fig. 1). The PI II protein has a molecular weight of approximately 24,000 daltons in its dimer form. Protomers (monomers) of PI II were present at approximately 10,000 to 12,000 daltons. Banding also was observed at molecular weights greater than 24 kd and is most likely the result of cross-linking of monomers, which results in trimers, quatramers, etc. The control 'Ogy' lanes also showed banding, though at slightly different molecular weights, by a protein in *Populus* that cross-reacts with anti-PI II antibody. The bands appearing in the 'Ogy' lane diverge slightly from the migration pattern of PI II, but do have many similarities in their banding patterns. The immunoreactive proteins have a molecular weights of approximately 24,000 daltons, as well as components in the 12-14,000 dalton range, and are capable of forming multimers like PI II.

Modifications in the Laemmli protocol of protein preparation resulted in differences in protein migration and subsequent banding for both the PI II containing transgenics, and the 'Ogy' material containing the native protein (Fig.1).

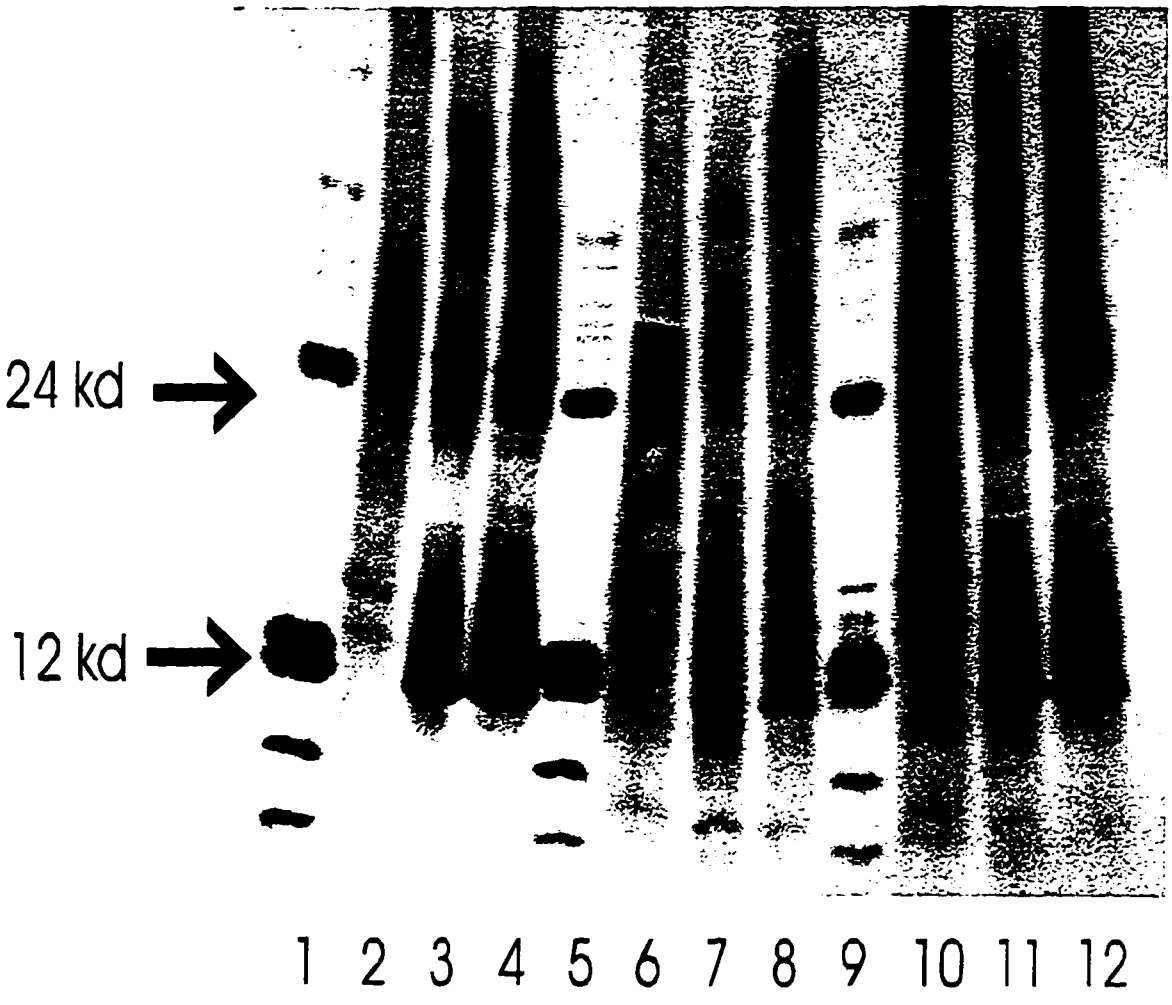

Fig. 1. Western blot of crude inhibitor protein extracts from transgenics and control 'Ogy'. Lanes 1,5, and 9 are purified PI II from potato. Lanes 2,6, and 10 are control 'Ogy'. Lanes 3,7, and 11 are TR 5. Lanes 4,8, and 12 are TR 10. Lanes 1-4 were run using the Laemmli protocol. Lanes 5-8 were run without beta-mercaptoethanol. Lanes 9-12 were run without heat-denaturation.

Visual comparisons with PI II standards show that the inhibitor protein in TR5 and TR10 may be expressed at a level of approximately 50 ug/gram leaf tissue (data not shown). The banding of protein from 'Ogy' was not as intense as the transgenic lanes containing the introduced PI II protein.

