RESISTANCE TO SEPTORIA MUSIVA IN HYBRID POPLAR

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ABSTRACT

Septoria leaf spot and stem canker, caused by the fungal pathogen *Mycosphaerella populorum* Thompson (Anamomorph = *Septoria musiva* Peck). An efficient greenhouse disease resistance screening is essential for the development of resistant clones. Fourteen clones of hybrid poplar were inoculated with spore suspension. A regression model with parameters (lesion number and proportion necrotic area) is consistently and accurately predicted field resistance categories of the most resistant and susceptible clones. In second experiments, the infection biology of *S. musiva* was examined at several time points by scanning electron microscopy (6 h, 12 h, 24 h, 72 h, 1-week and 3-week) and histological analysis (3-week, 5-week and 7-week). Results indicated that there are differences occur following penetration between the resistant and susceptible. Those differences provide the first clues elucidating resistance mechanism in hybrid poplar stems. The results from this thesis will be used to improve resistance to Septoria canker in breeding programs.

ACKNOWLEDGMENTS

DEDICATION

I wish to dedicate this research to my mother, Yu Ding. It is only with the love of my

mother that I was able to endure this long process of continual education. My schooling and

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Also, I would like to dedicate this thesis to the Qin family who passed away, including

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LITERATURE REVIEW

Populus genus

The genus name of *Populus* was thought to be derived from the expression *arbor populi* "the people's tree" due to the frequent planting of poplar trees in public places and their common use in many parts of Europe (Collingwood *et al.* 1964, Rupp 1990). All poplar trees are members of the *Salicaceae* (the willow family) which consists of two genera, *Populus* and *Salix* (Heilman *et al.* 1995, Eckenwalder 1996). The genus *Populus* comprises approximately 29 species divided among six taxonomic sections: *Populus* (formerly section *Leuce*), to which *P. grandidentata* Mich. and *P. tremuloides* Mich. belongs; *Tacamahaca*, to which *P. balsamifera* L. and *P. trichocarpa* Torr. & A. Gray belongs; *Aigeriros*, to which *P. deltoides* Marsh. and *P. nigra* L. belongs; *Leucoides*, to which *P. heterophylla* L. belongs; *Turanga*, to which *P. euphratica* Oliv. belongs; and *Abaso*, to which *P. mexicana* Wesm. belongs (Little 1971, Peterson and Peterson 1992, Eckenwalder 1996, Dickmann 2001). In North America, the common names of this genus include cottonwood, trembling aspen, quaking aspen, popple, and balm-of-gilead (Peterson and Peterson 1992).

The life history of *Populus* is unique. Trees in this genus are able to reproduce both sexually and asexually. *Populus* species are dioecious, meaning the female and male catkins are produced on separate trees. When fully developed catkins are 10 to 15 cm long, the female catkins are seated in cup-shaped disks with 2 to 4 stigmas (Eckenwalder 1996). The longer male catkins comprise a group of 4 to 60 stamens inserted on a similar disk (Eckenwalder 1996). Poplar trees flower when they are between five to ten years of age (Eckenwalder 1996). Flowering occurs in the early spring, from February to May, and wind pollination occurs shortly thereafter (Cooper 1990). Seed development takes approximately two months and seed dispersal occurs from May

to June (DeBell 1990, Zasada *et al.* 1990). *Populus* seeds contain no endosperm, as a result, germination and establishment must occur shortly after seed dispersal. The mode of asexual reproduction is varied and depends on species (Wilcox *et al.* 1967, Ying and Bagley 1977, Zasada and Phipps 1990). For example, species in the sections, *Aigeiros*, *Tacamahaca*, and *Leucoides* can grow from cuttings (Eckenwolder 1996) whereas white poplar (*Populus alba* L.) commonly reproduces by root sprouting (Welsh *et al.* 1987). These attributes allow a large number of genetically identical and phenotypically desirable individuals to be produced with relative ease, making species of *Populus* a common choice for high yield short rotation plantation forestry (Rood *et al.* 1994).

The anatomy of *Populus* stems and branches is similar to other hardwood species, and can be subdivided into bark and woody tissue (Raven *et al.* 1981) (Fig. 1.1). The term "bark" refers to all tissues external to the vascular cambium, including inner bark and outer bark (Srivastava 1964, Esau 1965, Dickison 2000). The outer bark is the dead tissue on the surface of the stem, commonly called "rhytidome" (Esau 1965). The living inner bark includes periderm, cortex and phloem derived from the primary vascular cambium (Dickinson 2000). Periderm is a protective tissue of secondary origin which replaces the epidermis in stems (Srivastava 1964, Esau 1965, Fahn 1967). Periderm consists of phellem, phellogen, and phelloderm (Esau 1965). Phellogen cells are meristimatic producing phellem towards the outside and phelloderm towards the inside (Raven *et al.* 1981). These cells are characteristically thin-walled, with protoplasts, having an irregular shape, and may contain starch and chloroplasts (Raven *et al.* 1981). Within the periderm are lenticels, which are used for gas exchange, new lenticels are formed within the cracks of the phellem layers as the bark develops (Esau 1965, Raven *et al.* 1981).

Between the bark and the wood is a thin layer of living cells known as the vascular cambium which produces phloem cells towards the outside and xylem cells towards the inside (Raven *et al.* 1981). The phloem is the living tissue responsible for transporting nutrients and is the innermost layer of bark (Raven *et al.* 1981). Xylem can be subdivided into sapwood and heartwood (Raven *et al.* 1981, Dickison 2000). Sapwood is comprised of living cells, which are similar to a pipe, transporting water from the roots to the shoots through. Interior to the sapwood is heartwood, which is made up of dead cells and forms the central support structure of a tree (Esau 1964, Raven *et al.* 1981).

Hybrid poplar

Hybridization, as with many wind pollinated tree species, is extremely common in the genus *Populus*, resulting in many naturally occurring hybrids throughout North America (Stettler *et al.* 1996). These hybrids may be inter-sectional such as *Populus × jackii* Sarg, a natural hybrid between *P. balsamifera* females and *P. deltoides* males, which occurs wherever the range of the two species overlap. Hybrids may also be intra-sectional, for example, *Populus × smithii* B. Boivin, a natural hybrid between *P. tremuloides* and *P. grandidentata*. Moreover, a large number of hybrid poplar genotypes (clones) have also been produced by artificial hybridization. Both natural and artificial hybrids exhibit a phenomenon known as hybrid vigor, whereby, the progeny of intra- and inter-specific hybridization exhibit growth rates superior to that of either parent (Mohrdiek 1983, Stettler *et al.* 1996). Growth rates of hybrid poplar range from 1.2 m to 1.8 m per year under favorable conditions, with total yields ranging from 20 to 43 Mg/ha of biomass per year (Sannigrahi *et al.* 2010).

To date, hybrid poplar breeding has focused on the hybridization of a few select species in the sections *Aeigeros* and *Tachamahaca* (Riemenschneider *et al.* 2001, Sannigrahi *et al.*

2010). The objective of these breeding programs is to develop cultivars of *Populus* exhibiting

hybrid vigor while taking advantage of its clonal nature to produce large numbers of genetically

identical individuals. In 2012, a report by the International Poplar Commission (2012) estimated

that there were over 83.6 million ha of poplar plantations world-wide (FAO 2012). In the United

States, over 50,000 ha are planted in the Pacific Northwest, 15,000 ha in the North Central

region, and 25,000 ha planted in the Mississippi river valley (Stanturf *et al.* 2003, Revels *et al*.

2009). These plantations are used to supply a variety of industries including pulp and paper,

electricity production, manufactured wood products, and biofuel (Stoffle 1998, Balatinecz and

Kretschmann 2001, Brown 2003, Zalesny *et al*. 2008). The U. S. Department of Energy has

increasingly shown interest in hybrid poplar as a feedstock for bioenergy (Stoffle 1998). Brown

(2003) reported that the higher heating value (HHV) of hybrid poplar (19.38 MJ/kg), when it

undergoes complete combustion is comparable to other biofuels including corn stoves (17.65

MJ/kg), wheat straws (17.51 MJ/kg), and switch grass (18.64 MJ/kg). However, widespread

adoption of hybrid poplars in many regions of North America has been limited by its well

documented susceptibility to a variety of pests and pathogens (Ostry *et al.* 1985).

Diseases of hybrid poplars in North America

To date, there are four major diseases affecting *Populus* in North America: Leaf rust

caused by *Melampsora* spp., stem and leaf spot caused by *Septoria musiva* Peck, leaf and shoot

blight caused by *Venturia* spp., and leaf spot caused by *Marssonina* spp. (Hiratsuka 1987,

Peterson and Peterson 1992, Newcombe *et al.* 2001). In the north central region of United States,

Septoria canker is considered the most serious disease of hybrid poplar plantations due to the

lack of effective disease management, the widespread distribution of pathogen, and susceptibility

of many important commercial clones to this disease (Waterman 1954, Long *et al*. 1986,

Newcombe *et al.* 2001). For example, Ostry and McNabb (1983) estimated there was a 66% loss of biomass in susceptible trees compared to non-infected controls, due to Septoria canker infection in Michigan. The range of this pathogen has expanded west of the Rocky Mountains in Canada and into South America (Sivanesan 1990, Callan *et al.* 2007). This disease is becoming increasingly important as the pathogen has expanded to other poplar production regions.

Septoria musiva

Septoria canker and leaf spot is caused by the fungal pathogen: *Mycosphaerella populorum* Thompson (Anamomorph = *Septoria musiva* Peck) (Bier 1939, Thompson 1941, Waterman 1954, Newcombe *et al*. 2001). The imperfect stage of this fungus produces pycnidia (21.5 - 56 µm × 4 µm) and conidia (17.2 - 54 µm × 3 - 4 µm) (Thompson 1941, Waterman 1954). Pycnidia are produced below the epidermis of the leaf and are globose to depressed globose with ostioles (Waterman 1954). Conidia are cylindrical and straight to curved, with one to four septations and released from ostioles in pink masses of spore tendrils (Bier 1939, Thompson 1941). The sexual stage produces pseudothecia (48 - 80 µm × 48 - 96 µm) and ascospores (16 - 28 µm × 4.5 - 6 µm). Pseudothecia are dark brown, globose, with several cylindric-clavate asci (46 - 65µm × 10 - 16 µm) and eight hyaline ascospores (Thompson 1941, Niyo *et al*. 1986). Colonies of *S. musiva* grown on V-8 juice agar are olive-green to gray, occasionally with white margins, producing pinkish pycnidia under high humidity (Spielman *et al*. 1986, Stanosz and Stanosz 2002).

There are two characteristic symptoms caused by this pathogen (Fig. 1.2): leaf spots and stem canker, both can adversely impact tree growth and survival (Waterman 1954, Long *et al.* 1986, Spielman *et al.* 1986). Severe foliar infection can result in premature defoliation reducing photosynthesis and predisposing trees to subsequent insect attack and pathogen invasion (Bier

1939, Waterman 1954, Ostry *et al* 1985, Newcombe 1996). Typically leaf spots are irregular in shape, dark brown to black, becoming whitish in color with yellow margins (Bier 1939, Waterman 1954). Cankers begin as lesions with a dark water soaked appearance, eventually becoming longitudinally and concentrically ellipsoid. The black fruiting bodies, pycnidia, are occasionally formed in the center of stem lesions (Thomason 1941, Waterman 1954). Stem and branch cankers weaken the affected tree increasing the likelihood of wind breakage (Ostry 1987).

The life cycle of *S. musiva* is similar to other *Dothideomycete* fungi (Ostry 1987 Luley and McNabb 1989). The pathogen overwinters on leaf debris, producing pseudothecia releasing ascospores, as the primary inoculum in the spring (Bier 1939, Ostry 1987). Ascospore dissemination by wind, begins in early April in the mid-west, and can continue until mid-summer resulting in new leaf and stem infections (Thompson 1941, Ostry 1987, Luley and McNabb 1989). These infections produce pycnidia and conidia, the secondary inoculum, which are dispersed by rain splash (from May to mid-October). Under ideal conditions, multiple cycles of infection, spore release and re-infection will occur during a single growing season (Ostry 1987). Both ascospore and conidia can cause leaf spot and stem canker. Ascospore and conidia produce germ tube to penetrate host leaves through stomata (Niyo *et al.* 1986, Luley and McNabb 1989). No information is available on the mode of infection resulting in Septoria stem and branch cankers (Ostry and McNabb 1985).

Disease etiology

Plant pathologists have developed methods for pathogen identification, detection and characterization, aimed at developing appropriated disease management strategies. New and improved techniques (microscopy, electrophoresis, histology, protein and nucleic acid characterization, as well as genetics) are constantly being adopted to study the etiology of plant

pathogens. Among these techniques, scanning electron microscopy (SEM) is often employed to observe the early stages of pathogen infection (Smith and Oatley 1955, Mims 1991, McMullan 1995). Cleary *et al.* (2013) found the ascospores of *Hymenoscyphus pseudoalbidus* Gray developed germ tubes, followed by appressoria formation and direct penetration of epidermal cells on common ash (*Fraxinus excelsior* Linn.) leaves and petioles. Hsieh *et al.* (2001) observed the infection process of *Botrytis elliptica* (Berk.) Cooke on lily (*Lilium* spp.) leaves by collecting different time points and observing whether *B. elliptica* penetrated the host via appressoria formation or through stomata. However, SEM observations of cells and tissues are limited to the surface of the infected plant. In order to examine host parasite interactions following infection, a histological examination needs to be conducted. For example, Hsieh *et al.* (2001) conducted the histological observations using of fluorescent microscopy to visualize sub-epidermal hyphae after successful penetration (Hsieh *et al.* 2001). Similar techniques have been used to elucidate host parasite interactions in many pathosystems.

