MOLECULAR AND HISTOLOGICAL CHARACTERIZATION OF SPHAERULINA MUSIVA- POPULUS SPP. INTERACTION

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Sphaerulina musiva, the causal agent of leaf spot and stem canker, is responsible for critical yield loss of hybrid poplar in agroforestry. This research examined quantification of *S. musiva* in host tissue, and infection of leaf tissue, plus gene expression between resistant and susceptible poplar genotypes. This study reports the first use of a multiplexed hydrolysis probe qPCR assay for faster and accurate quantification of *S. musiva* in inoculated stems of resistant, moderately resistant and susceptible genotypes of hybrid poplar at three different time points -1 wpi (weeks post-inoculation), 3 wpi and 7 wpi. This assay detected significant differences in the level of resistance among the different clones at 3 wpi ($p < 0.001$) and significant differences among isolates at 1 wpi ($p < 0.001$), that were not detected by visual phenotyping. Histological and biochemical comparisons were made between resistant and susceptible genotypes inoculated with conidia of *S. musiva* in order to study the mode of leaf infection and defense response of hybrid poplar. Leaf infection was examined at 48 h, 96 h, 1 wpi, 2 wpi and 3 wpi using scanning electron microscopy (SEM) and fluorescent and laser scanning confocal microscopy. Infection process of *S. musiva* on *Populus* spp. was further characterized by transforming *S. musiva* with red fluorescent protein through *Agrobacterium tumefaciens.* Results indicated that there was no difference in pre-penetration processes, however, differences were observed in post-penetration between resistant and susceptible genotypes. The host response was also studied by examining the accumulation of hydrogen peroxide (H_2O_2) using fluorescent microscopy after DAB staining, and a significant difference ($p < 0.0001$) was observed by 2 wpi. The molecular mechanism underlying host-pathogen interaction was elucidated by studying temporal differentially expressed genes of both the interacting organisms, simultaneously, using RNA-seq. Genes involved in cell wall modification, antioxidants, antimicrobial compounds, signaling pathways,

ROS production and necrosis were differentially expressed in the host. In the pathogen, genes involved in CWDE, nutrient limitation, antioxidants, secretory proteins and other pathogenicity genes were differentially expressed. The results from this research provide an improved understanding of poplar resistance/susceptibility to *S. musiva*.

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CHAPTER 1. LITERATURE REVIEW

Populus Genus

Intriguing characteristics of the genus *Populus* make it one of the most widely studied model organisms (DiFazio et al. 2011). Species of *Populus* are members of the *Salicaceae* family. In the most recent taxonomic analysis of the Angiosperms (Angiosperm Phylogeny Group 1998), the families *Salicaceae*, *Flacourtiaceae* and 29 others were placed under the order Malpighiales (Bradshaw et al. 2000). This genus consists of 30-40 species that are classified under six botanical sections: *Aigeiros*, *Tacamahaca*, *Turanga*, *Abaso*, *Populus* and *Leucoides* (Eckenwalder 1996). Based on fossil records and phylogenetic analyses, Eckenwalder (1996) hypothesized that this genus could have originated during the Paleocene, in North America or tropical Asia and the sections *Populus*, *Aigeiros* and *Tacamahaca* evolved rapidly during the Miocene. Economically important species are found primarily in the sections *Populus*, *Tacamahaca* and *Aigeiros* (Farrar 1995). For example, *P. deltoides* Marsh. and *P. nigra* L. are in section *Aigeiros*, while *P. balsamifera* L. and *P. trichocarpa* Torr. & A. Gray are in the section *Tacamahaca*. These species are widely used in various sectors like manufacturing, energy, agriculture and land management.

Life History

General morphology

Species belonging to the genus *Populus* exhibit high variation in morphology, biotic resistance, tolerance to abiotic stress and growth (Stettler et al. 1996). Traditionally, species belong to a section based on their reproductive and morphological characteristics, as well as their interspecific crossability (Eckenwalder 1996, Zsuffa 1975). All species of this genus are deciduous, soboliferous and single-trunked (Eckenwalder 1996). They are one of the fastest growing temperate trees. After bud burst, poplar shoots exhibit indeterminate growth beginning

with preformed (early leaves) and continuing late into the fall with neoformed (late leaves) leaves. Considerable variation is seen between preformed and neoformed leaves in terms of texture, shape and toothing pattern in *Populus* spp. Preformed leaves differ among sections and are used for taxonomic classification. They are more resilient than neoformed leaves, as they are present in frost prone early spring. Since more favorable conditions are experienced by neoformed leaves, they may boost the photosynthetic rates (Dickmann 1971, Donnelly 1974, Regehr et al. 1974). In some poplars, photoperiods can influence bud formation and cessation of growth, thus affecting hardening off in autumn and potentially resulting in damaged shoots during the winter (Eckenwalder 1996).

A significant difference is observed for leaf traits among different species and hybrids of poplar (Marron et al. 2005, Monclus et al. 2005, Rae et al. 2004). For example, the leaves of *P. trichocarpa* genotypes are thick and their abaxial surface appears to be white, due to thick loosely packed spongy mesophyll cells (Ceulemans 1990, Figliola 1986, Ridge et al. 1986, Van Splunder et al. 1996, Zavitkovski 1981), and they have a small number of large stomata that are only present on the lower surface of their leaves. In contrast, the leaves of genotypes belonging to section *Aigeiros* are thin and small with reduced spongy parenchyma thickness. The abaxial and adaxial leaf surfaces of *P. deltoides* genotypes are green because of the bilateral palisade parenchyma layers (Ceulemans 1990, Figliola 1986, Ridge et al. 1986, Van Splunder et al. 1996, Zavitkovski 1981). They also have a large number of small stomata on both sides of the leaves (Ceulemans 1990, Figliola 1986).

The leaf morphological traits of hybrids between the sections *Tacamahaca* and *Aigeiros* are typically intermediate to those described above. For instance, hybrids of *P. trichocarpa* and *P. deltoides* predominantly inherit stomatal characteristics from *P. trichocarpa*, but the stomatal

densities are similar in the abaxial and adaxial surfaces (Al Afas et al. 2006, Figliola 1986). Adding to the variability in leaf morphology are the anatomical and physiological changes resulting from environmental stimuli (Ferris et al. 2002, Marron et al. 2005, Monclus et al. 2005, 2006). Leaf characteristics, like morphology, anatomy, stomatal density and conductance, can be affected by light (Al Afas et al. 2005, Casella and Ceulemans 2002, Niinemets et al. 1999, 2004, Orlović et al. 1998, Tichá 1982), or may even be impacted by environmental conditions present during the growing season (Kajba et al. 2004).

The structure of *Populus* stems and branches can be subdivided into bark and woody tissue (Raven et al. 1981). All tissues exterior to the vascular cambium are collectively called bark (Dickison 2000, Esau 1965, Srivastava 1964). The dead tissue on the surface of the stem is the rhytidome, or outer bark (Esau 1965). Periderm, cortex and phloem, which are derived from primary vascular cambium, make up the living inner bark (Dickison 2000). Periderm is made up of three layers: phellem, phellogen and phelloderm. As the bark develops, cracks form in the phellem, within which new lenticels are formed, through which gas exchange takes place (Esau 1965, Raven et al. 1981). Vascular cambium cells found between the wood and bark are the source of xylem and phloem cells. Nutrients are transported by the phloem, which forms the innermost layer of the bark (Raven et al. 1981). Xylem is divided into heartwood and sapwood (Dickison 2000, Raven et al. 1981). Sapwood consists of cells that transport water from roots to shoots. Heartwood is found interior to sapwood. It is the main support structure of a tree and is made up of dead cells (Esau 1965, Raven et al. 1981).

Habitat

Populus species are known for their phenotypic plasticity, i.e. they have the ability to change their phenotype in response to different environmental conditions. They are

predominantly found in the Northern hemisphere (Bradshaw et al. 2000, DiFazio et al. 2011). They are typically riparian, but also occur in semi-arid areas that are usually sandy and rocky. They are found in infertile sands and clays, in regions experiencing temperatures ranging from -45°C to 46°C and in areas with more than 200 frost-free days during the growing season (DiFazio et al. 2011, Richardson et al. 2014). For example, *P. nigra* is found throughout Europe, the Middle East, central Asia and into west Africa. Even though they are common in temperate climates with rainfall in the spring and autumn in medium textured soils, they can also survive on soils ranging from stony to heavy clay and they tolerate dry summers (Richardson et al. 2014). In western North America, the largest hard wood tree is *P. trichocarpa* (Dickmann and Kuzovkina 2014). They grow well in semi-arid to humid conditions, in temperatures ranging from 16°C to 47°C, but can also endure temperatures ranging from 0°C to -47°C. They favor moist sandy or gravelly soil (Richardson et al. 2014).

Within the *Populus* genus, the largest native range is occupied by *P. tremula*. They grow from eastern Europe to central Siberia, China and the central islands of Japan and also in North Africa. They are adapted to a wide range of environmental conditions. They are capable of growing on a wide variety of soils from rocky, loamy sand, to heavy clay, and they will also grow on nutrient poor soil. They can also survive with rainfall of less than 40 mm for up to two months (Richardson et al. 2014).

Biology

Populus species are dioecious; however, the existence of cosexual species has been reported (Cronk 2005, Rottenberg et al. 2000, Rowland et al. 2002, Slavov et al. 2009, Stettler 1971). Under favorable conditions, *Populus* trees take 10-15 years to reach reproductive maturity; however, it takes just 4-8 years under intensive poplar plantation conditions (Stanton

and Villar 1996). In a population, the pollination period extends for up to two months, and can be effective over large distances, as pollen is dispersed by wind (Braatne et al. 1996, Lexer et al. 2005, Slavov et al. 2009, Tabbener and Cottrell 2003, Vanden Broeck et al. 2006). Usually, during early spring, flowering occurs before leaf emergence, but the period of flowering depends on factors like temperature, latitude and climatic conditions (Braatne et al. 1996, DeBell 1990, Perala 1990, Zasada and Phipps 1990). When pollen lands on a stigma, fertilization occurs within a day (Braatne et al. 1996). After fertilization capsules dehisce in 4-6 weeks, seed development generally takes 2-3 weeks, but may take up to 3-5 months, depending on species and location (DiFazio et al. 2011). A tree produces more than 25 million seeds per year. Due to their small size and cotton like appendages, they are dispersed over large distances, with the help of wind and water. For many species, annual flooding creates beneficial sites for seed establishment, as the timing of seed dispersal coincides with annual flooding (Braatne et al. 1996, Johnson 1994, Karrenberg et al. 2002). In natural systems, seeds can be viable for up to 2 weeks, and under moist and warm conditions, germination occurs within 24 hours (Braatne et al. 1996, Karrenberg et al. 2002). In most establishment sites, if conditions are severe, mortality of seeds during the first year can be high (Braatne et al. 1996, Dixon 2003, Dixon and Turner 2006, Karrenberg et al. 2002).

One of the traits that allow *Populus* species to survive in various habitats, and wildfire, is their ability to reproduce vegetatively. This type of reproduction makes *Populus* dominant over other temperate trees, thus making them an ideal model system that can be utilized for research purposes (DiFazio et al. 2011). Vast variation is seen among sections of this genus in terms of reproduction. Species from the *Tacamahaca* section spread vegetatively when a whole tree trunk, or even just a branch, has fallen during floods or storms, and once the vegetative shoot has

rooted, a new tree arises (Barsoum et al. 2004, Braatne et al. 1996, Rood et al. 2003, 2007, Smulders et al. 2008). Cladoptosis, an atypical mode of vegetative propagation occurring in this section, occurs when live short shoots self-prune, and act as propagules, which are dispersed by wind or rain (De Wit and Reid 1992, Galloway and Worrall 1979). However, this mode of reproduction is thought to be infrequent (Rood et al. 2003).

Less extensive vegetative propagation is seen with trees from section *Aigeiros*, when compared to other sections (Braatne et al. 1996). In *P. deltoides* and *P. fermontii*, vegetative propagation, which occurs by coppicing of broken or killed shoots, rarely happens (Braatne et al. 1996, Gom and Rood 1999, Rood et al. 2003, Schweitzer et al. 2002). In contrast, *P. nigra* shows high levels of vegetative propagation by sprouting extensively from branches, roots and broken stems (Arens et al. 1998, Barsoum et al. 2004, Legionnet et al. 1997). Some trees from the *Populus* section display higher levels of vegetative propagation. *P. tremuloides* propagates itself primarily by sprouting from adventitious buds, which are present on lateral roots, in a process called suckering (Perala 1990).

Many studies have revealed that sex ratios are highly variable in natural populations of *Populus*, with some showing either male or female biased ratios, while others show no deviation from a 1:1 ratio (Braatne et al. 1996, Farmer 1996, Hultine et al. 2007, Rottenberg et al. 2000, Rowland et al. 2001, Stanton and Villar 1996). However, several of these recorded biases in sex ratios could depend on how the sexes respond to environmental conditions. For example, males are more common in extreme conditions and at high elevations, while female growth is superior in regions that have greater resources and more moisture (DiFazio et al. 2011).

Hybrids

Hybridization is one of the traits that commonly occurs in *Populus*. Hybridization falls into two main categories: natural and anthropogenic. Natural hybridization can be inter- or intra-sectional and occurs freely between the closely related sections *Tacamahaca* and *Aigeiros* (Hamzeh et al. 2007, Lexer et al. 2005). For example, the natural inter-sectional hybrid *Populus* x *jackii* Sarg. (*P. balsamifera* x *P. deltoides*) occurs whenever these two species co-occur. An example of an intra-sectional hybrid is *Populus* x *smithii* B. Boivin (*P. tremuloides* x *P. grandidentata*). Crossing of allopatric *Populus* species is considered anthropogenic hybridization (Stettler et al. 1996). Poplar breeders carry out this type of hybridization as a way in which they can combine desirable traits. Both natural and anthropogenic hybrids have greater growth rates compared to either of the parents. This is called hybrid vigor (Mohrdiek 1983, Stettler et al. 1996). Hybrid poplar is said to have superior growth, when there are favorable combinations of traits like morphology of stomata (Tschaplinski et al. 1994) and leaves, leaf growth (Ridge et al. 1986) and photosynthesis of leaf, as well as the whole tree (Isebrands et al. 1988) and canopy.

Populus breeders mainly focus on a few species belonging to the *Tacamahaca* and *Aigeiros* sections for hybridization. The main purpose of a breeding program is to develop clones (genotypes) with hybrid vigor. It was estimated by the IPC in 2012 that there were 83.6 million ha of *Populus* plantations worldwide (FAO 2012), out of which, in the US alone, 25,000 ha are planted in the Mississippi river valley, 15,000 ha in the North Central region and over 50,000 ha are planted in the Pacific Northwest (Revels 2009, Stanturf et al. 2003). These *Populus* plantations are focused on production of pulp, paper, wood products, biofuel and electricity (Balatinecz and Kretschmann 2001, Brown 2003, Stoffel 1998, Zalensy et al. 2008). However, the extensive adoption of poplar plantations has been hindered in many parts of North America

due to susceptibility of many commercial hybrids to various pests and pathogens (Ostry and McNabb 1985, Whitham et al. 2008).

Populus – A Model Forest Tree

Among plants, trees are distinct, as they have characteristics of long life spans and the ability to generate woody biomass from secondary xylem (Taylor 2002). In the industrialized world, one of the most valuable commodities is wood. In less developed countries, wood harvested from more than half of the annual yield worldwide is used as fuel (Bradshaw et al. 2000). Although many characteristics of tree biology can be studied by using *Arabidopsis thaliana*, there are certain unique aspects, such as anatomy, physiology and disease resistance, that are more informative when studied in the trees themselves. For example, features of tree biology, like survival on a long time scale, generation of wood from vascular cambium, patterns and activity of dormancy as it depends on interactions between the environment and plant signals, flower phenology, cold hardiness, reallocation of nutrients during different seasons and juvenile to mature phase change, cannot be adequately studied with Arabidopsis (Bradshaw et al. 2000, Taylor 2002). Ideally, a model tree should have the capability to be easily manipulated using molecular approaches. *Populus*, more so then most woody plants, meets this criterion. The ease of breeding poplars is another major advantage. In the greenhouse, poplars are bred with pollen on detached female branches, and pollen can be stored for several years. In addition, hundreds of seeds are obtained from each pollination event within only four to eight weeks. Seeds do not need to be stratified and germinate within 24 hours. Within the same year, these seeds give rise to one to 2-meter tall seedlings (Zsuffa et al. 1996).

Rapid growth features allow the study of short-term biotic and abiotic responses on juvenile trees. This characteristic has led to physiological studies over short time periods. In

addition, QTLs regulating disease resistance (Cervera et al. 1996, Newcombe and Bradshaw 1996, Newcombe et al. 1996, Villar et al. 1996), phenology (Frewen et al. 2000) and morphology (Bradshaw and Stettler 1995) have been mapped. Biomass production of *Populus* has been studied from a physiological perspective. QTLs have also been identified for these traits (Bradshaw and Stettler 1995).

Furthermore, for a tree, the genome of poplar is relatively small, around 550 million bp, which is only four times larger than the genome of *Arabidopsis thaliana*. Due to its small size, it makes performing genetic analyses simpler. It is also an effective target for positional cloning, due to a ratio of physical and genetic distance similar to that of Arabidopsis (Bradshaw and Stettler 1993). Poplar was also one of the first woody systems to be transformed (Han et al. 2000). To date, molecular transformation mainly focuses on three areas: flowering, quality and quantity of lignin, and engineering of *Bacillus thuringiensis* (Bt) and glyphosate resistance (Meilan 2006). In spite of all these strengths, two of the major drawbacks are that *Populus* is not self-pollinating and it lacks well-planned breeding programs.

Diseases of *Populus*

A wide range of pathogens constantly challenges populations of *Populus* in the northern hemisphere, as well as in plantations worldwide. Two major reasons for this are intensive poplar cultural practices and the introduction of exotic species because of international trade (Newcombe 1996). Species belonging to *Populus*, *Tacamahaca* and *Aigeiros* sections are the main concern regarding fungal and bacterial infections, because of their use in commercial plantations (Newcombe 1996). In North America, there are four major diseases: leaf rust caused by *Melampsora* spp., leaf spot and stem canker disease caused by *Sphaerulina musiva*, leaf and shoot blight caused by *Venturia* spp. and leaf spot caused by *Marssonina* spp. (Hiratsuka 1987,

Newcombe and Ostry 2001, Peterson and Peterson 1992). Even though the majority of pathogens infecting *Populus* are fungal in nature, stem canker caused by *Xanthomonas populi* is a notable bacterial disease occurring in poplar (Ridé and Ridé 1978).

Melampsora spp., like *M. larici-populina* Kleb., cause severe economic losses in European poplar plantations, while *M. medusae* causes losses in eastern North America and the Northwestern US. Damages include defoliation, decreased photosynthesis and increased susceptibility to other pests and pathogens, ultimately leading to the death of young trees (Gérard et al. 2006, Newcombe et al. 1994). Recently, Frey et al. (2005) reported that new virulent strains of *M. larici-populina* have overcome the resistant poplar cultivars in Europe. Additionally, damage from rust disease in the Pacific Northwest is quite severe because of the length of the growing season, but in the prairies and in the province of Quebec the damage is less severe, likely as a result of the long cold winters (Feau et al. 2010, Newcombe 1998).

Marssonina spp. are also responsible for major leaf spot epidemics in Europe, Asia, Australia and more recently in China, apart from North America (Han et al. 2000). Defense responses to this pathogen are currently under study (Yuan et al. 2008, Zhang et al. 2007). Another major poplar pathogen is *Venturia* spp., which causes shoot blight and growth reduction on susceptible genotypes, and are mainly found in Europe, Asia and North America. Dominant and recessive resistance genes have been identified for resistance to *Venturia* spp. (Newcombe 2005, Newcombe and van Oosten 1997). Other pathogens that cause stem diseases on poplar trees are *Cytospora chrysosperma*, *Entoleuca mammata* and *Discosporium populeum* (Cellerino 1999, Newcombe 1996, Newcombe and Ostry 2001, Pinon and Valadon 1997). However, the most important disease is Septoria leaf spot and canker disease.

Sphaerulina musiva

A significant barrier, which is limiting the use of poplar species and hybrid poplar for wind breaks, culture plantations and fuel and fiber production, is canker and leaf spot disease caused by *Sphaerulina musiva* (Peck) Quaedvlieg, Verkley & Crous (syn. *Septoria musiva* Peck; teleomorph- *Mycosphaerella populorum* Thompson). This disease is considered the most serious disease of hybrid poplar plantations in the north central region of the US (Long et al. 1986, Waterman 1954). Recently, *S. musiva* was found in South America and west of the Rocky Mountains. Other closely related species of *S. musiva*, such as *S. populi* and *S. populicola*, cause leaf spot on poplar trees, and under rare circumstances, *S. populicola* also causes cankers on *P. balsamifera* (Newcombe and Ostry 2001, Zalasky 1978). It can be isolated from cankers and leaf spots during the growing season, and can be cultured *in vitro* (Bier 1939, Waterman 1946, 1954). Disease severity varies among sites, either due to highly virulent isolates or highly susceptible genotypes, and cankers formed lead to crown and stem breakage within 5-10 years of planting (Mottet et al. 2002). It is characterized by ascospores in pseudothecia. They are slightly constricted at the septum and range in size from 13-24 x 4-6 µm (Niyo et al. 1986). Ascospores are present in *Populus* plantations for most of the growing season, or for a period of 3-4 months, generally related to the frequency and amount of precipitation in a particular growing season. The asexual fruiting body, or pycnidia, bears multiseptate conidia which arise from holoblastic conidiogenous cells (Feau et al. 2010). These fruiting bodies are filled with rod shaped, hyaline conidia, which measure 28-54 x 3.5-4 µm with 1-4 septa (Sivanesan 1990). Under moist conditions, the conidia exude as pink masses or as long spore tendrils, which become white upon drying. Phylogenies constructed using rDNA have revealed the strong polyphyly characterizing

the genus *Sphaerulina* within the monophyletic genus *Mycosphaerella* (Crous et al. 2001, Goodwin et al. 2011).

In spring, primary infection occurs by means of ascospores. These ascospores penetrate the leaves through the stomata. The initial lesions appear 3 to 4 weeks after bud burst and are confined to leaves on the lower branches. Ascospore discharge occurs from the time the bud opens until the development of new growth of branches is complete. There have been no confirmed reports of ascospores infecting stem tissue (Luley and McNabb 1989). During summer, pycnidia are formed in lesions on both leaf surfaces and in cankers (Ostry 1987, Sivanesan 1990). Conidia released from these fruiting bodies are dispersed by the rain or wind, causing additional infections on stems and leaves by penetrating through petioles, lenticels, stomata or even at wound sites. The sexual stage begins in autumn with the formation of spermagonia on fallen leaves. In these leaves, numerous hyaline continuous rod shaped spermatia, measuring 4-6 µm x 1.5 µm, are produced. These give rise to fruiting bodies called pseudothecia in the fall. Even though the mechanism is not clear, it has been hypothesized that pseudothecia could possibly act as a survival structure for the fungus during the winter (Luley and McNabb 1989, Niyo et al. 1986, Ostry 1987). The cycle concludes with the meiotic formation of ascospores in pseudothecia.

The occurrence of leaf spot varies among genotypes. In some species, spots are small and multitudinous, while in others they are larger and sporadic. These differentiating features allows discrimination between resistant and susceptible genotypes. They can be circular, irregular or narrow, measuring from 1-15 mm in diameter. The color ranges from reddish brown to black, typically with yellow margins. The center region is mostly white, but sometimes pycnidia are visible. These spots eventually coalesce in susceptible genotypes, leading to severe damage of

leaf tissues, a reduction in photosynthesis and ultimately defoliation (Krupinsky 1989, Lo et al. 1995, Ostry and McNabb 1985).

Septoria canker in the US was first reported in 1923, in the Northern part of the US (Waterman 1954). These cankers are perennial. The etiology begins with tissue discoloration, followed by a sunken area of necrotic tissues, ultimately leading to stem deformities, and sometimes resulting in tree mortality, depending on the severity of infection (Stanosz and Stanosz 2002). A canker can predispose the tree to wind breakage in the area above the infection site. There are many factors that increase the incidence of cankers, such as increasing soil depth, tree height, levels of P, K and Mg and decreased levels of Fe and Al (Abebe and Hart 1990). High humidity near the ground favors the development of serious basal cankers (Waterman 1954). The new twigs and branches on highly susceptible trees are prone to infection, but formation of lesions will be less extensive. In stem infections, there is rapid invasion of the infected area by secondary parasites, which masks the presence of *S. musiva*. On resistant hosts, lesions are formed, but the pathogen grows slower and eventually stops. The lesion cracks are gradually healed or closed by means of callus deposition.

Sphaerulina musiva **and the** ***Populus*** **Pathosystem**

For disease to occur the interaction among three elements is required: host, pathogen and environment. Disease on species like *P. deltoides*, *P. tremuloides* and *P. grandidentata* is less severe. The incidence and severity of the disease also varies among genotypes. However, susceptibility to leaf spot and canker is mainly observed in those plantations where susceptible hybrids, between the sections *Aigeiros* and *Tacamahaca*, are planted. Numerous studies have reported on host range, incidence and severity of the disease in poplar (Bier 1939, Hansen et al. 1994, Ostry and McNabb 1985, Thompson 1941, Waterman 1954). However, some authors are

very critical of reporting canker damage from field surveys, because other pathogens besides *S. musiva* can be present (Lo et al. 1995).

A small number of studies have been conducted to assess population diversity and the geographic distribution of *S. musiva* (Feau et al. 2005, Krupinsky 1989, LeBoldus et al. 2008, Sakalidis et al. 2016). Krupinsky (1989) evaluated variability among isolates collected from local and regional areas. He found differences in severity of leaf spot likely due to variability among isolates. In a more recent study, the genetic structure of the pathogen population was shown to be consistent with spread mediated by anthropogenic activities, rather than natural spread of spores (Sakalidis et al. 2016). This type of spread was noted in the western part of North America, and the possible explanation for this is the continuous exchange of plant material between growers and breeders, ultimately leading to gene flow between distant geographic areas (Carnegie and Cooper 2011, Goss et al. 2009, Sakalidis et al. 2016).

The origins of *S. musiva* in the eastern and central US are consistent with published reports. *Sphaerulina musiva*'s endemic host is *P. deltoides* (Dhillon et al. 2015). The largest number of unique SNPs, and the greatest nucleotide diversity values, were observed for *S. musiva* populations in the eastern US (Sakalidis et al. 2016). This pattern is consistent with the belief that genetic diversity is greatest within a species endemic range (Dlugosch and Parker 2008, Nei et al. 1975, Robert et al. 2012). In the midwestern US, the *S. musiva* population is also highly diverse, appears to be involved in sexual reproduction, and is highly admixed. This can be explained by the presence of host species in riparian areas and shelterbelts acting as a reservoir of the pathogen. The genetic diversity of the population in this region has also been artificially inflated due to the introduction of infected plant material on dormant cuttings by the poplar industry (Dhillon et al. 2015, Sakalidis et al. 2016).

Quebec, Alberta, British Columbia and Saskatchewan are the main locations in Canada where breeding programs occur. The exchange of plant material among these breeding programs has likely resulted in the spread of this pathogen into western Canada. This spread is facilitated by the ability of *S. musiva* to colonize the host asymptomatically (Ostry and McNabb 1985, Waterman 1954, Waterman and Aldrich 1952). In Alberta, severe cankers on *P. balsamifera* were first reported in 2009 (LeBoldus et al. 2009). Based on only a few isolates, it has been speculated that this pathogen population has low diversity and was apparently clonal. The genetic structure and existence of the pathogen in Alberta could also be explained by the single introduction of cuttings, or as an association with plant debris from Saskatchewan (Sakalidis et al. 2016). Similar to Alberta, it is only recently that *S. musiva* has been reported in British Columbia, but the pathogen population there has higher levels of genetic diversity. Possible explanations for this pattern include: (i) *S. musiva* was present but undetected for a long period of time; (ii) multiple introductions from numerous sources; or (iii) the pathogen may be reproducing sexually (Sakalidis et al. 2016). More studies need to be conducted to get an in depth understanding of the relationship between genetic structure and virulence.

The third major factor affecting disease development is environmental. Two studies have reported the effects of water stress on the *S. musiva* and *Populus* pathosystem (LeBoldus et al. 2007, Maxwell et al. 1997). In both studies, there was no clone * isolate * water stress interaction for the majority of genotypes (LeBoldus et al. 2007, Maxwell et al. 1997). Recently, it was reported that fields in which NM6 clones were growing in Wisconsin (Weiland et al. 2003) and Minnesota were severely infected with canker. The reason for this is still unclear, but environmental conditions during disease outbreak could be one possible explanation (Qin et al. 2014). Newcombe et al. (2001) reported the lack of symptom development on canker

suppressive sites, in spite of the presence of susceptible hosts and inoculum. They concluded that this was a result of unfavorable environmental conditions. Thus, more studies need to be conducted for an in depth understanding of the role of environmental conditions on this disease. Other epidemiological factors recently reported by Dunnell and LeBoldus (2016), including host tissue differences (leaf or stem), leaf age effects, difference in sporulation and clone * isolate interactions also require further study.

Krupinsky (1989) detected a clone * isolate interaction by examining leaf spot aggressiveness, but the effects of clone and isolate explained more of the variation than the clone * isolate interaction. Ward and Ostry (2005) also detected a significant clone * isolate interaction. To date, the most in depth study where a significant clone * isolate interaction was detected was when 14 clones of hybrid poplar were inoculated with 19 isolates of *S. musiva* (LeBoldus et al. 2008). However, an examination of each clone * isolate combination indicated the absence of any rank order changes (LeBoldus et al. 2008). Dunnell et al. (2016) also reported significant clone * isolate interactions when disease severity score was used as the response variable. Thus, clone * isolate studies play a role in effective selection of resistant clones.

Due to recent advances in molecular techniques, there are new ways to study host–pathogen interactions. So far, two RNA-seq studies have been conducted to elucidate the host genes expressed during infection. Some of the genes that were differentially expressed by the host included lignin biosynthesis genes, chitinases, β-glucosidase, kunitz trypsin inhibitor, transcription factors such as WRKY 5 and WRKY 70, salicylic acid pathway and peroxiredoxin (Foster et al. 2015, Liang et al. 2014). Differentially expressed genes in the pathogen grown on leaf extract and wood chips media were studied using RNA-seq. Some of the genes that were expressed in *S. musiva* grown on poplar leaves and wood chips were NRPS (non-ribosomal

peptide synthetase) – PKS (polyketide synthase) cluster, lignolytic enzyme, lytic polysaccharide, copper oxidases and oligo peptidase. In addition, cutinase genes were expressed upon interaction with leaves, whereas those on wood chips were carbohydrate active enzymes, lytic polysaccharide monooxygenase enzymes, esterases, transmembrane sugar, α-glucan cluster, trypsin, peptidases, peptides and amino acid transporters. These results provide strong evidence that the pathogen relies on proteolysis for nutrition and colonization of bark (Dhillon et al. 2015).

Management

Sphaerulina musiva can cause early season defoliation and stem breakage of susceptible clones, leading to plantation failure (Ostry 1987). Several management strategies, including disease resistance, cultural practices and biological and chemical control methods, have been used to prevent adverse effects from this disease. Cultural practices, like removing leaf litter before leaf emergence in spring and pruning of infected branches, are recommended. However, this strategy seems to be effective only in nurseries and stool beds that serve as temporary places for growing plant material in which the inoculum comes from nearby locations, as opposed to plantations that are more permanent locations where inoculum can build up over time (Filer et al. 1971, Ostry et al. 1988). Chemical controls include fungicide treatments, like Bordeaux mixture 4-4-50, benomyl, ceresin M and semesan (Carlson 1974). However, the effectiveness of chemical controls may be reduced under increased inoculum and stem density (Ostry et al. 1988, Waterman 1954). In addition, they are expensive, as multiple applications over many years are required (Ostry and McNabb 1983, 1985).

Biological control methods could provide an alternative management approach. Some *Streptomyces* strains have demonstrated the ability to inhibit the growth of *S. musiva*, both *in vitro* and in field conditions. There was an 83% reduction of leaf disease, when a combined

mixture of *Streptomyces* and tergitol solution was applied (Gyenis et al. 2003). Another agent that has been used, *Phaeotheca dimorphospora* DesRoch. and Ouell., also has antibiotic activity against *S. musiva*. This fungus was able to reduce pathogen growth, *in vitro* and in the greenhouse, by reducing fungal growth and disease severity on leaves, respectively (Yang et al. 1994). Selection and deployment of resistant clones would provide the best chances for the survival of poplar plantations, as the management methods mentioned above are only partially effective. The present strategy is to cultivate a large diversity of unrelated, and partially resistant, clones from different lineages. One recommendation suggests taking six to eight clones from different regions to achieve a high level of variability in resistance (Périnet 1999, 2007).

In order to select resistant clones, it is necessary to conduct long-term field trials. These field trials occur at different sites, using different clones, over the course of several years. For example, 40-80 clones were tested for their performance at 41 sites throughout the Midwest, thus enabling selection of clones with lower disease severity (Hansen et al. 1994). Abrahamson et al. (1990), tested 54 hybrid clones and recommended superior clones based on their growth, height, width and canker severity. Even though these studies have proven to be effective in the selection of resistant clones from field experiments, one of the major problems with field-testing is disease escape (Abrahamson et al. 1990). In order to prevent problems of disease escape, many authors have developed screening assays for young trees under field and/or greenhouse conditions using artificial inoculation (Cooper and Filer 1976, Filer et al. 1971, Krupinsky 1989, LeBoldus et al. 2010, Long et al. 1986, Newcombe 1998, Ostry and McNabb 1985, Qin et al. 2014, Spielman et al. 1986, Weiland et al. 2003, Zalasky 1978).