Serine Proteinase Inhibition Assay

Compared to the protein extract from the untransformed 'Ogy', degradation of the protein substrate by serine proteases was inhibited to a higher degree when extracted protein from TR5 was added to the assay system. The transformant TR5's extract reduced proteolysis of the casein substrate 23.4% when trypsin was introduced, and 24.3% when chymotrypsin was the protease, compared to the control 'Ogy' extract (Fig. 2). The control 'Ogy' extract was effective in reducing proteolysis of the substrate by 20.5% and 14.5% compared to the negative control (without plant extract) containing trypsin or chymotrypsin respectively.

Discussion

Western blot analyses were initially obtained with considerable difficulty because of the PI II protein's biochemical properties. The PI II protein and the native proteinase inhibitor of 'Ogy' have proved to be difficult proteins to work with.

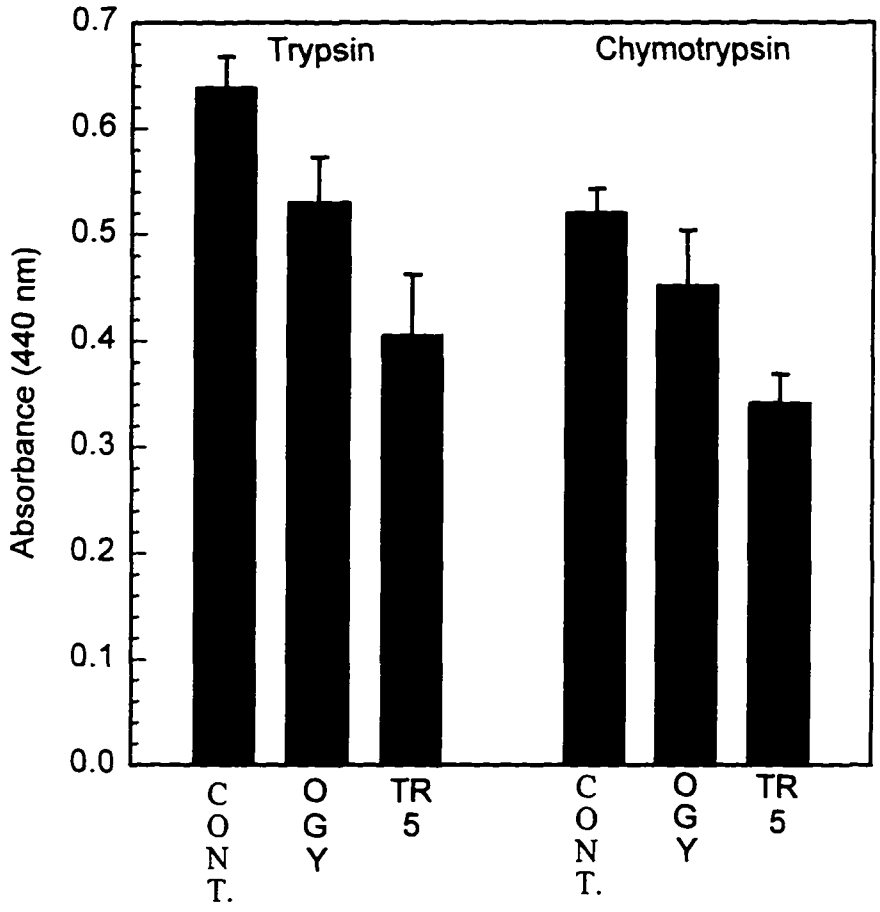

Fig. 2. Results of the enzyme inhibition assay for trypsin and chymotrypsin activity using protein extracts of TR5 and control 'Ogy'. Controls (CONT.) contained 'Ogy' extract, but no enzyme. One unit of proteolytic activity was defined as an increase in absorbance of 0.01 at 440 nm. Error bars represent standard deviation.

The formation of multimers is a real problem with the proteins and their protomers. The functional PI II dimer is composed of two protomers (monomers) that can vary in their molecular weight and isoelectric point (pI). Four different protomers; A, B, C, and D have been observed (Bryant et al. 1976). Protomer A has not been characterized because of problems in obtaining pure fractions of this protomer. The remaining three have pIs of 5.7 (B), 7.2 (C), and 8.2 (D) (Bryant et al. 1976). Any two of these protomers may combine to form one of 10 functional PI II dimers. The gel presented (Fig. 1) shows differences in banding based on sample treatment, and in each case the transgenics show novel bands compared to the control 'Ogy'. The center group of lanes (run without beta-mercaptoethanol) do not show nearly as much multimer formation. The reduction of the protein in the presence of beta-mercaptoethanol seems to allow the protomers to reassociate in a plethora of combinations. We doubt that the formation of these multimers is representative of what occurs *in planta*, because such severe reducing conditions do not occur in the plant. *In planta* the protomers likely associate with one another as folded tertiary proteins to form the active dimer, not linear molecules as in the reducing gel conditions. The protomers in their reduced state may also be forming hybrids or interacting with proteins from their plant host. The large number and variety of multimers are not seen in the "pure PI II" lanes or in the control lanes, suggesting that there is an interaction between the reduced PI II protein and 'Ogy' proteins. We have added pure PI II to control 'Ogy' extracts and have seen the multimers form in those

samples (data not shown). Variability in protein migration and banding were perhaps a result of the variable isoelectric properties of the different combinations of protomers under different gel conditions. The 'Ogy' protein detected in the control material exhibits many of the characteristics of PI II. Because the "PI II-like" protein in 'Ogy' is isolated by the protein isolation technique described in the methods, shows that it is quite heat stable and remains soluble at low pH values. The similarities between PI II and the native 'Ogy' protein are; their approximate molecular weights, migration patterns, heat stability, acid solubility, immunoreaction, and serine protease inhibition, and these suggest a commonality between the proteins.