In woody plants anatomical and histo-chemical changes to bark tissue following wounding and subsequent pathogen penetration have been well characterized (Biggs *et al.* 1983a, 1983b and 1984, Biggs 1984 and 1986, Hebard *et al.* 1984, Enebak *et al*. 1997). These changes in most cases involve the development of new periderm, called necrophylactic periderm (NP), to protect living host cells in the tissue surrounding the wound (Bloch 1952, Esau 1965). Biggs (1992) modified a model, which was originally proposed by Mullick (1977), describing tree responses to wounding leading to NP formation (Mullick 1977, Biggs 1992). He outlined three scenarios of nonspecific host response following injury (Biggs 1992). In the first scenario, a superficial injury or penetration of the bark disrupts the phellogen resulting in the nearby phellem and/or cortex cells becoming amorphous followed by subsequent lignification and

suberization. Biggs (1986 and 1992) called these areas ligno-suberized boundary zones stating that they were a prerequisite to the formation of NP (Hudler 1984, Biggs *et al.* 1984, Biggs 1986 and 1992). The second scenario, describes a wound that affects the vascular cambium, but is external to the xylem (Mullick 1977, Hudler 1984, Biggs 1986 and 1992). In this scenario a complete NP develops and the vascular cambium may regenerate a new layer of phellogen below the NP (Mullick 1977, Hudler 1984, Biggs 1986 and 1992). In the third scenario, the penetration goes beyond the vascular cambium into the xylem and the regeneration of phellogen does not occur (Mullick 1977, Hudler 1984, Biggs 1986 and 1992). The vascular cambium also becomes non-functional following occlusion of the adjacent xylem vessels (Shigo 1979, Hudler 1984). The three scenarios described above are generalized and are initiated by the disruption of phellogen. This disruption can be caused by mechanical damage, insect injury or pathogen invasion (Bloch 1953, Mullick 1977, Biggs 1984, 1986 and 1992). Biggs *et al.* (1984) suggested that a pathogen may be able to alter hosts response to phellogen disruption breaching either the ligno-suberized boundary zone or NP (Biggs *et al.* 1984 and 1986).

Several histo-pathological studies of canker diseases of trees have been conducted in order to examine host resistance responses. The chestnut blight pathogen, *Cryphonectria parasitica* (Murrill) Barr., infects stem wounds, accumulating mycelium in the necrotic tissue prior to lesion expansion (Brambe 1936, Hebard *et al*. 1984). This mycelium turns into a "fan-shape", and the "mycelial fan" penetrates living cells. The advancing margin of this mycelial fan develops just beneath the natural periderm (Brambe 1936, Hebard *et al*. 1984). The plant cells in advance of the fungus appear dead prior to colonization and NP layers (wound periderm in paper) form beneath the deepest point of the colonized wound and necrotic tissue (Hebard *et al*. 1984). Hypoxylon canker of aspen also requires a wound in order for infection to occur (Bier

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1940, Rogers and Berbee 1964). Interestingly, the presence of this pathogen has been demonstrated to delay wound closure in both resistant and susceptible ramets of quaking aspen (*Populus tremuloides* Michx.). Enebak *et al.* (1997) also indicated there was no significant difference between resistant and susceptible clones in terms of NP formation. Canker pathogens are typically associated with wounds and as a result the majority of studies examining their infection biology have relied on wounding in order to incite disease.

In contrast to these pathogens, very little is known about how *S. musiva* infects woody tissue and incites stem canker development. Weiland and Stanosz (2007), in the only study examining etiology of Septoria canker, demonstrated that the development of a thick and continuous NP layer was correlated with *S. musiva* resistance. Although an NP layer developed on both the resistant and susceptible clones they inoculated, it appeared to be repeatedly circumvented by the pathogen in the susceptible clone. In contrast there was no apparent disease development beyond the NP layer in the resistant clone, eight weeks following inoculation. As with the studies described above host response to inoculation is confounded with host response to wounding. As a result, it is difficult to determine the role of NP development in disease resistance to canker pathogens.

Disease management

There are four methods which have been tested for the management of Septoria leaf spot and stem canker. Cultural control including sanitation of infected leaf debris prior to leaf emergence in the spring and pruning of cankered branches can reduce the inoculum level in plantations (Filer *et al*. 1971, Ostry *et al.* 1989). However, the ability of the pathogen to disperse over long distances limits the effectiveness of this strategy (Filer *et al*. 1971, Ostry *et al.* 1989). Chemical control of Septoria leaf spot and stem canker has also been tested. Benomyl applied

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monthly (three times per growing season) or on a bimonthly schedule (five times per growing season) beginning at leaf flush can reduce the incidence of Septoria canker (Ostry 1987). However, the costs of these treatments are prohibitive over the length of a rotation (~15 years). Biological control, by using *Phaeotheca dimorphospora* DesRoch. & Ouell. and *Streptomyces* strains have also been tested in the greenhouse (Yang *et al.* 1994, Gyenis *et al.* 2003). Although they both reduced the severity of leaf spot disease, treatments needed to be applied weekly to be effective. As a result, planting resistant clones appears to be the best means of managing these diseases (Ostry 1987, Mottet *et al*. 1991, Newcombe and Ostry 2001).

In order to select disease resistant clones long term field trials have typically been conducted. These field trials last the length of a hybrid poplar rotation. For example, Schreiner (1972) based his selections on the results of a 15-year clonal test (Schreiner 1972). These trials are typically conducted at several sites using many different clones. Hansen *et al*. (1994) reported the field performance of 40 to 80 clones at 41 sites scattered in the Midwest selecting clones with lower disease severities across sites (Hansen *et al.* 1994). In another study Abrahamson *et al*. (1990) recommended superior clones based on tree growth, estimated from diameter at breast height and tree height, in combination with canker severity. They tested a total of 54 hybrid poplar clones (Abrahamson *et al*. 1990). Although these studies provide valuable information on the adaptation of different clones to specific environments disease escape is frequently a problem when attempting to determine relative levels of disease resistance (Abrahamson *et al*. 1990).

To avoid the issue of disease escape described above many breeding programs have adopted a screening assay involving artificial inoculation of young trees under field or greenhouse conditions, in order to evaluate disease resistance (Filer *et al.* 1971, Cooper and Filer

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1976, Zalasky 1978, Long *et al*. 1986, Ostry and McNabb 1985, Spielman 1986, Krupinsky 1989, Newcombe 1998, Weiland *et al.* 2003 and 2005, LeBoldus *et al.* 2010). These assays have typically involved some form of stem wounding to incite disease. Although the wounding procedure may circumvent some resistance mechanisms this procedure has been shown to accurately predict disease resistance, under field conditions, of the most resistant and susceptible clones (Weiland *et al.* 2003 and 2005). These studies used a similar inoculation procedure where stems of juvenile hybrid poplar clones had a leaf removed, a plug of sporulating mycelium placed on the wound which was then wrapped in parafilm (Filer *et al.* 1971, Cooper and Filer 1976, Zalasky 1978, Long *et al*. 1985, Ostry and McNabb 1985, Spielman 1986, Krupinsky 1989, Newcombe 1998, Weiland *et al.* 2003 and 2007, LeBoldus *et al.* 2010).

Recently, an inoculation protocol that does not rely on stem wounding to incite disease has been developed (LeBoldus *et al*. 2010). This protocol uses a conidial suspension to induce stem canker development and has three advantages over the wounding protocol: (i) relatively fast (resistance can be measured 3 weeks after inoculation); (ii) multiple isolates can be used simultaneously; and (iii) resistance mechanisms to the early stages of infection are not circumvented. Although this non-wounding inoculation procedure has produced results correlated with clonal response to wound inoculation (LeBoldus *et al.* 2010) the relationship of results from the non-wound inoculation and long term field performance are still unknown.

Study rationale and objectives

Due to the necessity for disease resistant clones in order to manage Septoria leaf spot and stem canker the majority of the research has focused on the selection of resistant clones (Feau *et al*. 2010). This has resulted in the development of a widely used resistance screening protocol which relies on artificial wounding. The recent development of the non-wound inoculation

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protocol by LeBoldus *et al*. (2010) may represent a significant improvement in the disease resistance screening process if responses are predictive of long term field performance (LeBoldus *et al.* 2010). Furthermore, this new assay will allow us to determine the mode of infection of *S. musiva* into host tissue and examine the role of NP in disease resistance when wounding is not used to incite disease.

The overall objective of this study was to develop a better understanding of the hybrid poplar-*S. musiva* pathosystem. The specific objectives were (i) test the predictive ability of this inoculation protocol for prediction of long-term canker damage categories; (ii) determine which disease severity parameters are best for prediction; (iii) describe the mode(s) of infection of *S. musiva* into nonwounded hybrid poplar stems; (iv) compare histological responses of resistant and susceptible clones following infection; and (v) determine when, in the infection process, differences in resistance to *S. musiva* occur.
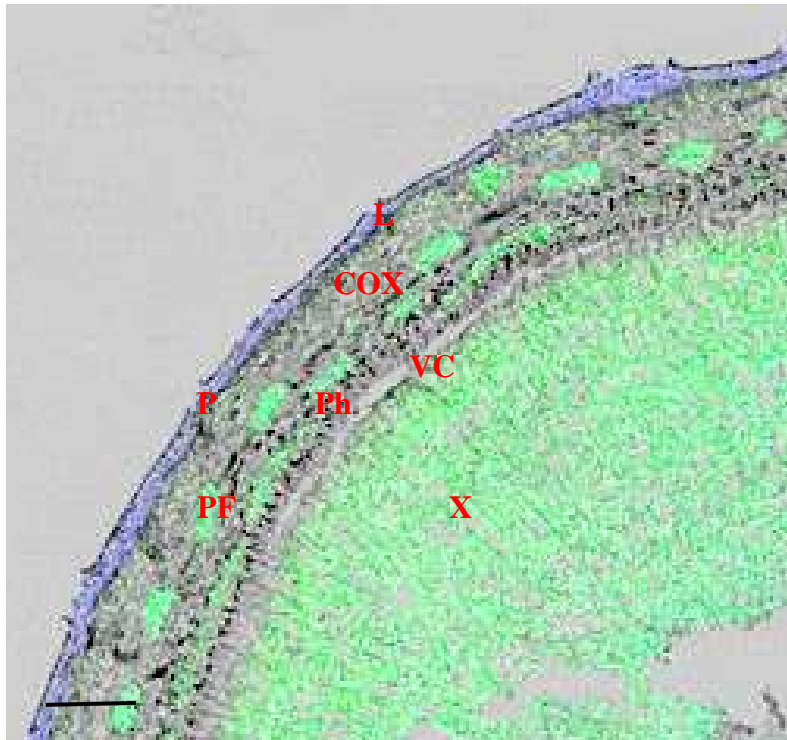
Figure 1.1. Anatomy of hybrid poplar clone DN74 (*Poplus deltoides* Marsh × *Poplus nigra* L.) under fluorescent microscope. COX = Cortex; L = Lenticel; P = Periderm; Ph = Phloem; PF = Phloem fiber; VC = Vascular cambium; X = Xylem. Scale bars = 200 µm.

Figure 1.2. Typical symptoms caused by *Septoria musiva* on juvenile tissue. (**A**): leaf spot with whitish center and black margin; and (**B**) stem canker with fruiting bodies embedded in whitish tissue and with dark brown margin.

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CHAPTER 1. A NON-WOUND GREENHOUSE SCREENING PROTOCOL FOR PREDICTION OF FIELD RESISTANCE OF HYBRID POPLAR TO SEPTORIA CANKER

Abstract

Populus species and their hybrids are short-rotation woody crops which supply fiber to a diversity of industries in North America. The potential of hybrid poplars has been limited by the fungal pathogen *Septoria musiva*, the cause of leaf spot and stem canker of *Populus* species. An inoculation protocol that does not rely on stem wounding to achieve infection was recently developed to screen poplar clones for resistance to Septoria canker. Young ramets of 14 clones of hybrid poplar that were previously assigned to long term canker damage categories (Low, Intermediate and High) were inoculated with a conidial suspension of three isolates of *S. musiva* under greenhouse conditions. Three weeks post-inoculation lesion number, lesions cm^{-1} stem length, area of stem that was necrotic, and proportion of stem area that was necrotic were measured. Logistic regression with lesion number and proportion necrotic area correctly predicted long term disease impact categories for 11/14 clones tested, including the most resistant (NM6) and the most susceptible (NC11505) clone demonstrating that this screening protocol is a promising method for prediction of long term disease impact of the most resistant clones.

Introduction

Populus species and their hybrids are an important forest resource in North America (Bier 1939). Trees in this genus are ideal for short rotation woody cropping (SRWC) systems due to their ease of propagation, phenotypic uniformity, and high

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growth rates (Dickmann 2001, LeBoldus *et al*. 2009, Stettler *et al.* 1996). Plantations of

Populus are currently being used for pulp and paper, value-added forest products, and are

a potential biofuel feedstock (Hansen *et al.* 1994, Eckenwalder 1996, Stettler *et al.* 1996,

Stoffle 1998, Balatinecz and Kretschmann 2001, Mercker 2007, McNeil Technologies

Inc. 2009, Sannigrahi *et al.* 2010). However, the growth and yield of *Populus* species and

their hybrids grown in SRWC systems are often impacted by several important diseases

(Waterman 1954, Long *et al.* 1986, LeBoldus *et al.* 2009). In the north-central and

eastern regions of North America the leaf spot and canker diseases caused by

Mycosphaerella populorum Thompson (Anamorph = *Septoria musiva* Peck) have had the

greatest impact (Bier 1939, Thompson 1941, Waterman 1954, Newcombe and Ostry

2001). The recent expansion of this pathogen into poplar producing regions such as the

Fraser Valley in British Columbia, Canada (Callan *et al.* 2007) and South America

(Sivanesan 1990) further highlight the importance of this pathogen.

Septoria leaf spot can impact growth and yield by reducing the leaf area available

for photosynthesis, and may cause premature defoliation of highly susceptible genotypes

(clones) (Bier 1939, Thompson 1941, Waterman 1954, Newcombe and Ostry 2001).

Cankers may kill distal portions of branches and stems. Stem cankers are defects that

reduce economic value, increase the risk of breakage, and may result in plantation failure

by killing highly susceptible clones (Bier 1939, Waterman 1954, Ostry and McNabb

1985, Newcombe and Ostry 2001). The importance of Septoria canker has resulted in

several studies evaluating the effectiveness of different management strategies. These

studies indicated that cultural, biological, or chemical controls are either too expensive or

have limited efficacy (Ostry *et al.* 1989, Yang *et al.* 1994, Gyenis *et al.* 2003). Therefore,

the selection and deployment of disease resistant clones is the most effective way to manage this disease (Ostry *et al.* 1989).

Early observations by Bier (1939) and Waterman (1954) indicated a relationship between response of clones to inoculation and Septoria canker damage in the field (Bier 1939, Waterman 1954). More recently, Weiland *et al.* (2003) inoculated young stems of poplar clones by placing mycelial plugs on wounds produced by removing a leaf. Clones had been categorized according to previously observed Septoria canker damage severity (Weiland *et al.* 2003). Results were predictive of long-term damage categories in both field (24 of 27 clones) and potted trees in the greenhouse (14 of 15 clones) experiments.