For these screening assays, two inoculation protocols have been developed. One is by wound inoculation, which is done by removing a leaf and placing a plug of sporulating mycelium

on the stem wound, which is then wrapped with Parafilm (Cooper and Filer 1976, Filer et al. 1971, Long et al. 1986, Newcombe 1998, Ostry and McNabb 1985, Spielman et al. 1986, Weiland et al. 2003, Zalasky 1978). This wounding procedure has proven to accurately predict resistant and susceptible clones under field conditions, even though it may circumvent some resistance mechanisms (Weiland et al. 2003). The second inoculation protocol involves inoculation of nonwounded trees with a spore suspension (Krupinsky 1989, LeBoldus et al. 2010, Qin et al. 2014). Screening assays using this method are much faster, as multiple isolates can be used and resistance mechanisms are not circumvented. Many authors have also suggested the use of highly virulent isolates to identify the most resistant and susceptible genotypes (Dunnell et al. 2016, LeBoldus et al. 2008, Ostry et al. 1988, Qin et al. 2014, Weiland et al. 2003). LeBoldus et al. (2007), suggest variation among host genotypes plays a major role in disease resistance, more than pathogen variability or abiotic factors. However, a combination of strategies is likely required for better management of the disease.

One such approach is the use of transgenic trees resistant to *S. musiva*. The transformation of *Populus* began in the mid 1980's (Fillatti et al. 1987). The first report of transgenic poplar were mutations to the *aroA* gene encoding 5-enolpyruvyl-3-phosphoshikimate synthase, which conferred herbicide resistance. In terms of disease resistance, synthetic peptides like MIM^{TM} and antimicrobial peptide ESF12 appear to inhibit the growth of *S. musiva*. *In vitro* characterization of the toxicity of MIM^{TM} and antifungal activity has demonstrated the potential of using such peptides to confer resistance to *S. musiva* infection (Jacobi et al. 2000, Powell et al. 1995). In a similar fashion, the introduction of the gene encoding for the antimicrobial peptide ESF12 into susceptible hybrid clone 'Ogy' enhanced resistance to leaf spot disease (Liang et al. 2001). The same cultivar showed increased resistance to *S. musiva*, because of increased latency

in the development of foliar necrosis caused by *S. musiva*, when transformed with the wheat oxalate oxidase gene (Liang et al. 2002). Trees expressing Bt transgenes were developed for resistance against chrysomelid beetles and lepidopteran caterpillars, as both are susceptible to insecticidal proteins (Knowles and Dow 1993). Transgenic poplars have also been developed for early flowering, increased biomass and tolerance of various environmental conditions (Ye et al. 2011). Although transgenic research with *Populus* has shown progress, it remains far behind Arabidopsis and rice, as transformation efficiency remains low, storing transgenic seeds is difficult and there is low seed viability (Ye et al. 2011).

Most of the research on the *S. musiva – Populus* spp. pathosystem is focused on selection and deployment of resistant genotypes, in order to reduce the impact of Septoria leaf spot and stem canker disease on poplar plantations. Septoria canker resistance screening is conducted on juvenile trees in the greenhouse by wounding or nonwounding approaches. These methodologies have been demonstrated to accurately identify the most resistant and susceptible clones and to predict their long-term field performance (Qin et al. 2014, Weiland et al. 2003). Another efficient strategy is to prevent the introduction of this pathogen, as it has the ability to asymptomatically colonize the host tissue. The recent development of molecular techniques like qPCR allows, not only the detection of very low levels of pathogen from symptomatic and asymptomatic tissue (Acevedo et al. 2010, Oliver et al. 2008), but also may have the potential to improve the speed and accuracy of phenotyping. Although these studies have paved the way for selecting resistant genotypes, a greater depth of understanding of the interaction between *S. musiva* and *Populus* spp. is required.

The overall objective of the work discussed in this thesis will help further elucidate this pathosystem. The specific objectives are: 1) develop a qPCR assay to: a) improve speed and

accuracy of phenotyping by quantifying the ratio of pathogen to host DNA; and b) evaluate the potential of the qPCR assay as a diagnostic tool; and 2) conduct comparative studies of resistant and susceptible *Populus* genotypes inoculated with *Sphaerulina musiva* using histological, biochemical and molecular approaches to: a) examine the pre-penetration processes of conidial germination, hyphal growth and mode of penetration of *S. musiva* on *Populus* leaves using scanning electron microscopy (SEM); b) examine the post-penetration process of colonization of leaf tissue with *AsRed* transformed *S. musiva* using confocal microscopy; c) describe the role of H_2O_2 in disease development; and d) identify genes expressed in host and pathogen during their interaction using RNA-seq.

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CHAPTER 2. DETECTION AND QUANTIFICATION OF HYBRID POPLAR STEMS INFECTED WITH *SPHAERULINA MUSIVA* USING QPCR

Introduction

Forests around the world are under threat from the introduction of exotic plant diseases (Dutech et al. 2012, Geils et al. 2010). This threat is a result of many factors, including the movement of plant material infested with fungal or bacterial pathogens. This is particularly true of hybrid poplar culture, where dormant cuttings of productive clones have been freely exchanged among private and public breeding programs, and across international borders, for decades. This movement has resulted in the spread of *Sphaerulina musiva* (Peck) Quaedvlieg, Verkley & Crous (syn. *Septoria musiva*), the cause of Septoria leaf spot and stem canker, across North America (Callan et al. 2007, Sakalidis et al. 2016), and as far away as the Russian Caucasus (Cellerino 1999) and Brazil (Santos et al. 2010).

Once established in *Populus* plantations, the impacts of *S. musiva* are well documented and severe (Feau et al. 2010). The leaf-spot disease can reduce a trees' photosynthetic area and cause premature defoliation, decreasing annual growth (Krupinsky 1989, Lo et al. 1995, Ostry and McNabb 1985). Stem cankers can reduce growth, predispose the tree to colonization by secondary organisms, girdle the main stem, or cause stem defects increasing the risk of wind breakage (Lo et al. 1995, Ostry 1987, Weiland et al. 2003). Examples of the impacts include a survey conducted in northern Wisconsin, where 10,000 ha of a single susceptible variety were planted, >90% of trees were infected and 74% had major stem damage (Weiland and Stanosz 2003). In Michigan, 86% of the trees in a plantation had Septoria cankers and two years later 69% had broken tops, just 5 years after planting (Ostry et al. 1989). A trial, conducted in northern Alberta with 56 clones of *P. balsamifera*, resulted in all trees with at least one canker

per stem 7 years after planting (LeBoldus et al. 2009). Management strategies, including cultural practices, chemical control and biological control, have been attempted but are only partially effective or too expensive to use in commercial poplar plantations (Feau et al. 2010, Gyenis et al. 2003, Lo et al. 1995, Ostry and McNabb 1983, Ostry et al. 1988, Yang et al. 1994).

Reducing the impact of this disease on hybrid poplar plantations hinges upon two key approaches: (i) preventing new introductions of *S. musiva*; and (ii) using disease resistant clones to reduce damage by this pathogen (Bernier et al. 2003, Mottet et al. 1991, Newcombe and Ostry 2001, Ostry 1987, Ostry and McNabb 1983). The most efficient strategy for preventing the introduction of a pathogen is early detection. Historically, this has involved plating of symptomatic tissue and morphological identification of the fungal colonies that develop. This can be problematic in the case of fungi like *S. musiva*, which can be present in asymptomatic tissue (Ostry and McNabb 1983, Waterman 1954, Waterman and Aldrich 1952). As a result, polymerase chain reaction (PCR) based approaches, which increase the speed and accuracy of pathogen detection in plant tissue, have been developed. More recently, quantitative PCR (qPCR) has been used to detect a pathogen at extremely low levels in symptomatic and asymptomatic tissue (Acevedo et al. 2010, Oliver et al. 2008, Pasche et al. 2013, Zurn et al. 2015). The incorporation of hydrolysis probes in these assays has further improved their sensitivity and specificity by allowing simultaneous quantification of both the host and pathogen DNA (Acevedo et al. 2010). This technique has improved pathogen detection and is currently being used as a tool for high-resolution phenotyping in several pathosystems (Boyle et al. 2005, Hietala et al. 2003, Huang et al. 2014, Luchi et al. 2013, Oliver et al. 2008). The second effective strategy for managing Septoria canker, using disease resistant clones, typically involves screening of juvenile trees by wounding or nonwounding approaches (LeBoldus et al. 2010,

Weiland et al. 2003). These methodologies have been demonstrated to accurately identify the most resistant and susceptible clones and predict their long-term field performance (Qin et al. 2014, Weiland et al. 2003). A qPCR-based approach has the potential to further improve the speed and accuracy of this phenotyping. With this in mind, the objectives of this study were to: (i) develop a specific, sensitive and accurate qPCR assay to detect and quantify *S. musiva* and *Populus* DNA from artificially (=inoculated samples) and naturally infected (=environmental sample) hybrid poplar; and (ii) test the potential of the qPCR assay as a tool for disease resistance phenotyping.

Materials and Methods

Primer design and validation for *Sphaerulina musiva*

Beta tubulin sequences of *S. musiva* (DQ026399.1 and DQ026398.1), and the closely related species *S. populicola* (DQ026389.1, DQ026388.1 and DQ026387.1), were obtained from NCBI-GenBank and aligned using the program BioEdit (Ibis Biosciences, Carlsbad, CA, USA). The primer pair specific to *S. musiva* (NABtF: 5'-CGACCTGAACCACCTTGTCT-3' and NABtR: 5'-CACGGTAACAGCGCGGAACGA-3') was identified using a variable region of the *beta tubulin* gene with a 200 bp fragment as the target amplicon. All DNA extractions were conducted using the Qiagen DNeasy plant mini kit (Valencia, CA, USA) following the manufacturer's protocol. The PCR reactions were conducted in an Applied Biosystems Veriti 96 well thermal cycler (Life Technologies, Grand Island, NY, USA) in a total volume of 25 µl containing 12.5 µl of 2X GoTaq Green Master Mix (Promega, Madison, WI, USA), 1 µl of a 5 µM suspension of each primer, 9.5 µl of H_2O and 1 µl of template DNA. The reaction conditions were an initial denaturation step at 94°C for 4 min, 35 cycles of 94°C for 10 s, 58°C for 20 s and 72°C for 30 s, followed by 72°C for 10 min of extension.

Primer specificity was evaluated using two approaches. Initially, non-specific amplification using genomic DNA extracted from a variety of fungi (*Fusarium* sp., *Alternaria* sp., *Epicoccum* sp., *Cladosporium fulvum*, *Penicillium* sp., *Venturia* sp., *S. populicola* and *Marssonina* sp.), as well as *Populus* DNA extracted from uninfected tissue. Specificity of beta tubulin primers were further validated by amplifying DNA from the pathogens mentioned above with ITS1 and ITS4 primers using the previous conditions but with an annealing temperature of 60°C. The PCR products were visualized on a 1.5% agarose (VWR, Radnor, PA, USA) gel stained with GelRed™ nucleic acid stain (Biotium, Hayward, CA, USA). The second approach used a high resolution melting curve analysis using the CFX96 to confirm that a single *S. musiva* PCR product was being amplified.

Primer design and validation for hybrid poplar

The *Populus* specific primers were based on the eukaryotic translation initiation factor (*eIF4AII*) gene (eIF4FI: 5'-TGGGGCCTCTATTTAGCATGGAT-3' and eIF4RI: 5'-CTGCACCCGAAATGGGATTGACC-3'; Hamel et al. 2011). Primer specificity was evaluated using the two approaches described above.

Probe design

The amplicons from NABtF/NABtR and eIF4FI/eIF4RI were sequenced (GenScript, Piscataway, NJ, USA) and used to design fluorescent probes for the pathogen and the host, respectively. The pathogen probe was labeled with 6-carboxyfluorescein (6-FAM; 5'-TCCCACGTCTCCACTTCT TC-3'; Applied Biosystems, Foster City, CA, USA) and the host probe was labeled with VIC (5'-GCAGGTTTGGTTCTGAGCAT-3'; Applied Biosystems, Foster City, CA, USA).

Multiplexed qPCR assay efficiency

The Taqman assay was performed on the Bio-Rad CFX96 Touch thermal cycler using 96 well plates. A five point standard curve was prepared for the host using a tenfold dilution (60 ng μl^{-1}, 6 ng μl^{-1}, 0.6 ng μl^{-1}, 0.06 ng μl^{-1} and 0.006 ng μl^{-1}) of *Populus* DNA, and for the pathogen using a fivefold dilution (50 ng μl^{-1}, 10 ng μl^{-1}, 2 ng μl^{-1}, 0.4 ng μl^{-1} and 0.08 ng μl^{-1}) of *S. musiva* DNA. Initially, the assays were conducted separately as simplex reactions to determine the quantification cycle (Cq) values for the host and pathogen. A Cq value is the PCR cycle number at which the fluorescence from a tested sample is significantly greater than that of the background fluorescence. Other synonyms for Cq found in the literature are C_p, C_t and take-off point (TOP). All of these terms represent the same value obtained from the thermal cycler (Bustin et al. 2009). Higher Cq values denote a lower quantity of target DNA in the tested sample.

The assay was then multiplexed to confirm its efficiency when host and pathogen DNA were combined in a single reaction. In the multiplexed reaction, the total volume was 22.5 µl containing 12.5 µl of Taqman gene expression master mix (ABI, Grand Island, NY, USA), 1.5 µl of 5 µM pathogen primers and probes, 1 µl of 5 µM host primers and probes and 2 µl each of host and pathogen template. Reaction efficiency was calculated using the formula $E = 10^{(-1/slope)} - 1$. Amplification conditions were initial incubation at 50°C for 2 min, initial denaturation at 95°C for 10 min, amplification for 50 cycles at 95°C for 15 s and elongation at 58°C for 30 s. For the quantification of inoculated and environmental samples, the reaction mixture and conditions were the same as described above. For all qPCR experiments, three technical replicates of each sample were included on a plate, distilled water was used as a negative control

and variation among plates was evaluated by including reference samples of a known concentration of DNA on each plate (60 ng μl^{-1} of host DNA and 50 ng μl^{-1} of pathogen DNA).

Phenotyping assay

Hybrid poplar propagation

A total of sixty dormant hardwood cuttings were collected from six different clones of hybrid poplar during February 2014 at a field site located at North Dakota State University (NDSU) in Fargo, ND. These clones were selected based on their resistance as reported in the literature (Qin et al. 2014, Weiland et al. 2003; Table 2.1). The hardwood cuttings were cut to a length of 10 cm. They were then placed in water for two days in small trays at room temperature, and subsequently, planted in SC10 Super cone-tainers (Stuewe & Sons Deepots D40 cell, Stuewe & Sons Inc., Tangent, OR, USA) using SunGro growing medium (SunGro Professional Mix #8, SunGro Horticulture Ltd., Agawam, MA, USA) combined with slow release nutricote fertilizer (15-8-12) (Scotts Osmocote Plus, Scotts Company Ltd., Marysville, OH, USA). Rooted cuttings were transferred into pots (22-cm deep by 22.5-cm in diameter; Stuewe & Sons Tree Pot CP59R, Stuewe & Sons Inc., USA) containing SunGro growing medium. Slow release fertilizer was supplemented with a bi-monthly application of 20-20-20 liquid fertilizer (Scotts Peters Professional, Scotts Company Ltd., Marysville, OH, USA). All trees were grown in a greenhouse with an 18 h photoperiod augmented with sodium lamps and a 20°C (day)/16°C (night) temperature regime.

Table 2.1. Classification of clones based on susceptibility against *Sphaerulina musiva*.

			Susceptibility class	
Clone	**Parentage**	**Field rating**	**Wound inoculation (Field and greenhouse)[1]**	**Spray inoculation (Greenhouse)[2]**
NM6	*P. maximowczii* x *P. nigra*	L	L	L
DN177	*P. deltoides* x *P. nigra*	L	L	L
MWH5	*P. deltoides* x *P. maximowczii*	H	H	L
NC11505	*P. maximowczii* x *P. trichocarpa*	H	H	H
NC11432	*P. deltoides* x *P. trichocarpa*	H	H	H
NE351	*P. deltoides* x *P. nigra*	H	H	H

1. Weiland et al. 2003
2. Qin et al. 2014

Pathogen propagation

Two isolates of *S. musiva*, MN14 and MN20, were isolated from hybrid poplar cankers collected near Garfield, MN during the winter of 2012. Cankers were surface sterilized by soaking them in a 5% bleach solution (Homelife Bleach Regular Scent, KIK Custom Products Inc., Chicago, USA) for 2 min and rinsed twice in sterile distilled water. Subsequently, the bark was removed from the margin between necrotic and healthy tissue and a 4 mm-long piece of wood was placed on V8 juice agar plates (1.5 g of $CaCO_3$, 137 ml of V8 juice, 15.2 g of Difco agar and 625 ml of deionized water). The necrotic tissue was incubated for 1 week at room temperature and any fungal growth on the petri plates was sub-cultured to obtain pure colonies.

Confirmation of the species was based on morphology of the conidia and multilocus genotyping (LeBoldus et al. 2015, Sivanesan 1990). The isolates were stored in 1 ml of a 50% glycerol solution at -80°C. To prepare inoculum for the experiments outlined below, the isolates were retrieved from storage, poured onto five V8 juice agar plates and allowed to grow for 1 week at room temperature prior to sub-culturing onto 10 plates. These two isolates were selected for the experiments described herein based on their known ability to incite stem cankers.

Experimental design, wound inoculation and DNA extraction

The experimental design was a randomized complete block design with five blocks. There were six hybrid poplar clones, two isolates and three time points. Each clone * isolate * time point combination occurred once per block. There were a total of 270 trees in the experiment, including non-inoculated controls. Wound inoculations were conducted as previously described in the literature (Mottet et al. 1991). Briefly, the fifth leaf from the shoot apex was excised and the wound was inoculated with a 5 mm diameter plug of sporulating mycelium. Control trees were inoculated in a similar manner with a sterile V8 juice agar plug. There was one control tree per genotype per block. The inoculation point was wrapped with Parafilm. After 48 h, Parafilm was removed and the samples for the qPCR experiment were collected at 1 week, 3 weeks and 7 weeks post-inoculation (wpi). Cankers were rated for disease severity at the time of collection using a 1 to 5 scale (1- no callus formation and wound is healed, 2- callus formation, 3- necrosis contained by callus, 4- spread of necrosis beyond the callus, 5- stem girdled by necrosis; LeBoldus et al. 2008); rating 0 was given for the clones at 1 wpi. Clones with a rating less than or equal to 3 were considered resistant, as the necrotic area was enclosed by callus preventing further disease development. Disease severity scores greater than 3 were considered susceptible, because there was either no callus development or the necrosis had

spread beyond the callus. The samples for DNA extraction were collected by pruning the stem at 1.5 cm above and below the inoculation point. DNA was extracted from a 3-cm long by 1-cm wide piece of bark and cambium tissue centered on the inoculation point. The experiment was repeated once.

The IC values were calculated by using the Cq's for both the host and pathogen in the following equation: IC = (*Cq* of pathogen/*Cq* of host), and was used as a measure of resistance. We interpreted it to mean that the higher the IC value the lower the amount of pathogen DNA in host tissues indicating resistance and vice versa.

Testing of field samples

To test the qPCR assay described above as a tool for molecular diagnosis of *S. musiva* from field samples, cankers were collected from two locations in the summer of 2016. Thirteen trees were sampled at the NDSU Horticultural Research Farm and 2 trees were sampled at the NDSU Seed Farm. From each tree, two cuttings (10-15 cm long) with diameters ranging from 0.5-3 cm were collected. One of the cuttings had a canker and the second cutting had no canker. Upon harvesting, the samples were stored at -20°C until they were processed. Further processing involved dividing them into two equal parts. One part was used for the qPCR assay and the second part was used in a cultural assay. DNA extraction and the qPCR assay was performed as described in the previous sections. For the cultural assay, each sample was surface sterilized and the bark was removed as described above. Four subsamples were plated on V8 juice agar plates amended with streptomycin (100 mg/L) and chloramphenicol (246 mg/L). These cultures were incubated at room temperature for seven to 10 days and observed for *S. musiva* growth.

Statistical analysis

The mixed procedure in SAS 9.3 (Littell et al. 2006) was used to analyze the effects of clone, isolate, time, and their interactions on the two response variables: disease severity and the Infection Coefficient (IC). These IC values were averaged across the three technical replicates of each sample. Model selection was conducted in a two-stage process. Initially, the significance of the random factors (experiment, block, and block * experiment) were tested using a likelihood ratio chi-square test. Subsequently, the significance of the fixed effects (clone, isolate, time, clone * isolate, clone * time, isolate * time, and clone * isolate * time) were tested using the significance of the default Z-test for fixed effects in SAS. The repeated statement in SAS was used to model unequal variances as needed. In all cases statistical significance was assessed at $\alpha = 0.05$.

In order to examine the relationship between the IC values at the early time points (1 wpi and 3 wpi) with disease severity at 7 wpi, the final models for disease severity and IC, in combination with the ESTIMATE statement in SAS, were used to generate the mean IC and disease severity values for each clone * isolate * time point combination. The mean IC values at 1 wpi and 3 wpi were each correlated separately to the mean disease severity at 7 wpi and Pearson correlation coefficients were estimated using the CORR procedure in SAS.

Finally, in order to establish a qPCR cut-off between resistant and susceptible clones, the IC values with the highest correlation to disease severity at 7 wpi were plotted and a simple linear regression was fit to the data (R Core Team 2015). The equation for the linear model was then used to determine the IC value cut-off between resistance and susceptibility by solving for Y when a value of 3 was used as the disease severity cut-off. The rationale for this disease severity cut-off was described above.

Results

Taqman qPCR assay development

Primer specificity and validation

Using the primers NABtF/NABtR, a 200 bp fragment of the *beta tubulin* gene from *S. musiva* was successfully amplified and sequenced. Sequence comparison indicated 100% identity to the *S. musiva beta tubulin* gene, confirming that the correct product was amplified. The NABtF/NABtR primer pair failed to amplify any non target fungal or host DNA (Table 2.2; Fig. A1, A2). The ITS1 and ITS4 primers successfully amplified all fungal DNAs (Fig. A3). Melting curve analysis indicated the presence of a single peak for the NABtF/NABtR amplicon at 82°C (±2) (Fig. A5).

Table 2.2. Evaluation of specificity of beta tubulin primers on different fungal species.

Pathogen tested	Beta tubulin primers
Sphaerulina musiva	+
***Epicoccum* sp.**	-
***Alternaria* sp.**	-
Cladosporium fulvum	-
***Fusarium* sp.**	-
***Penicillium* sp.**	-
***Venturia* sp.**	-
***Marssonina* sp.**	-
S. populicola	-

Primers (eIF4FI/eIF4RI) targeting the *eIF4AII* gene were used to amplify *Populus* DNA. The amplicon was 110 bp with 100% identity to the *Populus eIF4AII* gene. When these primers were tested for specificity, using similar reaction conditions and *S. musiva* as a template, no amplification was observed (Fig. A4). Furthermore, the high resolution melting curve analysis indicated a single peak with a mean temperature at 88°C (±2) (Fig. A5).

Testing qPCR assay efficiency

Amplification efficiency in the multiplex reaction was determined to be 91.2% for the pathogen and 101.9% for the host (Fig. 2.1). A strong correlation between Cq values and template concentration was observed, with $R^2 = 0.99$ for the host (*eIF4AII*) and $R^2 = 0.98$ for the pathogen (*beta tubulin*).

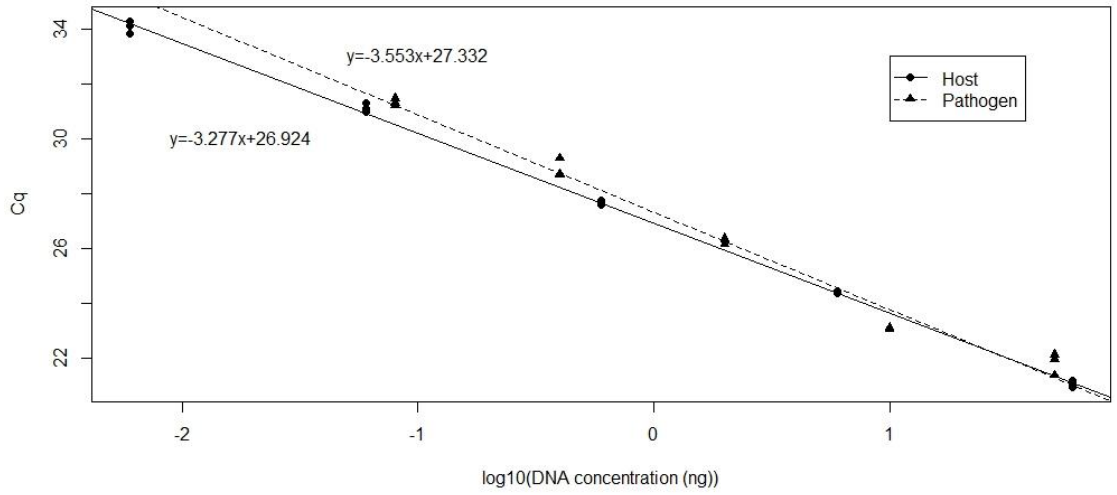

Figure 2.1. Estimated amplification efficiency curve for *S. musiva* (5-fold serial dilution, $R^2 = 0.98$, slope = -3.55, y-int = 27.332) in the presence of *Populus* DNA (10-fold serial dilution, $R^2 = 0.99$, slope = -3.27, y-int = 26.924).

Phenotyping assay

Disease severity of inoculated and control trees was evaluated at 1 wpi, 3 wpi and 7 wpi (1 - 5 scale). No symptoms were evident on samples collected at 1 wpi. At 3 wpi, necrotic lesions were observed, with no visible distinctions among the six inoculated clones. At 7 wpi, differences were apparent among clones in terms of disease severity.

Unequal variances between the two experiments ($p < 0.001$) were modeled using the repeated statement in SAS and the data was subsequently combined into a single analysis. This analysis indicated that there were differences among clones ($p < 0.001$), time points ($p < 0.001$), and that there was a significant clone * time interaction ($p < 0.001$; Table 2.3). No differences between isolates ($p = 0.163$) were detected, nor was there a significant isolate * time ($p = 0.498$) or clone * isolate * time interaction ($p = 0.473$; Table 2.3). Differences in mean disease severities among clones across the 3 time points were not observed until 3 wpi, when NC11505 had a greater disease severity than all other clones. There was also a notably higher disease severity on NE351 when compared to NM6, MWH5, DN177 and NC11432. In addition, MWH5 had less disease than DN177 and NM6 (Fig. 2.2). At 7 wpi, NC11505 had a substantially greater disease severity compared to all other clones. NC11432 and MWH5 had similar and less disease when compared to NE351, DN177 and NM6. However, no difference was detected between NM6 and DN177 (Fig. 2.2).

Table 2.3. Analysis of variance of the response variables disease severity and IC value showing significance of the fixed effects and their interactions partitioned among sources.

Effect	df_N	df_D	Disease severity	IC value
CLONE	5	137	<0.001[a]	<0.001
ISOLATE	1	288	0.163	0.0005
CLONE*ISOLATE	5	137	0.868	0.694
TIME	2	288	<0.001	<0.001
CLONE*TIME	10	137	<0.001	<0.001
ISOLATE*TIME	2	288	0.498	0.003
CLONE*ISOLATE*TIME	10	137	0.473	0.569

[a.] *p*-value

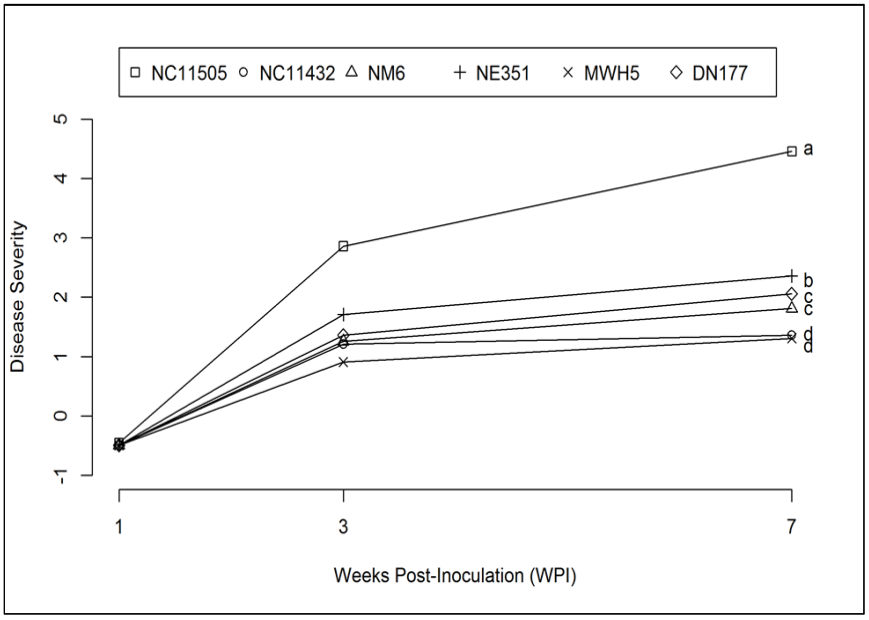

Figure 2.2. Mean disease severities between clones at 1 wpi, 3 wpi and 7 wpi.
Note: Statistical differences among clones in terms of disease severity at 3 wpi are listed in the text.

qPCR assay

Significant differences in IC values among clones ($p < 0.001$), time points ($p < 0.001$), as well as a clone * time interaction ($p < 0.001$; Fig.2.3) were detected. In addition, there were differences between isolates at 1 wpi ($p = 0.005$) and there was a significant isolate * time interaction ($p = 0.003$; Table 2.3). There was no clone * isolate * time interaction ($p = 0.568$). Comparisons of IC values among clones at 1 wpi indicated that there were no significant differences. However, at 3 wpi, a reduction in IC values across all the clones indicated an increase in pathogen biomass relative to host biomass. By 7 wpi, only the IC values of NC11505 continued to decline. Across 3 wpi and 7 wpi time points, only the IC value for NC11505 was significantly different ($p < 0.001$) when compared to all other clones.

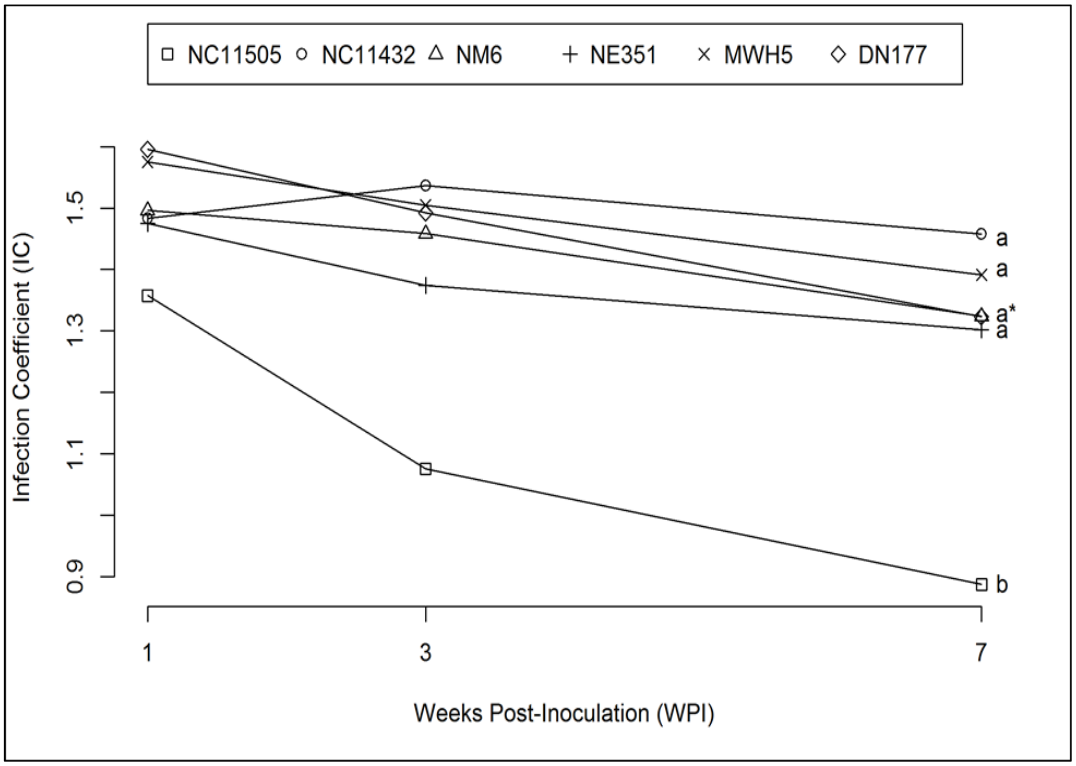

Fig 2.3. Mean of infection coefficient between clones at 1 wpi, 3 wpi and 7 wpi.
Note: Statistical differences among clones in terms of infection coefficient at 3 wpi are listed in the text.