Similar background signal problems with the controls were observed in other assays using immunodetection. ELISA tests had extensive problems with high background signal in the 'Ogy' control wells, and immunological radial diffusion assays were unable to quantify PI II expression accurately because of immunoreaction in the control material. The native protein must possess relatively close homology to PI II, based on the specificity of the immunoreaction and the evidence of possible hybridization with the protomers of PI II. The affinity purified antibody does not cross react with Kunitz or other protease inhibitors. The double affinity-purified polyclonal antibody was very useful in finding this native proteinase inhibitor because it was composed of a population of IgG-PI II antibodies that had a multitude of PI II epitopes that could be recognized. Extensive affinity purification of the antibody against control proteins resulted in less background but did not eliminate the binding

with specific proteins of the control 'Ogy' material. A single site specific monoclonal antibody would possibly have eliminated many of the problems incurred in proving the trees were transformed, but may not have allowed the discovery of this native inhibitor in 'Ogy'.

Accurate estimates of proteinase inhibitor expression are difficult to assess. One possible problem is that the antibody may have greater affinity to PI II than the native inhibitor, thus making quantification via immunodetection unreliable. Another problem results from the difficulty of determining how much of the banding intensity in the transgenics is caused by PI II and how much is from the native inhibitor. There is also the possibility that, because of strong homology between the inhibitors, there may be hybrid inhibitors composed of the protomers of PI II and the native inhibitor. This may help explain the complicated banding patterns observed. We also believe the components of PI II may not be migrating true to standard molecular weight size markers despite SDS treatment, because of their wide-ranging isoelectric points and strong cross-linking properties. This may explain the consistant appearance of two protomers at less than 12 kd. Historically, researchers have presented ever-changing molecular weights for PI II from potato. PI II dimer sizes of 19.5 kd (Iwasaki et al. 1972), 21 kd (Bryant et al. 1976), and 24.6 kd have been reported. Despite the problems with size quantification and banding patterns, it is clear that there are consistently unique bands in the transgenics vs. the control 'Ogy' (Fig.1).

Although the control 'Ogy' protein extract has serine proteinase inhibition activity, the transformant provides better inhibition of trypsin and chymotrypsin (Fig. 2). This is most likely an additive effect of the introduced PI II protein and the native protein. The inhibition of proteolysis seen in the enzyme inhibition assay by the control 'Ogy' extract may impart some resistance to pests. The native pests of *Populus* may have developed tolerances, over evolutionary time, to the native 'Ogy' inhibitor. The increased reduction in proteolysis by the transgenic extracts was not dramatic, but it may provide added resistance to pests and pathogens. Many insect pests, such as *Chrysomela scripta*, are multivoltine. If the inhibitors can reduce nutrient uptake, and thus growth, in the insect, there may be one less generation of larvae in a growing season. This may result in a substantial reduction of leaf area consumed, and reduced vigor resulting in reduced resistance to parasites as well as reduced vigor and numbers of overwintering adults that would start next year's infestation. The subtle inhibition may also work well for woody-plant fungal pathogens that use serine proteases. Reduced pathogen reproduction may decrease disease levels over time. Canker diseases of woody plants, that occur over multiple growing seasons, may have the rate of canker enlargement slowed by the proteinase inhibitor, thereby increasing the effectiveness of the natural wound response of the tree.

The results of this study supports the theory that 'Ogy', and perhaps *Populus* as a genus, already have a PI II type serine proteinase inhibitor because of divergent

or convergent evolution. Because the PI II gene (*pin2*) was originally isolated from potato, an evolutionarily distant non-woody plant, it is unlikely that both proteins would have exactly the same amino acid sequence because of mutations over evolutionary time. When our laboratory began transformations with the *pin2* gene in 1986 (Chun et al. 1988), the PI II protein was viewed as a "novel" plant defense from the Solanaceae. Since then, proteinase inhibitors have been observed in many genera of plants (Ryan, 1990) and seem to perhaps be the rule in plants rather than the exception. Our laboratory intends to determine further the similarity between the 'Ogy' inhibitor and PI II by analyzing the sequence homology between the two proteins, identifying possible differences in isoelectric properties, and continuing to investigate the nature of these proteins in disease and pest resistance.

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CHAPTER 5. ASSESSING CANKER DEVELOPMENT OF *SEPTORIA MUSIVA* ON *POPULUS*, AND POTENTIAL RESISTANCE OF CLONAL SELECTIONS

Introduction

Septoria musiva Peck. (teleomorph *Mycosphaerella populorum* Thompson) is an endemic fungal pathogen of *Populus* in North America. *S. musiva* is the causal organism in Septoria canker disease of poplar in North America. Septoria canker disease has become a serious problem (Bier 1939; Ostry and McNabb 1983, 1985; Spielman et al. 1986; Thompson 1941; Waterman 1954) since susceptible inter and intraspecific hybrids have been developed and introduced in North America. Stem infections can girdle the bole and sylleptic stems resulting in partial or complete dieback of the tree. Few researchers have used a standardized rating protocol to assess canker disease in poplars. Those who have, have used protocols that were strongly subjective or only took into consideration canker incidence (Lo et al. 1995). Artificial inoculation techniques for the induction of Septoria canker disease have been developed (Mottet et al.1990; Maxwell et al. 1997) using mycelial plugs as the inoculum. Because the mass of mycelium does not represent the natural infection process and may provide an

advantage to the fungus in cambial colonization, an alternate technique using both conidia and ascospores was developed for this study.