Although the feasibility and potential benefit of screening juvenile poplar clones was demonstrated, the procedures of Weiland *et al.* (2003) have possible disadvantages (Weiland *et al.* 2003). Numbers of clones and replicates are limited by available space, and responses to inoculation were evaluated after many weeks or months. Inoculum plugs bore a single pathogen isolate. Inoculation of wounds circumvents potential resistance mechanisms. LeBoldus *et al.* (2010) described an inoculation protocol in which very young, non-wounded ramets were inoculated with a conidial suspension in a greenhouse (LeBoldus *et al.* 2010). This allowed multiple isolates to be used simultaneously, and produced results correlated with clonal responses to wound inoculation in a relatively short period of time (LeBoldus *et al.* 2010). However, the ability of this protocol to predict long-term field performance of resistant and susceptible clones was not tested.

The current study was conducted using 14 clones of hybrid poplar assigned to long-term canker damage categories (Table 2.1) by Weiland *et al.* (2003 and 2005), based

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on multiple observations of these clones across regions (Hansen *et al.* 1983, Ostry and McNabb 1985, Long *et al.* 1986, Ostry 1987, Ostry *et al.* 1989, Strobl and Fraser 1989, Abrahamson *et al.* 1990, Hansen *et al.* 1994, Lo *et al.* 1995, Netzer *et al.* 2002). Clones were not wounded and were inoculated with a multiple-isolate conidial suspension. The goal was to improve and increase the practicality of greenhouse screening of hybrid poplar clones for resistance to Septoria canker. The specific objectives were to: (i) test the predictive ability of this inoculation protocol for prediction of long-term canker damage categories; and (ii) determine which disease severity parameters are best used for prediction.

Materials and methods

Host plant propagation

Fourteen hybrid poplar clones categorized according to previously observed Septoria canker damage categories (Weiland 2003 and 2005), were propagated from dormant branches collected in February 2012 at the University of Wisconsin-Madison Arlington Agricultural Research Station (Arlington, WI; Table 2.2). Cuttings 10-cm-long were soaked in distilled water for 48 h at room temperature (21°C) and then planted in SC10 Super cone-tainers (Stuewe & Sons® Deepots D40 cell; Stuewe & Sons Inc., Tangent, OR) containing SunGro® growing medium (SunGro® Professional Mix #8; SunGro Horticulture ® Ltd., Agawam, MA) amended with 12 g of nutricote slow release fertilizer (15-9-12) (N-P-K) (7.0% NH_3-N, 8.0% NO_3-N, 9.0% P_2O_5, 12.0% K_2O, 1.0% Mg, 2.3% S, 0.02% B, 0.05% Cu, 0.45% Fe, 0.23% chelated Fe, 0.06% Mn, 0.02% Mo, 0.05% Zn; Scotts® Osmocote Plus; Scotts Company Ltd., Marysville, OH). A 500 ppm solution of 20-20-20 liquid fertilizer (3.94% NH_3-N, 6.05% NO_3-N, 10.01% $CO(NH_2)_2$,

20.0% P_2O_5, 20.0% K_2O, 0.05% Mg, 0.0068% B, 0.0036% Cu, 0.05% Chelated Fe, 0.25% Mn, 0.0009% Mo, 0.0025% Zn; Scotts® Peters Professional; Scotts Company Ltd., Marysville, OH) was subsequently provided weekly. When trees reached a height of 30 cm, they were transplanted into pots (22 cm deep × 22.5 cm diameter; Stuewe & Sons® Treepot CP59R: Stuewe & Sons Inc., Tangent, OR) and fertilized as described above. Trees were grown in a greenhouse with an 18 h photoperiod supplemented with 600W high pressure sodium lamps and a 20°C/16°C (day/night) temperature regime.

Pathogen propagation and inoculation

Septoria musiva isolates, MN7, MN11, and MN23 were isolated from individual branch cankers collected from three different *Populus* trees (*Populus maximowiczii* A. Henry*, Populus trichocarpa* Torr. & A. Gary × *Populus deltoides* Marsh*,* and *P. deltoides* × *P. trichocarpa*) located in different plantings near Garfield, MN in 2012. The cankers were soaked in 5% bleach (NaClO 6%; Homelife ® Bleach Regular Scent; KIK Custom Products Inc., Houston, TX) for 2 minutes and rinsed twice with sterile distilled water. Bark was removed to expose the margin between healthy and necrotic tissue. From this area a 4-mm-long sliver of necrotic tissue was removed and placed on V-8 juice agar (137 ml V-8 juice, Campbell Soup Company, Camden, NJ; 1.5 g $CaCO_3$, ReagentPlus®, Research Organics Inc., Cleveland, OH; 15.2 g agar Difco, Franklin Lakes, NJ and 625 ml de-ionized water) in Petri plates. Subsequently, Petri plates were sealed with Parafilm and incubated at room temperature in constant light 30-cm below Gro-Lux full spectrum fluorescent bulbs (Sylvania; Osram Gmbh, Munich, Germany). After approximately 1 week, transfers were made to a second V-8 juice agar plate. Identity of pure cultures was

confirmed on the basis of conidial morphology (Sicanesan 1990). Isolates were stored at -80°C in vials containing 1 ml of a 50% glycerol solution.

Inoculum of each of the three isolates was produced from vials removed from cold storage. Each vial was poured onto a Petri plate containing V-8 juice agar and placed under light, as described above, for 5 d. Then four 5-mm-diameter sporulating masses of mycelium were aseptically transferred to each of 13 new plates of V-8 juice agar for each isolate. These plates were then placed under light as described above to induce sporulation. Conidia were harvested after 14 days by flooding each plate with 5 ml of sterile distilled water, rubbing the surface with a sterile loop, and removing the resulting spore suspension with a micropipette. The spore suspension for all 13 plates of each isolate was combined and the concentration adjusted to 1×10^6 conidia ml^{-1}. Equivalent volumes of each of the three conidial suspensions (MN7, MN11 and MN23) were then combined to conduct bulk inoculations. Inoculum for both trials was prepared in an identical manner.

Approximately 2 weeks after transplanting, stem height was measured and then trees were inoculated. Trees were removed from the greenhouse and each stem was sprayed with the conidial suspension and placed in separate black plastic bags at 21°C. After 48 h, plants were removed from the bags and returned to the greenhouse (LeBoldus *et al.* 2010). Twenty-one days after inoculation disease severity was evaluated. Lesion number (Lesion#) was a count of the number of necrotic lesions per stem. Lesions cm^{-1} was calculated by dividing canker number by the height of the tree at the time of inoculation. The necrotic area (NA) was determined by tracing lesions onto transparencies, digitizing the transparencies, and using Assess 2.0 software (APS, St.

Paul, MN) to measure the necrotic area in cm^2. The proportion necrotic area (PNA) was calculated by first estimating the stem surface area with the following formula: surface area = height × circumference. The NA was then divided by the surface area to determine PNA.

Experimental design

The experimental design was a randomized complete block design with six blocks among 14 clones. Each clone occurred once per block. Five blocks were inoculated with the conidial suspension and one block was a mock inoculated control (5 inoculated + 1 control block). A second trial was conducted with 10 blocks (8 inoculated + 2 control blocks) using the same methods. In both trials controls were used to confirm the absence of symptom development and subsequently discarded and not used in any statistical analysis.

Statistical analysis

Data from the inoculation trials were analyzed using SAS 9.2 (SAS Institute, Cary, NC) and significance was assessed at $\alpha = 0.05$. Initially, data from both trials was combined. The MIXED procedure in SAS (Littell *et al.* 2006) with clone and trial as fixed effects and block as a random effect was used to test for: equality of variances between trials, a significant interaction between clone and trial, and significant differences among main effects. Subsequently, data from each trial was analyzed separately and analyses of each of the four disease severity parameters (Lesion#, Lesions cm^{-1}, NA and PNA) were conducted independently. The MIXED procedure in SAS (Littel *et al.* 2006) was used to calculate the mean for each clone-disease severity parameter combination. The models used for mean estimation had clone as a fixed effect,

block as a random effect, and the repeated statement was used to model heterogeneous variances among clones. The means were then averaged across the two trials and used in the subsequent multinomial logistic regression analysis.

Fifteen separate models (Table 2.3) were compared in a two stage process: (i) fit of the model; and (ii) predictive power of the fitted model. In the first stage, models were compared using the deviance statistic. This statistic is used to compare a reduced model to the saturated model. It is assumed that the saturated model perfectly fits the data and that the reduced model will fit the data to a greater or lesser extent depending on the parameters included in that model. The larger the *P*-value of the deviance statistic the closer the reduced model is to the saturated model and the better the corresponding fit. In the second stage, the predictive power of the models were compared using the proportion of accurately predicted clones, percent concordance (% CC), percent discordance (% DC), Gamma, Tau-a, Somer's D, and the *c* statistic. Percent concordance and percent discordance represent how the predicted values are associated with the observed values (Allison 2012). The *c* statistic, Tau-α, Gamma, and Somer's D are statistics used to assess the predictive power of the model. For all four statistics the larger the value the higher the predictive power of the model.

Results

Necrotic lesions were first observed 2 to 3 weeks following inoculation. Latent period varied among clones and was shorter for more susceptible clones. Lesions were similar in appearance to incipient Septoria cankers observed in the field, and first appeared as small, elliptical areas of water-soaked cells on the surface of stems. These water-soaked areas became necrotic and coalesced to girdle the stem of susceptible

clones. Occasionally, the lesions bore pycnidia in light tan centers surrounded by black margins. The majority of lesions developed on the lower third of inoculated trees in association with lenticels and stipules. On highly susceptible clones; however, cankers also developed apparently due to direct penetration (i.e., at sites other than lenticels or stipules) on stems. Approximately 2 weeks after inoculation, swelling developed along the margin of necrotic and healthy tissue preventing further canker expansion on stems of resistant clones. No symptoms developed on control trees. Controls were not included in any further analysis.

The variances of the two trials were statistically similar and there was no significant clone by trial interaction across the four parameters. However, there were significant differences between trial 1 and 2 for Lesion# ($P < 0.001$), Lesions cm^{-1} ($P < 0.001$), and NA ($P = 0.002$), but not PNA ($P = 0.114$). As a result each trial was analyzed separately and the means of each clone-disease severity parameter combination were estimated (Table 2.2). The analysis indicated that there were significant differences among clones across all four parameters in both trials ($P_{Lesion\#} < 0.001$; $P_{Lesions/cm} < 0.001$; $P_{NA} < 0.001$; and $P_{PNA} < 0.001$). Overall, NM6 was the most resistant clone and NC11505 was the most susceptible clone regardless of the disease severity parameter (Table 2.2). The average number of lesions per clone ranged from 1.6 for NM6 to 39.6 for NC11505; the mean number of lesions cm^{-1} ranged from 0.1 for NM6 to 1.5 for NC11505; the mean NA ranged from 0.6 cm^2 for NM6 to 40.8 cm^2 for NC11505; and the PNA ranged from 0.01 for NM6 to 0.14 for NC11505 (Table 2.2). In order to develop a model to predict long term disease severity categories based on the non-wound inoculation protocol means for each clone-disease severity parameter combination were averaged across both trials.

Across the 15 tested models, four low damage category clones (DN74, DN34, MWH5 and NM6) and five high damage category clones (NC11432, NC11505, NC5271, NE308 and NE351) were correctly predicted to be resistant and susceptible, respectively (Table 2.4). The model with Lesion# and PNA as explanatory variables had the best fit, relative to the saturated model ($P = 0.63$; Table 2.3). Somer's D (0.78), Gamma (0.78), Tau-a (0.54), and the c-statistic (0.89) indicated that this model had the highest predictive power relative to all but one of the other models. Only the full model (Lesion# + Lesion^{-1} cm + NA + PNA) had greater predictive ability (Table 2.3). However, the proportion of accurately predicted clones, for the model with Lesion# + PNA (11/14) was greater than the full model (10/14).

Discussion

Inoculation of poplars with conidia of *S. musiva* has produced varying results in previous studies. Lesions that developed in the current study were similar in appearance to those observed on inoculated *P. balsamifera* trees by Zalasky (1978) and LeBoldus *et al.* (2010). Bier (1939) used similar methods to induce stem lesion development at the base of leaves and surrounding lenticels of non-wounded hybrid poplar clones. Krupinsky (1989) reported that a small number of cankers developed on young succulent tissue of the most susceptible clones. In contrast, cuttings of NE338 (NC11505 in this study), a highly susceptible clone, did not develop stem lesions after inoculation with a conidial suspension by Long *et al.* (1986). A consistent difference, based on the above authors' descriptions, between three of the studies in which stem lesions developed (Zalasky 1978, Krupinsky 1989, LeBoldus *et al.* 2010) and the one that they did not (Long *et al.* 1989) was the age of the trees. Long *et al.* (1989) inoculated 25-week-old trees whereas Zalasky

(1978), LeBoldus *et al.* (2010), and Krupinsky (1989) all inoculated trees ≤ 12 weeks of age. Bier (1939) did not report the age of the trees at the time of inoculation. One possible explanation for the lack of canker development on older trees is the period of time, lasting several weeks, where trees lose their epidermis and develop periderm and phloem fibers (Esau 1969). During this period of development the bark (epidermis) is relatively thin and may be more easily penetrated by *S. musiva* (Zalasky 1978). As the tree ages the thickening periderm might prevent infection without prior wounding.

To this author's knowledge, this study and that of LeBoldus *et al.* (2010) are the only studies where statistically significant differences in susceptibility among clones were detected for trees inoculated by conidial suspension (Table 2.2). Neither Bier (1939) nor Krupinsky (1989) reported differences in severity on inoculated trees. Although, Zalasky (1978) indicated that differences in response to inoculation were not apparent among inoculated seedlings, he was unable to make statistical comparisons due to a lack of replication. In this study not only were significant differences among clones apparent and consistent across the two trials, but the results were also predictive of long term field performance.

An examination of the predictive models tested in this study indicates that a measure of both lesion severity and lesion number result in the greatest number of accurately predicted clones. The combination of Lesion# and PNA (Table 2.3) allows the model to take both the number of lesions and the size of those lesions into consideration when predicting long term damage categories. For example, a model with PNA only would place clones with an equal number of small lesions and a single large lesion in the same category. However, the clone with a large number of small lesions may be resistant

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and survive whereas the clones with a single large lesion likely would not. These results are consistent with Weiland *et al*. (2003) who found that the parameters of canker incidence and girdle had the greatest predictive ability in both the greenhouse and field studies in which seedlings were wound-inoculated.