Correlation of disease severity with IC values

A significant Pearson's correlation (r = 0.93; $p < 0.001$) was detected between the mean disease severity at 7 wpi with the IC at 3 wpi. In contrast, the correlation was not significant when IC values at 1 wpi were correlated to the mean disease severity at 7 wpi. In order to establish an IC cut-off value between resistant and susceptible clones, a linear regression ($y = -6.331x + 11.724$) was fit to the plot of the IC values at 3 wpi versus the mean disease severity at 7 wpi. Using a threshold disease severity rating of 3, the difference between resistant and susceptible clones, the linear regression equation was used to establish an IC value of ≥ 1.37 at 3 wpi as the cut-off between resistance and susceptibility at 7 wpi (Fig. 2.4).

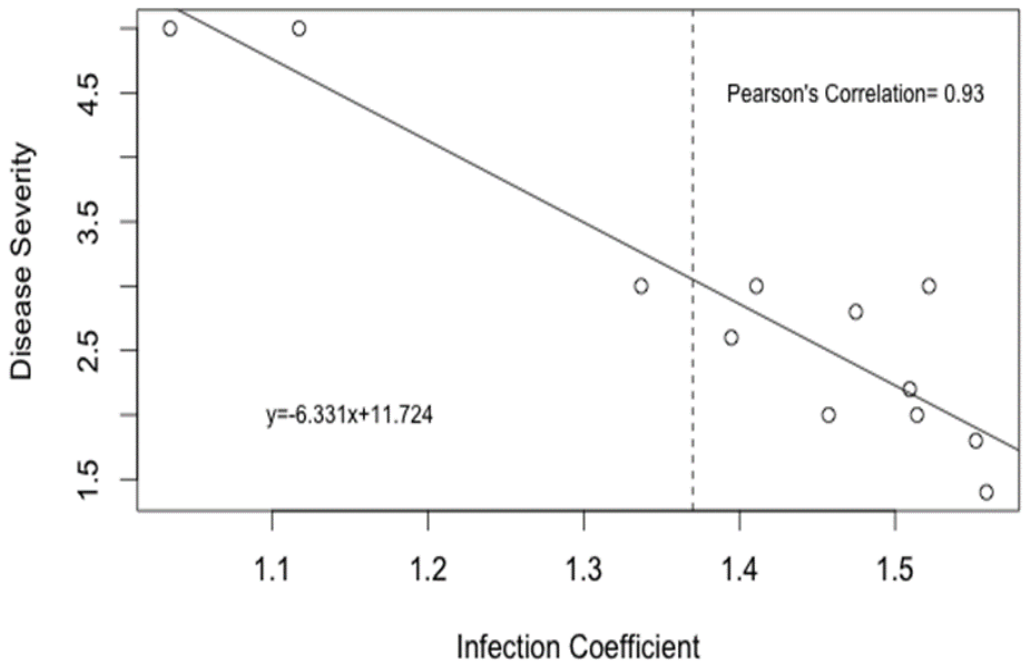

Figure 2.4. A Pearson's correlation of 0.93 was obtained when disease severity was correlated to IC value. An IC value of ≥ 1.37, represented by the dotted line, at 3 wpi, and then clones are predicted to be resistant.

Detection of pathogen in field samples

Samples collected from the NDSU Horticultural Research Farm resulted in 12/13 cankers which tested positive for *S. musiva*. The Cq values for these samples ranged from 28 – 46 (Table 2.4, Table A1). Both canker samples collected at the NDSU Seed Farm were positive for *S. musiva*, with Cq values of 34 and 41.7. No Cq values were observed from healthy samples collected at either location at NDSU. *Sphaerulina musiva* was not isolated using the cultural assay from any of the sampled cankers collected at either location.

Table 2.4. Detection of *S. musiva* from field samples by qPCR.

Location	**Number of trees sampled**[a]	**No. of samples with Cq values**	**Cq value range**[b]
Absaraka	26		
	Stem with canker (13)	12	28-46
	Stem without canker (13)	0	UD[c]
NDSU Seed Farm	4		
	Stem with canker (2)	2	34; 41.7
	Stem without canker (2)	0	UD[c]

[a.] In each sampled tree, two stem cuttings were collected, one with canker and another without canker. [b.] Cq value is the average of three technical replicates. [c.] UD- undetermined due to lack of fungal material in the sample. Individual values and canker age provided in Table A1.

Discussion

The primary objective of this research was to develop a qPCR assay to detect and quantify *S. musiva* in artificially and naturally infected hybrid poplar stems and branches. Historically, diagnosis has been problematic using cultural based approaches (Stanosz and Stanosz 2002, Waterman 1954). This difficulty is exacerbated by a decrease in isolation success

with canker age (Weiland and Stanosz 2006). Initial improvements included the creation of a semi-selective media for *S. musiva* (Stanosz and Stanosz 2002) and the development of a PCR assay (Weiland and Stanosz 2006). However, in a 36-week long experiment comparing these two approaches (cultural vs. PCR) the rate of *S. musiva* detection dropped to 66% of inoculated samples, with no real difference between cultural and PCR based detection (Weiland and Stanosz 2006). In fact, there was a lack of congruence between samples where *S. musiva* was identified by cultural based approaches and where it was identified by PCR based detection, suggesting results needed to be interpreted with caution.

The specific and sensitive qPCR assay described herein is a marked improvement on both the cultural and PCR based approaches developed to date. *Sphaerulina musiva* was detected from all greenhouse-inoculated samples at all time points and from all symptomatic environmental samples. It is important to note that cultural approaches, similar to those described by Weiland and Stanosz (2006), were also attempted. However, *S. musiva* was never detected from the field-collected samples. Given the range of canker ages of 1 to 6 years old (Table A1), the lack of isolation success from field samples is not surprising, and is consistent with the literature (Stanosz and Stanosz 2002, Waterman 1946, 1954).

The importance of *S. musiva* detection and quantification in woody tissue is largely due to two factors. The first factor is that *Populus* trees are typically propagated by dormant cuttings and that pathogen spread is linked to the movement of such plant material (Carnegie and Cooper 2011, Goss et al. 2009, Sakalidis et al. 2016). The second factor is the histological evidence that fungal hyphae can be found in the xylem of susceptible clones (Krupinsky 1989, Qin and LeBoldus 2014, Stanosz and Stanosz 2002, Waterman 1954, Weiland and Stanosz 2007) and that *S. musiva* has been reported to be present in asymptomatic woody tissue (Ostry and McNabb

1983, Waterman and Aldrich 1954). The qPCR assay described above could be used as a tool to help detect *S. musiva* in asymptomatic tissue preventing the continued spread of this pathogen.

The main criteria for diagnostic application of qPCR is its specificity combined with the possibility of detecting low levels of target DNA in the presence of non-target DNA (Bustin et al. 2009). Our assay was able to detect < 0.08 ng of pathogen DNA in the presence of ~ 0.6 ng of poplar DNA. Higher Cq values obtained for some infected samples indicate the presence of low amounts of pathogen DNA. Studies on *Phytophthora ramorum* detection using qPCR in field samples resulted in a majority of the samples having C_t values < 35 (Tomlinson et al. 2005). This has been attributed to infection with a low level of pathogen or due to samples containing nonviable pathogen (Hayden et al. 2004, Tomlinson et al. 2005). Apart from this, our results from conventional PCR further validated the specificity of the NABtF/NABtR primer pair, as it failed to amplify gDNA of *Fusarium* sp., *Alternaria* sp. or *Epicoccum* sp., which is important as these are some of the secondary parasites which commonly colonize cankers.

The second objective of this work was to determine if the qPCR assay could be used as a way to improve the resolution of disease resistance phenotyping in the greenhouse. To date, two different greenhouse assays, one using wounding (Weiland et al. 2003) and a second, inoculating non-wounded trees with a spore suspension (LeBoldus et al. 2010), have been developed and predict long-term field performance of resistant and susceptible clones (Qin et al. 2014, Weiland et al. 2003). However, prediction of the field performance of moderately resistant clones is less consistent. In other pathosystems, improving the resolution of phenotyping has reduced these inconsistencies and identified novel resistance phenotypes. In the case of poplar leaf rust, researchers successfully improved disease resistance phenotyping by increasing the number of disease development parameters that they measured (Jorge et al. 2005). This resulted in the

identification of several new quantitative trait loci conferring resistance to different aspects of disease development (Jorge et al. 2005). A qPCR based approach, quantifying pathogen biomass, has been used in other systems (Acevedo et al. 2010, Almquist and Wallenhammar 2014, Hayden et al. 2004, Oliver et al. 2008, Pasche et al. 2013) and has the potential to improve phenotyping accuracy for Septoria canker resistance.

In the two greenhouse experiments described above, we compared clones in terms of disease severity and IC value at 1 wpi, 3 wpi and 7 wpi. There were significant differences among clones for both parameters at 3 wpi and 7 wpi (Table 2.3). There was also a strong positive correlation ($r = 0.93$) between IC at 3 wpi and disease severity at 7 wpi. These results suggest that it may be possible to predict disease severity of a particular clone as early as 3 wpi in the greenhouse. However, it is important to note that at 7 wpi only 3 of the 6 clones tested, NC11505 ($\overline{x} = 5$), NM6 ($\overline{x} = 2.9$) and DN177 ($\overline{x} = 2.5$), had disease severities consistent with their damage categories as reported in the literature (Qin et al. 2014, Weiland et al. 2003). In contrast, clones MWH5 ($\overline{x} = 1.9$), NC11432 ($\overline{x} = 1.7$) and NE351 ($\overline{x} = 3$), were not consistently ranked with reports in the literature (Qin et al. 2014, Weiland et al. 2003). In fact, the results from these two experiments indicate that they are resistant, when in fact the literature consistently reports that they are susceptible under field conditions (Hansen et al. 1994, Ostry and McNabb 1985, Schreiner 1972, Weiland et al. 2003). There are several possible reasons for the failure to accurately rank these clones. It is possible that the cankers did not have sufficient time to develop in the 7-week time course of this experiment. Alternatively, the isolates used in the different experiments, in combination with the use of a single point inoculation rather than a whole tree inoculation, may explain the observed differences. Regardless of the reason, this

assay failed to predict the widely accepted field performance of 50% of the tested clones, indicating that this assay needs further refinement prior to its use as a phenotyping tool.

One potential area of improvement would be the ability to quantify *S. musiva* in whole trees inoculated by spore suspensions (LeBoldus et al. 2010). Qin et al. (2014) indicated that a measure of the number of cankers, and the canker severity, were essential for accurate prediction of field performance. In that study, the authors traced the necrotic area of all inoculated trees with a transparency, scanned these images, and used visual assessment software to estimate the proportion necrotic area. This procedure is extremely time consuming and logistically impossible for screening large numbers of clones. Initially, the authors attempted to use the qPCR assay described above, to circumvent this difficulty, and estimate canker severity on trees inoculated with a spore suspension. To do this, infected bark was removed from the tree and DNA was extracted and subjected to the qPCR assay. Although it was possible to detect the pathogen, detection was not consistent across samples and it was not possible to distinguish between clones in terms of their resistance (unpublished data). Further work needs to be conducted to refine a procedure that uses the qPCR assay in combination with non-wounded whole tree inoculations to compare trees in terms of their resistance.

Although this qPCR assay, in its current form, does not accurately predict field performance of certain clones, there are other aspects of *S. musiva* biology that it could help inform. For example, the qPCR assay detected a significant isolate * time interaction (Table 2.3). This interaction is the result of differences between isolates MN14 and MN20 in terms of IC value at 1 wpi. These differences disappeared by 3 wpi, resulting in no significant isolate * time interaction. These differences were not apparent with traditional disease severity phenotyping approaches (Table 2.3). This result suggests differences in the ability of isolates to colonize host

tissue in the early stages of infection and could have important implications for disease resistance. For example, it has recently been reported that differences among plant genotypes in terms of resistance are largely due to the timing and amplitude of the host's defense response (Muchero et al. 2016, *unpublished*). Isolates of a pathogen able to colonize host tissue more quickly, may be able to take advantage of a delayed defense response more readily than isolates that colonize host tissue more slowly.

In conclusion, this assay can quantify *S. musiva* with high sensitivity, specificity and reproducibility, in both inoculated and environmental samples. This suggests that the assay: (1) has the potential to be used as a diagnostic tool; (2) could be used to prevent the spread of the pathogen across provincial, state or international boarders; and (3) may be used to compare aggressiveness among isolates in the early stages of the disease.

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CHAPTER 3. A HISTOLOGICAL, BIOCHEMICAL AND MOLECULAR COMPARISON OF MODERATELY RESISTANT AND SUSCEPTIBLE *POPULUS* GENOTYPES INOCULATED WITH *SPHAERULINA MUSIVA*

Introduction

The economic importance of *Populus* spp. and their hybrids is a direct result of their rapid growth capacity, ability to grow in different climatic and edaphic conditions, and the numerous uses for their wood (Ahuja 1987, Stettler et al. 1996). Today, poplars are grown on a commercial scale under intensive cultivation for 6-8 year rotations. Volume growth rates with hybrid poplar range from 17-30 Mg/ha/yr of dry woody biomass, similar to that of corn (Zsuffa et al. 1996). However, a significant barrier limiting the use of hybrid poplar is the canker and leaf spot disease caused by *Sphaerulina musiva* Peck. This fungus causes necrotic lesions on the leaves and cankers on stems and branches (Moore and Wilson 1983). Leaf spot infection reduces the photosynthetic area and causes premature defoliation (Krupinsky 1989, Lo et al. 1995, Ostry and McNabb 1985). The cankers on the main stem can reduce growth and predispose the tree to colonization by secondary organisms, resulting in girdling and/or breakage of the main stem (Ostry and McNabb 1983).

The first symptoms observed in the spring are leaf spots. They are mainly confined to the leaves on lower branches. Later in the growing season, conidia are produced from these leaf spots. Under favorable conditions, conidia cause secondary infections on leaves and stems. The size and number of the infections varies depending on the genotype infected, and the number of cycles of secondary infection which occur (Feau et al. 2010, Luley and McNabb 1989, Ostry 1987, Sinclair et al. 2005). Cankers, are typically associated with plantations where trees are exposed to large amounts of inoculum from infected leaves (Feau et al. 2010). Very little is

known about the infection biology of *S. musiva* and *Populus* leaves, and no comparisons between the modes of penetration and colonization by this pathogen between moderately resistant and susceptible genotypes have been made, nor has a careful comparison of molecular responses been conducted.

In order to understand the underlying molecular mechanisms involved in the *S. musiva* and *Populus* interaction, it is important to identify the differentially expressed genes that are involved in pathogenicity and defense. The recent development of high–throughput sequencing technologies provide an opportunity to study these genes by sequencing RNA. This mode of transcriptome analysis (RNA-seq) is widely used, as it is highly sensitive, provides extensive coverage of the genome and gives an impartial estimate of the transcript's expression level (Liang et al. 2014, Xu et al. 2011). Several studies using RNA-seq have been conducted that reveal genes expressed during the interaction between poplar and *S. musiva* (Foster et al. 2015, Liang et al. 2014), as well as several other fungal pathogens of *Populus*, including *Melampsora* spp. and *Marssonina brunnea* (Azaiez et al. 2009, Miranda et al. 2007, Zhu et al. 2012). Dhillon et al. (2015) also conducted an *in vitro* study using RNA-seq, which revealed genes expressed in *S. musiva* when colonizing media containing leaf extract and wood-chips. However, these patterns of gene expression have not been linked to *S. musiva* during the infection process *in planta*.

The purpose of this research was to study differences in the infection process of *S. musiva* on moderately resistant and susceptible *Populus* leaves. The objectives were to analyze: 1) Pre-penetration process: conidial germination, hyphal growth and mode of penetration of *S. musiva* on *Populus* leaves using scanning electron microscopy (SEM); 2) Post-penetration process: colonization of leaf tissue with *AsRed* transformed *S. musiva* using confocal microscopy; 3) The

role of H_2O_2 in disease development; and 4) To contrast global expression patterns in moderately resistant and susceptible genotypes during their interaction with *S. musiva* using RNA-seq.

Materials and Methods

To study the pre-penetration process, and differentially expressed genes from host and pathogen using RNA-seq, we used genotypes DN99 (moderately resistant) and BESC121 (susceptible). DN99 is *P. deltoides* x *P. nigra* hybrid and BESC121 is *P. trichocarpa.* In a second set of experiments, post-penetration and H_2O_2 production using 3,3′-diaminobenzidine (DAB) staining procedure, we used DN34 (a moderately resistant hybrid similar to DN99) and NC11505 (*P. maximoiczii* x *P. trichocarpa*). The genotypes used in the second experiment were different than those in the first experiment because the initial genotypes were no longer available. Resistance and susceptibility of these genotypes were previously reported in the literature (Qin et al. 2014, Weiland et al. 2003).

Pre-penetration process- scanning electron microscopy (SEM)

Spore germination and mode of penetration were observed and compared on moderately resistant genotype DN99 and susceptible genotype BESC121. A total of eighteen dormant hardwood cuttings for each genotype were obtained from the US Department of Energy. These hardwood cuttings were cut to a length of 10 cm. They were placed in small trays of water and kept at room temperature for two days. Subsequently, cuttings were planted in SC10 Super cone-tainers (Stuewe & Sons Deepots D40 cell, Stuewe & Sons Inc., Tangent, OR) filled with SunGro growing medium (SunGro Professional Mix #8, SunGro Horticulture Ltd., Agawam, MA) combined with slow release nutricote fertilizer (15-8-12) (N-P-K) (9.0% P_2O_5, 12.0% K_2O, 0.45% Fe, 0.23% chelated Fe, 1.0% Mg, 2.3% S, 0.02% B, 0.05% Cu, 0.05% Zn, 7.0% NH_3-N,

8.0% NO_3-N, 0.06% Mn, 0.02% Mo; Scotts Osmocote Plus, Scotts Company Ltd., Marysville, OH). Rooted cuttings were transferred to pots (depth: 22 cm by diameter: 22.5 cm; Stuewe & Sons Tree pot CP59R, Stuewe & Sons Inc.), which contained SunGro growing medium, when they reached a height of approximately 30 cm. Trees were fertilized bi-monthly with 20-20-20 liquid fertilizer (3.94% NH_3-N, 0.05% Mg, 0.0068% B, 0.0036% Cu, 6.05% NO_3-N, 0.0025% Zn, 10.01% $CO(NH_2)_2$, 20.0% P_2O_5, 20.0% K_2O, 0.05% chelated Fe, 0.25% Mn, 0.0009% Mo; Scotts Peters Professional, Scotts Company Ltd., Marysville, OH) and grown in a greenhouse with an 18 hour (hr) photoperiod augmented with sodium lamps and a 20°C (day)/16°C (night) temperature regime.

S. musiva isolates MN14, MN32 and MN23 were collected from hybrid poplars located at different planting sites near Garfield, MN during the winter of 2012. Cankers were surface sterilized by soaking them in a 5% bleach solution (6% NaClO; Homelife Bleach Regular Scent, KIK Custom Products Inc.) for 2 minutes, followed by rinsing with sterile distilled water twice. Subsequently, the bark was removed from the margin between necrotic and healthy tissue and a 4-mm-long piece was removed from the cambium and placed on V8 juice agar plates (1.5 g of $CaCO_3$ [ReagentPlus®, Research Organics Inc.], 137 ml of V8 juice [Campbell Soup Company], 15.2 g of agar [Difco] and 625 ml of deionized water). The necrotic tissue was incubated for 1 week at room temperature and then putative *S. musiva* isolates were sub-cultured until pure colonies were obtained. Confirmation of the species was based on morphology of the conidia and multilocus genotyping (LeBoldus et al. 2015, Sivanesan 1990). The isolates were stored in 1 ml of a 50% glycerol solution at -80°C. To prepare inoculum, the isolates were recovered from storage, poured onto five V8 juice agar plates and allowed to grow for 1 week prior to sub-culturing onto 10 plates of V8 juice agar. These 10 cultures were incubated for seven days at

room temperature, then conidia were harvested by a sterile loop and a spore suspension was made with sterile distilled water (LeBoldus et al. 2010).

The experimental design was a complete randomized block design with a total of 36 trees including non-inoculated controls. Each host genotype had three replicates per sample time point. Trees were inoculated, two weeks after planting in larger pots, using a conidial suspension of 1 x 10^6 conidia/ml. The inoculum was sprayed over the entire tree until the spore suspension was dripping from the leaves. Control trees were sprayed with sterile distilled water. Inoculated and non-inoculated trees were then immediately covered in black plastic bags to maintain moisture and moved to the head-house and kept at room temperature. After 48 hrs, trees were removed from the plastic bags and returned to the greenhouse.

To examine the pre-penetration process of *S. musiva*, segments of leaves (2 x 2 cm) were collected from the fourth leaf from the top and fourth leaf from the bottom of each plant, i.e. two samples per replicate. Samples were collected at five different time points, 48 hrs, 96 hrs, 1 week (wk), 2 wks and 3 wks. Control leaves were collected at only the first time point. The samples collected were fixed in 2.5% glutaraldehyde in sodium phosphate buffer (Tousimis, Rockville, MD). After washing the leaves in water and buffer, they were desiccated using a graded alcohol series ranging from 30% to 100% ethanol. Using an Autosamdri-810 critical point drier (Tousimis, Rockville, MD) samples were critical-point dried with liquid carbon dioxide as a transitional fluid. The whole leaf samples were attached to aluminum mounts with silver paint (SPI Supplies, West Chester, PA) and sputter coated with gold (Balzers SCD 030, Balzers Union Ltd., Liechtenstein). Imaging was done using a JEOL JSm-6490LV scanning electron microscope.

Post-penetration process- cryofracture SEM and confocal microscopy

Cryofracture SEM

Leaves collected from control and infected trees were cut into squares (2 x 2 cm) and fixed in 2.5% glutaraldehyde in sodium phosphate buffer (Tousimis, Rockville, Maryland, USA) and stored at 4°C. They were rinsed in buffer and water, dehydrated using a graded alcohol series from 30% to 100% ethanol. At the 100% stage, leaves in alcohol were frozen in liquid nitrogen and then fractured (Moore and Payne 2012). Following an additional change of 100% ethanol, the leaves were critical-point dried, and leaf fragments were attached to aluminum mounts with silver paint. The fractured edge was exposed, and then sputter coated with gold. Images were obtained as described above.

Agrobacterium tumefaciens-mediated transformation

Isolate MN14 was cultured on five plates of V8 juice media for seven days at room temperature in order to harvest enough conidia for the transformation protocol. Strain EHA105 of *A. tumefaciens* carrying the binary vector SK2245:pSK2238 containing the *AsRed* gene and a *hygromycin B* resistance gene was used for *S. musiva* transformation. We followed the procedure of Khang et al. 2007 with the following modifications: concentration of acetosyringone 200 mM, hygromycin 250 μg/ml, 60 hrs of incubation in co-cultivation media and the selection media used was oatmeal agar (OMA) (Foster et al. 2014). After two to three weeks of incubation at room temperature, presumed transformants were screened for red fluorescent protein expression under a Ziess AxioObserver Z1 microscope equipped with an LSM 700 confocal laser scanning microscope (Ziess, Thornwood, NY) using 570 emission and 555 excitation. Only colonies expressing *AsRed* were transferred with sterile toothpick to 24 well microtiter plates containing V8 media with hygromycin B and incubated for five to seven days at room temperature.

Sporeswere harvested from each well containing transformants by single spore isolation method in order to obtain pure cultures.

Selection of true transformants

True transformants were selected based on a pathogenicity test, stability test and phenotypic characterization. For the pathogenicity test, detached leaves from susceptible genotype NC11505 were inoculated with a spore suspension (2 x 10^6 spores/ml) of transformants. The positive control was a spore suspension (2 x 10^6 spores/ml) of MN14 (wild type) and the negative control was water. Leaves were washed with sterile distilled water twice and then placed in petri dishes that contained wet tissue paper. There were three replicates of leaves per transformant. All the leaves were sprayed three times with the inoculum to ensure uniform coverage. The samples were incubated at room temperature for two weeks.

To select stable transformants and to analyze growth, colony morphology and spore production from actively growing cultures of transformants and the wild type, 4 mm diameter mycelial plugs taken from the edges of the above mentioned cultures were transferred to V8 juice agar plates. These plates were incubated for 1 wk at room temperature, after which four subsequent subcultures were made at 1 wk intervals using the same method. Mycelium from the last sub-culture was grown in liquid media for 1 wk at room temperature, and subsequently, used for DNA extraction. A PCR test was conducted to confirm the stability of these transformants by detecting the gene. The primer pair used was F-TCAGCTTCGATGTAGGAGGG and R-TTCTACACAGCCATCGGTCC (El Hadrami et al. 2015). The PCR reactions were conducted in an Applied Biosystems Veriti 96 well thermal cycler (Life Technologies, Grand Island, NY, USA) in a total volume of 25 µl containing 12.5 µl of 2X GoTaq Green Master Mix (Promega, Madison, WI, USA), 1 µl of 5 µM of each primer, 9.5 µl of H_2O and 2 µl of template DNA. The

reaction conditions included an initial denaturation step at 94°C for 4 min, 35 cycles of 94°C for 10 s, 62°C for 20 s, 72°C for 30 s, followed by final extension step at 72°C for 10 min.

Infection and colonization were observed and compared on moderately resistant genotype DN34 and susceptible genotype NC11505. A total of ten dormant hardwood cuttings were collected from each genotype from a research field at North Dakota State University (NDSU) in Fargo, ND. These cuttings were grown as described previously. The experimental design was a randomized complete block design. There were a total of 20 trees, including non-inoculated controls. Each genotype had five replicates per time point (one leaf per tree at each time point). Transformants were spray inoculated onto five moderately resistant and five susceptible genotypes with a spore suspension of 2×10^6 spores/ml. Control trees were sprayed with sterile distilled water. The inoculation procedure was done in the same way as described previously. Leaves were collected haphazardly from the middle section of each tree (one leaf per tree) at time points 48 hrs, 96 hrs, 1 wk, 2 wks and 3 wks. Control leaves were only collected at the first time point. Observations and images were taken with a Zeiss LSM700 Confocal Laser Scanning Microscope (CLSM) using a Plan-Apochromat 40x/1.3 oil immersion lens (Zeiss, Thornwood, NY). To visualize pathogen colonization, Z-stacks of 40 µm deep optical sections of leaf tissues, including pathogen, stomata, epidermal palisade and spongy mesophyll tissues, were obtained. To visualize leaf tissues, a 639 nm laser was used and emission was set at 668 nm. For the RFP tagged pathogen, excitation and emission spectra were 555 nm and 570 nm, respectively. Images were analyzed for presence of the pathogen in leaf tissue by using the Zeiss LSM image browser (Bitplane, South Windsor, CT).

In addition, germination of conidia on the moderately resistant and susceptible genotypes was compared at 48 hpi (hours post infection). For each of the five leaves, three separate

confocal images were recorded from each of two different leaf sections, germination was determined from each image and the average germination of conidia calculated per leaf. A conidium was considered germinated if growth of a germ tube was seen from one or more of the cells of the spore.

DAB staining

Two segments of leaves (2 x 2 cm) were collected from the same genotypes and replicates at all the time points described above, placed in DAB solution (0.1%, pH 3.8) and incubated in the dark for 4 hrs. The leaves were cleared overnight in an ethanol/chloroform (3:1 v/v) TCA solution (0.15%) (Orczyk et al. 2010). A Ziess Imager.M2 microscope using Fluor 5x/0.25 objective and Ziess AxioCam HRc camera (Ziess, Thornwood, NY) was used to visualize hydrogen peroxide accumulation in the infected leaf tissues. There were five leaf samples per genotype, and for each sample two leaf sections were viewed for DAB and four separate images were taken from each of the two samples. Those results were averaged over the eight subsamples for a single value per leaf sample. Distribution of positively labeled cells and area images were analyzed using Image-Pro Premier software (Ver. 9.0.1, Media Cyberbetics Inc., Silver Spring, MD), where the stained section of leaf tissue was expressed in percentage area. The stained section of leaf tissue was recorded as area in mm^2.

Statistical analysis

Germination of conidia on moderately resistant and susceptible genotypes was compared using an unequal variance T-test. SAS 9.4 software was used for the analysis with $p = 0.05$.

For DAB comparison, between moderately resistant and susceptible genotypes and within genotypes over time, the area stained was analyzed as a split plot in time ANOVA. Genotypes were considered as the whole plot and time as sub-plot.

RNA-seq and Illumina sequencing

Moderately resistant genotype DN99 and susceptible genotype BESC121 were used to study differentially expressed genes. The same inoculated trees as described in the section on the pre-penetration process were used for this gene expression study. From those trees, at 1 wpi, 2 wpi and 3 wpi, leaf samples showing leaf spots were collected and four individual leaf spots were cut from leaves and placed in lysing matrix tubes. The same was done for each replication. The samples collected were immediately frozen in liquid nitrogen and stored at -80°C. mRNA was extracted directly from crude samples using mRNA Dynabeads Direct Kit (Ambion, Life Technologies, NY, USA), as per the manufacturers protocol with two modifications. Tissue, flash frozen in liquid N_2, was disrupted in a bead beater 4 times for 18 s each, tissue was refrozen in liquid N_2 each time. An additional step was added to the extraction, adding 900 µl of chloroform:isoamyl alcohol to the supernatant obtained after centrifuging with lysis binding solution. This was further vortexed for 30 s and centrifuged for 5 minutes at 14,000 rpm. The supernatant obtained from this step was transferred to tubes containing Dynabeads and the remaining protocol was followed.

The quality and integrity of the mRNA was analyzed using a 2100 Bioanalyzer (Agilent Technologies, CA, USA) before preparing cDNA libraries. Illumina TrueSeq RNA Library Prep Kit v2 (CA, USA) was used for library construction. A total of 18 cDNA samples were individually barcoded and run in Illumina NextSeq 500. The genome and gff annotation file of *Populus trichocarpa* v3.0/v10.1 were downloaded from popgenie.org and genome and gff annotations of *S. musiva* S02202 v1.0 were downloaded from fungi.ensembl.org/Sphaerulina_musiva_so2202. Analysis of the sequences was done with CLC genomics workbench 8.0 using its default parameters. All the differentially expressed genes (DE)

were subjected to blast using BLAST2GO. Corrected *p*-values were obtained by using the EDGE bioconductor package available in CLC genomics workbench. Heat maps were constructed using R program version 3.1.1.1 and gplot package was used. Venn diagram was constructed using Venny website (Oliveros 2007).

In conventional gene expression studies, genes are selected based on significant differences between treatments. However, Jung et al. 2011 reported that due to stringent FDR adjusted *p*-values, there are chances of disregarding genes that have higher fold change but lack significance, and these genes may not be irrelevant in the study of particular biological system. Thus, we have chosen host genes that are significantly upregulated and downregulated (FDR < 0.01), along with genes based on fold change in order to explain the *S. musiva – Populus* pathosystem. FDR values were not used in reporting on genes expressed in *S. musiva*.

Results

Pre-penetration process

Scanning electron microscope images revealed that the surface morphology of moderately resistant and susceptible genotypes was similar. There were no differences between pre-penetration processes (attachment, germination and mode of penetration) on moderately resistant and susceptible genotypes (Fig. 3.1 A, B). At 48 hpi, conidia and developing hyphae were surrounded by a mucilaginous substance called the extracellular matrix (ECM). ECM has a fibrillar structure and was mainly seen at the interface between conidia and the leaf surface, or between growing hyphae and the leaf surface, indicating a potential role in adhesion (Fig. 3.2A). Germination of conidia and hyphal growth was seen on the adaxial surface of the leaf by 48 hpi (Fig. 3.2C). There was no difference in germination or hyphal growth observed between the young and old leaves of the plant. Conidial germination occurred either from one or both the

cells at the ends of the spore, or from a cell in the center. There was no particular pattern in hyphal growth, as the hyphae grew over the leaf surface with multiple branching points (Fig. 3.2D). Frequently, evidence of enzymatic activity was seen, as the cuticle around the hyphae appeared to be degraded (Fig. 3.2E). Other events infrequently observed were swellings of the conidia (Fig. 3.2F) and anastomosis (Fig. 3.2D). From these swellings, germ tubes developed, but there was no evidence of direct penetration by these germ tubes.

There are two modes of penetration used by *S. musiva*: stomatal penetration and direct penetration (Fig. 3.3 A, E). Penetration of the stomata occurred either from the main conidial germ tubes or from lateral branches. Based on the pattern of growth there was no evidence that germ tubes or hyphae were directly attracted to stomata. Often hyphae grew across the edges or directly over the stomata without evidence of penetration. Occasionally, multiple penetrations were observed (Fig. 3.3C). At the point of direct penetration through the epidermis, ECM were observed, possibly indicating that this matrix contains cell wall degrading enzymes, thus enabling the penetration process (Fig. 3.2B). Samples observed at other time points appeared similar for moderately resistant and susceptible genotypes.