Towards better understanding this disease process and what qualities in hybrid clones provide resistance to Septoria canker disease, the following studies were implemented: 1) A canker rating system to allow *Populus* researchers to evaluate Septoria canker disease with more uniform analyses of disease incidence and severity, and 2) an artificial inoculation technique to allow testing of juvenile material for resistance to Septoria canker disease. Both studies are intended to define standards for the uniform analysis of clonal susceptibility to Septoria canker disease. These studies have provided valuable preliminary information for further studies and a future paper on assessment of Septoria canker development on *Populus*.

Materials and Methods

Clonal selections of native and hybrid *Populus*

Native clonal selections of *P. deltoides*, and hybrid clones of *P. deltiodes*, *P. trichocarpa, P. nigra, P. maximowiczii, P. alba, and P. grandidentata* crosses, were used in this study (Tables 2 and 3). The clones were selected or developed by the poplar research programs of Canada, France, and the USA (United States Forest

Table 2. Clonal selections of native and hybrid *Populus* clones used in the *Populus* Clonal Field Validation Trial at the ISU Fick Farm.

Clone	Parentage	Clone	Parentage
51-5	DD	211.37	DM
119.16	DD	313.23	DM
68J1700	DD	313.55	DM
9252.36	DD	412.63	DM
5910100	DD	502.37	DM
7300501	DD	NE19	NN
7300502	DD	NM6	NM
8000105	DD	23	NT
8000113	DD	NE285	NT
DN2	DN	57-276	TD
DN182	DN	Crandon	AG
IS31	DN	C147	AE
NC5326	DN	CAG204	(AE)(AG)
107.14	DN		

A= *P. alba*; D= *P. deltoides*; E = *P. tremuloides*; G = *P. grandidentata*; M = *P. maximowiczii*; N = *P. nigra*; T = *P. trichocarpa*

Table 3. Clonal selections of native and hybrid *Populus* used in the artificial inoculation Septoria canker screening test

Parentage of cross or species of selection	Resistance to *S. musiva* [a,b]	Identification numbers or trade names	Source of original cross or selection
Populus deltoides	HR	'8000105'	Illinois (clonal selection)
Populus deltoides	HR	'8000113'	Illinois (open pollinated)
Populus deltoides	HR	'51-5'	North Central Regional Project
Populus X *euramericana* [c]	MR	'DN 34' ('NC 5326') 'Eugenei'	France (natural hybrid)
P. nigra x *P. maximowiczii*	MR	'NM 6'	Canada
Populus X *euramericana* [c]	MR	'Ogy'	Belgium
Populus X *euramericana* [c]	MR	'DN 2'	Canada
P. nigra 'Charkowiensis' x *P. nigra* 'Caudina'	R/S	'NE 19'	Oxford Paper Company
P. deltoides x *P. maximowiczii*	MS	'313.55'	USFS Univ. of Minn., St. Paul.
P. deltoides x *P. maximowiczii*	MS	'502.37'	USFS Univ. of Minn., St. Paul.
P. nigra x *P. trichocarpa*	HS	'NE 285'	Oxford Paper Company
P. trichocarpa x *P. deltoides*	HS	'57-276'	Univ. of Washington

[a] Resistance values: HR = highly resistant, MR = moderately resistant, R/S = low resistance/susceptible, MS = moderately susceptible, HS = highly susceptible.

[b] Resistance values represent the observed resistance to Septoria canker disease, as determined by researchers (Dickman and Stuart 1983; McNabb and Ostry, personal communication).

[c] *Populus* X *euramericana* hybrids are crosses of *P. deltoides* and *P. nigra*.

Service [USFS], state universities, and private industry). The clonal genetic material was originally selected for growth potential, cold and drought tolerance, pest and disease resistance, and growth form (Mühle-Larson et al. 1980; Dickmann and Stuart 1983). The clones in this study represent a range of susceptibilities to Septoria canker disease.

Introduced Belgian *Populus* clones

In 1985, twenty-nine hybrid clones of *Populus* were imported from Belgium, under quarantine, to the Iowa State University (ISU) Department of Forestry. The clones were tissue cultured explants of selected individuals from the breeding program of Vic Steenackers at the Poplar Research Station (Rijksstation voor Populierenteelt) in Geraardsbergen, Belgium. After quarantine procedures, the material was propagated and maintained in the forestry greenhouse. The material was imported to ISU so that disease evaluations could be conducted on the clones, in a field setting, with endemic pathogens of eastern North America. Twenty-one clones were advanced to a field trial at the ISU Rhodes Farm in central Iowa (Table 4).