There are several possible explanations for the lack of complete predictive ability of this study with the previously assigned damage categories. In field studies, clones NC5271, NC5260, and NE222 displayed a range of disease severities across regions (Hansen *et al.* 1983, Lo *et al.* 1995, Weiland *et al.* 2003 and 2005) potentially contributing to uncertainty as to whether their previously assigned canker damage category accurately reflected resistance or susceptibility to Septoria canker. This may have been exacerbated by the different Septoria canker rating methods used by various researchers. For example, Lo *et al.* (1995) used canker number to place clones into four classes; whereas, Hansen *et al.* (1983), used a different method to evaluate Septoria canker in the mid-west. As a result clones were placed in different categories based on these different rating systems, perhaps explaining placement in the Intermediate category by Weiland *et al*. (2003).

A second possibility for lack of correct prediction of the damage category may be related to the relative disease tolerance of specific clones. For example, clone A and clone B may exhibit similar disease severities following experimental inoculation. However, clone A may not show a corresponding reduction in growth, yield, or survival, whereas clone B does. This may be reflected in the assay's low predictive ability for clones in the intermediate damage category. These clones may have similar levels of disease severity 3-weeks following inoculation; however, field performance may differ

resulting in the final placement of some clones in the intermediate or low damage category, based on their field performance, and others assigned to the high damage category. A third possibility may be the presence of specificity in the pathogen population. Several authors (Krupinsky 1989, Ward and Ostry 2005, LeBoldus *et al.* 2010) detected a small but significant clone × isolate interaction in their inoculation assays. This may explain the discrepancy between the predictions of the wound inoculation protocol and the non-wound inoculation protocol both of which used different isolates. However, further exploration of the significance and magnitude of this effect needs to be conducted.

There are two main advantages of the conidial inoculation of non-wounded seedlings. Due to the time and space requirements of screening multiple clones with the wound-inoculation assay several authors (Lo *et al.* 1995, Ward and Ostry 2005, Weiland *et al.* 2003 and 2005, LeBoldus *et al.* 2010) have suggested that a single highly virulent isolate could be used to select the most resistant clones for field testing. However, Feau *et al.* (2005) found evidence of sexual reproduction in the pathogen population suggesting that *S. musiva* virulence may shift in response to the deployment of resistant clones. The ability of the pathogen to reproduce sexually and the potential presence of a clone × isolate interaction, described above (Kruspinsky 1989, Ward and Ostry 2005, LeBoldus *et al.* 2010), suggests that using multiple isolates to screen for resistance is more likely to select the most durable resistance under field conditions.

The second advantage is the cost savings associated with conducting a preliminary greenhouse screening. Disease resistance evaluation under field conditions requires thousands of trees, at replicated field sites, measured over several years (Table

2.1). Plantation establishment costs are proportional to the number of planted cuttings, routine maintenance, pest control, irrigation, and fertilization costs (Hansen *et al.* 1983). Savings can be achieved by reducing the overall number of clones tested by eliminating the most susceptible clones prior to field testing. The non-wound greenhouse screening protocol, described above, would facilitate this process.

The consistency in predictive ability of results obtained by inoculation of wounds by Weiland *et al.* (2003) and from conidial inoculation in this study may provide clues as to the type of resistance mechanism being evaluated. In both cases, it is likely that post penetration resistance among clones is being compared. If this is the case then both inoculation assays are likely to provide similar predictions of long term field performance. The results from this study and the correlation between wound and non-wound inoculations reported by LeBoldus *et al.* (2010) support this hypothesis. Furthermore, the reliability of the conidial inoculation protocol presents an opportunity, initially proposed by Newcombe and Ostry (2001), to elucidate the genetic mechanism of Septoria canker resistance. Newcombe and Ostry (2001), in a field study conducted in Minnesota and Iowa, evaluated Septoria canker resistance in a three generation pedigree of *P. deltoides* × *P. trichocarpa*. and found evidence supporting the recessive inheritance of Septoria canker resistance. However, due to disease escape and variation in pathogen virulence they could not conclude that resistance was conferred by a single gene. As a result they suggested that further testing of the hypothesis be conducted in a greenhouse. A greenhouse assay, similar to that described above, would ensure that trees were inoculated with a single spore isolate of known virulence, at a specific concentration, and

set volume of inoculum eliminating the sources of variability, disease escape and variation in pathogen virulence, described in the study by Newcombe and Ostry (2003).

Continued evaluation of clones selected for scale up based on field observations or responses to inoculations are warranted. For example, clone NM6 was placed in the low canker damage category by Weiland *et al.* (2003) based on previous reports and response to inoculation in the current study (Abrahamson *et al.* 1990, Hansen *et al.* 1994, Lo *et al.* 1995, Netzer *et al.* 2002). There has been a report of high incidence and severity of canker disease damage to that clone in the field in Wisconsin (Waterman 1954); however, and commercial plantings of NM6 have been severely impacted in Minnesota (personal communication, Jared LeBoldus). The reasons for these discrepancies are unclear. The role of environmental conditions in disease development may offer a potential explanation. For example, Maxwell *et al.* (1997) reported an effect of water stress on Septoria canker disease severity of clone NM6 (Maxwell *et al.* 1997). Similar effects of water stress on disease severity and response to wounding have been reported in other systems (Mullick 1977, Biggs *et al.* 1983, Long *et al.* 1986). The role of environmental conditions in disease development needs to be clarified in order to better understand this phenomenon; however, this was beyond the scope of this study.

Conidial inoculation of non-wounded seedlings can be integrated into hybrid poplar breeding programs to accurately predict the long term disease impact categories of the most resistant and susceptible clones. Compared to other methods, this procedure is faster, less resource intensive, allows combinations of isolates to be used simultaneously, and does not circumvent potential resistance mechanisms by wounding. This procedure is currently being used by the hybrid poplar breeding program at the University of

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Minnesota-Duluth to screen D $\times$ N hybrids prior to deployment in the field (LeBoldus *et al*. unpublished data).

Table 2.1. Summary of seven field studies, including number of field sites, number of clones, observation period and tree age.

Reference	Number of field sites	Numbers of clone	Observation period	Tree age
Schreiner (1972)	2	40	1970 to 1971	15- to 20-year-old
Hansen et al. (1983)	3	34	1976 to 1981	----
Hansen et al. (1994)	30	40 - 80	1991 to 1992	5- to 6-year-old
Abrahamson et al. (1990)	1	54	1986 to 1987	3-year-old
Lo et al. (1995)	1	54	1993	9-year-old
Netzer et al. (2002)	16	95	1987 to 1992	7- to 12-year-old
Ostry and McNabb (1985)	3	34	1976 to 1982	5-year-old

Table 2.2. Parentage, assigned damage categories (High, Intermediate, Low), mean and standard deviation of disease severity parameters (Lesion#, Lesion cm^{-1}, NA and PNA)[a] of 14 hybrid poplar clones. The clones were inoculated in the greenhouse with spraying conidial suspension of three different isolates (MN7, MN11 and MN23) mixture of *Septoria musiva*.

Clone	Par[b]	Assigned damage cat.[c]	Lesion#	Lesion cm^{-1}	NA (cm^2)	PNA
			Meand (SD)[e]	Mean (SD)	Mean (SD)	Mean (SD)
NM6	4×3	L	1.6 (3.0)	0.1 (0.1)	0.6 (1.0)	0.00 (0.00)
DN74	2×4	L	3.8 (3.4)	0.2 (0.2)	1.2 (0.8)	0.00 (0.00)
DN34	2×4	L	7.1 (11.6)	0.2 (0.3)	2.1 (1.8)	0.01 (0.01)
DN164	2×4	L	7.8 (10.2)	0.4 (0.4)	2.3 (1.2)	0.01 (0.01)
DN177	2×4	L	17.8 (13.0)	0.5 (0.3)	8.8 (5.5)	0.02 (0.01)
NC5260	9×1	I	4.6 (5.5)	0.4 (0.3)	1.7 (0.6)	0.01 (0.00)
NE222	2×5	I	9.2 (7.2)	0.3 (0.2)	6.2 (5.9)	0.02 (0.01)
NC5271	7×5	I	13.3 (10.6)	0.5 (0.2)	10.2 (7.8)	0.04 (0.03)
MWH13	2×3	H	7.0 (8.8)	0.3 (0.2)	4.1 (4.5)	0.01 (0.02)
MWH5	2×3	H	3.6 (3.4)	0.3 (0.1)	1.6 (1.1)	0.02 (0.00)
NE308	7×6	H	14.0 (7.8)	0.5 (0.2)	7.8 (3.4)	0.03 (0.02)
NC11432	2×8	H	23.4 (11.7)	0.8 (0.2)	15.0 (8.6)	0.05 (0.04)
NE351	2×5	H	24.5 (14.0)	0.6 (0.1)	14.7 (10.3)	0.05 (0.04)
NC11505	3×8	H	39.6 (8.8)	1.4 (0.2)	40.8 (24.4)	0.14 (0.07)

[a] Lesion# = Lesion number, Lesion/cm = $\dfrac{Lesion\ number}{Height\ (cm)}$, NA = Necrotic area and Proportion necrotic area (PNA)

$$= \dfrac{Necrotic\ area\ (cm2)}{Height\ \times Circusference\ (cm2)}.$$

[b] Par. = Parentage. Numbers refer to *Populus* spp. and hybrids as follows: 1 = *balsamifera*, 2 = *deltoides*, 3 = *maximowiczii*, 4 = *nigra*, 5 = *nigra* var. *caudina*, 6 = *nigra* var *incrassate*, 7 = *nigra* var. *plantierensis*, 8 = *trichocarpa* and 9 = *tristis*.

[c] Cat. = Disease damage categories of Weiland *et al*. (L = Low, I = Intermediate, H = High) (Weiland *et al*. 2003 and 2005).

[d] Mean was calculated from 13 individuals of each clone.

[d] SD = Standard deviation, calculated from 13 individuals of each clone.

Table 2.3. Goodness-of-fit (deviance) and predictive ability (overall proportion, percent concordant, percent disconcordant, Somer's D, Gamma, Tau-a and c-statistic) results from logistic regression analysis of data from 14 hybrid poplar clones. The clones were inoculated in the greenhouse with spraying conidial suspension of three different isolates (MN7, MN11 and MN23) mixture of *Septoria musiva*.

Model	Dev. $P > \chi^{2a}$	Overall Proportion[b]	% CC[c]	% DC[d]	SD[e]	Gamma[f]	Tau-a[g]	c[h]
Lesion# + Lesion cm^{-1} + NA + PNA	0.54	10/14	90.5	9.5	0.81	0.81	0.56	0.91
Lesion# + NA + PNA	0.58	10/14	88.9	11.1	0.78	0.78	0.54	0.89
Lesion# + Lesion cm^{-1} + NA	0.49	9/14	85.7	14.3	0.71	0.71	0.50	0.86
Lesion cm^{-1} + NA + PNA	0.55	9/14	84.1	15.9	0.68	0.68	0.47	0.84
Lesion# + Lesion cm^{-1} + PNA	0.60	11/14	88.9	11.1	0.78	0.78	0.54	0.89
Lesion# + NA	0.52	9/14	84.1	15.9	0.68	0.68	0.47	0.84
Lesion cm^{-1} + NA	0.49	8/14	77.8	22.2	0.56	0.56	0.39	0.78
NA + PNA	0.61	10/14	82.5	17.5	0.65	0.65	0.45	0.83
Lesion cm^{-1} + PNA	0.53	9/14	77.8	22.2	0.56	0.56	0.39	0.78
Lesion# + PNA	0.63	11/14	88.9	11.1	0.78	0.78	0.54	0.89
Lesion# + Lesion cm^{-1}	0.41	8/14	71.4	28.6	0.43	0.43	0.30	0.71
Lesion#	0.45	8/14	71.4	28.6	0.43	0.43	0.30	0.71
Lesion# cm^{-1}	0.46	7/14	69.8	28.6	0.42	0.42	0.29	0.71
NA	0.52	8/14	71.4	28.6	0.43	0.49	0.30	0.71
PNA	0.59	8/14	79.4	20.6	0.59	0.59	0.41	0.80

[a] *P*-value of the deviance of goodness-of-fit test. [b] The proportion of clones which are accurately predicted in greenhouse experiment.

[c] %CC = Percent concordant. [d] %DC = Percent discordant. [e] SD = Somer's D. Somer's D $= \frac{CC - DC}{CC + DC - T}$. [f] Gamma $= \frac{CC - DC}{CC + DC}$.

[g] Tau-a $= \frac{CC - DC}{N}$. N = Total number of pairs. [h] $C = c$-statistics. $c = 0.5 \times (1 + SD)$.

Table 2.4. The best linear unbiased estimates (MEANs) of final model (Lesion# + PNA) of 14 hybrid poplar clones. This model is used to predict the probability of placement in the low (L), intermediate (I), or high (H) categories contrasted with the assigned damage category (L, I, H). Fourteen clones were inoculated with conidial suspension of three different isolates (MN7, MN11 and MN23) mixture of *Septoria musiva*.

Clone	Assigned damage cat.[a]	Predicted damage cat.[b]	Prob. of placement in cat. (%)[c]		
			L	I	H
NM6	L	L	49	33	18
DN74	L	L	57	29	14
DN34	L	L	71	21	8
DN164	L	L	73	20	7
DN177	L	L	74	19	7
NC5260	I	L	57	29	14
NE222	I	I	35	36	29
NC5271	I	H	2	7	90
MWH13	H	H	26	36	38
MWH5	H	L	47	33	19
NE308	H	H	13	28	59
NC11432	H	H	6	17	77
NE351	H	H	2	6	92
NC11505	H	H	0	0	100

[a] Assigned damage categories of Weiland *et al.* (2003) (L = low, I = intermediate, H = high)

[b] Predicted damage categories were based on probability of placement (L = low, I = intermediate, H = high) given response to conidial suspension inoculation.

[c] Probability of placement in categories (%) (L = low, I = intermediate, H = high)

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CHAPTER 2. INFECTION BIOLOGY AND HOST RESPONSE OF HYBRID POPLAR STEMS INOCULATED WITH SEPTORIA MUSIVA

Abstract

Trees in the genus *Populus* and their interspecific hybrids are used across North America in shelter belts, fiber production, and as a potential source of biofuel. Plantations of these species are severely impacted by a fungal pathogen, *Mycosphaerella populorum* (Anamorph = *Septoria musiva*), the cause of leaf spot and stem canker. The majority of the research has focused on the development of disease resistant clones. An inoculation protocol that does not rely on stem wounding to achieve infection was recently developed. Using this protocol two experiments were conducted to examine the infection biology and disease etiology. In the first experiment, non-wounded stems of one resistant clone (NM6) and one susceptible clone (NC11505) were inoculated and examined by scanning electron microscope at six different times (6 h, 12 h, 24 h, 72 h, 1 week, and 3 weeks) post inoculation. The images indicate that the pathogen appears to enter host tissue through small openings and lenticels and that there are no significant differences in the penetration rate between resistant and susceptible clones at 12 h post inoculation. In a second experiment, a histological comparison of stem cankers for resistant clone DN74 and susceptible clone NC11505 were conducted at three time points (3 weeks, 5 weeks and 7 weeks) post inoculation. Distinct differences in disease etiology were apparent between the resistant and susceptible clones. The results from these two experiments support the hypothesis that resistance responses to stem infection may be occurring post penetration.