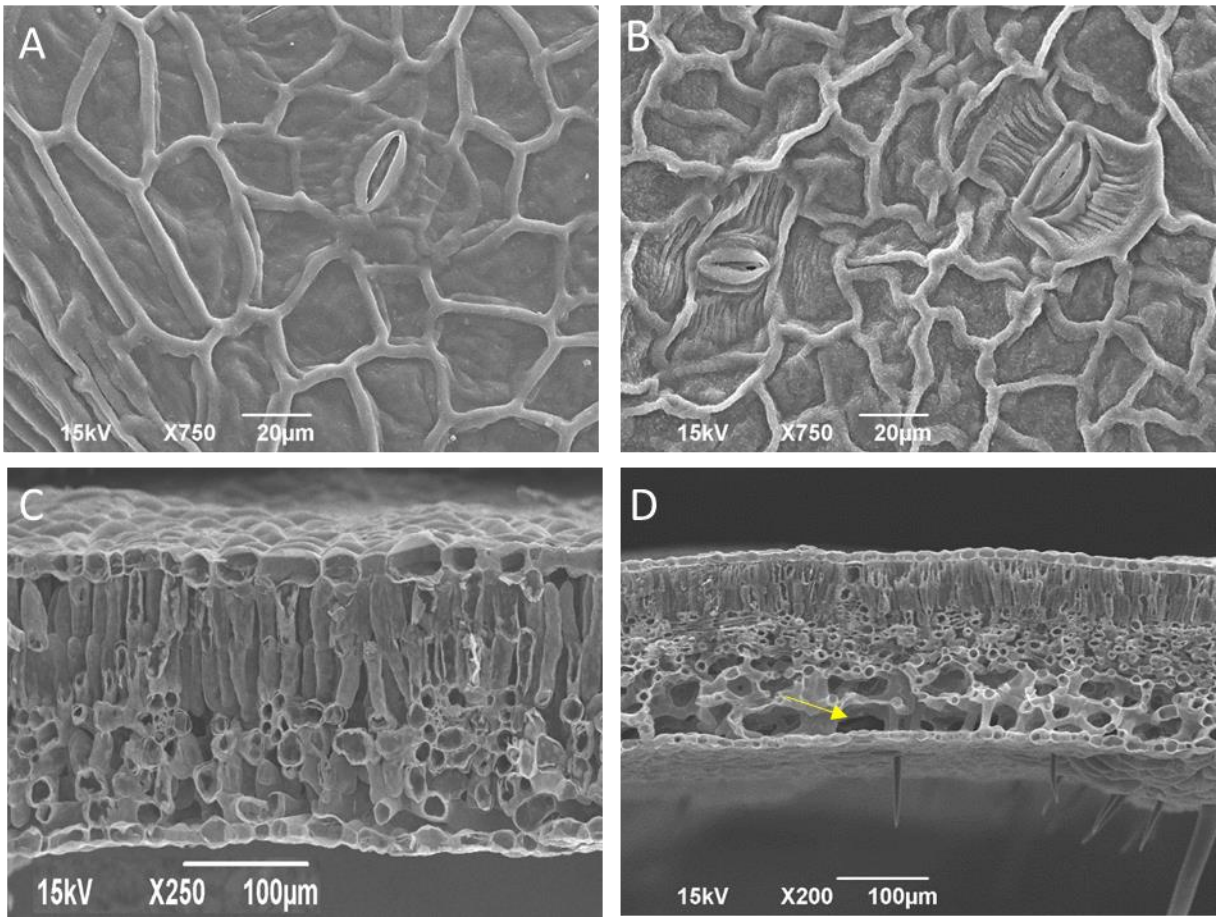

Figure 3.1. Scanning electron micrographs (SEM) of leaves of moderately resistant and susceptible genotypes of *Populus*. A. surface morphology and C. cross section of mock inoculated moderately resistant leaf. B. surface morphology and D. cross section of mock inoculated susceptible leaf. Evidence of clearly defined air pockets (yellow arrow) are observed in the susceptible genotype.

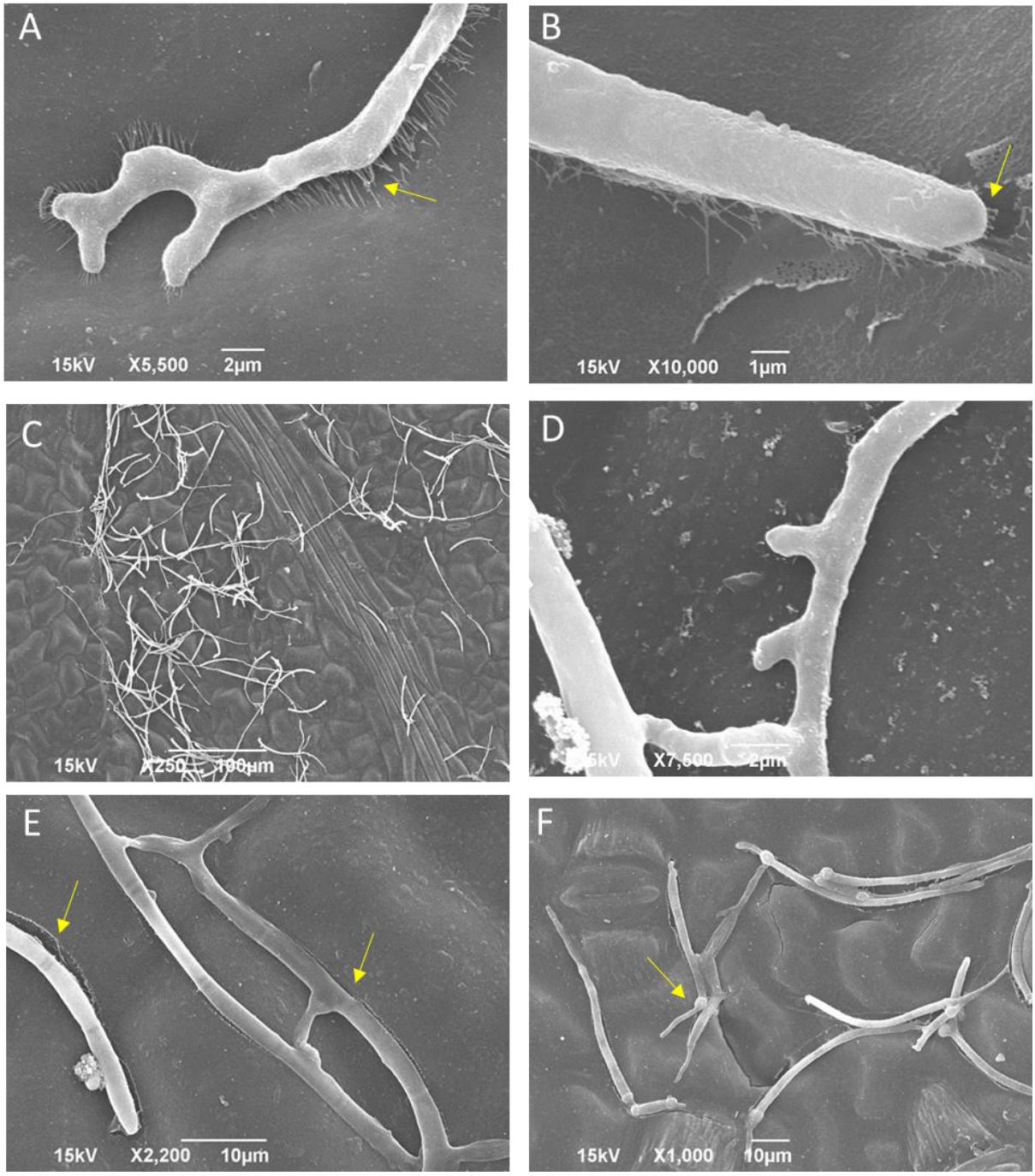

Figure 3.2. Scanning electron microscope (SEM) images of leaves of *Populus* genotypes inoculated with *Sphaerulina musiva*. A. Fibrillar ECM seen surrounding conidia or hyphae (yellow arrow). B. ECM at point of penetration, may indicate presence of cell wall degrading enzymes. C. Germination of conidia was noted at 48 hpi, with growth of germ tubes occurring from various cells of conidia. D. Hypha with multiple branches; anastomosis. E. Frequently, erosion of cuticle (yellow arrow) was observed during germination of conidia and growth of hyphae. F. Infrequently, swelling on one end of the conidia was observed, from which sometimes a germ tube arose, but they were observed not to penetrate the leaves.

Post-penetration process

Cryofracture SEM

The transverse sections of moderately resistant and susceptible leaves showed a single layer of upper and lower epidermal cells, two layers of palisade mesophyll cells, loosely packed spongy mesophyll cells and vascular bundles. However, transverse sections of susceptible leaves revealed the presence of well-defined air pockets just above the lower epidermis (Fig. 3.1 C, D). No evidence of the pathogen was detected in or on any control leaves.

Transformation of S. musiva

Transformation of *S. musiva* with EHA105 *A. tumefaciens* strain containing plasmid SK2245:pSK2238 was successful. All fourteen *AsRed* transformants were tested for intensity of red fluorescence using the confocal microscope, and calculated using the Axiovision software. Four of the fourteen transformants, based on the intensity of red fluorescence and colony morphology being similar to the wild type, were selected for pathogenicity testing. The four transformants were pathogenic, as necrotic lesions were formed on inoculated leaves after 10 days (Fig. B2). Stability in expression of red fluorescence of these four transformants was confirmed by PCR after four subsequent sub-culturing events (Fig. B3). Transformant R14 was selected to study the post-penetration events.

Post-penetration events at each time point are described as follows:

48 hpi

Observations made using confocal imagery were consistent with SEM images. In moderately resistant and susceptible genotypes, both modes of penetration were observed, but there was no evidence of tissue colonization at this time point. Occasionally, multiple penetration events were observed (Fig. 3.3 B, D, F). No growth of the pathogen was observed on control

leaves of either of the genotypes. A significant difference ($p < 0.03$) was found in germination of the conidia between the resistant (19.5±) and susceptible genotype (30.5±).

96 hpi

Colonization of palisade mesophyll cells began in both moderately resistant and susceptible genotypes. Hyphae were observed in the intercellular spaces. Observations made on transverse sections were consistent (Fig. 3.4 A, B).

1 wpi

In the moderately resistant genotype, no visible symptoms were seen on the leaves prior to sampling (Fig. B1). However, fungal invasion was observed, but was still confined to the palisade mesophyll cells. Hyphae were present in the intercellular spaces, but there was no evidence of cell death (necrosis) (Fig. 3.4 C, D). In contrast, in the susceptible genotype small necrotic lesions were seen on the leaves prior to sampling (Fig. B1). Under confocal microscopy, these necrotic lesions appeared red with hyphae visible on the surface of these lesions (Fig. 3.5D). Spongy mesophyll cells within these lesions were colonized intercellularly by *S. musiva*. Cell death was observed in only a few mesophyll cells (Fig. 3.4F). Similar results were seen with cryoSEM for both the moderately resistant and susceptible genotypes (Fig. 4 C, E).

2 wpi

In the moderately resistant genotype, small necrotic lesions were visible on infected leaves. Under confocal microscopy, hyphal growth was observed on a few lesions. Spongy mesophyll cells under some lesions showed complete cell death with no evidence of fungal colonization, whereas some lesions showing the beginning of cell death in spongy mesophyll cells had a few intercellular hyphae (Fig. 3.5B). In contrast, in the susceptible genotype most of the leaves were covered with necrotic lesions of various sizes where some large lesions were

likely the result of coalescing of smaller lesions. Transformed hyphae were visible on the surface of all the lesions. Hyphae continued to grow extensively in the spongy mesophyll tissues but remained intercellular (Fig. 3.5D). These observations on moderately resistant and susceptible leaves were further validated with cryofracture SEM.

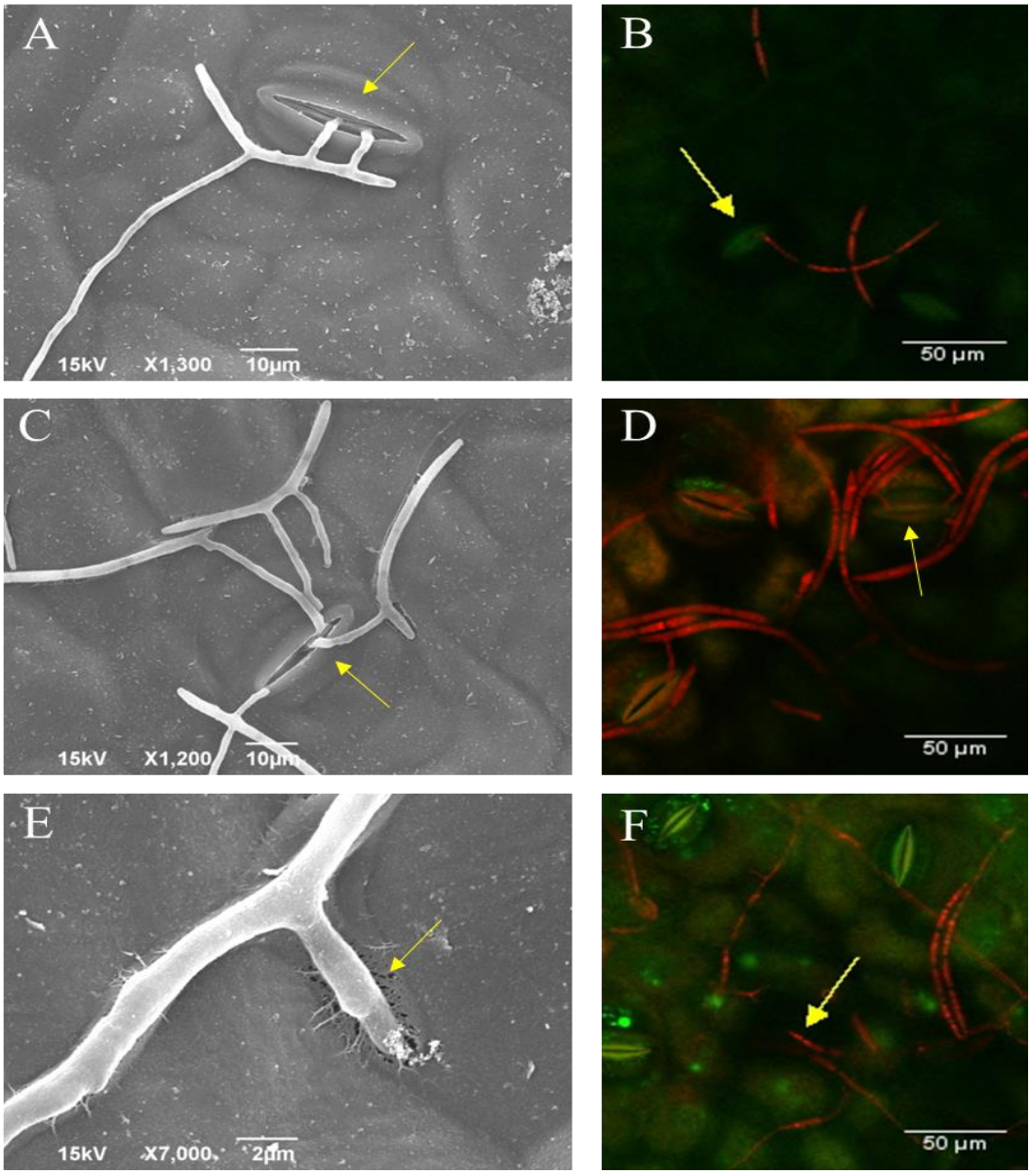

Figure 3.3. Mode of penetration of *Sphaerulina musiva* on inoculated leaves of moderately resistant and susceptible genotypes of *Populus* using SEM and confocal laser scanning microscopy. A and B. Stomatal penetration. C and D. Occasional multiple entry in single stomata was seen. E and F. Germ tubes and hyphae directly penetrating the leaves. Red hyphae in B, D and F are transformed *S. musiva* while green is the poplar leaf.

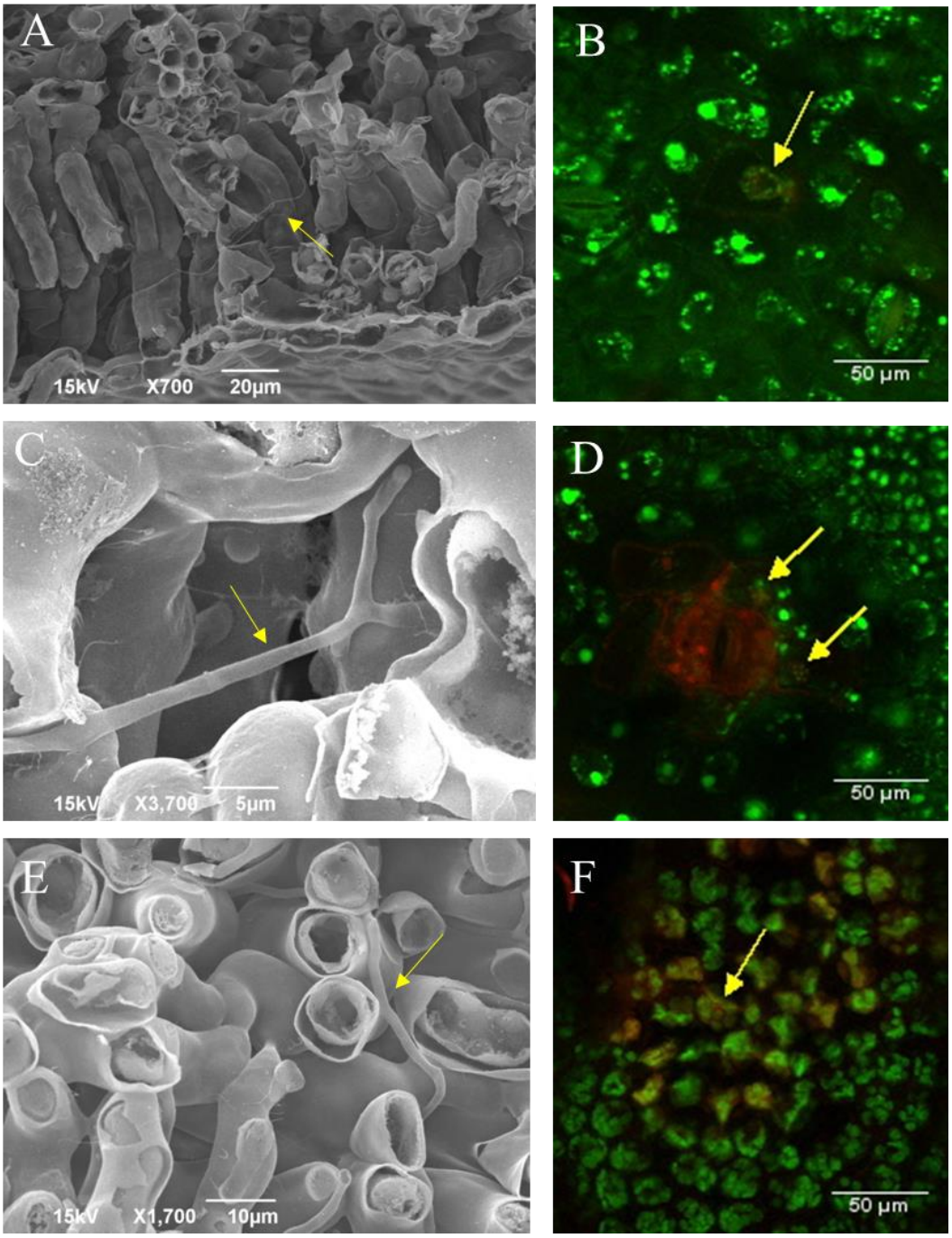

Figure 3.4. Post-penetration events of *Sphaerulina musiva* on inoculated leaves of moderately resistant and susceptible genotypes of *Populus* using CryoSEM and confocal laser scanning microscopy. A and B. At 96 hpi, intercellular hyphal growth was observed on palisade mesophyll tissues on both moderately resistant and susceptible genotypes. C and D. At 1 wpi, in the moderately resistant genotype hyphal growth was still confined to palisade mesophyll tissues. E and F. At 1 wpi, in the susceptible genotype colonization was observed in spongy mesophyll tissues. Consistent observations were seen in both the microscopes. Transformed *S. musiva* – bright red hyphae (B, D, F); uncolonized spongy mesophyll tissues- green.

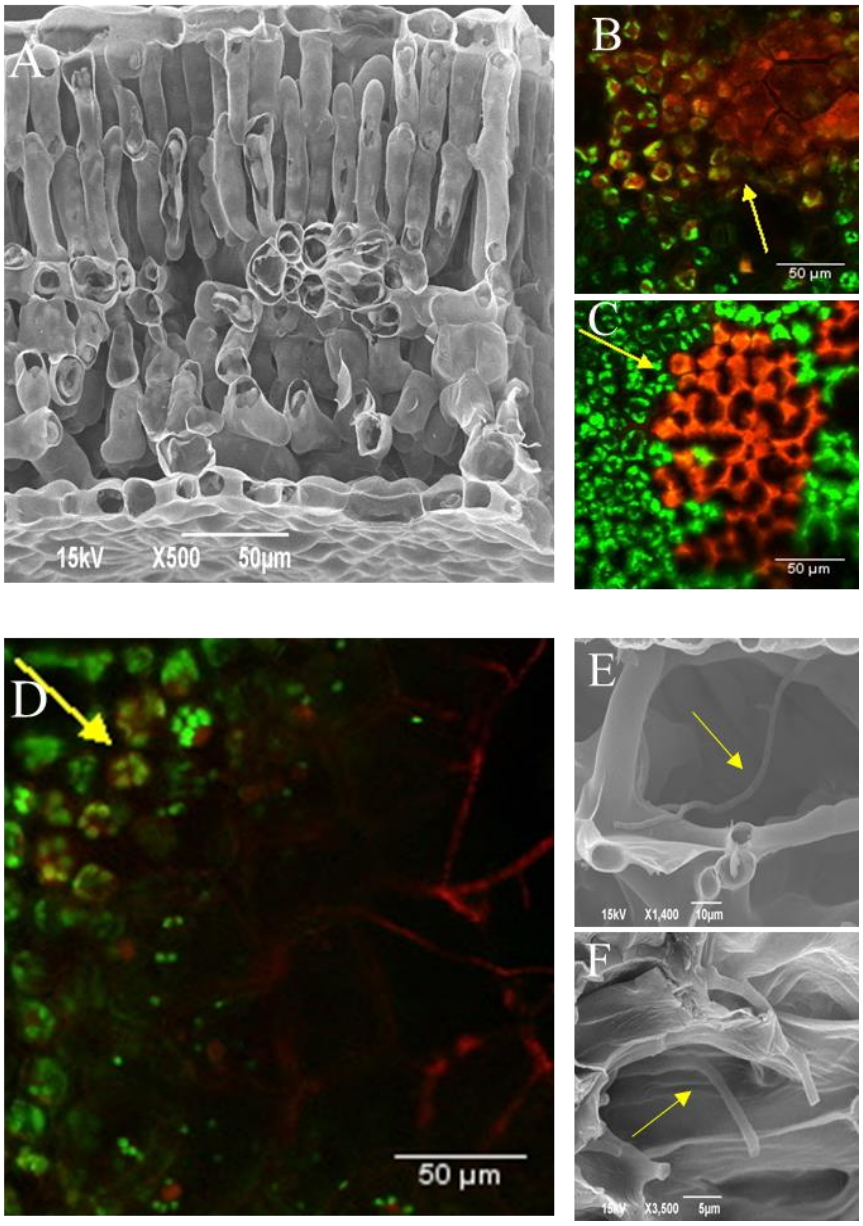

Figure 3.5. Post-penetration events of *Sphaerulina musiva* on inoculated leaves of moderately resistant and susceptible *Populus* genotypes using CryoSEM and confocal laser scanning microscopy. A. At 2 wpi, in the moderately resistant genotype, no hyphal growth was detected with CryoSEM in spongy mesophyll tissues. B. Confocal microscope revealed the beginning of cell death with very little colonization of spongy mesophyll by the fungus in the moderately resistant genotype. C. At 3 wpi, complete cell death was seen in spongy mesophyll tissues of the moderately resistant genotype. At 2 wpi and 3 wpi, intra- (F) and inter- cellular colonization of spongy mesophyll tissues beneath the overhead lesions (D) and air pockets were seen in the susceptible genotype (E). Transformed *S. musiva* – bright red hyphae; cell death in spongy mesophyll tissues- red color (B, D); uncolonized spongy mesophyll tissues- green.

3 wpi

In the moderately resistant genotype, necrotic lesions appeared slightly larger than the previous 2 wpi time point. However, at the microscopic level, hyphae were observed on the surface of very few lesions and cell death of spongy mesophyll tissues (reddish tissues) were observed, but without any detectable fungal colonization. Cross sections of moderately resistant leaves also revealed the same results (Fig. 3.5C). In contrast, in the susceptible genotype more coalesced lesions were visually observed on leaves. Greater intercellular colonization of spongy mesophyll cells occurred beneath the overhead lesions, however, no cell death was seen. These observations were validated with cryofracture of susceptible leaves, which also showed intracellular colonization and in the air pockets in the lower part of the leaf (Fig. 3.5 D, E, F).

DAB staining

DAB staining was used to visualize H_2O_2 production. A dark brown color associated with DAB was visible in the inoculated leaves, but was not observed in the control leaves (Fig. 3.6 A, B). There was no significant difference between the genotypes at 1 wpi, even though microscopically, brown precipitate was seen on both the genotypes (Fig. 3.6 C, D; Fig. 7). A significant difference was only observed at 2 wpi ($p < 0.0001$) and at 3 wpi ($p < 0.0001$), with a greater area showing H_2O_2 production in the susceptible genotype (Fig. 3.6 E, F).

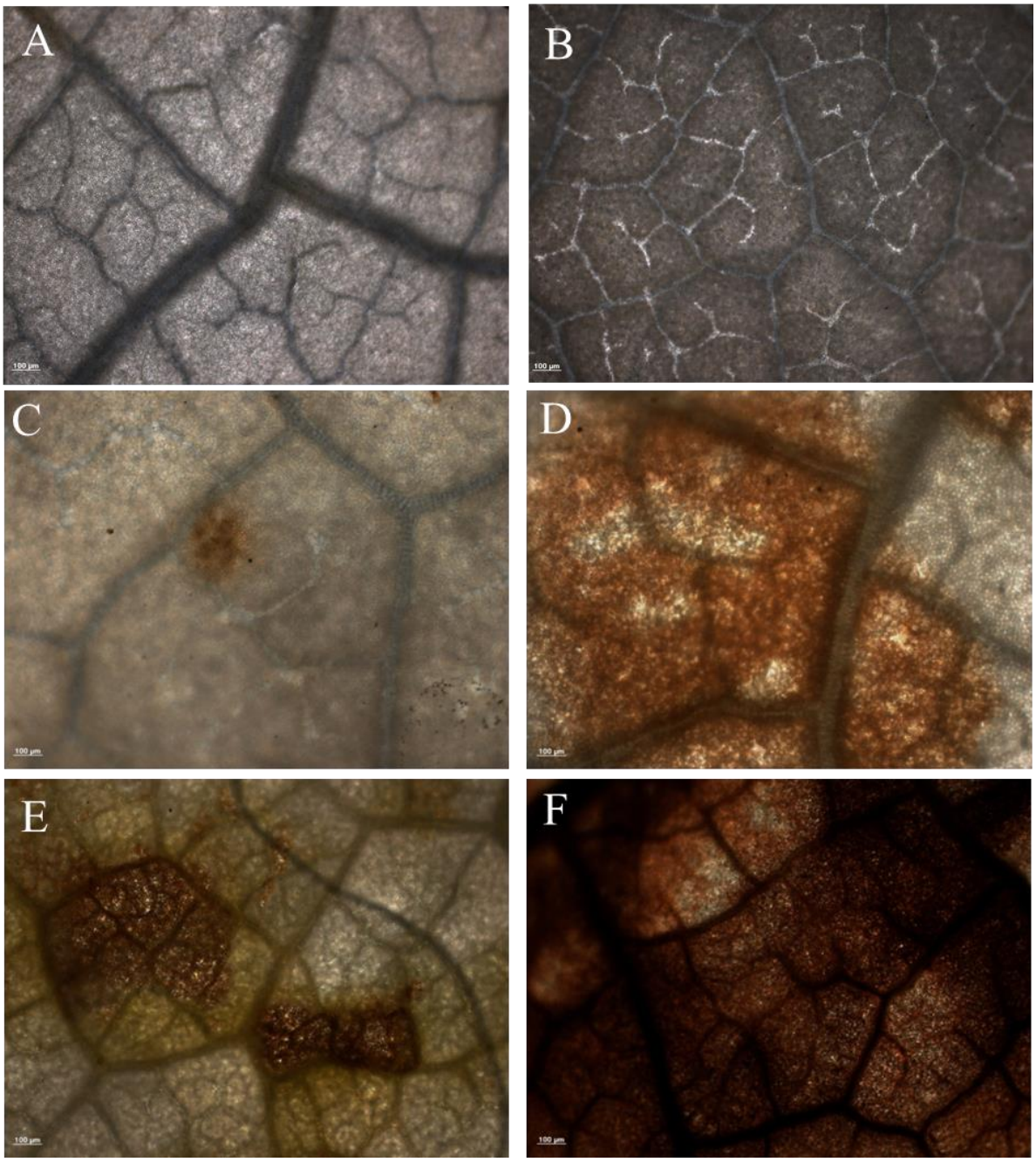

Figure 3.6. H_2O_2 production detected with DAB stain in *Populus* leaves inoculated with *Sphaerulina musiva*. A and B. Non-inoculated moderately resistant and susceptible genotypes, respectively. C and D. DAB staining on inoculated moderately resistant and susceptible leaves at 1 wpi. E and F. DAB staining on moderately resistant and susceptible leaves, respectively, at 3 wpi. The dark brown is the DAB staining of H_2O_2.

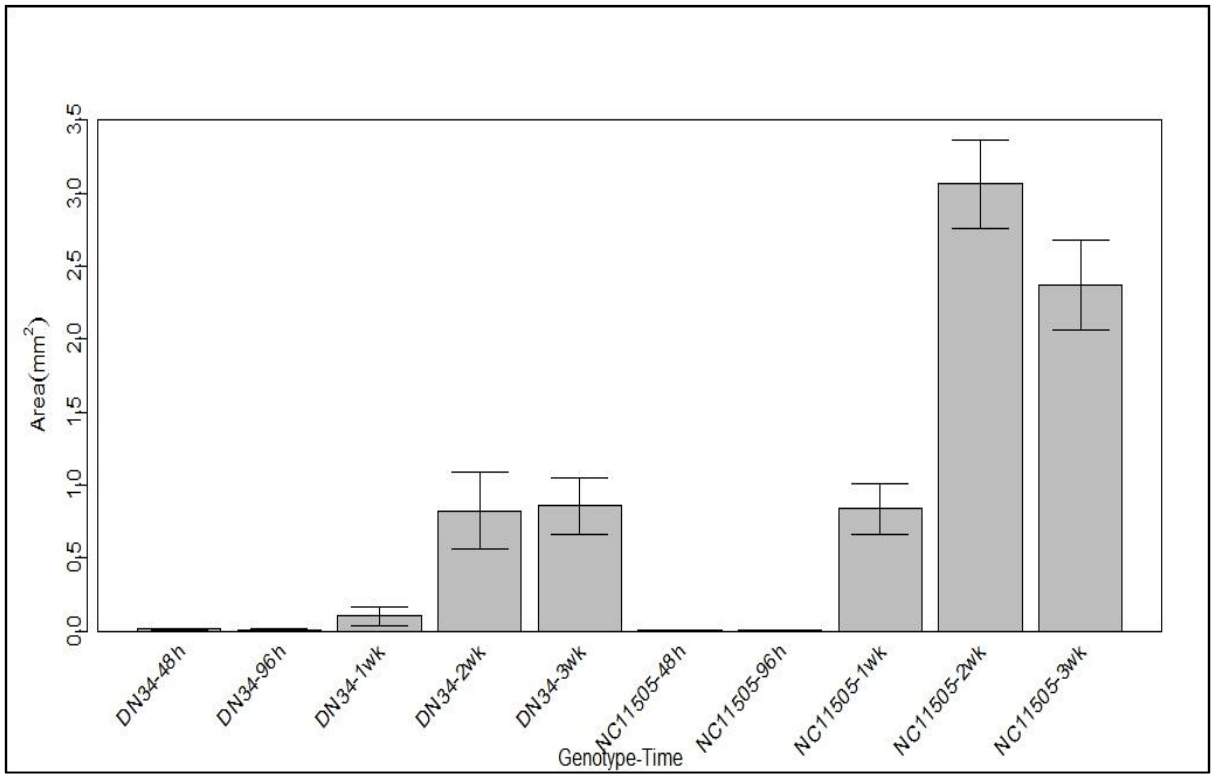

Figure 3.7. Mean area of H_2O_2 accumulation in moderately resistant genotype (DN34) and susceptible genotype (NC11505) at 48 hpi, 96 hpi, 1 wpi, 2 wpi and 3 wpi.

Differentially expressed genes of moderately resistant and susceptible *Populus* after *S. musiva* infection

At 1 wk, 2 wks and 3 wks infected leaf samples from moderately resistant and susceptible genotypes were collected to identify genes responsive to *S. musiva*. In total, 1.04 billion reads were generated using Illumina Nextseq 500, with an average read length of 150 bp. For each sample, 85% of the reads in the susceptible genotype and 74% of the reads in the moderately resistant genotype were mapped to the *Populus* reference genome. The total number of genes differentially expressed between the moderately resistant and the susceptible genotypes at 1 wpi were 10,984, at 2 wpi 6,654 genes and at 3 wpi 7,459. One thousand seven hundred and fifty one genes were commonly upregulated and seven hundred thirteen genes were commonly downregulated at all three time points (Fig. 3.8).