Canker rating system

A rating system for canker disease of *Populus* was developed with the goal of standardizing the assessment of disease incidence and severity. Previous rating systems varied with the institutional program that was assessing disease symptoms,

Table 4. Clonal selections of hybrid *Populus* from the Belgian breeding program

ISU Clone #	Trade name	Parentage
4	Unal	*P. trichocarpa* 'Fritzi Pauley' x [*P. deltoides* "V.5" x *P. deltoides* "V.1"]
5	Boelare	*P. trichocarpa* 'Fritzi Pauley' x [*P. deltoides* "V.5" x *P. deltoides* "V.1"]
6	Raspalije	*P. trichocarpa* 'Fritzi Pauley' x [*P. deltoides* "V.5" x *P. deltoides* "V.1"]
7	Beauprè	*P. trichocarpa* 'Fritzi Pauley' x [*P. deltoides* "V.5" x *P. deltoides* "V.1"]
8	Hunnegem	*P. trichocarpa* 'Fritzi Pauley' x *P. deltoides* "V.11 (from Missouri)
9	Primo	[*P. deltoides* "V.5" x *P. deltoides* "V.1"] x *P. nigra* (from 'Ghoy' farm, Belg.)
10	Ghoy	[*P. deltoides* "V.5" x *P. deltoides* "V.1"] x *P. nigra* (from 'Ghoy' farm, Belg.)
11	Gaver	*P. deltoides* (cross made in Belgium) x *P. nigra* (from 'Gaibecq' farm, Belg.)
12	Gibecq	*P. deltoides* (cross made in Belgium) x *P. nigra* (from 'Gaibecq' farm, Belg.)
13	Ogy	[*P. deltoides* "V.5" x *P. deltoides* "V.1"] x *P. nigra* (from 'Ogy' farm, Belg.)
14	Isieres	*P. deltoides* (from Wapello, Iowa) x *P. nigra* (from Isieres farm, Belg.)
15	S.724-101 (Trichobel)	*P. trichocarpa* 'Fritzi Pauley' x *P. trichocarpa* 'Columbia River'
17	76.028/5	Beauprè (see above #7) x *P. deltoides* (cross made in Belgium)
18	69.038/6	*P. trichocarpa* 'Fritzi Pauley' x *P. deltoides* (from Michigan)
20	72.029/4	*P. trichocarpa* 'Fritzi Pauley' x *P. deltoides* (cross made in Belg.)
21	70.045/1	*P. trichocarpa* 'Fritzi Pauley' x *P. deltoides* (from Michigan)
23	(unnamed)	*P. nigra* (from Essine, Belg.) x *P. trichocarpa* 'Columbia River'
24	S.907-1	*P. trichocarpa* 'Fitzi Pauley' x *P. maximowiczii* (from Japan)
25	71.106	*P. deltoides* (from Michigan) x *P. maximowiczii* (S.122-3 from Japan)
26	(unnamed)	*P. deltoides* x [*P. deltoides* x (*P. trichocarpa* x *P. maximowiczii*)]
28	(unnamed)	*P. trichocarpa* 'Fritzi Pauley' x *P. lasiocarpa* (from Asia)

and usually were based on presence or absence of canker and frequency (Lo et al. 1995). The system described here is based on the leaf disease rating system of Schreiner (1959), with modifications of the rating indices and symptoms assessed (Table 5).

The canker rating system uses two numbers to indicate the incidence and severity of disease. The first numerical rating indicates incidence per clone, and the second number represents severity of the disease interaction. For this system, incidence is defined as the number of canker infection events observed on the previous year's hardened growth for an individual clone. Severity, is based on a visual assessment of the host-pathogen interactions that characterize the canker (Table 5). The aggressiveness of the pathogen's colonization of host tissues and the amount of callus production by the host are criteria for the visual severity rating.

Field trials

North Central Regional *Populus* clonal field validation trial. A *Populus* clonal field validation trial of 27 native or hybrid *Populus* selections (Table 2), was planted in 1992 at the Iowa State University Fick Observatory. Four tree plots of each clone (two hardwood cuttings, and two rooted barbatels) were planted adacent to each other in a row, and randomized within the experimental block of clones. The experimental field planting contained four replications in the randomized block design. The blocks were encircled by a one-tree border of clone NC 5326

Table 5. Canker rating system for incidence and severity

Severity Rating	Canker Type	Host / Pathogen Interaction
1	Subdued	Wound healed or healing quickly / canker compartmentalized
2	Obstinate	Wound slow-healing / canker active, slowly being overtaken by callus
3	Aggressive	Wound healing response inadequate, canker is slowly growing in area
4	Lethal	Wound not healing, little to no callus response. Canker grows rapidly

Incidence Rating	Number of Cankers Observed
0	no cankers present
1	1 or 2 cankers per tree
2	3 to 5 cankers per tree
3	6 or more cankers per tree

('Eugenei'). Clones were rated for canker disease with the afore mentioned canker rating system (Table 5). Data were compared to previous consensus of clone resistance to Septoria canker disease, or susceptibility of parental lines.

Hybrid *Populus* from the Belgian breeding program. A field trial of 21 hybrid *Populus* selections (Table 4), was planted in 1990 at the Iowa State University Rhodes Farm. The test plot consisted of uneven replications (2-8 reps) of each clone, randomized within the plot. Uneven replication of clones was because of poor propagation success which resulted in limited numbers of some clones. The plot was located next to an old *Populus* trial plot which was severely damaged by Septoria canker disease.

Clones in the field trial were rated for incidence and severity of canker disease using the canker rating system. Data was compared to the previous consensus of the clonal parental lines and their susceptibility to Septoria canker disease (Table 3).