Introduction

Mycosphearella populorum Thompson (Anamorph = *Septoria musiva* Peck) causes Septoria leaf spot and stem canker diseases of poplar species and their hybrids in eastern and

central North America (Waterman 1954, Cellen *et al.* 2007). Severe leaf spot disease can result

in premature defoliation, and stem and branch infections can lead to stem defects, breakage, and

mortality (Bier 1939, Waterman 1954, Ostry *et al* 1985, Newcombe 1996). In many cases, severe

outbreaks of stem canker limit the commercial viability of poplar plantations (Bier 1939,

Newcombe 1996). Resistant genotypes (clones) are typically considered the most effective way

to manage this disease (Ostry and McNabb 1985, Newcombe and Ostry 2001). The identification

of these clones has been conducted by wound inoculations in both the greenhouse (Filer *et al.*

1971, Weiland *et al.* 2003 and 2005, LeBoldus *et al.* 2010) and field (Zalasky 1978, Long *et al*.

1985, Ostry and McNabb 1985, Spielman 1986, Krupinsky 1989, Newcombe 1998, Weiland *et

al.* 2003 and 2005). Evaluation of disease severity in naturally infected field plantings has also

been used to evaluate disease resistance (Hansen *et al.* 1983, Ostry and McNabb 1985, Long *et

al.* 1986, Ostry 1987, Ostry *et al.* 1989, Strobl and Fraser 1989, Abrahamson *et al.* 1990, Hansen

et al. 1994, Lo *et al.* 1995, Netzer *et al.* 2002). Although these procedures have successfully

identified resistant clones, very little information is available regarding the mode of infection of

the pathogen into woody tissue and subsequent disease development (Ostry and McNabb 1985).

A review of the literature indicates that *S. musiva* is typically considered to be a pathogen

associated with wounds largely why the wound inoculation assay was developed as a screening

tool (Ward and Ostry 2005, Weiland *et al.* 2003 and 2005, LeBoldus *et al.* 2009, LeBoldus *et al.*

2010). However, some authors have also indicated that the pathogen may be able to penetrate

host tissue through natural openings including lenticels, stipule scars, and petioles (Bier 1939,

Waterman 1954, Zalasky 1978, Long *et al*. 1986). For example, Bier (1939) reported necrotic

lesion development surrounding lenticels, petioles and stipule scars of non-wounded hybrid

poplars. Similar findings were reported in other studies (Zalasky 1978, Long *et al*. 1986,

Krupinsky 1989). It has also been noted that on the most susceptible clones lesions seemed to develop without any association with natural openings (Zalasky 1978). The recent development of a consistent non-wound inoculation protocol permits the investigation of the infection biology of *S. musiva* (LeBoldus *et al.* 2010).

In similar studies examining the infection process, scanning electron microscopy (SEM) has been used to visualize the mode of infection of a fungal pathogen (Smith and Oatley 1955, Mims 1991, McMullan 1995). For example, Pegg *et al.* (2009) studied the mode of penetration of shoot blight on eucalyptus leaves by *Quambalaria* spp., Graca *et al.* (2009) verified the pattern of infection of *Cylindrocladium pteridis* into eucalyptus leaves and Roderick and Thomas (1997) examined the factors influencing the infection of three rust fungi on ryegrass. These studies provided valuable information on the infection biology and disease progress in the early stages of the host–parasite interaction. However, SEM is limited to an examination of tissue surfaces leaving many aspects of the interaction uncharacterized.

In order to examine anatomical changes to host tissue following infection a histological analysis is typically conducted (Biggs *et al.* 1983a, 1983b and 1984, Biggs 1984 and 1986b, Hebard *et al.* 1984, Enebak *et al*. 1997). In the hybrid poplar-Septoria pathosystem, Weiland and Stanosz (2007) wound inoculated the susceptible clone NC11505 (*Populus maximowiczii* A. Henry × *Populus trichocarpa* Torr. & A. Gary) and the resistant clone DN34 (*Populus deltoides* Marsh × *Populus nigra* L.). DN34 exhibited minimal necrosis with the development of continuous necrophylatic periderm (NP) in close proximity to the inoculation point, apparently limiting pathogen development. In contrast, the susceptible clone NC11505 developed extensive necrosis with several successive layers of thin NP, which were located further away from the point of infection and were often disrupted by phloem fibers (Weiland and Stanosz 2007). The

disruption of NP was absent in the resistant clone and the authors hypothesized that the pathogen was able to circumvent the NP by passing through these phloem fibers (Weiland and Stanosz 2007). The results are similar to other pathosystems where histological comparisons of resistant and susceptible trees inoculated by wounding were conducted (Soo 1977, Biggs *et al*. 1983b, Hebard *et al.* 1984, Biggs 1984 and 1986, Enebak *et al*. 1997).

The formation of NP is considered to be, by most authors, a non-specific host response resulting from disruption of the phellogen by mechanical injury, insect damage, or pathogen invasion (Mullick 1977, Soo 1977, Biggs *et al.* 1984, Biggs 1986a). Although some minor differences in the anatomy of NP formation have been described when wounds alone and wounds inoculated with fungal pathogens have been compared, it can be difficult to differentiate host response to mechanical injury from host response to pathogen invasion (Biggs 1986a). On the one hand, the comparative study of wounded and non-wounded inoculation protocol demonstrated that a potential resistance mechanism might occur post-penetration. As a result using non-wounded *Populus* trees inoculated with a conidial suspension could detail the host response in the absence of wounding. In this study, the author will compare resistant: DN74 (*P. deltoides* × *P. nigra*) & NM6 (*P. maximowiczii* × *P. nigra*) and susceptible (NC11505) clones to: (i) describe the mode(s) of infection of *S. musiva* into non-wounded hybrid poplar stems; (ii) compare histological responses of resistant and susceptible clones following infection without wounding; and (iii) determine when, in the infection process, differences in resistance to *S. musiva* occur.

Materials and methods

Part I. Infection biology

Host plant propagation

Dormant branches of the susceptible clone NC11505 and resistant clones NM6 and DN74 were collected in February, 2012 at the University of Wisconsin-Madison Arlington Agricultural Experiment Station (Arlington, WI) and cut into 10-cm lengths. Cuttings were initially soaked in distilled water at room temperature (21℃) for 48 hours, and then planted in SC10 Super cone-tainers (Stuewe & Sons® Deepots D40 cell; Stuewe & Sons Inc., Tangent, OR) containing SunGro® growing medium (SunGro® Professional Mix #8; SunGro Horticulture® Ltd., Agawam, MA) amended with 12 g of nutricote slow release fertilizer (15-9-12) (N-P-K) (7.0% NH_3-N, 8.0% NO_3-N, 9.0% P_2O_5, 12.0% K_2O, 1.0% Mg, 2.3% S, 0.02% B, 0.05% Cu, 0.45% Fe, 0.23% chelated Fe, 0.06% Mn, 0.02% Mo, 0.05% Zn; Scotts® Osmocote Plus; Scotts Company Ltd., Marysville, OH). This was supplemented on a weekly basis with a 500 ppm solution of liquid fertilizer (20-20-20) (N-P-K) (3.94% NH_3-N, 6.05% NO_3-N, 10.01% $CO(NH_2)_2$, 20.0% P_2O_5, 20.0% K_2O, 0.05% Mg, 0.0068% B, 0.0036% Cu, 0.05% Chelated Fe, 0.25% Mn, 0.0009% Mo, 0.0025% Zn; Scotts® Peters Professional; Scotts Company Ltd., Marysville, OH). Planted cuttings were placed on a greenhouse bench with an 18-hour photoperiod, supplemented with 600W high pressure sodium lamps, and a 20°C/16°C (day/night) temperature regime. Trees were transplanted into plastic pots (22 cm deep × 22.5 cm diameter; Stuewe & Sons® Treepot CP59R: Stuewe & Sons Inc., Tangent, OR) when they reached a height of 30 cm.

<u>Pathogen propagation and inoculation</u>

Septoria musiva was isolated from branch cankers, collected from hybrid poplars located near Garfield, MN. The cankers were soaked in a 5% bleach solution (NaClO 6%; Homelife® Bleach Regular Scent; KIK Custom Products Inc., Houston, TX) for 2 minutes and then rinsed twice with sterile distilled water. Bark was carefully removed from the canker margin and a 4-mm long sliver of tissue were placed on V-8 juice agar (137 ml V-8 juice, Campbell Soup Company, Camden, NJ; 1.5 g $CaCO_3$, ReagentPlus®, Research Organics Inc., Cleveland, OH; 15.2 g agar Difco, Franklin Lakes, NJ and 625 ml de-ionized water). Petri plates were sealed with Parafilm and incubated at room temperature (21℃) 30-cm below continuous light (Gro-Lux full spectrum fluorescent bulbs: Sylvania; Osram Gmbh, Munich, Germany). After one week, colonies resembling *S. musiva* were transferred onto a second V-8 juice agar plate and identified based on conidial morphology (Sivanesan 1990). Pure *S. musiva* cultures were stored at -80°C in vials containing 1 ml of 50% glycerol solution.

Each isolate (MN7, MN11, and MN23) was recovered from cold storage by pouring 1 ml glycerol solution onto one Petri plate containing V-8 juice agar. Three plates of each isolate were grown on the light bench described above. Five days later sporulating colonies were aseptically transferred onto 13 new V-8 juice agar plates and incubated on the light bench until sporulation occurred. Conidia were harvested by flooding the plates with 5 ml of sterile distilled water and lightly rubbing the surface of the plate with a sterile loop. For each isolate conidial suspensions harvested from each plate were combined and the concentration was adjusted to 1×10^6 conidia ml^{-1}. Equal volumes of each isolate were combined and the bulked spore suspension was used for inoculations. Four weeks after transplanting, the stems of each tree were inoculated, using a spray bottle and the spore suspension as described by LeBoldus *et al*. (2010). Twelve trees of the

susceptible clone NC11505 and Twelve trees of the resistant clone NM6 were inoculated. Two control trees from each clone were inoculated in an identical manner except that sterile distilled water was used rather than a spore suspension.

The spore germination rates of 4 time points (6 h, 12 h, 24 h and 72 h) were estimated by spraying inoculum onto 3 water agar (WA) plates (10 g agar Difco, Franklin Lakes, NJ and 500 ml de-ionized water) kept in the dark for 48 h.

Experimental design

The experimental design was a completely randomized design. At each of six time points (6 h, 12 h, 24 h, 72 h, 1 week, and 3 weeks) post inoculation (PI) two trees of each clone were harvested, with one exception. Only a single stem from each clone was harvested at 1 week PI. In addition, a single non-inoculated control tree of each clone was harvested at 3 weeks PI. Two cankers were randomly selected from the lower 15 cm of each stem. Cankers were sampled such that a 5 cm segment of stem, centered on the canker, was collected. In addition four 5-cm segments, from the lower 15 cm of the tree and the top 15 cm were harvested from each of the controls. All samples were fixed in a 2.5% solution of glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.4; Tousimis Research Corporation, Rockville, MD) and stored at 4°C for three days. A total of 4 cankers per time point and 8 control segments were examined for each clone.

Scanning electron microscopy

The fixed stems were split longitudinally, so that both surfaces could be observed, and then dehydrated in an ethanol series from 30% to 100%. The split samples were critical-point dried using an Autosamdri 810 critical point drier (Tousimis Research Corporation, Rockville, MD) with liquid carbon dioxide as the transitional fluid. Longitudinal sections were attached to aluminum mounts with silver paint (SPI Supplies, West Chester, PA) and sputter coated with

gold/palladium (Balzers SCD 030, Balzers Union Ltd., Liechtenstein). Images were obtained

using a JEOL JSM-6490LV SEM (JEOL Ltd., Japan) operating at an accelerating voltage of 15

kV. Comparisons between penetration rates on the resistant and susceptible clones were

conducted on images at 12 h PI. A total of 100 spores were counted in 2 to 3 fields of view, this

was repeated 3 times and a t-test was used to compare penetration rates between the two clones

($\alpha = 0.05$).

Part II. Host response to non-wound inoculations

Host plant propagation, pathogen propagation, and inoculation

Plants were propagated in a similar manner to that described above, with the following

exception: DN74 rather than NM6 was used as the resistant clone. Inoculum production and

inoculation were also conducted as described above.

Experimental design

The experimental design was a completely randomized design. A total of 6 stem

segments, approximately 5 cm in length and centered on cankers, were collected from each clone

at each of the following time points: 3 weeks, 5 weeks and 7 weeks PI. Six stem segments from a

mock inoculated control of each clone were also harvested at 7 weeks PI. In total 48 segments

(36 inoculated and 12 controls) were fixed in 10 ml of formalin-acetic acid-ethyl alcohol (FAA,

10:5:50) for one week at 21℃.

Histology

Fixed stem canker segments were dehydrated in an automated tissue processor (Leica

Microsystems Inc., Buffalo Grove, IL) following the manufacturer's instructions. The samples

were then embedded with Paraffin Plus (Fisher Scientific Co., Houston, TX) using a Leica

embedding machine (Leica Microsystems Inc., Buffalo Grove, IL). Longitudinal and transverse sections of the cankers were then made using a rotary microtome (Leica Microsystems Inc., Buffalo Grove, IL) set to a thickness of 20 µm. Three segments were sectioned transversely through the top, middle, and bottom of each canker and the other three were sectioned longitudinally through the center of each canker. Several sections were made at each location. All sections were placed on microscope slides (Fisher Scientific Co., Houston, TX), de-waxed in Histo-Clear (Fisher Scientific Co., Houston, TX), stained using a Safranin O-Fast green protocol (Gram and Jorgensen 1953) and mounted using Permount (Sigma-Aldrich Co., St. Louis, MO). Sections were examined by fluorescence and bright field microscopy using a Zeiss Axio Imager M2 microscope. Blue auto-fluorescence viewed with ultraviolet light (Excitation filter G 365, Beam Splitter FT 395, Emission filter BP 445/50) and green auto-fluorescence viewed with blue-green light (Excitation filter BP 450-490, Beam Splitter FT 510, Emission filter BP 515-565) were used to visualize host responses.