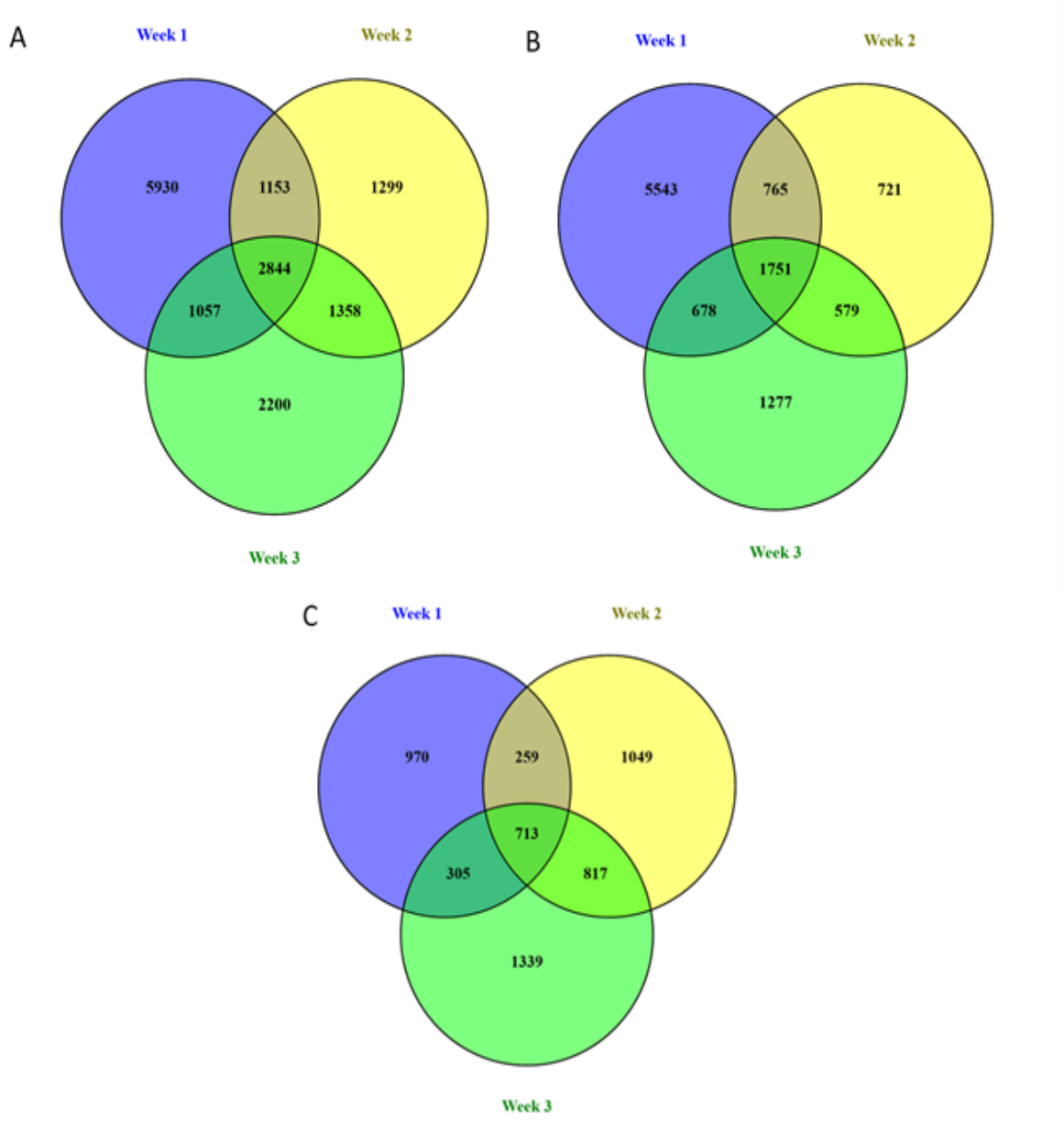

Figure 3.8. Venn diagram summarizing: A. total number of genes differentially expressed in both resistant and susceptible genotypes at each time point (1 wpi, 2 wpi, 3 wpi); B. total genes upregulated and overlap of differentially expressed genes at each time point; and C. total genes downregulated and overlap of differentially expressed genes at each time point.

Differentially expressed genes associated with cell wall modification

After *S. musiva* infection, several genes involved in cell wall modification were differentially expressed. Genes annotated for enzymes like cellulase 2, cellulase, cellulose synthase-like D1 and D5, eight genes of pectin lyase-like superfamily, six genes of plant invertase/pectin methylesterase inhibitor, two genes of polygalacturonase 4, two genes of rhamnogalacturonate lyase, hydroxyproline-rich glycoprotein family protein, 3-ketoacyl-CoA synthase, D-arabinono-1,4-lactone oxidase family protein, two genes encoding beta-xylosidase 2, six of cinnamoyl CoA reductase 1, cinnamyl alcohol dehydrogenase 9, 4-coumarate:CoA ligase 2, extension 3 and terpene synthase 21, were upregulated at 1 wpi in the moderately resistant genotype, while in the susceptible genotype only pectin methyl esterase, 4-coumarate:CoA ligase 2 and terpenoid cyclases family protein was upregulated. Genes with the same annotation – cellulose synthase-like D3 and xyloglucan endotransglucosylase/hydrolase 15, were upregulated in both the genotypes. At 2 wpi, the only genes upregulated were terpene synthase 21, pectin lyase-like superfamily, beta-xylosidase 2 and two hydroxyproline-rich glycoprotein family proteins, which were consistently upregulated from 1 wpi. However, by 2 wpi in the susceptible genotype, more genes were upregulated compared to the moderately resistant genotype. These genes included cellulase 2, four genes of pectin lyase-like superfamily, plant invertase/pectin methylesterase inhibitor, expansin A4, rhamnogalacturonate lyase, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase, cinnamyl alcohol dehydrogenase 9, 4-coumarate:CoA ligase 2 and terpenoid cyclases family protein. By 3 wpi, genes upregulated in the moderately resistant genotype were hydroxyproline-rich glycoprotein family protein, two plant invertase/pectin methylesterase inhibitor, two 4-coumarate:CoA ligase 2 and cinnamyl alcohol dehydrogenase 9, while in the susceptible genotype genes that were upregulated were

terpene synthase 21, three genes encoding pectin lyase superfamily protein, cellulase 2, plant invertase/pectin methylesterase inhibitor superfamily and 3-ketoacyl-CoA synthase (Table 3.1). In the moderately resistant genotype, pertaining to the pattern of gene expression, a peak in the regulation of genes annotating cell wall modelling enzymes was seen at 1 wpi, when compared to the susceptible genotype, with a gradual decrease by 3 wpi. In the susceptible genotype, an expression of more genes involved in cell wall modelling was seen by 2 wpi (Fig. 3.9).

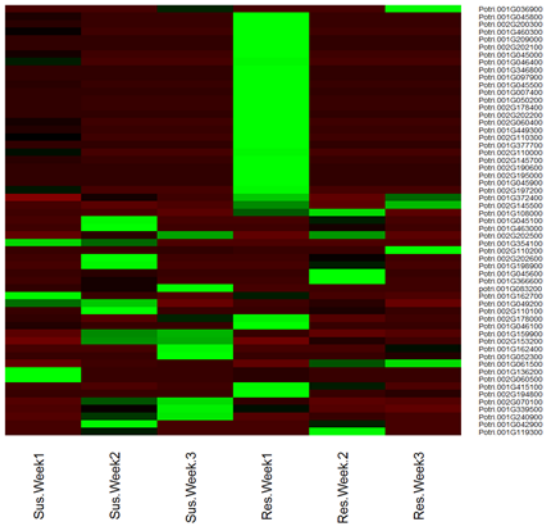

Figure 3.9. Heat map showing differentially expressed associated with cell wall modification across time points (1 wpi, 2 wpi, 3 wpi) in moderately resistant and susceptible genotypes. Green color- high expression, black color- intermediate expression and red color- low expression.

Differentially expressed genes in receptors and signal transduction

In the moderately resistant genotype at 1 wpi, upregulation of genes encoding three receptor-like kinase 1, four receptor kinase 3, ten leucine-rich receptor-like protein kinase family protein, leucine-rich receptor-like serine threonine kinase FLS2 isoform X1, leucine-rich repeat receptor-like serine threonine kinase GSO1, wall-associated receptor kinase 2-like and two wall-associated kinase like-2 was observed. PAMP signals were further mediated by upregulation of MAPK cascade-mitogen-activated protein kinase kinase kinase 3, MAP kinase kinase 10, Ca^{2+}

signaling pathway- CBL-interacting protein kinase 9, BR-signaling kinase 3 and phosphatidylinositol-4-phosphate 5-kinase family protein, which are downstream of these receptors. In the susceptible genotype, three leucine-rich receptor-like protein kinase, nine leucine-rich repeat transmembrane protein kinase, three leucine-rich repeat receptor-like serine threonine kinase GSO1, two wall-associated receptor kinase 2-like, calmodulin-binding receptor-like cytoplasmic kinase 2, cysteine-rich RLK 3, MAP kinase 4 and mitogen-activated protein kinase kinase kinase 19 were upregulated at 1 wpi.

Genes that were consistently expressed in the moderately resistant genotype from 1 wpi, with either an increase or decrease in the fold change at 2 wpi and 3 wpi, included *leucine-rich receptor-like protein kinase family protein* (*Potri.001G053400, Potri.001G064400*), receptor-like kinase 1 (*Potri.001G014100*), receptor kinase 3, phosphatidylinositol-4-phosphate 5-kinase family protein, CBL-interacting protein kinase 9, and three leucine-rich repeat transmembrane protein kinase (*Potri.001G386100, Potri.001G386500, Potri.001G384800*), leucine-rich receptor-like serine threonine kinase FLS2 isoform X1, leucine-rich repeat receptor-like serine threonine kinase GSO1 and wall-associated receptor kinase 2-like. Other genes upregulated at 2 wpi were lectin protein kinase family protein and mitogen-activated protein kinase 3.

However, in the susceptible genotype, genes that were commonly upregulated at 2 wpi and 3 wpi from 1wpi were four leucine-rich repeat transmembrane protein kinase (*Potri.001G082900, Potri.001G384700, Potri.001G385200, Potri.001G38600*), leucine-rich receptor-like protein kinase family protein (*Potri.002G008000*), calmodulin-binding receptor-like cytoplasmic kinase 2, three leucine-rich repeat receptor-like serine threonine kinase GSO1, wall-associated receptor kinase 2-like (*Potri.004G193100*) and mitogen-activated protein kinase

kinase kinase 19. There were a few genes upregulated at 2 wpi, including BR-signaling kinase 3, *ADF4* and LRR family protein (*Potri.001G3017500, Potri.009G154100*) (Table 3.1).

Differentially expressed genes in hormone metabolism

Genes involved in hormone metabolism were also expressed following *S. musiva* infection. At 1 wpi in the moderately resistant genotype, upregulation of different transcription factors involved in hormone metabolism and signaling were observed. Transcription factor myb domain protein 21 (*Potri.001G346600*), which is involved in jasmonic acid (JA) and gibberellic acid (GA) signaling pathways; myb domain protein 65 (*Potri.001G224500*), which was upregulated in response to ethylene and SA and is involved in the GA signaling pathway; myb domain proteins 9 and 52, which are involved in the abscisic acid (ABA) pathway; and myb domain protein 93, upregulated in response to SA, ABA and auxin. Other transcription factors induced upon infection were IAA amido synthetase GH3.9, three SAUR-like auxin-responsive protein family, and auxin response factor 2, which are involved in the auxin pathway; 1-aminocyclopropane-1-carboxylate synthase 8 and ethylene responsive element binding factor 1, which are involved in the ethylene signaling pathway; and highly ABA-induced PP2C gene 3, involved in ABA and JA signaling. At 2 wpi, genes that were still upregulated were 1-aminocyclopropane-1-carboxylate synthase 8, ethylene responsive element binding factor 1, two auxin-responsive factor 2 and myb domain-like protein 73, which are involved in SA, JA, ABA and ethylene. By 3 wpi, only myb domain-like protein 73 and auxin-responsive factor 2 were upregulated (Table 3.1).

However, in the susceptible genotype at 1 wpi, there was an upregulation in accumulation of transcripts involved in: auxin pathway- SAUR-like auxin-responsive protein family (*Potri.002G145300*), transcriptional factor B3 family protein/auxin-responsive factor AUX/IAA-

related; ethylene pathway- 1-aminocyclopropane-1-carboxylic acid synthase 6, ethylene binding factor 5; ABA pathway- homology to *AB12*; JA pathway- jasmonate-zim-domain protein 10, brassinosteroid hormone- brassinosteroid-responsive RING-H2; and also myb domain proteins 73 and 68 (response to SA, GA). At 2 wpi, apart from genes encoding jasmonate-zim-domain protein 10, homology to AB12 and myb 68 that were upregulated, other genes encoding enzymes- zeaxanthin epoxidase (ZEP), *ABA1*, ACC synthase 1, IAA amido synthetase GH3.9; and transcription factors- ethylene response factor 1 and myb domain-like protein 2 (response to ethylene, SA, ABA), were also upregulated. By 3 wpi, genes encoding ABI-1-like 1, brassinosteroid-responsive RING-H2, ethylene response factor 1, 1-aminocyclopropane-1-carboxylic acid synthase 6, ACC synthase 1 and myb domain-like proteins 2 and 68 were upregulated. A gene encoding homology to *AB12* was upregulated only 6 fold at 3 wpi, compared to a 22 fold change at 2 wpi (Table 3.1). The pattern of gene expression, concerning hormone metabolism in the moderately resistant genotype, had a peak in the regulation of the majority of the genes at 1 wpi, when compared to the susceptible genotype. At later time points, most of the genes were downregulated with very few upregulated. In the susceptible genotype, more genes where highly upregulated at 2 wpi and 3 wpi, when compared to the moderately resistant genotype (Fig. 3.10).

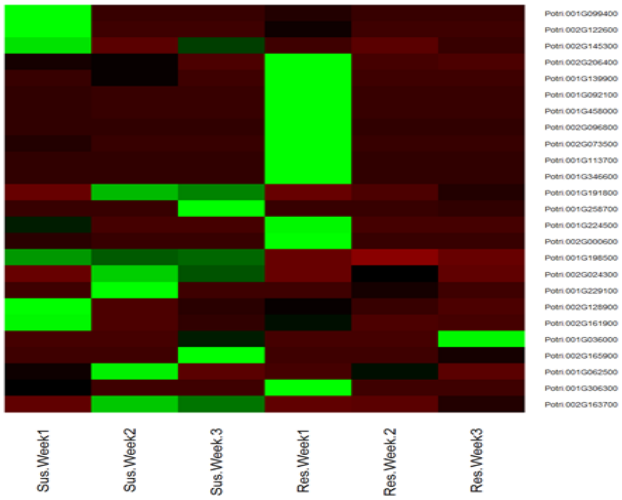

Figure 3.10. Heat map showing differentially expressed genes associated with hormone metabolism across time points (1 wpi, 2 wpi, 3 wpi) in moderately resistant and susceptible genotypes. Green color- high expression, black color- intermediate expression and red color- low expression.

Differentially expressed genes in detoxification and ROS production

Antioxidants play a major role in defense by scavenging ROS. In the moderately resistant genotype, a high increase in upregulation of genes encoding HSP20, HSP40, two glutaredoxin family protein, seven Laccase 17 and four peroxidase superfamily protein (*Potri.001G182400, Potri.001G458700, Potri.001G458900, Potri.002G0180000*) were seen at 1 wpi only. Genes upregulated at all the time points were alcohol dehydrogenase 1, thioredoxin (*Potri.002G017500*), six glutathione S-transferase TAU 19, two glutathione S-transferase TAU 25, carotenoid cleavage dioxygenase 1 (*Potri.001G265900*) and peroxidase superfamily protein (*Potri.001G011000*). Additional genes encoding plant L-ascorbate oxidase and aldehyde dehydrogenase 7B4 were also upregulated. In the susceptible genotype, antioxidants upregulated at all the time points were two thioredoxin (*Potri.001G281100, Potri.002G208900*). Genes encoding plant L-ascorbate oxidase, carotenoid cleavage dioxygenase 1 (*Potri.001G265600*),

glutathione S-transferase TAU 19 (*Potri.001G431300*) and aldehyde dehydrogenase 7B4 were expressed at 1 wpi and 2 wpi. Peroxidase 2 was differentially expressed only at 1 wpi and 3 wpi.

We also found ROS production in the moderately resistant and susceptible genotypes as a part of the defense mechanism. In the moderately resistant genotype, genes encoding FAD-binding Berberine family protein and plant L-ascorbate oxidase were upregulated, while in the susceptible genotype, genes encoding germin-like protein 1 and most of the FAD-binding Berberine family proteins were upregulated, primarily at 2 wpi (Table 3.1). In the moderately resistant genotype, the pattern of gene expression with regards to antioxidants had a peak in the regulation of the majority of the genes at 1 wpi, when compared to the susceptible genotype. At later time points, most of the genes were still expressed with very few downregulated. In the susceptible genotype, few genes were highly upregulated at 2 wpi, but most of the genes expressed remained the same at 3 wpi. With respect to ROS, in the moderately resistant genotype, a peak in expression was seen at 1 wpi and 3 wpi of the same genes, as well as different genes. However, in the susceptible genotype, most of the genes were highly upregulated at 2 wpi, with few highly upregulated at 3 wpi (Fig. 3.11).

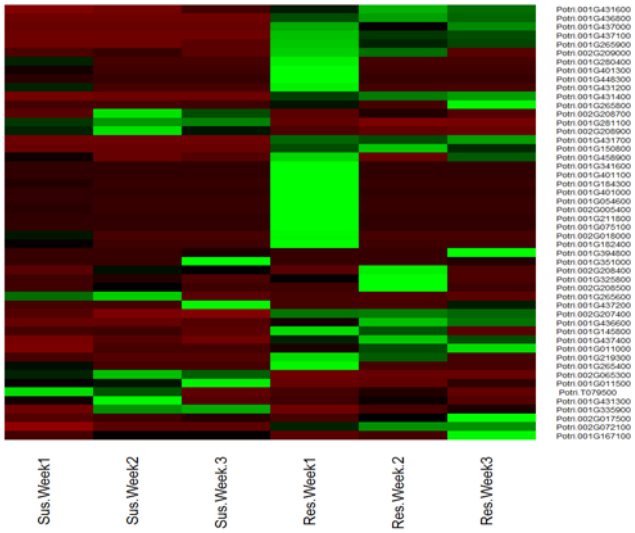

Figure 3.11. Heat map showing differentially expressed genes associated with antioxidants across time points (1 wpi, 2 wpi, 3 wpi) in moderately resistant and susceptible genotypes Green color- high expression, black color- intermediate expression and red color- low expression.

Differentially expressed genes related to defense

A greater number of upregulated genes involved in defense were observed at 1 wpi in the moderately resistant genotype, when compared to the susceptible genotype. These upregulated genes were three disease resistance-responsive (dirigent-like protein) family protein, Arabinogalactan protein 26, three seven transmembrane MLO family protein (*Potri.002G006700, Potri.001G402400, Potri.002G007000*), *MLO1, MLO4*, HMG (high mobility group) box protein, disease resistance RPMI, disease resistance gene NBS-LRR family, two NB-ARC domain-containing disease resistance protein, families of cytochrome P450, nudix hydrolase, autophagy 3, two pathogenesis-related family protein, pathogenesis-related gene 1, five pathogenesis-related thaumatin superfamily, two beta glucosidase 16, three beta glucosidase 17, suppressor of npr1 constitutive-like, three RNI-like superfamily protein, alpha/beta-hydrolases superfamily protein, Ras-related small GTP-binding family protein, photosystems I

and II, RIN4 family protein, kunitz family trypsin and protease inhibitor proteins, CC-NBS-LRR (*Potri.001G134500*), four TIR-NBS-LRR, four serine carboxypeptidase S28 family protein, *BCL-2*-associated athanogenes 1, 4 and 6 and Chalcone and stilbene synthase family proteins (*Potri.002G141400*). Among these genes, those upregulated continuously at 2 wpi and 3 wpi were three TIR-NBS-LRR (*Potri.002G056100, Potri.001G363200, Potri.001G307300*), suppressor of npr1 constitutive-like, *MLO4,* RNI-like superfamily protein, alpha/beta-hydrolases superfamily protein, Ras-related small GTP-binding family protein, photosystem II, disease resistance RPMI, two NB-ARC domain-containing disease resistance proteins, families of cytochrome P450, nudix hydrolase, beta glucosidase 16 (*Potri.001G226100*), serine carboxypeptidase S28 family protein (*Potri.001G213400, Potri.001G213200, Potri.001G213000*) and seven transmembrane MLO family protein (*Potri.002G006700*). However, PR1 (*Potri.001G288400*), three pathogenesis-related thaumatin superfamily (*Potri.001G221700, Potri.001G222000, Potri.001G2110400*), two alpha/beta-hydrolases superfamily protein and autophagy 3 were upregulated only at 3 wpi after 1 wpi (Table 3.1).

In the susceptible genotype at 1 wpi, genes that were upregulated included disease resistance-responsive (dirigent-like protein) family protein (*Potri.001G096500*), with a fold change of only 9.6, Arabinogalactan protein 1, two Avr9 Cf-9 rapidly elicited genes, Cf-4 9 disease resistance-like, five disease resistance RGA4, NB-ARC domain-containing disease resistance protein, *MLO1*, photosystem II subunit R, nudix hydrolase homolog 12, *BCL-2*-associated athanogene 3, CLP protease proteolytic subunit 1, plantacyanin, two osmotin 34, CC-NBS-LRR (*Potri.001G134500*), TIR-NBS-LRR (*Potri.001G363300*), Chalcone and stilbene synthase family proteins (*Potri.001G051500*), two beta glucosidase 27 and serine carboxypeptidase-like 18. Genes that were upregulated from 2 wpi were alpha/beta-hydrolases

superfamily protein, PR1 (*Potri.001G288600*), four pathogenesis-related thaumatin superfamily (*Potri.001G221100, Potri.001G221500, Potri.001G221800, Potri.002G020500*), pathogenesis-related family protein (*Potri.001G389800*), two beta glucosidase, osmotin 34 (*Potri.001G107800*), disease resistance-responsive (dirigent-like protein) family protein (*Potri.001G214600*), kunitz family trypsin and protease inhibitor proteins (*Potri.001G309900*) and three beta glucosidase 17 (*Potri.001G223300, Potri.001G222900, Potri.001G222800*). By 3 wpi, only a few genes were still upregulated (*Potri.001G451500, Potri.001G382100*, *Potri.002G020500*, *Potri.001G389800*, *Potri.001G222900, Potri.001G222800*, *Potri.001G102400*, *Potri.001G290900, Potri.001G295300*), *MLO1*, NB-ARC domain-containing disease resistance protein, photosystem II subunit, two alpha/beta-hydrolases superfamily protein, three disease resistance *RGA4, Cf-4 9*, and two *Avr9-Cf9*.

Out of the sixteen WRKYs induced upon pathogen attack, notable were WRKY DNA-binding proteins 75 and 23, which were upregulated only at 3 wpi in the moderately resistant genotype. However, in the susceptible genotype, WRKY40 was upregulated at all the time points, whereas WRKY 28 and 75 were upregulated only at 2 wpi (Table 3.1). In the moderately resistant genotype, pertaining to the pattern of gene expression, a peak in the regulation of genes annotating defense related was seen at 1 wpi, when compared to the susceptible genotype. At later time points, up and down regulation of some other genes and the same genes were observed. In the susceptible genotype, expression of more genes or a peak in defense related genes were seen by 2 wpi, and at later time points up and down regulation of some genes and the same genes were observed (Fig. 3.12).

Other genes that were up and downregulated during the infection process in moderately resistant and susceptible genotypes are listed in Table B1.

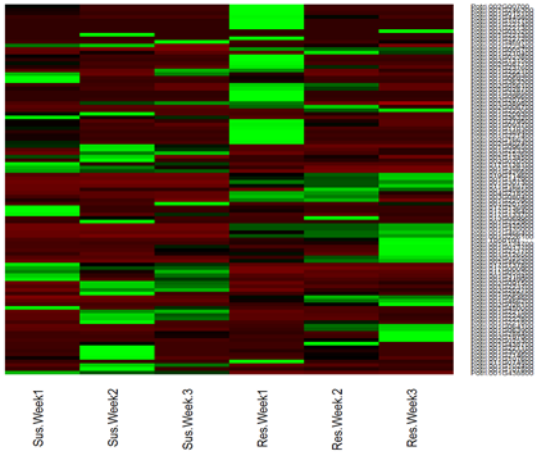

Figure 3.12. Heat map showing differentially expressed genes associated with defense across time points (1 wpi, 2 wpi, 3 wpi) in moderately resistant and susceptible genotypes. Green color- high expression, black color- intermediate expression and red color- low expression.

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva*.

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Cell Wall Modification				
Potri.001G083200	Cellulase 2	4	-5	-36.5
Potri.001G097900	Cellulase (glycosyl hydrolase family 5) protein	∞		
Potri.001G050200	Cellulose synthase-like D1	87.6		
Potri.001G136200	Cellulose synthase-like D3	-8.5		
Potri.001G449300	Cellulose synthase-like D3	14.8		
Potri.002G200300	6-Cellulose synthase-like D5	20		
Potri.001G108000	Pectin lyase-like superfamily protein	6	18	
Potri.001G159900	Pectin lyase-like superfamily protein	3	-23	-11
Potri.001G007400	Pectin lyase-like superfamily protein	∞		
Potri.001G346800	Pectin lyase-like superfamily protein	∞		
Potri.001G377700	Pectin lyase-like superfamily protein	∞		
Potri.001G190600	Pectin lyase-like superfamily protein	∞		
Potri.001G339500	Pectin lyase-like superfamily protein	5	-8	-323.7
Potri.001G367800	Pectin lyase-like superfamily protein	5	-5.8	
Potri.001G463000	Pectin lyase-like superfamily protein		-8	
Potri.001G052300	Pectin lyase-like superfamily protein			-12
Potri.001G052300	Pectin methylesterase 3			-3.9
Potri.001G162700	Pectin methylesterase 1	-3	-3.8	
Potri.002G145700	Plant invertase/pectin methylesterase inhibitor superfamily	26		
Potri.001G209000	Plant invertase/pectin methylesterase inhibitor superfamily	∞		
Potri.001G209200	Plant invertase/pectin methylesterase inhibitor superfamily	∞		
Potri.002G145700	Plant invertase/pectin methylesterase inhibitor superfamily	9.8		18
Potri.002G202500	Plant invertase/pectin methylesterase inhibitor superfamily		4	-23
Potri.002G194800	Plant invertase/pectin methylesterase inhibitor superfamily	∞		∞
Potri.002G202600	Plant invertase/pectin methylesterase inhibitor superfamily		-5	
Potri.002G195000	Plant invertase/pectin methylesterase inhibitor superfamily	∞		

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G119300	Plant invertase/pectin methylesterase inhibitor superfamily		3	
Potri.002G202100	polygalacturonase 4	∞		
Potri.002G202200	polygalacturonase 4	∞		
Potri.002G110300	Rhamnogalacturonate lyase family protein	5		
Potri.002G110100	Rhamnogalacturonate lyase family protein		-11	
Potri.002G110000	Rhamnogalacturonate lyase family protein	3.8		
Potri.002G110200	Rhamnogalacturonate lyase family protein			62
Potri.001G460300	Hydroxyproline-rich glycoprotein family protein	5		
Potri.001G061500	Hydroxyproline-rich glycoprotein family protein		5.8	7.7
Potri.001G098900	Hydroxyproline-rich glycoprotein family protein		-3.3	
Potri.001G366600	Hydroxyproline-rich glycoprotein family protein		7	
Potri.002G060400	Xyloglucan endotransglucosylase/hydrolase 15	7.5		
Potri.002G060500	Xyloglucan endotransglucosylase/hydrolase 15	-18.7		
Potri.001G354100	Beta-xylosidase 2	∞	∞	
Potri.002G197200	Beta-xylosidase 2	3.5		
Potri.002G178000	3-ketoacyl-CoA synthase 1	9		-4.6
Potri.002G153200	Endoxyloglucan transferase A3		-3.5	-6
Potri.002G070100	extensin 3	3	-26	-26
Potri.001G240900	expansin A4		-5.8	-33
Potri.002G178400	D-arabinono-1,4-lactone oxidase family protein	66		
Signal Recognition and Transduction				
Potri.001G039400	Wall-associated kinase-like 2	25.7	-3.7	
Potri.001G039900	Wall-associated kinase-like 2	3.5	-5.4	
Potri.002G075900	Wall-associated kinase-like 2			3
Potri.009G157200	Wall-associated receptor kinase 2-like	7.5	23	10

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.009G154500	Wall-associated receptor kinase 2-like		12	6
Potri.002G075900	Wall-associated receptor kinase 2-like			3
Potri.009G154600	Wall-associated receptor kinase 2-like	-3		
Potri.004G193100	Wall-associated receptor kinase 2-like	-22	-19	-19
Potri.009G154100	Wall-associated receptor kinase 2-like		-2.8	-3
Potri.001G442000	Lectin protein kinase family protein		5	34.6
Potri.001G064400	Leucine-rich receptor-like protein kinase family protein	14.8	43.6	25.1
Potri.001G052500	Leucine-rich receptor-like protein kinase family protein	∞		
Potri.002G095700	Leucine-rich receptor-like protein kinase family protein	∞		
Potri.002G008000	Leucine-rich receptor-like protein kinase family protein	-13.7	-7.5	-3.3
*Potri.002G008*100	Leucine-rich receptor-like protein kinase family protein	-5		
Potri.002G008400	Leucine-rich receptor-like protein kinase family protein	-3		
Potri.001G053400	Leucine-rich receptor-like protein kinase family protein		3	5
*Potri.002G007*900	Leucine-rich receptor-like protein kinase family protein			3
Potri.001G430500	Leucine-rich receptor-like protein kinase family protein	∞		
Potri.002G019900	Leucine-rich receptor-like protein kinase family protein	∞		
Potri.002G027400	Leucine-rich receptor-like protein kinase family protein	∞		
Potri.001G042500	Leucine-rich receptor-like protein kinase family protein	4		
Potri.001G0465800	Leucine-rich receptor-like protein kinase family protein	8.6		
Potri.001G0467300	Leucine-rich receptor-like protein kinase family protein	3.6		
Potri.002G063300	Leucine-rich receptor-like protein kinase family protein	19		

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.005G007900	Leucine-rich receptor-like serine threonine kinase FLS2 isoform X1	6.6	9	5
Potri.005G013100	Leucine-rich receptor-like serine threonine kinase GSO1	-10	-3.6	-4
Potri.005G013400	Leucine-rich receptor-like serine threonine kinase GSO1	-5	-3	-3
Potri.005G016000	Leucine-rich receptor-like serine threonine kinase GSO1	-6	-21	-12
Potri.005G00970	Leucine-rich receptor-like serine threonine kinase GSO1	3	10	6
Potri.001G384700	Leucine-rich repeat Transmembrane protein kinase	-37.9	-29.3	-31.9
Potri.001G385500	Leucine-rich repeat Transmembrane protein kinase	∞		
Potri.001G082900	Leucine-rich repeat Transmembrane protein kinase	-7	-4	-5
Potri.001G385200	Leucine-rich repeat Transmembrane protein kinase	-4.7	-4	-4
Potri.001G385300	Leucine-rich repeat Transmembrane protein kinase	-3.8		4
Potri.001G385600	Leucine-rich repeat Transmembrane protein kinase	-9		
Potri.001G385900	Leucine-rich repeat Transmembrane protein kinase	-17.6		
Potri.001G386000	Leucine-rich repeat Transmembrane protein kinase	-7	-3.7	-4.7
Potri.001G386100	Leucine-rich repeat Transmembrane protein kinase	-6.6	3.5	4
Potri.001G386300	Leucine-rich repeat Transmembrane protein kinase	-15		
Potri.001G384800	Leucine-rich repeat Transmembrane protein kinase		∞	∞
Potri.001G386500	Leucine-rich repeat Transmembrane protein kinase		9	21.5
Potri.001G414300	Receptor kinase 3	79	64.7	62
Potri.001G414200	Receptor kinase 3	4.5	-3.2	
Potri.001G413800	Receptor kinase 3	43	25.6	11.7
Potri.001G413400	Receptor kinase 3	33.7	9	40.8
Potri.001G409300	Receptor kinase 3		5.5	

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G014100	Receptor-like protein kinase 1	∞	8	22.8
Potri.001G153800	Receptor-like protein kinase 1	43	-22.5	-7
Potri.001G014600	Receptor-like protein kinase 1	∞		11
Potri.001G014300	Receptor-like protein kinase 1	-11.8		-4.4
Potri.001G014400	Receptor-like protein kinase 1	-17		-4
Potri.001G014700	Receptor-like protein kinase 1	12		
Potri.001G228200	Receptor-like protein kinase 1	-3.5		-3.5
Potri.001G014800	Receptor-like protein kinase 1		∞	∞
Potri.001G014500	Receptor-like protein kinase 1		-9	-3
Potri.002G161600	Calmodulin-binding receptor-like cytoplasmic kinase 2	-20.6	-5	-5
Potri.001G042400	Mitogen-activated protein kinase kinase kinase 19	-17	-3	-7
Potri.002G073100	Mitogen-activated protein kinase kinase kinase 3	∞		
Potri.001G271700	Mitogen-activated protein kinase 3		4	
Potri.001G138800	MAP kinase kinase 10	3.8		
Potri.002G162500	MAP kinase 4	-4.8		
Potri.001G027900	Phosphatidylinositol-4-phosphate 5-kinase family protein	59	8	
Potri.001G236500	Actin depolymerizing factor 4		-4	-5.6
Potri.002G158400	BR-signaling kinase 3	3.6	-3.6	-20
Potri.002G177900	CBL-interacting protein kinase 9	6.3	6	5
Potri.001G276600	Cysteine-rich RLK (RECEPTOR-like protein kinase) 3	-5		
Hormone Metabolism				
Potri.001G191800	myb domain protein 2		-12	-3.6
Potri.001G346600	myb domain protein 21	∞		
Potri.001G258700	myb domain protein 46			-41
Potri.002G073500	myb domain protein 52	16		
Potri.001G224500	myb domain protein 65			
Potri.001G036000	myb domain protein 65			3
Potri.001G113700	myb domain protein 68	-3	-12	-7