An artificial screen for Septoria canker disease

Septoria isolates. Four isolates (MP1-4) of S. musiva were screened for conidial germination efficiency, mycelial growth rate, and sporulation potential. The asexual pathogen propagules (conidia) were collected from hybrid poplar field plots in Iowa (MP1), Wisconsin (MP2), North Dakota (MP3), and Minnesota (MP4). Isolate MP1 was obtained from a local *Populus* plantation by our laboratory (Lulley 1986),

and isolates MP2, MP3, and MP4 were collected from other plantations in the northcentral states by cooperators in the USDA Forest Service, St. Paul, MN. Of the four isolates, isolate MP1 was selected for field inoculations because of its consistently good results in the laboratory screening tests, and field observations as a highly aggressive isolate. Conidia of isolate MP1 were collected from sporulating cultures on V-8 PPA medium and suspended in ddH_20 at a concentration of 10^3 conidia/ml for immediate use in field inoculations. Percentage germination of conidia was tested on water agar covered microscope slides.

Ascospore suspensions were pooled populations of ascospores from the field, representing the natural variation in pathotypes found in the field. Ascospores were collected from a population of overwintering leaves of hybrid *Populus* clones near the test site. The fallen leaves had been collected the previous autumn and stored in a 4 C cold-room. To obtain ascospores for the inoculations; leaves were thoroughly washed with ddH_2O containing 0.01% Tween 20, rinsed in a solution of 10% chlorine bleach and 10% ethanol, hydrated for 6 hr in ddH_2O, drained and arranged to cover the bottom of sterile disposable petri plates, and incubated for 18 hr at room temperature. Leaves and the inner surface of the petri plates were washed with 5 ml of ddH_20 which was sequentially transferred from plate to plate to increase the spore count. The rinse solution was checked for the quantity of ascospores it contained and

when the concentration reached 10^3 ascospores/ml it was used for inoculations. Percentage germination of ascospores was tested on water agar covered microscope slides.

Artificial Inoculation of stems for Septoria canker disease. An artificial inoculation technique, using spores of *S. musiva,* was used to determine differences in canker establishment and colonization of young stems in clonal *Populus* selections. The field study was conducted at the Hind's farm of Iowa State University, and included twelve clonal selections of *Populus* (Table 3) that represent a range of resistance to Septoria canker disease.

The field study consisted of 120 trees from 12 clonal selections. There were 10 trees of each clone and the plot was divided into two replication blocks. Five trees of each clone were randomly distributed in each of the two blocks.

Three-month-old stems (~1 cm diameter) of the hybrid poplar clones were inoculated with conidial and ascospore suspensions of *M. populorum* at a concentration of 10^4 spores/ml ddH_2O. The inoculation sites on the test clones received either conidia, ascospores, or ddH_20. Fresh leaf scars were the site of inoculation. The 20 ul droplets of inoculum were placed on the leaf scar of a freshly removed petiole and the area wrapped with paraffin film to maintain high humidity at the infection court. There were three inoculation sites per replication. One site was inoculated with conidia, one with ascospores and one with distilled water (control).

Inoculation sites were at every other node and at approximately 120 degree angles to one another. Inoculum type was alternated among the three possible sites of each replication to minimize interference. Canker growth and pathogen sporulation was monitored externally at bi-weekly intervals for 10 replicates of each clone.

Quantification of canker development. Six months after inoculation, while in winter dormancy, the cankered stems were harvested for analysis. The inoculated section of the main stem from each clone was cut 4 cm above and below visibly diseased tissue or wounds at inoculation sites one and three. The stem sections were labeled and bagged, and placed in a 4° C cold-room. Sites of inoculation were identified, and the resulting canker or wound response was measured using a millimeter tape measure. Axial length and lateral width of cankers or healed wounds were determined by measuring from the leading edge of the canker's necrotic depression, the discolored diseased stem tissue, or the callus tissue around the canker margin, depending on the type of host-pathogen interface observed. Estimated area (cm^2) of cankered tissue was obtained by using the formula for area of an ellipse (1/2 W X 1/2 L X 3.14) and the canker's axial and lateral measurements

Results

Field trials for canker rating system

North Central clones. The new canker rating system provided ratings that were consistent with the consensus of field susceptibility by previous researchers. Clones that had previously shown good field resistance to canker disease exhibited host-parasite reactions consistent with strong defenses to infection. Clones that had previously shown poor or questionable field resistance to canker disease exhibited host-parasite reactions that suggested a weak host defense response or an inadequate barrier to initial infection.

Clones with pure *P. deltoides* (DD) parentage did not show symptoms of canker disease. Hybrids with *P. alba* parentage (AE and AG) occasionally showed signs of canker infection, but never developed a perenial canker. The clones with pure *P. nigra* parentage (NN) and *P. nigra* x *P. maximowiczii* (NM) exhibited moderate levels of severity and incidence. Ratings for clones that had a *P. deltoides* crossed with another *Populus* species were wide ranging. If the other parent was *P. nigra*, the clone tended to have low severity and low to moderate incidence. If the other parent was *P. maximowiczii* or *P. trichocarpa*, the clone tended to have moderate to high severity and low to high incidence.

Belgian clones

The new canker rating system provided novel information on the susceptibility of the Belgian clones to North American canker diseases. The majority of the Belgian clones exhibited host-parasite reactions that showed high susceptibility to North American canker diseases. Clones that had previously shown excellent resistance to bacterial canker (*Xanthomonas populi*), and the leaf diseases of Europe, became ravaged with cankers or were killed outright by basal cankers. Only a few clones showed acceptable levels of canker incidence and severity.