Results

In both experiments necrotic lesions were first observed 2 and 3 weeks following inoculations, on the susceptible and resistant clones respectively. Initially, lesions appeared as areas of water soaked cells on the surface of the stem. The majority of the lesions developed on the lower 15-cm of inoculated trees and were rarely observed on the top 15-cm section. Disease etiology differed between resistant and susceptible clones. Water soaked areas on the two resistant clones (DN74 and NM6) developed swollen margins. Seven weeks PI any necrosis that developed on resistant clones was completely contained by these swollen margins. In the case of the susceptible clone NC11505, the water soaked areas became necrotic with a dark brown to black appearance. Three weeks PI necrotic lesions developed tan centers with pycnidia oozing

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pinkish spore tendrils. At 7 weeks PI necrotic lesions had coalesced completely girdling the stem of the susceptible clones. No symptoms developed on control trees in either experiment.

Part I. Infection biology

Conidia had 2- to 4-septations and ranged in size from 28 - 54 × 3.5 - 4 µm. The average germination rates of the conidia at the four time points (6 h, 12 h, 24 h and 72 h) increased from 10% to 98.3%. No spores were visible on the surface of the control trees and no symptoms had developed by the end of the experiment (Fig. 3.1A and 3.1B). An examination of the upper (15-cm) and lower (15-cm) revealed the lack of lenticels or small openings on the upper 15-cm. This observation was consistent across both the resistant (NM6) and susceptible (NC11505) clones (Fig. 3.1C and 3.1D). At 6 h PI, spores had adhered to the stem surface and had begun to germinate on both clones (Fig. 3.2A and 3.2B). At 12 h PI, Germ tubes appeared to have entered host tissue through either lenticels or small openings on both resistant and susceptible clones. However, infection structures were not visible (Fig. 3.2C and 3.2D). The majority of the germ tubes had entered host tissue at 24 h PI, but the germ tubes did not grow towards the nearest opening but appeared to meander across the surface of the inoculated stem entering openings at random (Fig. 3.3A and 3.3B). The images at 72 h PI, 1 week PI and 3 weeks PI were similar for both the resistant and susceptible clones (Fig. 3.3C and 3.3D).

A t-test comparing the mean number of germ tubes appearing to have penetrated host tissue indicated no significant difference ($P = 0.41$) between the resistant clone (NM6) 35.75% and susceptible (NC11505) clone 42.75% (Table 3.1).

Part II. Host response to non-wound inoculations

    <u>Histology of control plants</u>

    The anatomy of DN74 and NC11505 controls resembled the descriptions of NC11505 and DN34 made by Weiland and Stanosz (2007). The transverse sections can be subdivided into three layers (periderm, cortex, and xylem) containing primary phloem fibers, phloem, vascular cambium and xylem vessels. The epidermis was typically 1 to 2 cell layers thick and appeared blue-green under fluorescence microscopy (Fig. 3.4). Lenticels were visible throughout the periderm. Cortex was located adjacent to periderm beyond phloem tissue. Sometimes, phloem fibers with thick cell walls were located within the cortex and appeared bright blue under fluorescent microscopy. Disease did not develop on control plants and no hyphae were visible (Fig. 3.4).

    <u>Histology 3 weeks post-inoculation</u>

    Susceptible clone NC11505. Symptomatology was similar to that described previously for the susceptible clone. Transverse sections through the midpoint of each cankers revealed light brown necrosis of the vascular cambium. Both fluorescent and bright field images of transverse sections indicated the presence of an impervious tissue (IT) layer (Fig. 3.5A and 3.5B), which was chromophilic and amorphous under blue UV fluorescence. Hyphae were clearly visible in the cortex 3 weeks PI (Fig. 3.7A). No evidence of NP formation was observed.

    Resistant clone DN74. Disease development was similar to that described for NM6. Swelling developed along the margin of necrotic tissue. Fungal invasion appeared to be restricted to lenticels and adjacent cortex by the rapid formation of NP visible in transverse sections (Fig. 3.6A and 3.6B). Hyphae were not observed in the cortex of the resistant clone.

Histology 5 weeks post-inoculation

Susceptible clone NC11505. After 5 weeks, necrotic lesions had enlarged longitudinally and multiple cankers had coalesced. Without magnification, transverse sections of cankers at 5 weeks PI were observed to have yellowish to brown staining of the xylem tissue and pycnidia were observed forming at the stem surface. Several cankers had developed NP at an oblique angle from the periderm to the xylem by this time (Fig. 3.5C and 3.5D). The NP appeared discontinuous under fluorescent light and was interrupted at several locations by phloem fibers (Fig. 3.5C). Hyphae were present in the periderm and cortex adjacent to the vascular cambium (Fig. 3.7B).

Resistant clone DN74. The swollen margins of the canker were larger and appeared to have completely contained the small necrotic area. The NP layer was close to the epidermis restricting necrosis from the vascular cambium (Fig. 3.6C and 3.6D). Under fluorescent light the NP layer was visible in the cortex and had become thicker than at 3 weeks PI (Fig. 3.6C).

Histology 7 weeks post-inoculation

Susceptible clone NC11505. Coalesced cankers had girdled the stem at multiple locations. The NP layer was rarely invisible at this time point fluorescing weekly under UV light (Fig. 3.5E). The majority of the periderm and cortex had collapsed (Fig. 3.5F) and hyphae were visible throughout the periderm, cortex and xylem (Fig. 3.7C).

Resistant clone DN74. The majority of cankers examined at 7 weeks PI were similar to those at 5 weeks PI. The necrotic area was contained by the NP and no further disease development had occurred. However, in several cankers (four out of six segments) necrosis had developed from the periderm all the way to the vascular cambium and xylem. In these cases two

successive layers of NP were evident (Fig. 3.6E and 3.6F). The first appearing to be incomplete, extending into the cortex, and the second complete, extending from the periderm to the xylem (Fig. 3.6E and 3.6F). Xylem cells in close proximity to the NPs were occluded and the vascular cambium appeared to be regenerating xylem tissue (Fig. 3.6F). No hyphae were visible in longitudinal sections of the xylem.

Discussion

Septoria musiva, a necrotrophic pathogen, is frequently reported in the literature to cause cankers in association with wounded stems and branches (Waterman 1954). However, several studies inoculating non-wounded stems of *Populus* spp. with *S. musiva* have also incited disease (Bier 1939, Zalasky 1978, Long *et al.* 1986, Krupinsky 1989, LeBoldus *et al.* 2010). In these studies cankers have typically developed at stipule scars (Zalasky 1978), lenticels (Bier 1939, Long *et al.* 1986, Krupinsky 1989), the base of leaves (Zalasky 1978), and on petioles (Bier 1939, Zalasky 1978). Disease development following inoculation is similar across all studies. Initially, small water soaked lesions appeared on stems of inoculated trees 2 to 3 weeks PI, and these water soaked areas rapidly became necrotic. On susceptible clones, necrotic lesions coalesced with no visible macroscopic host response eventually girdling the tree. On resistant clones, the margins of the necrotic lesions become swollen as the tree recovers from the infection. Disease progress was similar in this study to what has been previously reported in terms of both the location of canker development and the responses of the resistant (DN74 and NM6) and susceptible (NC11505) clones.

Disease development was similar between resistant and susceptible clone in early stage of infection. For example, at 12 h PI there was no significant difference between the resistant and susceptible clones in terms of the number of spores that had found a lenticel or small opening

(Fig. 3.2 and 3.3) to enter. Furthermore, there was no evidence of direct penetration or the formation of infection structures on either the resistant or susceptible clones. In both the resistant and susceptible clone, the mode of infection appears to be limited to lenticels and wounds. A similar mode of infection has been reported for *Quambalaria* spp. causing leaf and shoot blight of *Eucalyptus* spp. (Pegg *et al.* 2009). This pathogen was able to enter host tissue via stomata and small wounds and did not produce any infection structures (Pegg *et al.* 2009). A second similarity between *S. musiva* and *Quambalaria* spp. is the haphazard pattern of growth exhibited by the germ tubes (Pegg et al. 2009). In many cases the germ tube would grow over a nearby infection court (stomata/ lenticel/ wound) and penetrate a similar infection court further away. This pattern of growth may be related to a chemotrophic rather than thigmotrophic mechanism of attraction. For example, Peterson (1969) reported that *Dothistroma pini* Hulbary germ tubes were attracted to particular stomata by an emitted chemical stimulus that was not common to all stomata. A similar phenomenon was hypothesized by Patton and Spear (1978) in the infection of Scotch pine by *Scirrhia acicula* (Dearn.) Siggers.

In this inoculation experiment, and others conducted by our research group, the majority of cankers appear to develop on the lower 15 cm of the inoculated trees (Bier 1939, Waterman 1954, Zalasky 1978, Krupinsky 1989). There are two possible explanations for this phenomenon. The first possibility may be due to the developmental stage of the host. The youngest tissue at the top of the tree is covered by a thin epidermis and lacks lenticels and other natural openings limiting entry of *S. musiva* into host tissue (Fig. 3.1). As the periderm and lenticels begin to form the epidermis initially stretches and then splits open, resulting in the formation of crevices and lenticels providing potential infection courts for the pathogen (Fig. 3.2 and 3.3). A second possible explanation for this phenomenon is the movement of spores along with dripping water

following inoculation. Although the tree may be uniformly sprayed at the time of inoculation it is possible that water dripping down from the top of the tree may carry spores with it, resulting in infections on the lower portion of the stem.

In the previous chapter Qin *et al*. (2013) hypothesized that the correlation in the predictive ability of the wound compared to the spray inoculation protocol suggests that resistance may occur post penetration. The lack of differences described above, in terms of penetration frequency at 12 h PI, support this hypothesis. Once the fungus has gained access to the interior of the stem colonization of host tissue appears to occur in the developing periderm (phelloderm, phellogen, and phellem) and cortex below the epidermis. At this point, differences in host response are observed between resistant and susceptible clones. Host response to pathogen invasion is typically characterized by the development of a layer of impermeable tissue (IT) followed by the development of NP. In the resistant clones, the rapid development of NP in close proximity to the epidermis occurred within 3 weeks PI. This is similar to what was reported by Weiland and Stanosz (2007) for resistant clone DN34. In their experiment a full layer of NP had developed in response to both wounding and wounding with inoculation within 7 weeks PI with no further pathogen development observed (Weiland and Stanosz 2007).

The similarity in host responses of the resistant clones can be contrasted with the response of the susceptible clone NC11505 in the two studies. Weiland and Stanosz (2007) reported the development of a continuous NP layer in the wounded control and multiple successive NP layers in the wounding with inoculation treatments 7 weeks PI. The development of multiple successive NP layers indicates that the pathogen may be able to circumvent the defense response of the host, triggering the formation of a new NP layer. In the susceptible clones, the NP layer did not begin to develop until 5 weeks PI and was discontinuous and only

one cell layer thick (Fig. 3.5C). At 7 weeks PI, there was limited evidence of NP development in any of the sectioned cankers (Fig. 3.5E). In this study, symptom development was more severe than in the descriptions provided by Weiland and Stanosz (2007). Although there were differences in the age of the inoculated trees and the environment, the lack of wounding in our inoculation study coupled with the failure of the susceptible clones to generate a full NP layer by 7 weeks PI suggests that the wounding conducted by Weiland and Stanosz (2007) may have been the trigger for NP development. In the absence of wounding, NC11505 is unable to produce a complete NP layer suggesting that the production of NP may be a determining factor in resistance against fungal pathogens (Biggs *et al.* 1983b and 1984, Biggs 1984).

Forest pathologists usually consider the development of NP as part of a restorative process which serves to reestablish the integrity of the trees vascular system and the secondary meristem responsible for lateral growth (Zalasky 1964). Mullick (1977) stated that the development of NP was initiated whenever the phellogen is rendered non-functional. The reason for this loss of function was irrelevant and could be due to pathogen invasion, mechanical wounding, or insect damage. However, the results above suggest that the pathogen may be interfering in some way with this restorative process in NC11505. There are several possible explanations for this phenomenon. Firstly, there is potential for clonal differences in pathogen recognition and subsequent resistance. For example, DN74 may have a biochemical or molecular resistance mechanism that prevents pathogen development allowing the NP to form. NC11505 lacks this resistance mechanism allowing the pathogen to continue to grow at a faster rate than NP can develop. A second possibility may be related to the number of necrotic lesions that developed on the susceptible host. In this study the large number of cankers which developed on the susceptible clone may have compromised the host's ability to produce NP, in comparing to

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the study of Weiland and Stanosz (2007), where a single canker developed on each stem. This may have also occurred with the resistant clone where in some cases there was evidence of successive NP development following inoculation (Fig. 3.6). A third possibility may be related to the host - pathogen interaction. Biggs *et al.* (1986b) reported in the peach-*Leucostoma* system that the fungus is able to alter the location and structure of the NP layer. The production of a host selective toxin may be a potential mechanism by which the pathogen is able to achieve this.

To this author's knowledge the majority of studies examining canker development in woody tissue have relied on wounding in order to incite disease (Biggs *et al.* 1983a, 1983b and 1984, Biggs 1984 and 1986b, Hebard *et al.* 1984, Enebak *et al*. 1997). The results from this study indicate that wounding may be artificially triggering the development of NP. The lack of significant difference in the early stages of infection between the resistant and susceptible hosts support the idea proposed by Qin *et al.* (2013) that differences in resistance occur post penetration. These post infection differences are characterized by the lack of NP formation in the susceptible clone compared to the rapid development of NP in the resistant clone. These differences highlight the importance of using the non-wounded inoculation protocol for dissecting host parasite interactions in the *S. musiva* – hybrid poplar interaction.