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.002G122600	myb domain protein 73	-6.7		
Potri.002G128900	myb domain protein 73	-4	6	-22
Potri.001G139900	myb domain protein 9	77	∞	
Potri.002G096800	myb domain protein 93	∞		
Potri.001G099400	1-aminocyclopropane-1-carboxylic acid (acc) synthase 6	-15.7		
Potri.002G113900	1-amino-cyclopropane-1-carboxylate synthase 8	∞	∞	-3.8
Potri.001G092100	highly ABA-induced PP2C gene 3	63.7		
Potri.002G161900	brassinosteroid-responsive RING-H2	-3.6		-4.7
Potri.001G229100	Zeaxanthin epoxidase (ZEP) (ABA1)		-8.5	
Potri.002G165900	ABI-1-like 1			-8
Potri.001G198500	Homology to ABI2	-6.6	-22.7	-6
Potri.001G458000	SAUR-like auxin-responsive protein family	70		
Potri.001G306300	SAUR-like auxin-responsive protein family	5		
*Potri.002G000*600	SAUR-like auxin-responsive protein family	33.7		
Potri.002G145300	SAUR-like auxin-responsive protein family	-12		-4.5
Potri.002G024300	SAUR-like auxin-responsive protein family		-3	-27
Potri.002G206400	Putative indole-3-acetic acid-amido synthetase GH3.9	6	-7.8	
Potri.001G062500	jasmonate-zim-domain protein 10	-3	-3	
Potri.002G163700	ACC synthase 1		-35	-3.5
Transcription Factors				
Potri.001G079900	Ethylene responsive element binding factor 1	5	3	
Potri.001G154100	Ethylene responsive element binding factor 1		-2.9	3
Potri.001G154200	Ethylene responsive element binding factor 5	-16.9		
Potri.002G039100	Ethylene response factor 1			
Potri.002G039000	Ethylene response factor 1		-3.5	-8

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.002G024700	Transcriptional factor B3 family protein/auxin-responsive factor AUX/IAA-related	-2.9		
Potri.002G207100	Auxin response factor 2	15.6	19	34
Potri.002G207000	Auxin response factor 2	6	23	22
Potri.002G266200	Auxin response factor 2			-4
Potri.002G172500	Auxin response factor 11	13.5	4	4.9
Potri.001G352400	WRKY DNA-binding protein 28		-3	
Potri.001G328000	WRKY DNA-binding protein 75		-3.9	
Potri.001G058800	WRKY DNA-binding protein 75			8
*Potri.001G002*400	WRKY DNA-binding protein 51			-14
Potri.002G193000	WRKY DNA-binding protein 23			3
Potri.001G044500	WRKY DNA-binding protein 40	-9.7	-4.9	-4.9
Defense Related				
Potri.002G091500	AGD2-like defense response protein 1	-7	-3	
Potri.002G131500	Disease resistance-responsive (dirigent-like protein) family protein	23		
Potri.001G096600	Disease resistance-responsive (dirigent-like protein) family protein	∞		
Potri.001G023800	Disease resistance-responsive (dirigent-like protein) family protein	5.4		
Potri.001G096500	Disease resistance-responsive (dirigent-like protein) family protein	-9.6	-∞	-∞
Potri.001G214600	Disease resistance-responsive (dirigent-like protein) family protein		-5.9	
Potri.001G096500	Disease resistance-responsive (dirigent-like protein) family protein	-9.6	-∞	-∞
Potri.001G310400	Arabinogalactan protein 1	-15.8	-7	-12
Potri.002G207500	Arabinogalactan protein 26	83.6		
Potri.001G408500	Diacylglycerol kinase 5		5.4	3.6
*Potri.002G006*700	Seven transmembrane MLO family protein	40.9	77	19
Potri.002G402400	Seven transmembrane MLO family protein	3		
*Potri.002G000*700	Seven transmembrane MLO family protein	4.6		

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G005700	ACD1-like	5.8	7.7	9
Potri.001G069200	Nudix hydrolase homolog 12	-7		
Potri.011G165700	disease resistance RPM1-like	926	256	366
Potri.001G435000	disease resistance gene NBS-LRR family	1170	200	
Potri.001G093700	HMG (high mobility group) box protein	3.7	3.3	
Potri.002G033200	Arabidopsis defensin-like protein			∞
Potri.001G126500	Autophagy 3 (APG3)	3.25		4.8
Potri.001G151100	Subtilisin-like serine protease 3	74		
Potri.001G295300	CLP protease proteolytic subunit 1	-4		-13
Potri.001G209300	Plantacyanin	-13	-7	
Potri.001G110300	BCL-2-associated athanogene 1	3		
Potri.001G358200	BCL-2-associated athanogene 3	-4.6	-22	-44
Potri.001G279500	BCL-2-associated athanogene 4	3.6	3.6	-3.8
Potri.002G166300	BCL-2-associated athanogene 6	8.7		
Potri.001G469700	cytochrome c oxidase 15			-6
Potri.001G389400	Pathogenesis-related family protein	19		
Potri.001G389800	Pathogenesis-related family protein	3.4	-3.8	-76
Potri.001G288400	Pathogenesis-related gene 1	16.6		5.9
Potri.001G288600	Pathogenesis-related gene 1		-7	
Potri.001G221700	Pathogenesis-related thaumatin superfamily protein	19		4
Potri.001G222100	Pathogenesis-related thaumatin superfamily protein	18		-6
Potri.001G221400	Pathogenesis-related thaumatin superfamily protein	8		
Potri.001G237600	Pathogenesis-related thaumatin superfamily protein	6	5	
Potri.002G087100	Pathogenesis-related thaumatin superfamily protein	3.8		
Potri.001G221300	Pathogenesis-related thaumatin superfamily protein		-∞	

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G222000	Pathogenesis-related thaumatin superfamily protein		∞	
Potri.001G210400	Pathogenesis-related thaumatin superfamily protein		4	∞
Potri.001G221100	Pathogenesis-related thaumatin superfamily protein		-4.8	
Potri.001G221500	Pathogenesis-related thaumatin superfamily protein		-10.6	-7
Potri.001G221800	Pathogenesis-related thaumatin superfamily protein		-3	
Potri.002G020500	Pathogenesis-related thaumatin superfamily protein		-21	
Potri.001G015100	beta glucosidase 13	10.6		
Potri.001G226100	beta glucosidase 16	4.5	6.7	7
Potri.001G225900	beta glucosidase 16	3	6	
Potri.001G223400	beta glucosidase 17	-3		-3
Potri.001G227200	beta glucosidase 17	∞		
Potri.001G227300	beta glucosidase 17	∞		
Potri.001G227400	beta glucosidase 17	∞		
Potri.001G222800	beta glucosidase 17		-666.5	-6
Potri.001G222900	beta glucosidase 17		-60	
Potri.001G223300	beta glucosidase 17		-149.7	-3
Potri.001G223700	beta glucosidase 27		-462	-10
Potri.001G224000	beta glucosidase 27		-275	-4.6
Potri.002G025300	cytochrome P450, family 83, subfamily B, polypeptide 1	∞	7594	∞
Potri.002G025500	cytochrome P450, family 83, subfamily B, polypeptide 1	∞	4276	∞
Potri.002G026100	cytochrome P450, family 83, subfamily B, polypeptide 1	∞	709	∞
Potri.001G338500	RPM1-interacting protein 4 (RIN4) family protein	∞		
Potri.001G309900	Kunitz family trypsin and protease inhibitor protein	3.6	-9	
Potri.001G102400	Osmotin 34	-4.6	-33	-5.7
Potri.001G107700	Osmotin 34	-∞		

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G107600	Osmotin 34	-4.7	-4	
Potri.001G107800	Osmotin 34		-25	
Potri.001G435000	NB-ARC domain-containing disease resistance protein	1170	200	∞
Potri.001G134700	NB-ARC domain-containing disease resistance protein	8.5	6	27
Potri.001G426600	NB-ARC domain-containing disease resistance protein	-7	-6	-7
Potri.001G134500	Disease resistance protein (CC-NBS-LRR class) family	-3	-3.5	
Potri.001G445800	Disease resistance protein (CC-NBS-LRR class) family	22.8	27.8	
Potri.011G055200	Cf-4 9 disease resistance-like family	-19.5	-3	-7.8
Potri.001G450000	Avr9 Cf-9 rapidly elicited	-26		-8
Potri.001G449900	Avr9 Cf-9 rapidly elicited	-26		
Potri.017G137800	disease resistance RGA4	-33	-11	-16
Potri.017G138100	disease resistance RGA4	-25	-4	-11
Potri.017G133700	disease resistance RGA4	-24		-3
Potri.017G136900	disease resistance RGA4	-12		
Potri.017G136400	disease resistance RGA4	-9		
Potri.001G147900	Ras-related small GTP-binding family protein	∞	∞	∞
Potri.001G063900	RNI-like superfamily protein	36.9	221	84
Potri.001G064100	RNI-like superfamily protein	4	13.510	
Potri.001G064600	RNI-like superfamily protein	5	6	3
Potri.001G438800	photosystem II subunit R	-321.5	-18.6	-164
Potri.001G438700	photosystem II subunit R		4.5	
Potri.011G074800	photosystem II H (chloroplast)	1897	73.8	25.6
Potri.T050100	Suppressor of npr1 constitutive-like	66	820	1200
Potri.019G114600	Suppressor of npr1 constitutive-like	4	72	109
Potri.017G000900	MLO1	-68	-45	-24
Potri.013G069900	MLO1		4.5	
Potri.004G218500	MLO4	3		
Potri.003G080400	nudix hydrolase 8	9	16	6
Potri.001G466200	alpha/beta-Hydrolases superfamily protein	89.7	255	298

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G466300	alpha/beta-Hydrolases superfamily protein	46	50	47
Potri.001G466400	alpha/beta-Hydrolases superfamily protein	45	42.9	46.6
Potri.002G202700	alpha/beta-Hydrolases superfamily protein		-4	-4
Potri.001G013700	alpha/beta-Hydrolases superfamily protein			3.3
Potri.001G201500	alpha/beta-Hydrolases superfamily protein			-3
Potri.002G197900	alpha/beta-Hydrolases superfamily protein			-7.6
Potri.001G363200	Disease resistance protein (TIR-NBS-LRR class), putative	∞	∞	
Potri.001G307300	Disease resistance protein (TIR-NBS-LRR class), putative	9.9	11.5	
Potri.002G207400	Flavin-containing monooxygenase family protein	3	6	6
Potri.001G335900	Flavin-dependent monooxygenase 1	4	-5.9	-3
Potri.001G028700	Disease resistance protein (TIR-NBS-LRR class), putative	5	6	
Potri.001G363300	Disease resistance protein (TIR-NBS-LRR class), putative	-8		
Potri.002G056100	Disease resistance protein (TIR-NBS-LRR class), putative	8	5	
Detoxification and Antioxidants				
Potri.002G005400	HSP20-like chaperones superfamily protein	32		
Potri.001G211800	HSP40/DnaJ peptide-binding protein	∞		
Potri.002G072100	Alcohol dehydrogenase 1	6.8	3.6	3.7
Potri.001G448300	Glutaredoxin family protein	25		
Potri.001G280400	Glutaredoxin family protein	3		
Potri.002G209000	Thioredoxin superfamily protein	27	5.9	
Potri.001G281100	Thioredoxin superfamily protein	-5.7	-114.8	-29
Potri.001G325800	Thioredoxin superfamily protein	5.5	7.8	
Potri.002G017500	Thioredoxin superfamily protein	5.6	3.8	8
Potri.002G208900	Thioredoxin superfamily protein	-5	-68	

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.002G208400	Thioredoxin superfamily protein		3	-5.8
Potri.002G208500	Thioredoxin superfamily protein		5	
Potri.002G208700	Thioredoxin superfamily protein		-5.6	-24
Potri.001G431400	Glutathione S-transferase TAU 19	43	20	20
Potri.001G431300	Glutathione S-transferase TAU 19	-4	-4.8	
Potri.001G431700	Glutathione S-transferase TAU 19	∞	∞	∞
Potri.001G437000	Glutathione S-transferase TAU 19	∞	∞	94
Potri.001G437100	Glutathione S-transferase TAU 19	∞	∞	∞
Potri.001G431200	Glutathione S-transferase TAU 19	3		
Potri.001G436600	Glutathione S-transferase TAU 19	11.8	23	6.6
Potri.001G437400	Glutathione S-transferase TAU 19	9	6.7	9.4
Potri.001G437200	Glutathione S-transferase TAU 19			-3.7
Potri.001G431600	Glutathione S-transferase TAU 25	16	14	3.5
Potri.001G436800	Glutathione S-transferase TAU 25	∞	∞	∞
Potri.001G394800	Glutathione S-transferase TAU 25			9
Potri.001G265900	Carotenoid cleavage dioxygenase 1	76.6	33	7.9
Potri.001G265600	Carotenoid cleavage dioxygenase 1	-11	-26	
Potri.001G265400	Carotenoid cleavage dioxygenase 1	4		
Potri.001G265800	Carotenoid cleavage dioxygenase 1	21.6		35
Potri.001G145800	Peroxidase superfamily protein	13	5.6	
Potri.001G075100	Peroxidase superfamily protein	∞		
Potri.001G011000	Peroxidase superfamily protein	10	3	5.5
Potri.001G182400	Peroxidase superfamily protein	6		
Potri.001G458700	Peroxidase superfamily protein	3.7		11.7
Potri.001G458900	Peroxidase superfamily protein	3.5		8.3
Potri.002G018000	Peroxidase superfamily protein	3.4		
Potri.002G065300	Peroxidase superfamily protein	-7.9	-14	-8.7
Potri.001G351000	Peroxidase superfamily protein			-17
Potri.001G011500	Peroxidase 2	-3.8	3.5	-4
Potri.001G054600	Laccase 17	70		
Potri.001G341600	Laccase 17	∞		
Potri.001G401000	Laccase 17	∞		

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G401100	Laccase 17	∞		
Potri.001G184300	Laccase 17	13.7		
Potri.001G401300	Laccase 17	7.8		
Potri.001G219300	Plant L-ascorbate oxidase	14.5	51.6	6
Potri.T079500	Plant L-ascorbate oxidase	-11	-2	
Potri.001G167100	Aldehyde dehydrogenase 7B4	-2.9	-2.9	3.9
ROS				
Potri.001G169000	Germin-like protein 1	-3	-12	-42
Potri.001G440700	FAD-binding Berberine family protein	∞	29.5	72
Potri.001G462200	FAD-binding Berberine family protein	13.9	-163	
Potri.001G464800	FAD-binding Berberine family protein	∞	-38	14.8
Potri.001G461800	FAD-binding Berberine family protein	28		7.9
Potri.001G462400	FAD-binding Berberine family protein	-4		9
Potri.001G462500	FAD-binding Berberine family protein	4	5.5	45
Potri.001G463400	FAD-binding Berberine family protein	149		
Potri.001G459100	FAD-binding Berberine family protein		-20	
Potri.001G463300	FAD-binding Berberine family protein		-18	
Potri.001G463100	FAD-binding Berberine family protein		-7.8	-8
Potri.001G462000	FAD-binding Berberine family protein		-11.6	-4.7
Potri.001G461900	FAD-binding Berberine family protein		-6	
Potri.001G461700	FAD-binding Berberine family protein		-3	5.4
Potri.001G459500	FAD-binding Berberine family protein		-3	-3
Potri.001G462100	FAD-binding Berberine family protein	∞	-∞	

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G463500	FAD-binding Berberine family protein	∞		
Potri.001G464700	FAD-binding Berberine family protein	∞		
Potri.001G461800	FAD-binding Berberine family protein	28		7.9
Potri.001G462700	FAD-binding Berberine family protein		-3	
Potri.001G461500	FAD-binding Berberine family protein			18
Potri.001G462300	FAD-binding Berberine family protein			162
Potri.001G462800	FAD-binding Berberine family protein			-3
Potri.001G470100	FAD-binding Berberine family protein			-3
Secondary Metabolites				
Potri.001G051500	Chalcone and stilbene synthase family protein	-3	-6	
Potri.002G141400	Chalcone and stilbene synthase family protein	∞		
Potri.001G051600	Chalcone and stilbene synthase family protein		-3.3	
Potri.001G212800	Serine carboxypeptidase S28 family protein	49.6	15.9	
Potri.001G213000	Serine carboxypeptidase S28 family protein	∞		
Potri.001G213200	Serine carboxypeptidase S28 family protein	7	13	11.5
Potri.001G213400	Serine carboxypeptidase S28 family protein	6	10.6	11.8
Potri.001G212900	Serine carboxypeptidase S28 family protein		-3	-3
Potri.001G213300	Serine carboxypeptidase S28 family protein		4.5	
Potri.001G290900	Serine carboxypeptidase-like 18	-5.8	-4	-20
Potri.001G291300	Serine carboxypeptidase-like 18	3.7	4	
Potri.001G291300	Serine carboxypeptidase-like 18			-7.9

Table 3.1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G042900	Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase		-3	
Potri.001G036900	4-coumarate:CoA ligase 2			3
Potri.001G045500	Cinnamoyl CoA reductase 1	19		
Potri.001G045900	Cinnamoyl CoA reductase 1	∞		
Potri.001G045000	Cinnamoyl CoA reductase 1	8		
Potri.001G045800	Cinnamoyl CoA reductase 1	9		
Potri.001G046100	Cinnamoyl CoA reductase 1	13		
Potri.001G046400	Cinnamoyl CoA reductase 1	3		
Potri.001G045100	Cinnamoyl CoA reductase 1		-3	
Potri.001G045600	Cinnamoyl CoA reductase 1		19	
Potri.001G372400	Cinnamyl alcohol dehydrogenase 9	∞	-3	3
Potri.002G034800	Cystathionine beta-synthase (CBS) family protein	6		3.5
Potri.002G033700	Ornithine carbamoyltransferase	-5.9	-11	
Potri.001G415100	Terpene synthase 21	18.7	23.9	-7
Potri.001G049200	Terpenoid cyclases family protein	-5	-5	

*****Positive value indicates upregulation in moderately resistant genotype and negative value indicates upregulation in susceptible genotype.

∞: Means reads were only found in moderately resistant genotype; -∞: means reads were found only in susceptible genotype.

Differentially expressed genes in *S. musiva* during infection

A total of 36.6 million reads were mapped to the *S. musiva* reference genome during interaction with the susceptible genotype, and 23.5 million reads mapped during interaction with the moderately resistant genotype. The total number of genes differentially expressed in the pathogen at 1 wpi was 911, at 2 wpi 1,366 genes and 2,824 genes at 3 wpi during the interaction with the host. One hundred ninety eight genes were commonly upregulated and seven hundred fifty six genes were commonly downregulated at all time points (Fig. 3.13).

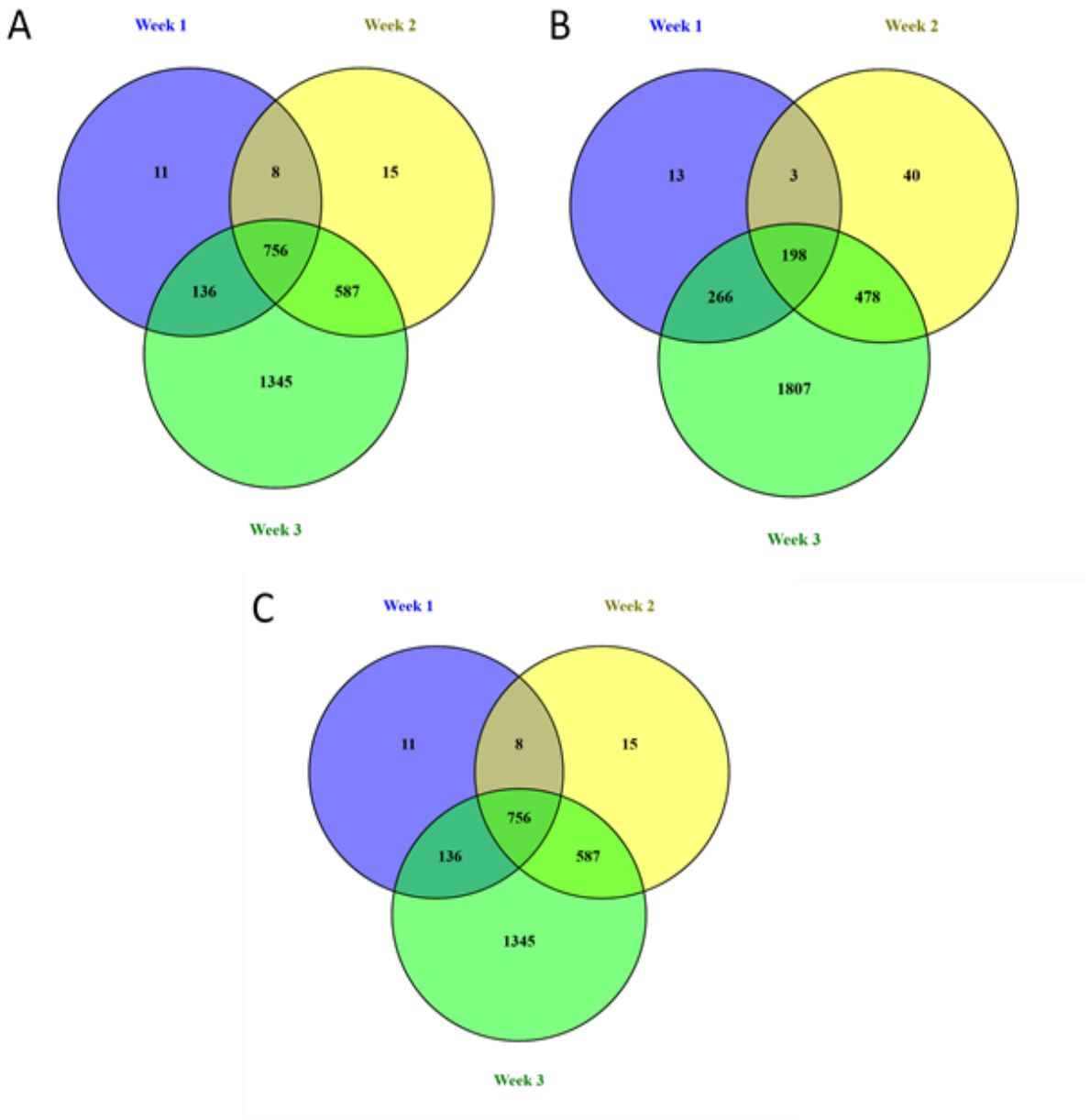

Figure 3.13. Venn diagram summarizing: A. total number of genes differentially expressed in pathogen during interaction with both resistant and susceptible genotypes at each time point; B. total genes upregulated and overlap of differentially expressed genes in pathogen at each time point; and C. total genes downregulated and overlap of differentially expressed genes in pathogen at each time point.

S. musiva cell wall degrading genes expressed in planta

It is well known that pathogens produce cell wall degrading enzymes to breach initial plant barriers, for cell-to-cell movement or even to deploy nutrients (Mathioni et al. 2011). Upregulation of genes involved in cell wall degradation was observed in *S. musiva*. The most prevalent carbohydrate activity enzymes (CAZymes) involved in cell wall degradation are carbohydrate esterase (CE) and glycoside hydrolases (GH) (Zhao et al. 2013). At 1 wpi, CE family 1 protein (*SEPMUDRAFT_145978*), tannase and feruloyl esterase, two GH family 3 protein (*SEPMUDRAFT_148250*, *SEPMUDRAFT_160784*), GH family 5 protein (*SEPMUDRAFT_83285*) and carbohydrate-binding module family 18 and 48 were upregulated while interacting with the moderately resistant genotype. Genes upregulated only at 2 wpi were carbohydrate-binding module family 50, CE family 5 protein, three cloroperoxidase (*SEPMUDRAFT_143621*, *SEPMUDRAFT_87490*), two GH family 16 protein and GH family proteins 5 and 43. By 3 wpi, genes annotated four GH family 3 (*SEPMUDRAFT_148250*, *SEPMUDRAFT_159337*, *SEPMUDRAFT_160784*), three GH family 5, GH family 16 and 43 protein (*SEPMUDRAFT_145598*), CE family 16, 1, 4 and 5, carbohydrate-binding molecule family 18, 20, 63 and 48, cloroperoxidase (*SEPMUDRAFT_20042*) and two tannase and feruloyl esterase were upregulated.

However, genes upregulated upon interaction with the susceptible genotype at 1 wpi were two cloroperoxidase (*SEPMUDRAFT_143621*, *SEPMUDRAFT_87490*), GH family protein 43 and 5 and two GH family 16 protein. By 2 wpi, genes belonging to CE family 1 protein, two GH family 3 (*SEPMUDRAFT_148250*, *SEPMUDRAFT_159337*), GH family 5 protein (*SEPMUDRAFT_84225*), carbohydrate-binding molecule family 20 and 28 and two tannase and feruloyl esterase were upregulated. Only GH family 16 (*SEPMUDRAFT_1463358*) and

carbohydrate-binding molecule family 50 were upregulated at 3 wpi (Table 3.2). While interacting with the moderately resistant genotype, the pattern of gene expression with regards to CWDEs indicated a gradual increase in the expression of the majority of the genes that peaked at 3 wpi. However, while interacting with the susceptible genotype, the majority of CWDE gene expression was seen at 2 wpi (Fig. 3.14).

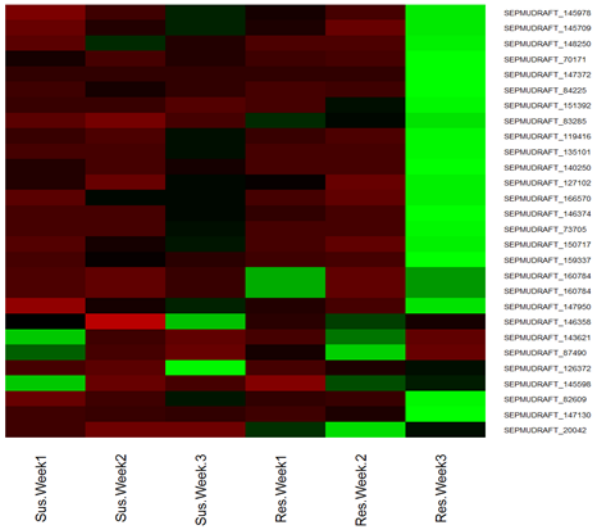

Figure 3.14. Heat map showing differentially expressed genes in pathogen (cell wall degrading enzymes) across time points (1 wpi, 2 wpi, 3 wpi) while interacting with moderately resistant and susceptible genotypes. Green color- high expression, black color- intermediate expression and red color- low expression.

Changes in expression of S. musiva genes involved in nutrient limitation in planta

In many pathogens, during conditions of starvation or limited nutrients, activation of genes responsible for nitrogen or carbon metabolism is noticed. In *S. musiva*, five aldehyde dehydrogenase and alcohol oxidase *(SEPMUDRAFT_128186)* genes were upregulated at all the time points, which are usually activated during carbon starvation. Other genes induced at 3 wpi were *SEPMUDRAFT_61928*, *SEPMUDRAFT_148931*, *SEPMUDRAFT_128186*, *SEPMUDRAFT_151105*, *SEPMUDRAFT_154708* and *SEPMUDRAFT_124691*. In the

susceptible interaction, only glucose-repressible alcohol dehydrogenase transcriptional effector was upregulated at 1 wpi. Aldehyde dehydrogenase (*SEPMUDRAFT_164222*), alcohol dehydrogenase and two alcohol oxidase (*SEPMUDRAFT_154708, SEPMUDRAFT_61928*) were upregulated at 2 wpi. It was also noted that transcription factor SNF2_N was upregulated in the moderately resistant interaction at all the time points, while it was upregulated only at 2 wpi in the susceptible interaction.

Genes induced during nitrogen starvation at 1 wpi in the moderately resistant interaction were glutamine synthetase, nitrate reductase and glutamate dehydrogenase. Of these, glutamine synthetase and glutamate dehydrogenase were upregulated at all time points. At 2 wpi, upregulation of xanthine dehydrogenase/oxidase and amino acid permease (*SEPMUDRAFT_166403*) was seen. By 3 wpi, genes annotated amino acid permease (*SEPMUDRAFT_151121*, *SEPMUDRAFT_163519*, *SEPMUDRAFT_166403*), nitrate reductase, xanthine dehydrogenase/oxidase and choline transport protein were upregulated. Other genes having a role in the initial stages of infection included ornithine aminotransferase and 3-ketoacyl-CoA thiolase B. However, *S. musiva* was not deprived of nitrogen in the interaction with the susceptible genotype (Table 3.2). The pattern of starvation gene expression indicated that *S. musiva* was devoid of nutrients at all the time points, especially at 3 wpi, as all genes were highly upregulated. However, in the susceptible genotype very few changes in expression patterns were observed with very few genes showing high expression at 2 wpi (Fig. 3.15).

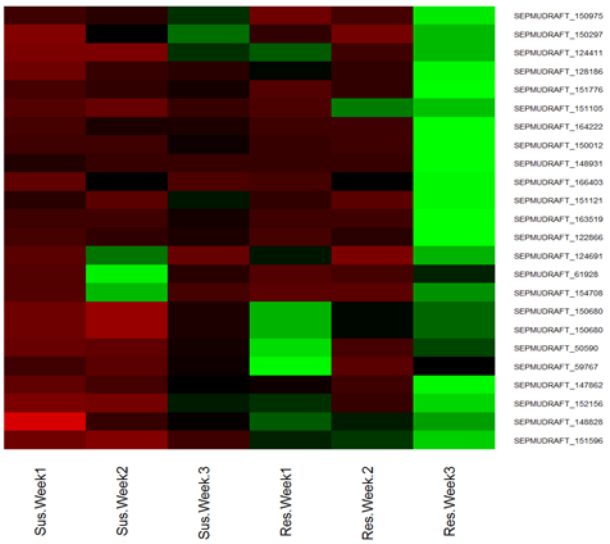

Figure 3.15. Heat map showing differentially expressed genes in pathogen (C/N starvation) across time points (1 wpi, 2 wpi, 3 wpi) while interacting with moderately resistant and susceptible genotypes. Green color- high expression, black color- intermediate expression and red color- low expression.

S. musiva* detoxification and ROS genes expressed *in planta

In response to plant defense mechanisms, many transcripts encoding fungal ROS and ROS detoxifying proteins were upregulated. In the analysis at 1 wpi, glutathione S-transferase *(GST)* (*SEPMUDRAFT_14537*), carotenoid oxygenase and NADP-dependent mannitol dehydrogenase were upregulated in the moderately resistant interaction, while in the compatible interaction, only catalase domain containing protein, CAT1 catalase and thioredoxin (*SEPMUDRAFT_15135*) were upregulated. At 2 wpi, CAT1 catalase and GST (*SEPMUDRAFT_14537*) were induced during the moderately resistant interaction, while in the susceptible genotype more antioxidants were upregulated, such as carotenoid oxygenase, manganese and iron superoxide dismutase, glutathione peorxidase 1, peroxiredoxin-6, NADP-dependent mannitol dehydrogenase and two thoredoxin (*SEPMUDRAFT_15135*, *SEPMUDRAFT_150926*). By 3 wpi, there were no notable antioxidants induced while

interacting with the susceptible genotype, but genes annotated to NADP-dependent mannitol dehydrogenase, carotenoid oxygenase, manganese and iron superoxide dismutase, CAT1 catalase, catalase domain containing protein, four GSTs, peroxiredoxin-6, two thioredoxin and L-ascorbate oxidase were expressed with the moderately resistant genotype interaction. Other stress related genes that were induced in both genotypes were HSP70 and stress response transcription factor SrrA/Skn7.

ROS produced by *S. musiva* were induced from 2 wpi, or were induced only at 3 wpi, in the moderately resistant interaction, except for NADH-ubiquinone oxidoreductase. Those genes were amine oxidase, Berberine-like protein, extracellular dioxygenase and quinone oxidoreductase, although in the susceptible interaction only upregulation of amine oxidase was observed (Table 3.2). The pattern of ROS and antioxidant gene expression revealed that there was an increase in expression of the majority of genes from 1 wpi to 3 wpi while interacting with the moderately resistant genotype. However, in the susceptible genotype there was a slight increase in gene expression from 1 wpi to 2 wpi, and some genes were expressed more in 3 wpi (Fig. 3.16).