The clones with pure *P. trichocarpa* parentage (TT) and with *P. trichocarpa* x *P. maximowiczii* hybrid parentage exhibited moderate to high levels of severity and moderate to high incidence. *P. nigra* x *P. trichocarpa* crosses tended to have high severity and moderate to high incidence ratings. *P. nigra* x *P. maximowiczii* crosses had low severity and low to high incidence ratings. Ratings for clones that had *P. deltoides* crossed with another *Populus* species as their parents varied greatly. If the other parent was *P. nigra*, the clone tended to have low severity and low to moderate incidence. If the other parent was *P. maximowiczii*, the clone tended to have high severity and low to moderate resistance. Hybrid clones with *P. trichocarpa* parentage generally died within 3 yr because of a lethal basal canker, or if they did survive, had both high incidence and severity ratings.

Artificial field screening for canker disease

Artificial inoculation of hybrid clones of *Populus* at the ISU Hinds farm resulted in canker formation, though high variability was present within and between clones. Because of high levels of variance and missing data points, no statistically different comparisons could be determined. Ascospores and conidia each induced cankers, but each treatment had numerable canker induction failures and canker size did not show any statistical trends. Rep 1 of the field experiment produced slightly more cankers, and resulting cankers were typically larger than in Rep 2. Determination of whether ascospores or conidia are a better source of inoculum in canker formation could not be determined in this experiment.

Discussion

The canker rating system should enable researchers to evaluate canker diseases of *Populus* clones with a standarized protocol. Previously, data on canker disease have been collected and recorded according to the protocols of each poplar program or researcher. Many of these systems are highly subjective and do not have specific criteria for their susceptibility ratings. This system is intended as a starting point towards standardized assessment of fungal canker disease of *Populus*. It clearly

defines both the amount of incidence and the severity of the infection process. Determining the severity rating, based on the host-pathogen reaction, provides crucial information in determining the efficacy of the host defense response and the possible nature of susceptibility. Having evaluated a wide range of *Populus* clones with this system, it seems that resistance to fungal canker disease operates on at least two levels. The first level of resistance is to initial infection and the second level can be described as the ability of the host to defend itself from fungal colonization. A 1-4 rating, which shows low severity and high incidence, may indicate a clone that possesses a strong physiological defense response but has poor resistance to infection. A 4-1 rating, which shows a high severity and low incidence rating, may indicate a clone with few defenses against collonization by the fungus, but a strong barrier to initial infection. The system is intended to be simple in its design. This should allow it to be implemented by all types of personnel, while still providing enough detailed information on host reaction to researchers for analyses of possible resistance mechanisms.

Analyzing the canker formation of both the pre-selected North Central material and the non-selected Belgian materials has provided some indications of which parental clones may contribute to canker susceptibility. The least favorable parental species seems to be *P. trichocarpa* and *P. maximowiczii*. Using *P. nigra* as a parent usually did not seriously exacerbate canker susceptibility, and in a few examples (NM2 and NM6) may have enhanced resistance levels. *P. deltoides* was, as expected, the best source of

resistance to canker disease. We are continuing research in the area of clonal susceptibility characteristics, and will focus on non-hybridized selections of the above species.

There were considerable problems with the artificial screening system. Field screening has proved to be very difficult because of the effect of environment on disease development. Environmental stress was recently shown to affect the development of Septoria canker on *Populus* (Maxwell 1997). Trees that were subjected to greater amounts of drought stress had increased incidence and severity of canker. This effect could explain why field replication 1 had better canker induction and development. Rep 2 was adjacent to a greenbelt of trees that provided shade in the late afternoon hours. Rep 1 was in full sun until a few hours before sunset. The trees of Rep 1 may have been under more water stress than those in Rep 2.

Another problem with the system may have been the delivery of the spores to the wound tissues. When removing the leaf, most wounds were the exposed cells of typical leaf traces. But, in removing the leaves, some wounds had slight tears or were deeper than others. This may have bypassed some barriers to infection in some of the inoculations.

Variation within and between clones was so high that many statistical tests failed. This variation may be unavoidable when trying to compare different genotypes. Within-clone variation may have been because of microsite differences or differences in inoculation technique.

This test is being redesigned to try and compensate for these possible sources of variation. Future modifications of the artificial canker inoculation protocol may include: inoculating ramets of a coppiced clone to reduced within clone variability, applying spores to deep wounds and comparing infection frequency with surface applied spores.

CHAPTER 6. GENERAL CONCLUSIONS

General Discussion

A major goal of this dissertation was to begin to standardize the methodology used by researchers studying *S. musiva*. At the time these studies were begun, there were few (if any) standard protocols for the culture of, and disease assessment of *S. musiva*. The need for a standard medium in growth and sporulation studies is crucial. Krupinsky's V-8 PPA medium provides the best growth and sporulation of all previously used media. The only problem with the medium is that its composition is uncontrolled by the manufacturer. Different batches of V-8 can contain different types and ratios of the vegetables it is composed of. This variability could cause consistency problems in studies of the biochemical processes involved in pathogenicity. While the V-8 PPA medium provides fairly consistent results, a more controlled medium should be adopted. The CCM medium developed in this dissertation provided excellent growth and sporulation that rivaled the V-8 PPA medium. The synthetic medium proved to be an ideal growth medium for both agar plate culture and liquid shaker cultures of *S. musiva*. Unfortunately, it is a time-consuming medium to prepare because of casein's solubility problems in neutral to

acid solutions. The CCM medium should work well as a standard medium for *S. musiva*, but additional research with other protein sources may increase its usability.