Table 3.1. Means of the penetration rate of mixed isolates (MN7, MN11, and MN23) of *Septoria musiva* into lenticels and small openings on hybrid poplar clones NC11505 (*Populus maximowiczii × Populus trichocarpa*) and NM6 (*Populus maximowiczii × Populus nigra*). Average were determined by counting 100 spores from 2 to 3 field of view under scanning electron microscope. ($\alpha = 0.05$)

Clone	Percentage of entering (%)		
	Mean	Range	Std. deviation
NC11505	42.75 a	35 - 55	8.80
NM6	35.75 a	28 - 45	8.10
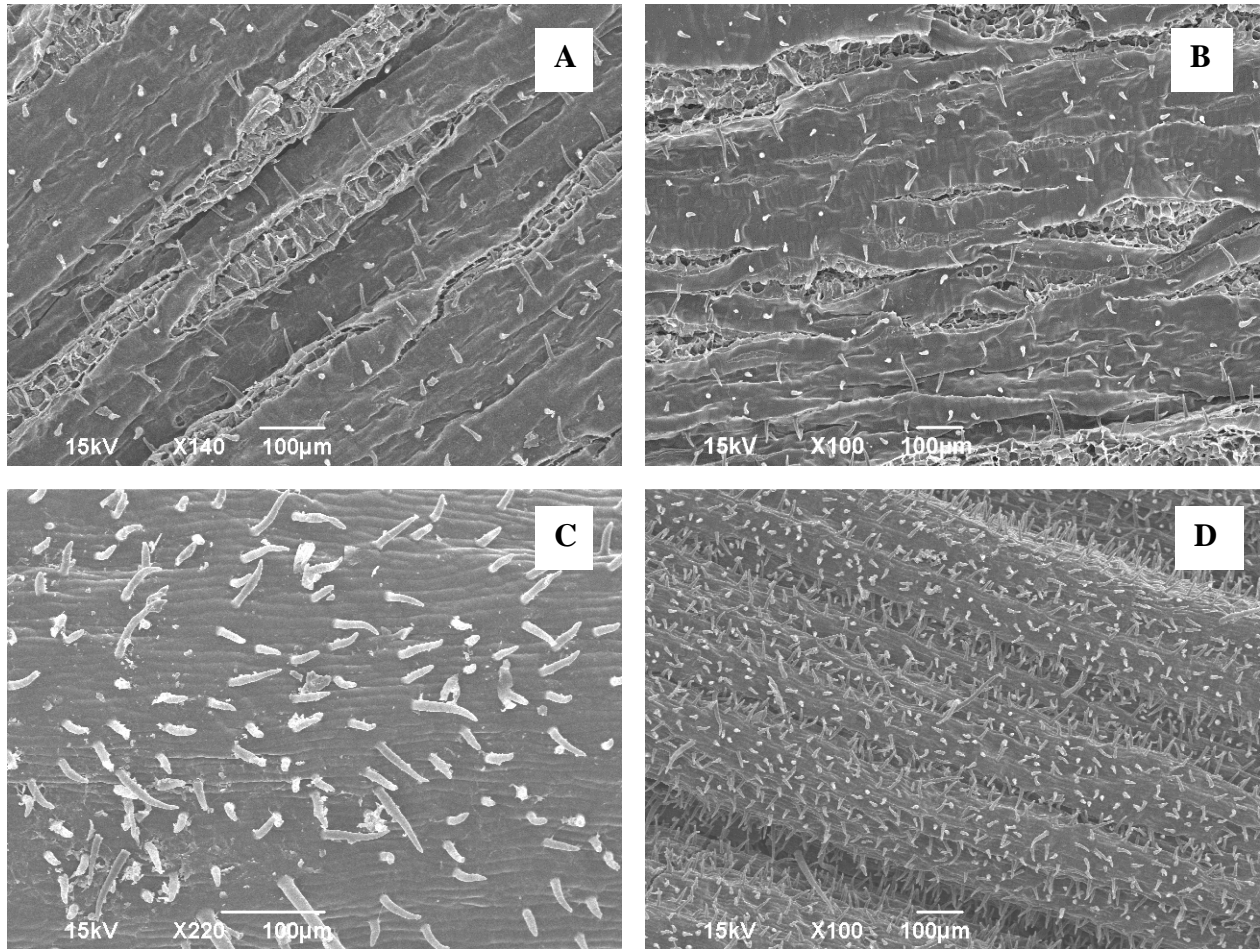
Figure 3.1. Scanning electron microscope images of non-wound and mock inoculated hybrid poplar clone NC11505 (*Populus maximowiczii* × *Populus trichocarpa*) and NM6 (*Populus maximowiczii* × *Populus nigra*). **A**, lower 15-cm-section of clone NC11505, lenticels and small openings randomly appeare on the surface. **B**, lower 15-cm-section of NM6 stem, lenticels and small openings appear on stem surface. **C**, top 15-cm-section of NC11505, without lenticels and small openings. **D**, top 15-cm-section of NM6, without lenticels and small openings.
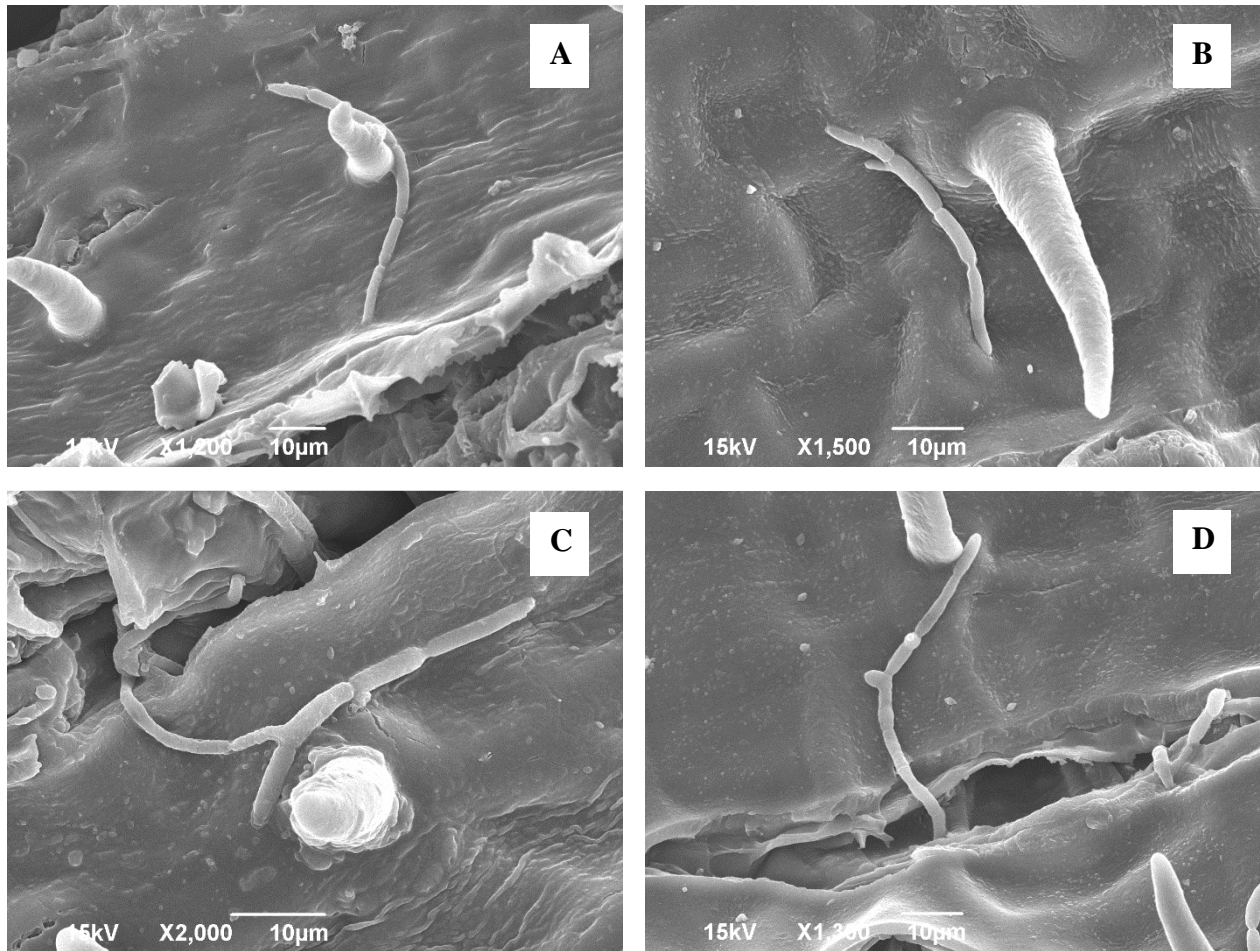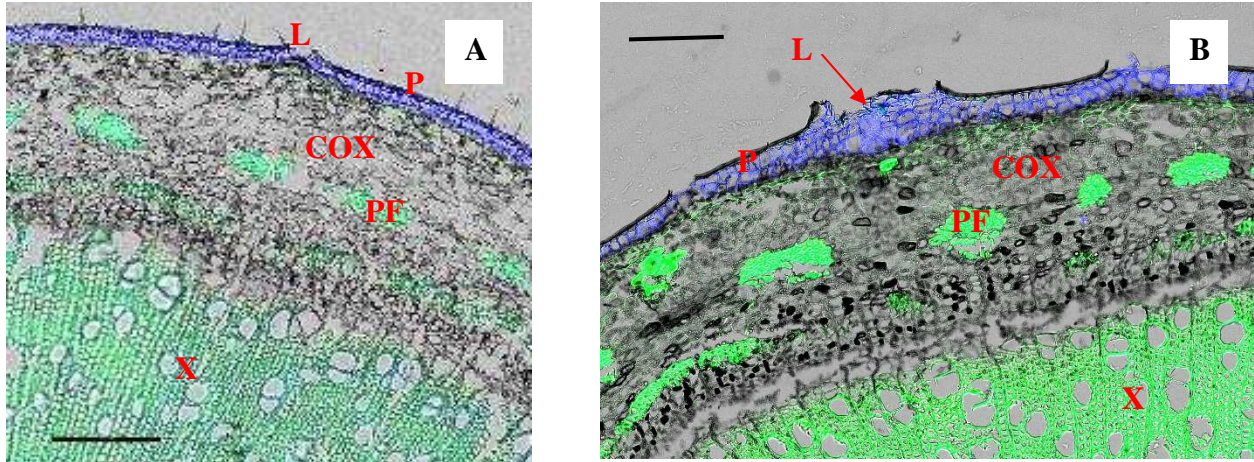
Figure 3.2. Scanning electron microscope images of non-wounded inoculate hybrid poplar clone NC11505 (*Populus maximowiczii* × *Populus trichocarpa*) and NM6 (*Populus maximowiczii* × *Populus nigra*) at 6 h PI and 12 h PI. Inoculations were conducted by spraying a mixed conidial suspension (MN7, MN11 and MN23) of *Septoria musiva* and harvesting stem sections at two time points (6 h PI and 12 h PI). **A**, Spore on clone NC11505 surface near trichome at 6 h PI, without a visible germ tube. **B**, Spore on clone NM6 surface near trichome at 6 h PI with germ tube visible. **C**, Germ tube entering a lenticel in clone NC11505 at 12 h PI. **D**, Germ tube entering crevice small opening on the surface of clone NM6 at 12 h PI.
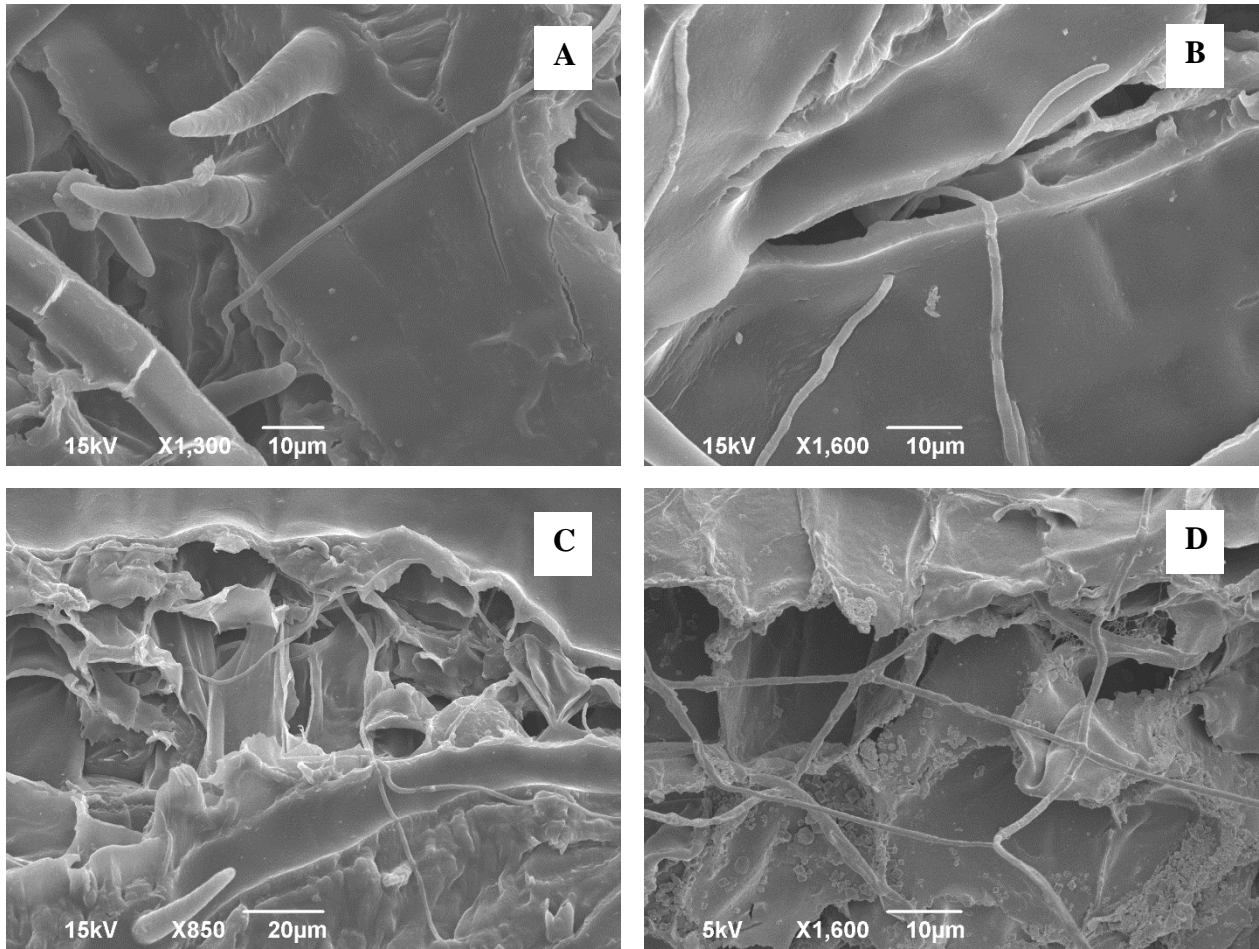
Figure 3.3. Scanning electron microscope images of non-wounded stems of hybrid poplar clone NC11505 (*Populus maximowiczii × Populus trichocarpa*) and NM6 (*Populus maximowiczii × Populus nigra*) at 24 h PI and 72 h PI. Stem sections were harvested at two time points (24 h PI and 72 h PI). **A**, *S. musiva* hyphae on clone NC11505 stem surface and entering lenticels and small openings at 24 h PI. **B**, *S. musiva* hyphae entering small openings on NM6 at 24 h PI. **C**, hyphae entering lenticels on clone NC11505 at 72 h PI. **D**, extensive hyphal development on clone NM6 at 72 h PI.
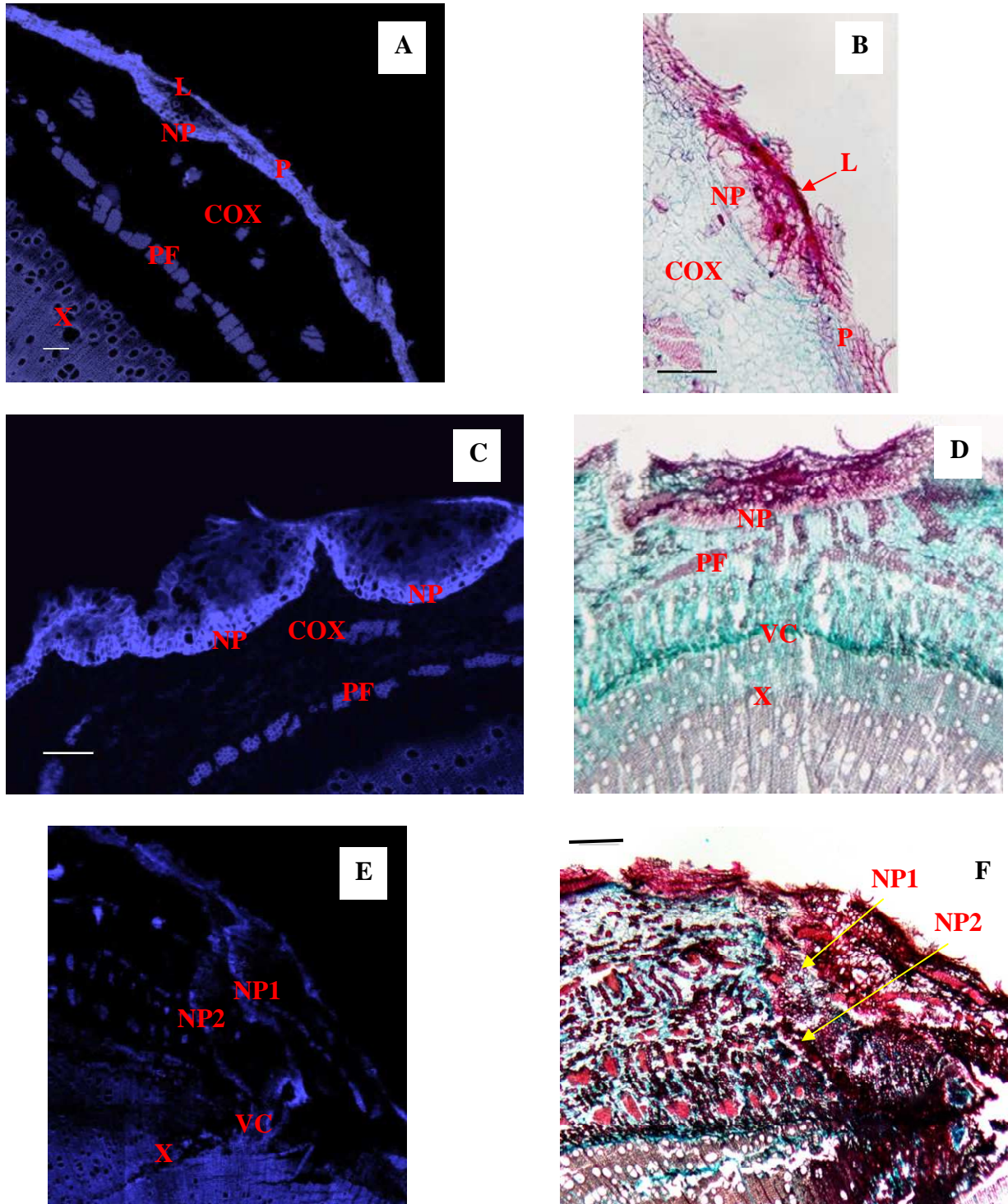
Figure 3.4. Fluorescenct micrographs of transverse section of non-wounded and mock inocualted hybrid poplar clones NC11505 (*Populus maximowiczii* × *Populus trichocarpa*) and DN74 (*Populus deltoides* × *Populus nigra*). **A**, anatomy of cross-section of clone NC11505 with lenticels, periderm, cortex, phloem fiber and xylem visisble. **B,** anatomy of cross-section of clone DN74 showing lenticel, periderm, cortex, phloem fiber and xylem. **COX** = Cortex, **L** = Lenticel, **P** = Periderm, **PF** = Phloem fiber, **X** = Xylem. Scale bars = 100 µm.
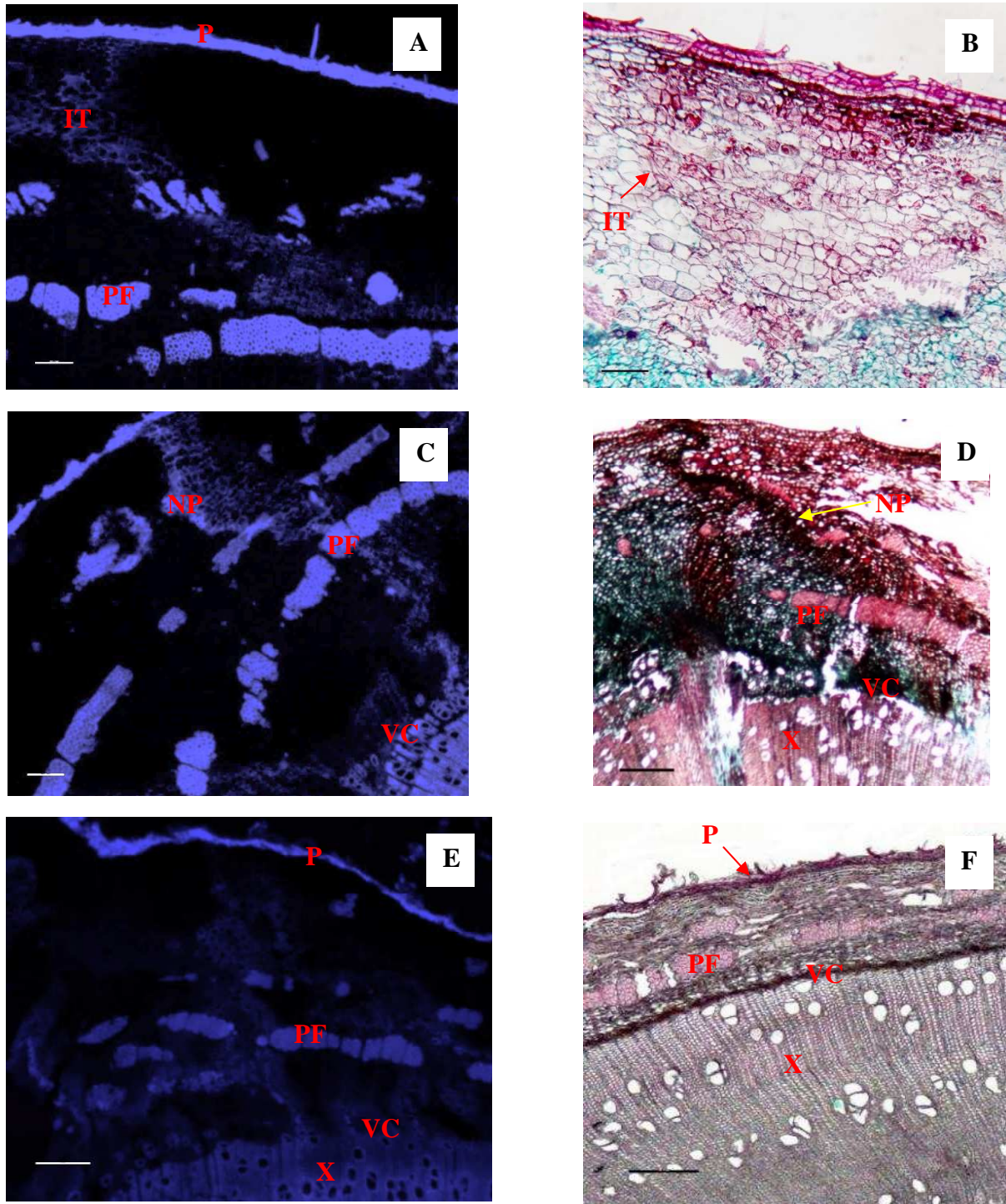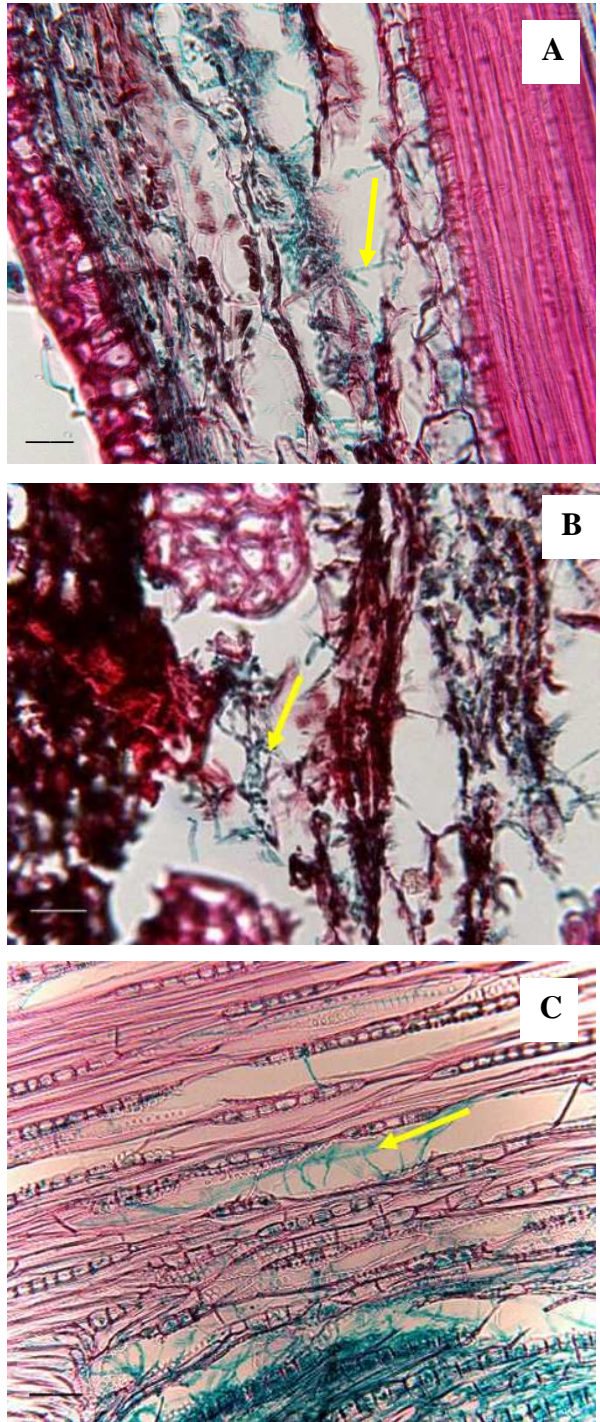
Figure 3.5. Fluorescent and bright field micrographs of transvers sections of inoculated hybrid poplar clone NC11505 (*Populus maximowiczii* × *Populus trichocarpa*). Stem sections were harvested at three time points (3 weeks PI, 5 weeks PI and 7 weeks PI). **A**, Fluorescent