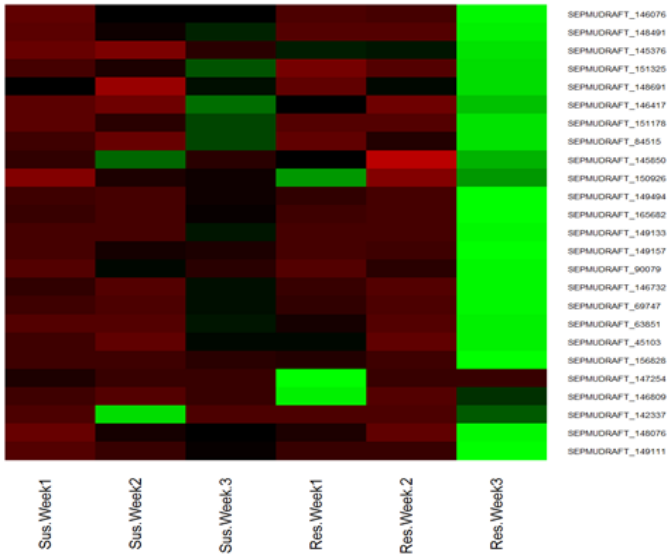

Figure 3.16. Heat map showing differentially expressed genes in pathogen (ROS and ROS scavengers) across time points (1 wpi, 2 wpi, 3 wpi) while interacting with moderately resistant and susceptible genotypes. Green color- high expression, black color- intermediate expression and red color- low expression.

S. musiva pathogenicity related genes expressed in planta

Genes (*SEPMUDRAFT_84059*, *SEPMUDRAFT_150789*) that facilitate cell adhesion and colonization were downregulated in the interaction with the moderately resistant genotype. Among the induced genes that could possibly play a role in pathogenicity, vacuolar serine protease, cullin-4B (ubiquitination), RmlC-like cupin, E3 ubiquitin-protein ligase bre1, serine/threonine-protein kinase cot1 (signaling), MFS transporter and cytochrome P450 (adapting to new conditions; Mathioni et al. 2011) were upregulated at all time points. Several other notable genes upregulated at later time points included Bax inhibitor family protein, subtilisin-like protein, ABC transporter, PR-1-like protein, LON domain serine protease, osmotin, thaumatin-like protein, three RmlC-like cupin, bet v1-like protein, S-glutamate dehydrogenase, transcription factor STE12, lectin family integral membrane protein and

autophagy_C-domain-containing protein. However, at 1 wpi in the interaction with the susceptible genotype, genes induced were lectin family integral membrane protein, PR-1-like protein, LON domain serine protease, ABC transporter, Bax inhibitor family protein and subtilisin-like protein. At 2 wpi, autophagy_C-domain-containing protein, MFS transporter and ABC transporter were upregulated. No notable pathogenicity related genes were upregulated at 3 wpi.

We also observed genes annotating enzymes that would disrupt host hormone metabolism. Genes *SEPMUDRAFT_132215*, *SEPMUDRAFT_151589*, *SEPMUDRAFT_65477* and *SEPMUDRAFT_146920* were upregulated in the moderately resistant genotype interaction, while only *SEPMUDRAFT_146920* was upregulated at 1 wpi in the susceptible genotype interaction. There were a large number of hypothetical secretory proteins expressed by *S. muisva*, of which 125 were predicted to have a signal peptide. Noteworthy were genes annotating extracellular protein 4 (*SEPMUDRAFT_148426*), upregulated at all time points, ECP2 (*SEPMUDRAFT_146583*), upregulated at 2 wpi and 3 wpi in the susceptible genotype interaction, and long chronological lifespan 2 (*SEPMUDRAFT_149673*), upregulated at 3 wpi in the moderately resistant genotype interaction (Table 3.2). Patterns of pathogenicity related gene expression revealed that there was an increase in expression of the majority of genes from 1 wpi to 3 wpi while interacting with the moderately resistant genotype. However, in the susceptible genotype there was a slight increase, as well as a decrease, in expression of some genes from 1 wpi to 2 wpi, and some genes were expressed more at 3 wpi (Fig. 3.17).

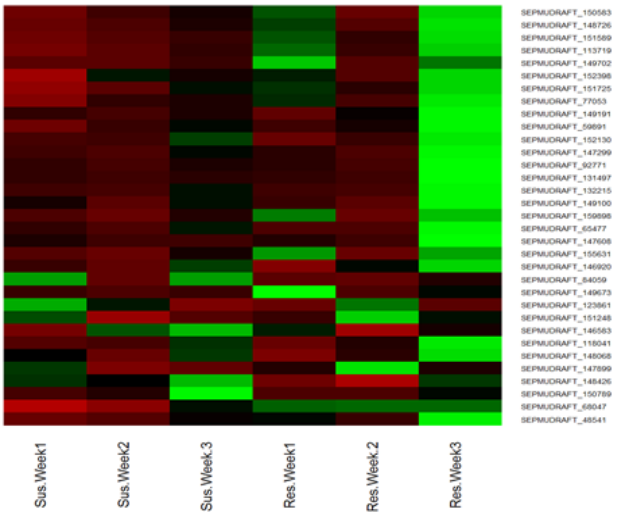

Figure 3.17. Heat map showing differentially expressed pathogenicity related genes across time points (1 wpi, 2 wpi, 3 wpi) while interacting with moderately resistant and susceptible genotypes. Green color- high expression, black color- intermediate expression and red color- low expression.

Table 3.2. List of genes expressed in *Sphaerulina musiva* during interaction with moderately resistant and susceptible *Populus* spp.

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Cell Wall Degrading				
SEPMUDRAFT_160784	Glycoside hydrolase family 3 protein	14		6
SEPMUDRAFT_148250	Glycoside hydrolase family 3 protein	3	-8.8	5.8
SEPMUDRAFT_159337	Glycoside hydrolase family 3 protein		-17.9	8.9
SEPMUDRAFT_160784	Glycoside hydrolase family 3 protein	14		6
SEPMUDRAFT_70171	Glycoside hydrolase family 5 protein	-5		8
SEPMUDRAFT_84225	Glycoside hydrolase family 5 protein		-13.5	19.6
SEPMUDRAFT_83285	Glycoside hydrolase family 5 protein	4	10.6	5
SEPMUDRAFT_151392	Glycoside hydrolase family 43 protein	-1	2	9.7
SEPMUDRAFT_145598	Glycoside hydrolase family 16 protein	-7	3	1.8
SEPMUDRAFT_146358	Glycoside hydrolase family 16 protein	-1	2.8	-1.7
SEPMUDRAFT_143621	Cloroperoxidase	-11.5	6	
SEPMUDRAFT_87490	Cloroperoxidase	-2	8.3	
SEPMUDRAFT_20042	Cloroperoxidase	5	2.8	1.7
SEPMUDRAFT_145978	Carbohydrate esterase family 1 protein	4	-1	2
SEPMUDRAFT_140250	Carbohydrate esterase family 1 protein			7
SEPMUDRAFT_119416	Carbohydrate esterase family 16 protein			3.7
SEPMUDRAFT_135101	Carbohydrate esterase family 4 protein			3.9
SEPMUDRAFT_147130	Carbohydrate esterase family 5 protein		2	16.7
SEPMUDRAFT_127102	Carbohydrate esterase family 5 protein			3
SEPMUDRAFT_146374	Carbohydrate esterase family 5 protein			4

Table 3.2. List of genes expressed in *Sphaerulina musiva* during interaction with moderately resistant and susceptible *Populus* spp. (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
SEPMUDRAFT_73705	Carbohydrate esterase family 5 protein			3.8
SEPMUDRAFT_82609	Carbohydrate-binding molecule family 18 protein	3	1	2
SEPMUDRAFT_150717	Carbohydrate-binding molecule family 20 protein		-5.6	2.6
SEPMUDRAFT_147950	Carbohydrate-binding molecule family 48 protein	5	-1	1.9
SEPMUDRAFT_126372	Carbohydrate-binding molecule family 50 protein		2	-2
SEPMUDRAFT_147372	Carbohydrate-binding molecule family 63 protein			∞
SEPMUDRAFT_166570	Tannase and feruloyl esterase	6	-4.9	2
SEPMUDRAFT_145709	Tannase and feruloyl esterase		-9.7	3
Carbon/Nitrogen Starvation				
SEPMUDRAFT_150680	Aldehyde dehydrogenase	3.7	3.5	1.7
SEPMUDRAFT_124411	Aldehyde dehydrogenase	3.6	1	1.5
SEPMUDRAFT_148828	Aldehyde dehydrogenase	4	1	1.5
SEPMUDRAFT_151596	Aldehyde dehydrogenase	3	5.5	3.4
SEPMUDRAFT_164222	Aldehyde dehydrogenase		-9.8	9
SEPMUDRAFT_128186	Alcohol oxidase	6.7	1	4
SEPMUDRAFT_148931	Alcohol oxidase			59
SEPMUDRAFT_151105	Alcohol oxidase		11	4
SEPMUDRAFT_154708	Alcohol oxidase		-101	9.4
SEPMUDRAFT_61928	Alcohol oxidase		-19	3
SEPMUDRAFT_151776	Glucose-repressible alcohol dehydrogenase transcriptional effector	-1	-1	3.7
SEPMUDRAFT_124691	Alcohol dehydrogenase		-10.5	6.7
SEPMUDRAFT_150680	Aldehyde dehydrogenase	3.7	3.5	1.7
SEPMUDRAFT_147862	Glutamine synthetase	3	1	2.8
SEPMUDRAFT_150012	Choline transport protein			6
SEPMUDRAFT_166403	amino acid permease		1	6.3
SEPMUDRAFT_151121	amino acid permease			3
SEPMUDRAFT_163519	amino acid permease			7.5

Table 3.2. List of genes expressed in *Sphaerulina musiva* during interaction with moderately resistant and susceptible *Populus* spp. (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
SEPMUDRAFT_150975	ornithine aminotransferase	-1.6	-1.2	1.8
SEPMUDRAFT_50590	ornithine decarboxylase	8.1	1.5	1.6
SEPMUDRAFT_59767	Nitrate reductase [NADPH]	13		1
SEPMUDRAFT_150297	3-ketoacyl-CoA thiolase B	5	-4	1
SEPMUDRAFT_122866	xanthine dehydrogenase/oxidase		1	3
SEPMUDRAFT_152156	NADP-specific glutamate dehydrogenase	3	1.7	1.8
ROS				
SEPMUDRAFT_156828	Berberine-like protein			15
SEPMUDRAFT_149157	amine oxidase		-3.5	6.5
SEPMUDRAFT_142337	Extracellular dioxygenase		-∞	24.5
SEPMUDRAFT_146809	NADH-ubiquinone oxidoreductase 105 kDa subunit			5
SEPMUDRAFT_84515	NADH-ubiquinone oxidoreductase 51 kDa subunit	-1	1.5	1.5
ROS Scavengers				
SEPMUDRAFT_146076	Glutathione peroxidase 1		-2	3
SEPMUDRAFT_149494	Glutathione S-transferase			5.7
SEPMUDRAFT_165682	Glutathione S-transferase			5.4
SEPMUDRAFT_63851	Glutathione S-transferase			3
SEPMUDRAFT_14537	Glutathione S-transferase	4	12	3.6
SEPMUDRAFT_146732	glyoxylase I			3
SEPMUDRAFT_148491	Manganese and iron superoxide dismutase		-4	2.5
SEPMUDRAFT_145850	Peroxiredoxin-6		-3	1.8
SEPMUDRAFT_151325	thioredoxin	-1.7	-1.5	1.5
SEPMUDRAFT_150926	thioredoxin		-4.9	2

Table 3.2. List of genes expressed in *Sphaerulina musiva* during interaction with moderately resistant and susceptible *Populus* spp. (continued).

		Fold Change*		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
SEPMUDRAFT_151178	Carotenoid oxygenase	3	-4	1.9
SEPMUDRAFT_90079	Catalase-domain-containing protein		-1.8	6
SEPMUDRAFT_148691	CAT1 catalase	-1.8	3.8	1.9
SEPMUDRAFT_45103	L-ascorbate oxidase			3
SEPMUDRAFT_148076	NADP-dependent mannitol dehydrogenase	4.8	-3.6	3
Pathogenicity Related				
SEPMUDRAFT_84059	Fasciclin-domain-containing protein	-27.8		-4
SEPMUDRAFT_150789	Hydrophobin		-∞	-4
SEPMUDRAFT_159898	RmlC-like cupin	9.5		4.5
SEPMUDRAFT_113719	Serine/threonine-protein kinase cot-1	5	1	3
SEPMUDRAFT_68047	Cytochrome P450	6.5	3.8	1
SEPMUDRAFT_149191	lectin family integral membrane protein	-1.7	1.6	3
SEPMUDRAFT_92771	ATP-dependent Clp protease proteolytic subunit			7.1
SEPMUDRAFT_148446	MFS general substrate transporter	3.7	-2.6	3
SEPMUDRAFT_146966	MFS general substrate transporter		-11	3
SEPMUDRAFT_145777	ABC transporter		3	2.6
SEPMUDRAFT_165275	subtilisin-like protein			∞
SEPMUDRAFT_152052	ABC transporter	-2	-12	3
SEPMUDRAFT_149673	Long chronological life span 2			4.6
SEPMUDRAFT_148426	Extracellular 4	-2	-2.7	-1.4
SEPMUDRAFT_146583	ECP2		-8	-2
SEPMUDRAFT_152398	HSP70-domain-containing protein	3.3	-1.6	2
SEPMUDRAFT_131497	Osmotin, thaumatin-like protein			16
SEPMUDRAFT_151429	glycosyltransferase family 2 protein	-18		3
SEPMUDRAFT_150583	glycosyltransferase family 32 protein	13	-2	3
SEPMUDRAFT_118041	transcription factor STE12	-1.4	1.4	1.9
SEPMUDRAFT_48541	vacuolar serine protease	4.6	1.5	2.9
SEPMUDRAFT_123861	PR-1-like protein	-3	1.3	1.3
SEPMUDRAFT_59891	cullin-4B	4.9	1	2.8

Table 3.2. List of genes expressed in *Sphaerulina musiva* during interaction with moderately resistant and susceptible *Populus* spp. (continued).

Gene ID	Gene Annotation	Fold Change*		
		1 wk	2 wk	3 wk
SEPMUDRAFT_151725	HCP-like protein	4	1	1.9
SEPMUDRAFT_132215	Isochorismatase hydrolase			3.8
SEPMUDRAFT_148726	Autophagy_C-domain-containing protein		-1	3
SEPMUDRAFT_146920	auxin efflux carrier	-2	1.9	1.5
SEPMUDRAFT_148068	Bax inhibitor family protein	-2	1.5	1.6
SEPMUDRAFT_147299	Bet v1-like protein			3.7
SEPMUDRAFT_149702	E3 ubiquitin-protein ligase bre1	3	1	2
SEPMUDRAFT_152130	E3 ubiquitin-protein ligase NEDD4	-1.6	1	
SEPMUDRAFT_149100	Stress response transcription factor SrrA/Skn7	-2		3
SEPMUDRAFT_147899	Subtilisin-like protein	-1.6	8.5	2
SEPMUDRAFT_148960	LON domain serine protease	-5	2	1
SEPMUDRAFT_159898	RmlC-like cupin	9.5		4.5
SEPMUDRAFT_147608	RmlC-like cupin			∞
SEPMUDRAFT_155631	RmlC-like cupin			3
SEPMUDRAFT_151589	Salicylate hydroxylase	10.6	1.7	4.6
SEPMUDRAFT_65477	Gibberellin 20 oxidase			3

*****Positive value indicates upregulation in *S. musiva* while interacting with the moderately resistant genotype and negative values while interacting with the susceptible genotype.

Discussion

This is the first detailed study of the moderately resistant and susceptible interaction of *Populus* leaves infected with *S. musiva*. Examination of the pre-penetration process using SEM elucidated the adhesion, germination and mode of penetration of *S. musiva*. Studies have examined the association of an extracellular matrix (ECM) with propagules and germ tubes, for example in *C. heterostrophus* (Braun and Howard 1994), *C. graminicola* (Sugui et al. 1998), *S. nodorum* (Zelinger et al. 2006) and *M. grisea* (Howard and Valent 1996). Many studies have reported that the ECM plays an important role, not only in pathogenesis, but also in reducing

desiccation and providing a favorable environment (Epstein and Nicholson 1997, Nicholson 1996).

In this study, mucilage was observed surrounding ungerminated conidia, as well as germ tubes and hyphae. The ECM of *S. musiva* appears to be a network of fibrils at the region of contact with the leaf, suggesting a role in adhesion (Castle et al. 1996). However, these fibrils did not show any directed growth, nor did they grow over a long distance, such as those observed in *B. sorokiana*, *B. cinerea* and *C. heterostrophus* (Apoga and Jansson 2000, Cole et al. 1996, Sugui et al. 1998). ECM of many plant pathogenic fungi, in addition to adhesives, also produce enzymes such as cutinases and esterases (Nicholson 1996). One of the events observed during the pre-penetration process was the erosion or dissolution of the cell wall (possibly cutin) where hyphae were present, indicating that ECM of *S. musiva* may contain degrading enzymes, thus enhancing adhesion. A possible enzyme that could facilitate this could be cutinase, as it was recently shown that *S. musiva* has a cutinase gene, which was obtained by horizontal gene transfer from basidiomycota (Dhillon et al. 2015). This mode of adhesion has been reported for several necrotrophs (Dickman et al. 1989, Kolattukudy et al. 1995). However, further studies are needed to determine the role of this mucilaginous substance, as neither the nature of adhesion nor its composition is known.

Generally, pathogenic fungi enter plants through wounds, natural openings (i.e. stomata) or by means of direct penetration. This study provides evidence for two modes of penetration, direct penetration and through stomata. It has previously been reported that pathogens that differentiate into infection structures require cell wall degrading enzymes for direct penetration (Mendgen 1996). *S. musiva* demonstrated the presence of ECM on the epidermis, perhaps indicating that these mucilaginous substances may contain cell wall degrading enzymes. Also, it

should be noted that Dhillon et al. (2015) verified the presence of genes encoding cell wall degrading enzymes in *S. musiva,* through an *in vitro* study on wood-chip medium using RNA-seq. To understand the specific role of ECM in the interaction of *S. musiva* and *Populus* spp., *in vivo* studies are needed. In this study, germ tubes of *S. musiva* did not appear to show any preference to contact or chemical stimuli, as they were observed growing undirected on leaf surfaces, similar to observations in *M. graminicola* (Kema et al. 1996). Similar to *Quambalaria* sp. (Pegg et al. 2009), *S. musiva* did not appear to be primarily attracted to stomata, as some hyphae grew right over the top or side without any attempted penetration, and even when they penetrated a stoma a branch would sometimes appear and grow away from the stomata. Perhaps, because stomata were closed at the time of germination, some hyphae grew right over stomata (Pegg et al. 2009). However, De Wit in 1977 and Rathaiah in 1976 provided evidence of stomatal penetration by pathogens, even when stomata are closed.

Events occurring *in planta* during *S. musiva* infection leading to resistance or susceptibility

Resistance and susceptibility of a plant depends on the kinetics and impacts of preformed and induced defenses, such as physical barriers, nutrient limitation, programmed cell death and antimicrobial compounds (Jones and Dangl 2006, Lumsden 1979, Thatcher et al. 2005). Differences in post-penetration events were evident at 1 wpi, due to a lack of colonization of spongy mesophyll cells in the moderately resistant genotype. There could be a combination of factors preventing this colonization.

Initially, a plant needs to recognize a pathogen, and this is mediated by receptors, which in turn are responsible for initiating a defense response (Azaiez et al. 2009). We found that in the moderately resistant genotype at 1 wpi, there were several differentially expressed RLKs, like

WAK2 (wall-associated kinase 2), LRR-RLK, RLK1 and RLK3, which conceivably could be involved in detecting PAMPs or MAMPs, thus initiating intracellular signaling for a defense response. WAKs are known to have a dual role as a signaling molecule and as linkers of cell wall and plasma membrane, and they can be induced by SA or INA (He et al. 1999). Other signaling components were also upregulated, such as BSK3, MAPKKK3 and MAPKK10. BSK3 is known to mediate signal transduction from an interaction with the BRI1 receptor in Arabidopsis (Tang et al. 2008), indicating the possibility of BSK3 interacting with a receptor to mediate a defense response in poplar leaves. However, in the susceptible genotype, LRR-RLK and several LRR-transmembrane protein kinases were differentially expressed at 1 wpi, in addition to receptors like cysteine-rich RLK3 and RLK1. This would suggest that the initiation of a complex defense response was lower when compared to the moderately resistant genotype. Most of the expression of WAK2 was detected at later time points, which could lead to the hypothesis that the kinetics of defense responses are slower or the accumulation of SA or INA is not fast enough to induce WAK2. However, since we did not take samples prior to 1 wpi, we are unable to elaborate the possible roles of RLKs in this pathosystem.

One of the outcomes of basal defense in plants is the induction of a physical barrier or cell wall modifications in order to prevent pathogen penetration or colonization (Hückelhoven 2007). Apart from genes annotated as plant invertase/pectin methyl esterase inhibitors, there were genes involved in cell wall modification like cellulase 2, cellulose synthase, pectin lyase, pectin methyl esterase and others that were also upregulated at 1 wpi (Table 3.1). This indicates that the moderately resistant genotype is constantly repairing or modifying its cell wall, thus reducing colonization. This change in gene expression correlates with Figure 3.4C. Moreover, genes annotating lignin biosynthesis and wax synthesis (3-ketoacyl-CoA synthase 1) were

upregulated, suggesting that there is lignin and wax deposition during *S. musiva* infection, further strengthening the cell wall (Azaiez et al. 2009, Todd et al. 1999).

At later time points (2 wpi, 3 wpi), modification of the cell wall was still occurring, as minimal colonization of the leaf tissue was observed in the moderately resistant genotype (Table 3.1; Fig. 3.5 B, C). However, in the susceptible genotype, the only genes that were upregulated at 1 wpi were xyloglucan endotransglucosylase/hydrolase 15, pectin methyl esterase and cellulose synthase-like D3 (Table 3.1). At later time points, activation of several genes annotated for cell wall modification and prevention of cell wall degradation were observed. Hypothetically, due to the apparent lack of a strong physical barrier at the early time points in the susceptible genotype, faster colonization of the pathogen in the leaf tissue occurred (Fig. 3.4F). Extended colonization by the pathogen at 1 wpi enabled it to overcome a late defense response in the form of cell wall modification in the susceptible genotype.

DAB staining is used to detect H_2O_2, as it precipitates in infected leaf tissues. It is known that ROS generation leads to programed cell death (PCD), cell wall strengthening and other defense responses (Wen et al. 2013). ROS production in plants provides resistance to biotrophs and hemibiotrophs by means of a hypersensitive response. In contrast, for necrotrophs, the production of ROS increases pathogenicity (Mengiste 2012). However, if induction of ROS occurs early in infection it can lead to resistance, as it can initiate other defense responses. Differences in ROS production, as measured by DAB staining, between moderately resistant and susceptible genotypes was not observed until 1 wpi. Genes annotated for ten FAD-binding Berberine family proteins were upregulated in the moderately resistant genotype. Berberine proteins generate not only H_2O_2, but are also known to produce antifungal compounds in poppy plants (Dittrich and Kutchan 1991). However, at early time points in the susceptible genotype,

only two FAD-binding Berberine family protein and germin-like protein 1 were upregulated. This suggests higher intensity and kinetics in the production of ROS in the moderately resistant genotype attempting to restrict the pathogen by inducing a ROS burst.

A question that arises is the following: if there were dead cells present would it promote easier pathogen colonization. There was upregulation at 1 wpi of several antioxidants in the moderately resistant genotype, including catalases, peroxidases, GST and thioredoxin, suggesting that these antioxidants are delaying the inception of cell death by detoxifying, which in turn slows the spread of *S. musiva*. Some of these enzymes, for example peroxidase, also help to generate ROS that are toxic to the pathogen (Arfaoui et al. 2007, Daayf et al. 2003, El Hadrami et al. 1997, Wen et al. 2013). This correlates with formation of smaller lesions observed in the moderately resistant genotype, even at later time points (Fig. 3.6E). In the susceptible genotype, lesions grew larger at later time points. Two explanations for this could be that: (i) most of the ROS production takes place at later time points, indicating a delayed defense response to *S. musiva* that could not have been toxic enough for the pathogen, thus enabling it to establish to a larger extent; and (ii) fewer antioxidants are upregulated, resulting in a lack of balance between ROS and antioxidant production (Table 3.1).

Plant hormones play an important role in the defense response. For example, SA is important for local resistance, and it can induce SAR to biotrophic and hemibiotrophic pathogens. Conversely, Ferrari et al. (2003) and Govrin and Levine (2002) demonstrated that SA provides local resistance to necrotrophs, such as *B. cinerea* and *S. sclerotiorum.* Whereas jasmonic acid (JA) and ethylene are the two main hormones involved in inducing systemic resistance to necrotrophs (Pieterse et al. 1998). In both the moderately resistant and susceptible genotypes, induction of transcription factors myb, and other precursors for hormones like SA,

ethylene and JA, suggests that there could be cross talk between these pathways during infection (Table 3.1). In many studies, a synergistic effect has been observed with JA and ethylene signaling to activate defense related genes such as *PR1b*, osmotin and MAPK signaling (Penninckx et al. 1998, Xu et al. 1994).

Mutational studies involving components in the JA or ethylene signaling pathways have led to susceptibility of *Arabidopsis* to various fungal pathogens, like *B. cinerea* and *Pythium* sp. Norman-Setterblad et al. (2000) demonstrated that JA and ethylene played a role in regulating defense genes that are activated by cell wall degrading enzymes. In this study, many differentially expressed genes for auxin signaling were upregulated in the moderately resistant genotype, moreso than in the susceptible genotype. Apart from having a role in plant development and growth, it also enhances PTI. For example, mutations in *axr1*, *axr2* and *axr6* lead to susceptibility of *Arabidopsis* to *B. cinerea* and *P. cucumerina* (Llorente et al. 2008).

Another hormone that can be a key positive and/or negative regulator of defense, which was also observed in poplar, was ABA. For example, mutations in genes controlling ABA synthesis leads to enhanced resistance in *F. oxysporum* and *B. cinerea*, either by enhancing PTI or inducing the JA/ethylene signaling pathways (Adie et al. 2007, Fujita et al. 2006, Ton et al. 2009). However, in *S. sclerotiorum* ABA is a positive regulator, as its role in stomatal closure leads to resistance (Guimarães and Stotz 2004). Upregulation of the highly induced *ABA PP2C* gene in the moderately resistant genotype, and *ABI2* and *ABI1* (negative regulators) in the susceptible genotype, were observed. Further studies are necessary to elucidate the exact role of these hormones in defense against *S. musiva.*

As predicted, upregulation of PR1, thaumatin and PR family proteins were seen in the moderately resistant genotype at 1 wpi, but not in the susceptible genotype. It is known that PR

proteins have antimicrobial activity, as they can hydrolyze pathogen cell walls, disrupt the membrane or even affect fungal physiology (Wang et al. 2005, 2006). Accumulation of PR proteins is due to activation of SA and JA (Spoel and Dong 2012). Other notable defense responses in the moderately resistant genotype seen at early time points were provided by antimicrobial compounds (beta glucosidase 13, 16 and 17, except *Potri.001G223400* and Serine carboxypeptidase-like 18 and 28), three dirigent-like proteins (lignin and lignans production) and diacylglycerol kinase 5.

An intriguing observation in the moderately resistant genotype was the activation of defense genes leading to cell death, but also genes leading to cell survival, suggesting the reason for smaller lesions at later time points. In addition to the upregulation of TIR-NBS-LRRs, disease resistance RPMI-like, NBS-LRR family and CC-NBS-LRR, genes that mediate cell death, like *ACD1* and Arabinogalactan protein 26, were also upregulated. However, Dangl et al. (1996) reported that cell death can lead to SA accumulation, which in turn can induce local and systemic resistance by activating downstream resistance mechanisms, suggesting these genes may also be indirectly involved in activation of TFs leading to SA accumulation. Cell survival in the moderately resistant genotype is likely mediated by upregulation of genes like *BCL-2*-associated athanogene, *APG3*, HMG box protein, *MLO1*, *MLO4*, nudix hydrolase and seven transmembrane MLO protein. Autophagy can be induced by ROS, starvation and mitochondrial toxin (Scherz-Shouval et al. 2007). A study done by Lai et al. (2011) revealed that autophagy has a role in resistance to necrotrophic fungi. The study found that fungal toxins induced autophagy, and induction of autophagy in uninfected cells can stop expansion of lesions. HMG box 3 protein can instigate basal defense, by activating the MAPK pathway, callose deposition and defense

related genes during necrotrophic infection. This protein can be released into the apoplast as soon as necrosis is induced by the pathogen (Bianchi 2009, Choi et al. 2016).

In the susceptible genotype, most of the PR protein upregulation occurred at later time points, except for osmotin which was upregulated at all time points. Other upregulated genes that have antimicrobial activity were plantacyanin and serine carboxypeptidase-like 18. We found only *MLO1*, nudix hydrolase and one *BCL-2*-associated athanogene upregulated at 1 wpi, which have a role as negative regulators of cell death, compared to upregulation of genes annotating *ALD1* and Arabinogalactan protein 1, which mediate cell death. Upregulation of putative *R* genes, like *Avr9/Cf9* elicited genes, disease resistance *RGA4* and *Cf-4 9* disease resistance-like family, were also observed. These genes probably play a role in *R* gene mediated cell death. Cytochrome c oxidase, also known for reducing apoptosis, was only upregulated at 3 wpi. This later expression in the susceptible genotype indicates a slower and less impactful defense response, and a lack of balance between cell death due to the defense response and cell survival. This in turn leads to larger lesions, which can be seen microscopically and macroscopically as early as 1 wpi, thus facilitating greater colonization of *S. musiva*.

Difference in infection biology of *S. musiva* in moderately resistant and susceptible interaction

For successful infection, spore germination, hyphal attachment, growth and penetration are required. Cell wall degrading enzymes (CWDEs) are secreted by pathogenic fungi to ease infection and colonization of the host tissue by weakening the cell wall (Hancock 1967, Lumsden 1969). In this RNA-seq study, genes annotating CAZymes, like glycoside hydrolase family (GH), carbohydrate-binding molecule family (CBM) and carbohydrate esterase family (CE), were upregulated while interacting with the moderately resistant genotype at 1 wpi. GH

and CE are known for roles in plant biomass decomposition and CBMs work along with enzymes that are involved in cell wall hydrolysis (Ospina-Giraldo 2010). The GH family includes cellulase, endomannase, beta glucosidase, beta D xylosidase, alpha L arbinase, 1,4 beta galactosidase and others. CE family includes xylan esterase, cutinases, cinnamyl esterase, trehalose 6-o mycolyl transferase and others (CAZypedia.com). However, in the susceptible genotype, only the GH family was upregulated. It could be hypothesized that due to the strong physical barriers formed in the moderately resistant genotype, more classes of CAZymes are required to break down the cell wall. Even at later time points, these genes were still upregulated in the moderately resistant interaction. During the interaction with the susceptible genotype, four GHs, one CE and two CBMs were upregulated at 2 wpi. This correlates with the upregulation of cell wall modification genes at 2 wpi in the susceptible genotype. Besides this, cloroperoxidase and tannase and feruloyl esterase genes were also upregulated to breakdown lignin. Differential expression of these genes were mainly seen in the interaction with the moderately resistant genotype (Table 3.2).

Establishment of disease is determined by the availability of nutrients from the host (Abawi et al. 1975, DeBary 1887, Lumsden and Dow 1973, Purdy 1958). Fungi require considerable amounts of energy to form hyphae and to grow inter- and intra- cellularly during infection (Lumsden 1979). One might assume that once lysis of the host cells occurs there would be readily available nutrients. CWDEs in *S. musiva*, apart from having a role in penetration and colonization, could also be providing C/N sources. It was very evident that *S. musiva* was devoid of nutrients in the moderately resistant genotype, as several genes like aldehyde dehydrogenase, alcohol oxidase and others were expressed. This would indicate carbon starvation that correlates with a strong physical barrier in the moderately resistant genotype, ultimately leading to limited

colonization (Fig. 3.4D). In addition to carbon, nitrogen is also a prerequisite for infection. During periods of nitrogen limitation when a pathogen is growing, transcription factors bind to the GATA promoter that transcribes genes involved in nitrogen metabolism (Donofrio et al. 2006). Glutamine synthetase, glutamate dehydrogenase and nitrate reductase were upregulated at 1 wpi during interaction with the moderately resistant genotype. However, it is also possible that *S. musiva* might be using nitrogen starvation for full expression of certain pathogenicity genes or secretory proteins that aid in its survival in the moderately resistant genotype. For example, *C. lindemuthianum* almost became nonpathogenic when transcription factor CLNR1 was deleted (Pellier et al. 2003) and expression of *Avr9* in *C. fulvum* was also seen during nitrogen starvation (Van den Ackerveken et al. 1994). In this study, the C/N starvation genes were still upregulated at later time points in the moderately resistant genotype.

However, in the interaction with the susceptible *Populus* genotype at 1 wpi, there was expression of ornithine aminotransferase and glucose-repressible alcohol dehydrogenase transcription factor, a gene expressed in the absence of glucose as it uses ethanol as a carbon source. Upregulation of genes that are expressed during carbon starvation was also observed by 2 wpi. As described previously, this may be due to late upregulation of cell wall modifying genes in the susceptible genotype, which may have reduced the availability of carbon sources to the pathogen. By 3 wpi, there was no upregulation of any C/N starvation genes (Fig. 5D), as colonization extended to the spongy mesophyll tissues and growth of hyphae was seen on the lesions.

In plants, production of ROS changes the redox state of the cell, which in turn creates a hostile environment for pathogens. In order to reduce any toxic effects, pathogens produce antioxidants (El-Bebany et al. 2010). Differentially expressed antioxidant genes were seen at 1

wpi and 3 wpi during the interaction with the moderately resistant genotype, while most were expressed at 2 wpi during the interaction with the susceptible genotype.