The system for the production and detection of the extracellular proteolytic enzymes of *Septoria musiva* gave consistent results in this study. Because of the poor growth seen in isolates MP2-4, it was apparent that for studies of extracellular enzyme production in liquid culture, it is important to screen isolates for their ability to adapt to liquid culturing. Proper fungal nutrition, including an appropriate C/N ratio, is a crucial factor in maintaining consistent proteinase production and minimizing growth-limiting effects. In the liquid culture growth studies, the C/N ratio of glucose to casein had an unexpected effect on glucose consumption. Cultures that started with lower glucose concentrations used their glucose at a faster rate than cultures with higher initial concentrations. The reason for this is unknown, but may relate to having to use more energy in sequestering nutrition from the media. This result illustrates how nutritional factors can have unexpected effects on the outcome of an experiment, and need to be closely controlled so that data between researchers is efficacious.

The SDS-PAGE protease activity gels provided a practical way to visualize the proteolytic activity of the two fungal proteinases found in this study. There may be other proteinases present in the culture filtrates, but this particular assay method may have selected against their activity. The pH conditions of the assay are neutral to basic and would tend to select against aspartic proteinases that are active in acid

environments. Another possible exclusion could be proteinases which are activated by other proteins or compounds. Proteinases separated on the gel, may be segregated from catalytic compounds that are normally available in the culture filtrate. Because of these possibilities it is likely that proteinases other than P1 and P2 may be present in the culture filtrates. Proteinase P1 is some type of serine proteinase based on the inhibition data. Further study is needed to determine if the activity of P1 is more closely related to trypsin or chymotrypsin proteinases. Based on inhibition data, proteinase P2 is not a serine proteinase. Because it is active in neutral to basic conditions, it may be a cysteine proteinase or a metallo-proteinase. Further studies (using other methods) will be conducted to determine the proteinase type of P2 and to determine if other proteinases are present. Further analysis of these proteinases should allow us to choose highly specific inhibitors against the proteinases of *S. musiva*, and learn more about the possible role of proteinases in pathogenicity.

The *in vitro* studies of the effect of PI II on *Septoria musiva*, demonstrated that PI II was capable of reducing serine proteinase activity in the culture filtrates of *S. musiva*. Proteolytic activity of culture filtrates was reduced by approximately 33% in the presence of the PI II protein. This demonstrates that serine proteinases are an active component of the culture filtrate. Other non-serine proteinases, or unbound serine proteinases may have been responsible for the remaining proteolytic activity.

PI II is a reversible inhibitor and its ability to inhibit serine proteinases follows an equilibrium constant. Because of this, there is always some unbound proteinase that may be active in PI II assays. PMSF is an irreversible inhibitor and permanently inactivates the proteinases with which it binds. This may help explain why PMSF provided such a dramatic reduction in proteolysis. In the *in vitro* tests with *S. musiva* conidia, PI II was effective in limiting germination, mycelial growth, and the sporulation of the fungus when incorporated into the culture medium.

Reduction in germination and the reduction in germtube elongation was substancial and was also observed by Lorito et al. with a serine inhibitor from cabbage. Lorito et al. (1994) detected cytoplasmic constituents issuing from hyphae in the presence of proteinase inhibitors. They proposed that some types of proteinase inhibitors may cause leakage in cell membranes. If cell leakage is occurring in this study, it could perhaps be the result of proteinase inhibitor induced amino acid deficiencies, possibly leading to protein deficient plasma membranes. This membrane leakage would further exacerbate any nutrient deficiencies in the cytosol that were induced by PI II's antinutrient properties. The strong effect of PI II on pycnidial development may be a result of slowed growth, rather than a direct inhibition of pycnidial development. Pycnidia that matured produced normal viable conidia. The observed reduction in mycelial growth may have slowed development of the reproductive structures and/or resulted in less stored nutrients for pycnidial development.

The *in vivo* tests, with *S. musiva* and transgenic *Populus* expressing PI II protein suggests a reduction in leaf lesion size when conidia were applied to transgenic leaves. The presence of the inhibitor in leaves did not significantly affect infection frequency.

The studies with transgenic *Populus x euramericana* 'Ogy', unexpectantly revealed a native proteinase inhibitor in *Populus*. The 'Ogy' inhibitor seems to have very close homology to PI II and appears to be able to form hybrid complexes of the monomers of PI II and itself. The inhibitor does provide some inhibition of serine proteinases, but pests of *Populus* may have developed resistance to this native inhibitor over evolutionary time. The ~24 kd protein will require further study to determine the amount of sequence homology that exists between it and PI II.

The studies with Septoria canker and *Populus* hybrids provided valuable information for future studies in this area. Although little reportable data was presented in this dissertation, the experiments provided valuable experience in inoculation technique and indentification of clone characteristics. The parental clones of these hybrids need to be obtained in order to truly tell what genetic lines contribute to resistance. The system for artificial stem inoculations needs to be redesigned to minimize variability, and plant water potential needs to be controlled in the experiments. The canker rating system appears to work well and will provide more consistent information to researchers in the future in assessing differences in canker susceptibility among clones. The new system will be presented at the 1997 IUFRO

meeting in Quebec City, Canada. The system will be presented to researchers for incorporation in their programs.

Proteinase inhibitors show great promise as a tool to help manage arthropod and pathogen pests of *Populus*. However, interpreting ecological interactions of proteinase inhibitors is complex. More studies are needed to evaluate the role of specific proteinase inhibitors in managing specific pests using intact plants under field conditions, where interactions with other pest management practices, other host resistance mechanisms, natural enemies, and less-favorable environments are expected. In addition, deployment strategies must be devised to maximize resistance stability and minimize adverse environmental effects.

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