micrograph of inoculated stem of clone NC11505 at 3 weeks PI, showing impervious tissue (**IT**). **B**, Bright field micrograph of inoculated stem of clone DN74 at 3 weeks PI showing necrotic area without necrophylactic periderm (NP) present. **C**, Fluorescent micrograph of clone NC11505 at 5 weeks PI, showing incomplete NP extending to the vascular cambium (**VC**). **D**, Bright field micrograph of clone DN74 at 5 weeks PI, showing NP development at an oblique angle from periderm to phloem fiber (**PF**). **E**, Fluorescent micrograph of clone NC11505 at 7 weeks PI, showing dead bark tissue and no NP layer. **F**, Bright field micrograph of clone NC11505 at 7 weeks PI, bark tissue is depressed and necrotic. **COX** = Cortex, **IT** = Impervious tissue, **NP** = Necrophylactic periderm, **P** = Periderm, **PF** = Phloem fiber, **VC** = Vascular cambium, **X** = Xylem. Scale bars = 200 µm.

Figure 3.6. Fluorescent and bright field micrographs of transverse sections of inoculated hybrid poplar clone DN74 (*Populus deltoides* × *Populus nigra*). Inoculations conductedwith mixed conidial suspension (MN7, MN11 and MN23) of *Septoria musiva*. Stem harvested sections at three time points (3 weeks PI, 5 weeks PI and 7 weeks PI). **A**, Fluorescent micrograph of clone

DN74 at 3 weeks PI, showing a complete NP layer below lenticels. **B**, Bright field micrograph of clone DN74 at 3 weeks PI, showing necrotic area restricted to lenticel. **C**, Fluorescent micrograph of clone DN74 at 5 weeks PI, showing thickened NP layer within cortex tissue. **D**, Bright field micrograph of clone DN74 at 5 weeks PI, showing NP layer forming below necrotic area. **E**, Fluorescent micrograph of clone DN74 at 7 weeks PI, showing 2 layers of NP extending to the vascular cambium (**VC**). **F**, Bright field micrograph of clone DN74 at 7 weeks PI, the NP layer acting as a barrier between healthy and necrotic tissue. **COX** = Cortex, **L** = Lenticel, **NP** = Necrophylatic peridem, **P** = Periderm, **PF** = Phloem fiber, **VC** = Vascular cambium, **X** = Xylem. Scale bars = 200 µm.

Figure 3.7. Bright field micrographs of transverse and longitudinal sections of inoculated hybrid poplar clone NC11505 (*Populus maximowiczii* × *Populus trichocarpa*). Inoculations conducted with a mixed conidial suspension (MN7, MN11 and MN23) of *Septoria musiva*. Stem sections harvested at three time points (3 weeks PI, 5 weeks PI and 7 weeks PI). Arrow indicates fungal hyphae. **A**, Hyphae of *S. musiva* colonizing cortex and surrounding phloem fiber in longitudinal

section at 3 weeks PI. **B**, Cross-section of clone NC11505 showing hyphae extending to the vascular cambium at 5 weeks PI. **C**, Longitudinal section of clone NC11505 showing hyphae in xylem vessels at 7 weeks PI. Scale bars=200 µm.

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