Fascilin protein and hydrophobin were expressed in *S. musiva* during the susceptible interaction. It is known that fascilin protein mediates cell adhesion. However, Liu et al. (2009) demonstrated a targeted disruption of *MoFLP1* (gene encoding fascilin-like protein in *M. oryzae*) leading to decreased pathogenicity on rice, as it affected adhesion, development and pathogenicity on the leaf. Hydrophobin, in turn, aids in successful colonization by masking the pathogen from the plants immune system (Bayry et al. 2012). The *MPGI* gene found in *M. grisea* encodes hydrophobin, and is considered to be a pathogenicity factor. Thus, it could be hypothesized that these proteins facilitate colonization in susceptible leaves. Other candidate virulence factors were induced *in planta*. Among these, secretory proteins, ECP2 and extracellular protein 4, were upregulated in the susceptible genotype, indicating a probable role in induction of ETI. Further studies will have to be done to deduce the role of these secretory proteins in pathogenicity.

Genes upregulated during the moderately resistant interaction clearly indicate that the pathogen is stressed and is trying to suppress the defense response. Expression of isochorismatase hydrolase and salicylate hydrolase genes were noted, suggesting that the pathogen was trying to inhibit SA; and also expression of gibberellin 20 oxidase, to manipulate the host defense response, as this hormone is a negative regulator for necrotrophs. Apart from this, expression of auxin efflux carrier protein was also observed. Auxin has been shown to enhance virulence in pathogens like *P. infestans* and *C. gloeosporioides* f. sp. *aeschynomene.* In potatoes, auxin is produced by *P. infestans*, which competitively binds to GST (Cohen et al. 2002, Hahn and Strittmatter 1994). Upregulation of Bax inhibitor was also found. Bax inhibitor

(BI) is a conserved protein that inhibits cell death. Fungal cells can undergo PCD due to defense responses produced by plants, such as PR proteins, phytoalexins, ROS and others. BI was upregulated in the susceptible genotype at 1 wpi, in order to protect itself from the outcome of the ETI response. However, there may be several reasons for the expression of BI at later time points in the moderately resistant genotype, and these could be: 1) the impact of disease resistance was so high at early time points that the majority of the hyphae have undergone PCD leaving very few to survive; and/or 2) these hyphae may recover, or surviving hyphae are expressing genes such as autophagy C, PR-1-like protein, osmotin, thaumatin, hormone inhibiting enzymes, HSP70, Berberine protein and others. Apart from this, at 3 wpi in the moderately resistant genotype, it was difficult to observe any RFP transformed hyphae in the lesions, due to the complete cell death in spongy mesophyll cells. These strategies have also been observed in other necrotrophs (Dickman et al. 2001, Govrin and Levine 2000, Shlezinger et al. 2011, van Kan 2006). In addition, it may also be possible that *S. musiva* can go into a stationary phase, as the expression of the *long chronological life span 2* gene was seen at 3 wpi.

To summarize the infection process, *S. musiva* upon adhering to *Populus* leaves (48 hpi), penetrates through stomata or by direct penetration, irrespective of the genotype infected. After penetration, colonization was observed in the palisade mesophyll cells (96 hpi). However, a difference in post-penetration events was visible between genotypes at 1 wpi, because there was no apparent colonization in spongy mesophyll cells in the moderately resistant genotype. The possible factors averting this colonization were analyzed using RNA-seq. At 1 wpi in the moderately resistant genotype, there was a peak in gene expression for those annotating for cell wall modelling, defense related, antioxidants, reactive oxygen species and hormone metabolism (SA, JA, ethylene). At later time points (2 wpi, 3 wpi), minimal colonization of spongy

mesophyll cells was observed. This can likely be attributed to the continuous expression of defense related genes, antioxidants, ROS and cell wall remodelling genes. However, in the susceptible genotype, colonization was already seen in spongy mesophyll cells at 1 wpi. A minimal defense response in the susceptible genotype, through a lack of defined cell wall modelling or antioxidants, led to cell death which facilitated pathogen colonization. At later time points (2 wpi, 3 wpi), expression of genes annotating cell wall remodelling, defense related, antioxidants and ROS were observed. However, by then the pathogen had already colonized the tissue and appeared to be unaffected. Thus, the kinetics and intensity of the defense responses were much more extensive in the moderately resistant genotype, when compared to the susceptible genotype, eventually leading to a hostile environment for the pathogen and preventing colonization. Although potential key genes involved in the infection process, and in the resistance to the pathogen, were observed, the results have highlighted the need for further studies to obtain a more in-depth understanding of this host-parasite interaction.

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APPENDIX A. QPCR SUPPLEMENTARY FIGURES AND TABLE

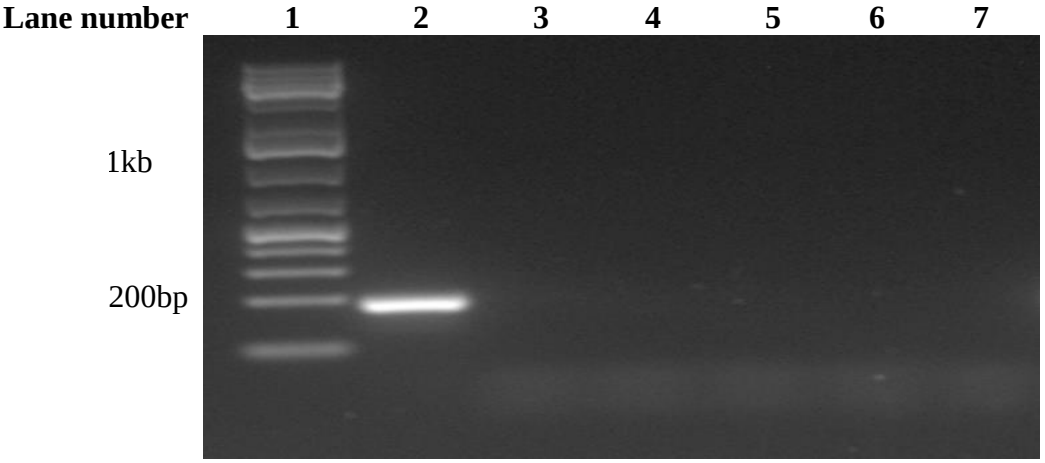

Figure A1. Evaluation of specificity of primers for the Septoria *beta tubulin* gene using gDNA of *S. musiva* (lane 2), *S. populicola* (lane 3), *Populus* (lane 4), *Venturia* sp. (lane 5) and *Marssonina* sp. (lane 6); Marker (lane 1), Negative control (lane 7).

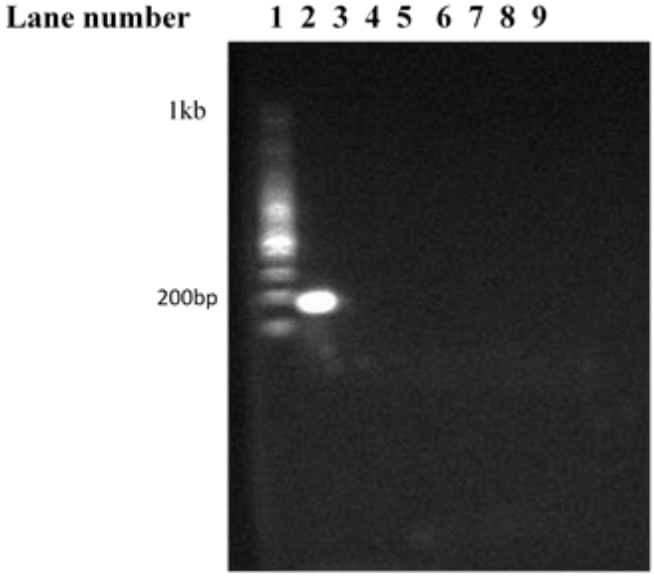

Figure A2. Evaluation of specificity of primers for the Septoria *beta tubulin* gene using gDNA of *S. musiva* (lane 2), *Fusarium* sp. (lane 3), *Trichoderma* sp. (lane 4), *Alternaria* sp. (lane 5), *Cladosporium fulvum* (lane 6), *Penicillium* sp. (lane 7) and *Epicoccum* sp. (lane 8); Marker (lane 1), Negative control (lane 9).

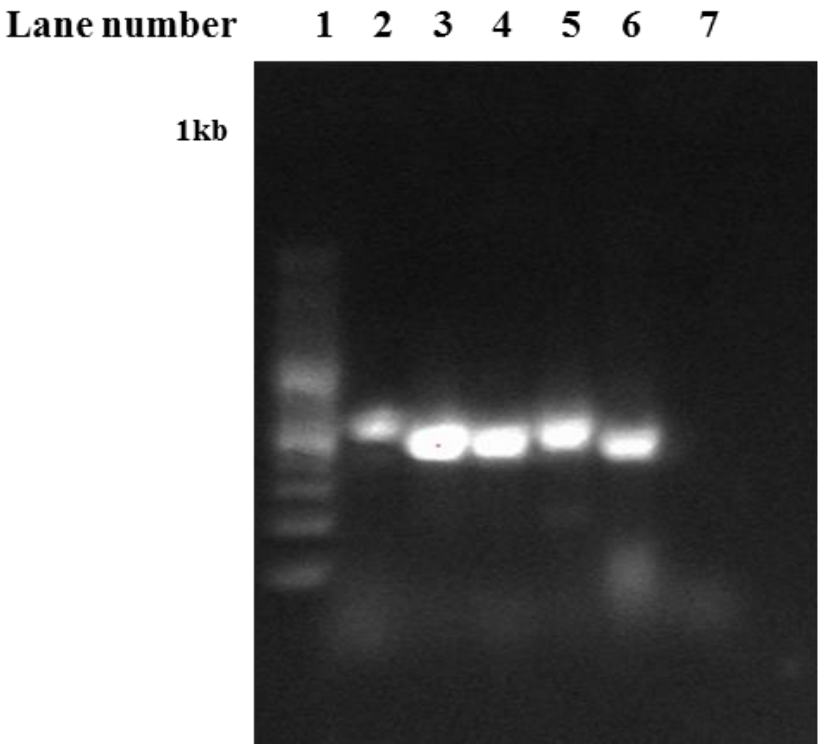

Figure A3. Specificity of Septoria beta tubulin primers were further validated using universal primers ITS1 & ITS4 on gDNA of *Fusarium* sp. (lane 2), *Cladosporium fulvum* (lane 3), *Epicoccum* sp. (lane 4), *Penicillium* sp. (lane 5) and *S. musiva* (lane 6); Marker (lane 1), Negative control (lane 7).

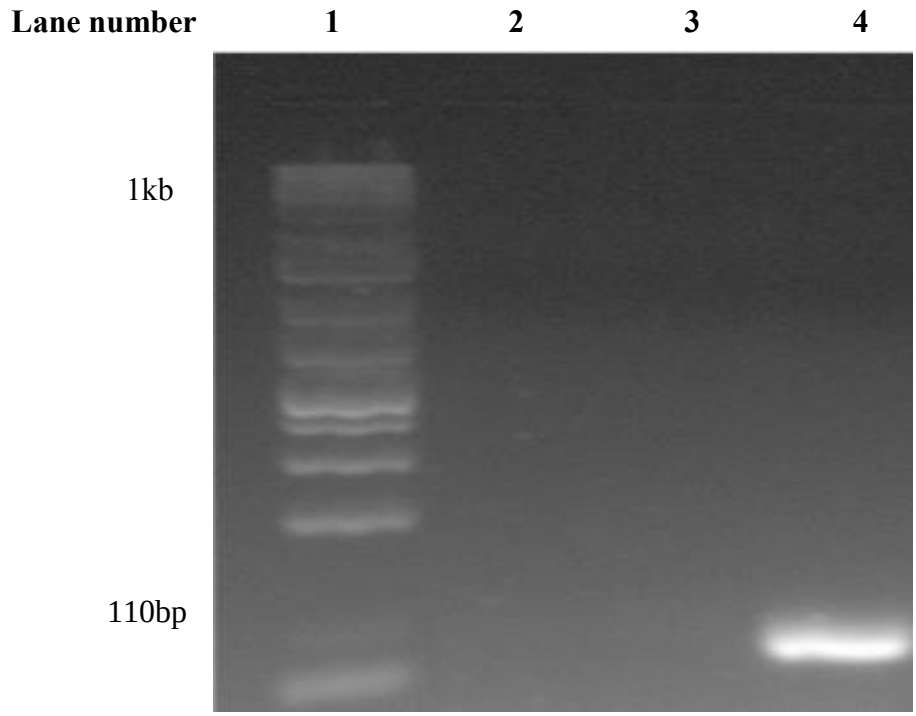

Figure A4. Evaluation of specificity of primers to the *eIF4AII* gene of *Populus* spp. (lane 4), compared to *S. musiva* (lane 2); Marker (lane 1), Negative control (lane 3).

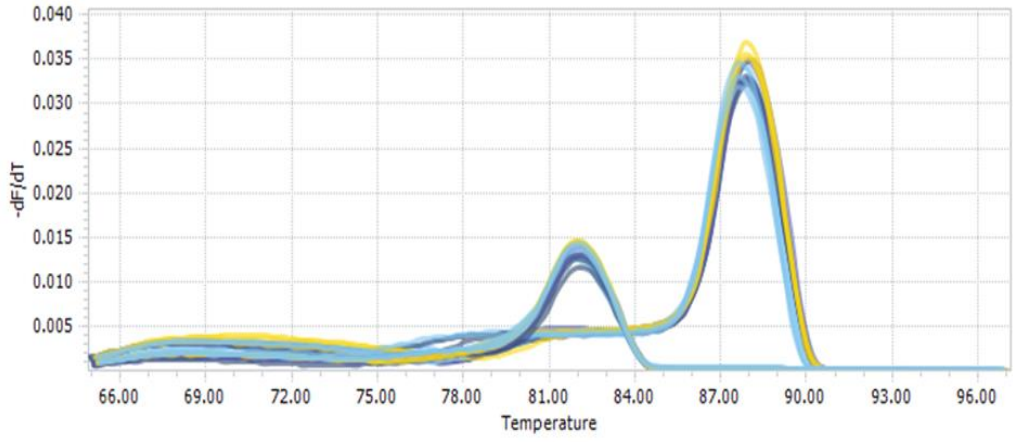

Figure A5. Meltcurve analysis of host and pathogen amplification to determine primer specificity using SYBR green.

Table A1. Average Cq value and canker age of each infected sample from Absaraka and the NDSU Seed Farm.

Location	Sample	Average Cq Value	Canker Age (Years)
Absaraka			
Infected	1	43.34	1
	2	33.2	2
	3	40.8	2
	4	0	1
	5	33.1	2
	6	28.3	1
	7	38.06	2
	8	46.7	1
	9	35.5	2
	12	40.4	1
	13	35.7	1
	14	32.4	1
	15	39.3	1
NDSU Seed Farm			
Infected	1	34.1	5
	2	41.7	6

APPENDIX B. RNA-SEQ SUPPLEMENTARY FIGURES AND TABLE

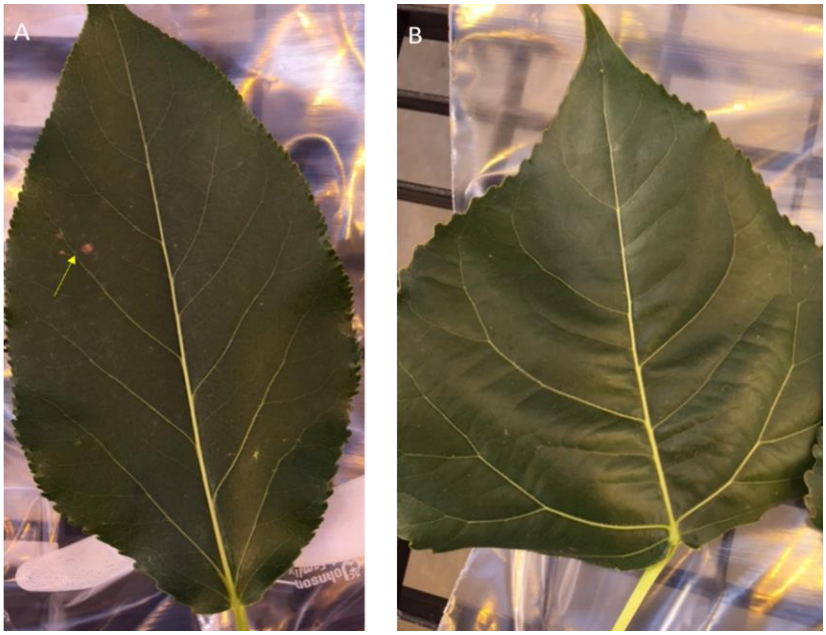

Figure B1. Lesion development on the susceptible genotype (A), and moderately resistant genotype (B), at 1 wpi.

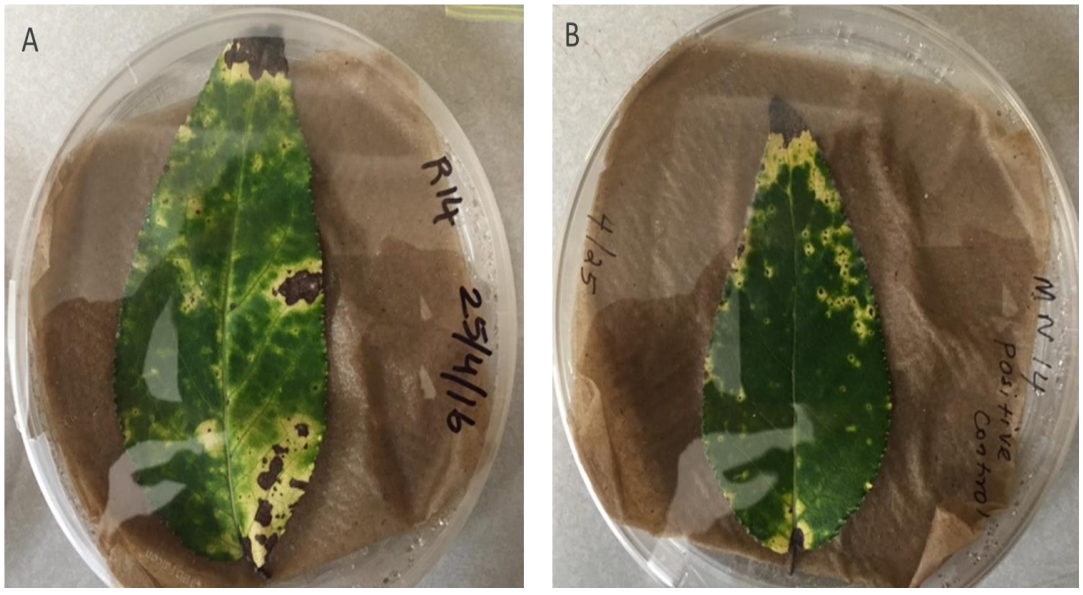

Figure B2. *In vitro* pathogenicity test with transformant R14 (A), and positive control MN14 (B), on susceptible *Populus* leaves.

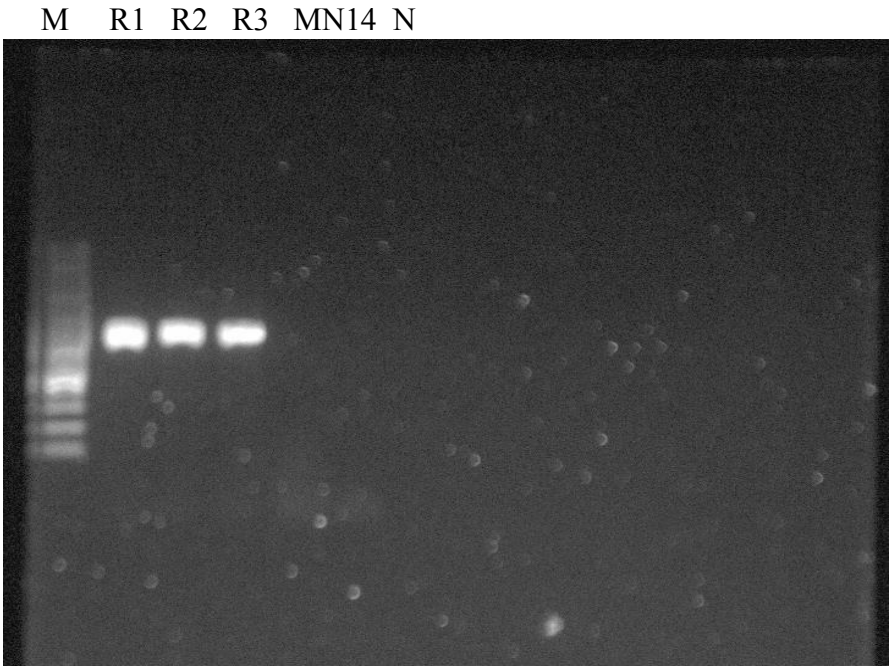

Figure B3. Stability of the transformant R14 was confirmed through hygromycin primers. M- Marker; R1, R2 and R3 are replicates of R14; MN14- wild type, N- negative control.

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva*.

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G176500	2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily protein	4	6.6	7
Potri.001G451300	2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily protein	-8	-4.7	
Potri.001G451900	2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily protein		-3.8	-35.8
Potri.001G150500	3-deoxy-d-arabino-heptulosonate 7-phosphate synthase			3
Potri.001G115000	A20/AN1-like zinc finger family protein			5.6
Potri.001G189500	ABC-2 and Plant PDR ABC-type transporter family protein	-6		
Potri.002G074800	ACT domain repeat 3		-5	
Potri.002G010600	acyl activating enzyme 1	41	32	11.8
Potri.002G196700	Adenine nucleotide alpha hydrolases-like superfamily protein	10	7	6
Potri.001G162800	alanine aminotransferase 2		-4.8	
Potri.002G072100	alcohol dehydrogenase 1	6.8	3.6	3.7
Potri.002G081800	aldehyde dehydrogenase 3H1		3.5	14
Potri.002G126300	alpha-amylase-like			4
Potri.001G403000	Aluminium induced protein with YGL and LRDR motifs			4.9
Potri.001G144300	aluminum-activated, malate transporter 12			3.8
Potri.002G112100	amino acid permease 6			4.5
Potri.001G422300	Aminotransferase-like, plant mobile domain family protein	10		119
Potri.001G105900	AMP-dependent synthetase and ligase family protein	-3.9		
Potri.001G293400	APS kinase	4	7	3.7
Potri.001G004100	Arabinogalactan protein 14			-7.8
Potri.002G081100	Armadillo/beta-catenin-like repeat; C2 calcium/lipid-binding domain (CaLB) protein	-7	-18	-24.8

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G142200	Basic helix-loop-helix (bHLH) DNA-binding family protein	-3.5		
Potri.002G176900	Basic helix-loop-helix (bHLH) DNA-binding family protein	-4.5	8	
Potri.001G270000	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein	4	5	5
Potri.002G159400	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein	-3	-16.8	-9
Potri.002G172100	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein	-5		
Potri.002G032400	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein		3	5.9
Potri.002G105300	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein			-7.5
Potri.001G414700	B-box type zinc finger family protein	3.8	6.5	
Potri.001G223600	beta glucosidase 27	-5.8	-98.7	
Potri.001G223900	beta glucosidase 27	-7	-238.0	
Potri.001G223800	beta glucosidase 27		∞	-26.8
Potri.001G409900	beta glucosidase 41			5.7
Potri.001G100200	beta-hydroxylase 1		-18	13.9
Potri.002G113600	branched-chain amino acid transaminase 2			5
Potri.001G102300	BTB/POZ domain with WD40/YVTN repeat-like protein	-5.6		
Potri.001G468700	BTB/POZ domain-containing protein		3	4
Potri.001G086100	BTB/POZ domain-containing protein			3
Potri.002G090700	bZIP transcription factor family protein			3
Potri.001G235800	C2H2 and C2HC zinc fingers superfamily protein	-27		-11.5
Potri.001G411800	Calcium-binding EF-hand family protein	-17.5		-5.5
Potri.002G182500	Calcium-binding endonuclease/exonuclease/phosphatase family		4	
Potri.001G301900	Calcium-dependent lipid-binding (CaLB domain) family protein	-5.8		

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.002G123200	Calcium-dependent lipid-binding (CaLB domain) family protein			7
Potri.002G001400	Calmodulin-like 37	-79		-13
Potri.002G127000	Calmodulin-like 37	-5		-3.79
Potri.001G332900	Calmodulin-like 38	-14.6	-12.5	-20
Potri.001G348900	carbonic anhydrase 1			-23
Potri.002G177900	CBL-interacting protein kinase 9	6	6	5
Potri.001G288000	CBS domain-containing protein with a domain of unknown function (DUF21)			6
Potri.002G090600	Chaperone DnaJ-domain superfamily protein			5
Potri.001G056600	Chaperone protein DnaJ-related	-3.8		
Potri.001G258100	Chaperonin-like RbcX protein			4
Potri.001G148900	chloroplast beta-amylase			3
Potri.001G116400	chloroplast ribosomal protein S15	30		
Potri.001G118200	Copper amine oxidase family protein		3.8	
Potri.001G416500	C-terminal cysteine residue is changed to a serine 1			16.8
Potri.001G441700	Curculin-like (mannose-binding) lectin family protein	-37.9	-39	-8
Potri.001G225800	cystatin B			3
Potri.002G158800	Cytidine/deoxycytidylate deaminase family protein	-7		
Potri.001G242600	cytochrome P450, family 707, subfamily A, polypeptide 2	11.5	3.6	
Potri.002G025400	cytochrome P450, family 71, subfamily B, polypeptide 36	586.5	3030.8	∞
Potri.001G422500	cytochrome P450, family 716, subfamily A, polypeptide 1	9.8	12	29
Potri.002G121400	cytochrome P450, family 81, subfamily D, polypeptide 3	-6	-4.5	
Potri.002G026300	cytochrome P450, family 83, subfamily B, polypeptide 1	∞	∞	∞
Potri.002G025800	cytochrome P450, family 83, subfamily B, polypeptide 1	63	215.6	354.7
Potri.001G270500	cytochrome P450, family 87, subfamily A, polypeptide 2	10.9		6.6

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G270400	cytochrome P450, family 87, subfamily A, polypeptide 2		3.6	19
Potri.001G270600	cytochrome P450, family 87, subfamily A, polypeptide 2		-11	-4
Potri.002G010500	cytochrome P450, family 87, subfamily A, polypeptide 2			-6
Potri.002G062200	Cytokine-induced anti-apoptosis inhibitor 1, Fe-S biogenesis			9.6
Potri.002G017200	DC1 domain-containing protein		-4.6	
Potri.001G186700	delta tonoplast integral protein	-4		-2.9
Potri.001G258600	dicarboxylate transporter 1		-3	-6
Potri.002G063500	DNAse I-like superfamily protein	-7		
Potri.001G074600	Domain of unknown function, (DUF23)	-8	-7	-21
Potri.001G164800	Dormancy/auxin associated family protein		3.7	6.9
Potri.001G334600	DSS1 homolog on chromosome V	-44	∞	-3355.54
Potri.001G398800	early nodulin-like protein 1		-3.8	
Potri.002G150600	early nodulin-like protein 18	-4	-7.8	-25
Potri.001G268600	elicitor-activated gene 3-1		-15	
Potri.001G435700	endoribonuclease L-PSP family protein		-4	-3
Potri.001G324200	Esterase/lipase/thioesterase family protein	-9.7	-4.6	-5
Potri.001G397200	ethylene responsive element binding factor 4	-11		-5
Potri.001G154200	ethylene responsive element binding factor 5	-16.9		
Potri.001G464300	ethylene-responsive nuclear protein/ethylene-regulated nuclear protein (ERT2)	-6		
Potri.002G054900	Eukaryotic aspartyl protease family protein	6.5	-4	11.9
Potri.001G234600	exocyst subunit exo70 family protein H2	-6.9	3	
Potri.001G311700	EXORDIUM-like 2		-4.21	
Potri.002G191600	galactinol synthase 1	-19	-216.6	-9.5
Potri.002G089800	Galactosyltransferase family protein	-6		

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.002G083700	GDSL-like Lipase/Acylhydrolase superfamily protein		-107	
Potri.001G010700	geminivirus rep interacting kinase 1	-10.9	-8	-5
Potri.002G007400	glutamate receptor 3.3			13
Potri.001G104500	glutamate-cysteine ligase			3
Potri.001G278400	glutamine-dependent asparagine synthase 1	-22.7	-12	
Potri.002G015100	glutathione S-transferase F11	4.6		
Potri.001G319800	glycine-rich RNA-binding protein 2	-3	-4.9	-5
Potri.001G255100	Glycosyl hydrolase superfamily protein		-35.65	-6.09
Potri.001G415200	GRAS family transcription factor	-7.6	3	
Potri.001G310500	GTP cyclohydrolase II	-3.7	-2.9	
Potri.001G104400	Haloacid dehalogenase-like hydrolase (HAD) superfamily protein			4.5
Potri.001G234700	Heavy metal transport/detoxification superfamily protein			9.9
Potri.001G350500	Heavy metal transport/detoxification superfamily protein			-4
Potri.002G163400	Heavy metal transport/detoxification superfamily protein			3
Potri.001G045200	high affinity K^+ transporter 5	-221.8	∞	-1172
Potri.002G026800	Histone superfamily protein			3
Potri.002G176300	homeobox 7			6.
Potri.001G314800	Homeodomain-like superfamily protein			-7.7
Potri.001G152500	HXXXD-type acyl-transferase family protein	-5.8	-4	-20
Potri.001G448000	HXXXD-type acyl-transferase family protein	3	5	9
Potri.001G448000	HXXXD-type acyl-transferase family protein	-22	-52	-85
Potri.001G061500	hydroxyproline-rich glycoprotein family protein		5.8	7.7
Potri.002G082400	IAA-leucine resistant (ILR)-like gene 6	-17		
Potri.002G044900	indole-3-acetic acid inducible 14	-4.5		

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G327700	Integral membrane HRF1 family protein		5.7	75
Potri.001G079600	Integrase-type DNA-binding superfamily protein	3	5.5	
Potri.001G079800	Integrase-type DNA-binding superfamily protein	-12		
Potri.001G092400	Integrase-type DNA-binding superfamily protein	-9		-3.5
Potri.001G099300	isoprenoid F			-3
Potri.001G123800	K^+ uptake permease 11			3.5
Potri.001G230600	KAR-UP F-box 1	-6		
Potri.001G058200	K-box region and MADS-box transcription factor family protein	12	11	5
Potri.002G105600	K-box region and MADS-box transcription factor family protein		3	
Potri.001G208000	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	-9	-1013	-263.5
Potri.002G165000	Late embryogenesis abundant protein	-28		-2.82
Potri.001G441900	lectin protein kinase family protein	∞	∞	∞
Potri.001G441300	lectin protein kinase family protein	3.5		22.121
Potri.001G113100	leucoanthocyanidin dioxygenase	-3.6	-9.5	-4
Potri.001G407100	light-harvesting chlorophyll B-binding protein 3			-4
Potri.001G384000	light-regulated zinc finger protein 1	-4		-11.8
Potri.001G015600	lipoxygenase 2	-3.5		-9.5
Potri.001G015400	lipoxygenase 2		3	-15
Potri.001G015500	lipoxygenase 2			-3
Potri.001G167700	lipoxygenase 3	-13.6		
Potri.001G335300	lysine histidine transporter 1	-3		
Potri.001G285300	Magnesium transporter CorA-like family protein	-4		
Potri.002G095900	Major facilitator superfamily protein	10	13	11
Potri.001G013400	Major facilitator superfamily protein			5.8
Potri.001G231500	Major facilitator superfamily protein			3

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.002G162500	MAP kinase 4	-4.8		
Potri.002G102100	MATE efflux family protein	3.8		
Potri.002G107200	MATE efflux family protein			7
Potri.002G059800	MEI2-like protein 5			3
Potri.001G271700	mitogen-activated protein kinase 3		4	
Potri.001G042400	mitogen-activated protein kinase kinase kinase 19	-17	-3	-7
Potri.002G105900	MSCS-like 3			3.7
Potri.001G095600	multidrug resistance-associated protein 14			15
Potri.001G362300	multidrug resistance-associated protein 3			-2.9
Potri.001G145900	myo-inositol polyphosphate 5-phosphatase 2	4.7	7.2	
Potri.001G404100	NAC (No Apical Meristem) domain transcriptional regulator superfamily protein	-3	-5.5	5
Potri.001G404400	NAC domain containing protein 2		7.7	15.8
Potri.001G080900	NAC domain containing protein 42			5.3
Potri.001G218800	NAC domain containing protein 61	-5	3	-6.6
Potri.001G325100	NAC domain containing protein 83	-7	-2.8	
Potri.001G061200	NAC domain containing protein 83			4
Potri.001G086900	NAD(P)-binding Rossmann-fold superfamily protein	∞	∞	∞
Potri.001G093300	NAD(P)-binding Rossmann-fold superfamily protein	-542.9	-757	-1561
Potri.001G349600	NAD(P)-binding Rossmann-fold superfamily protein	-19.8		
Potri.002G148000	NAD(P)-binding Rossmann-fold superfamily protein	4		3.7
Potri.001G327800	NAD(P)-binding Rossmann-fold superfamily protein		-5	
Potri.001G156300	NHL domain-containing protein			5
Potri.002G190800	NIM1-interacting 1	-4.8		-8
Potri.002G088600	nitrate reductase 1			-6.7

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.002G034400	NmrA-like negative transcriptional regulator family protein			-3
Potri.001G455000	NOD26-like intrinsic protein 5;1			4
Potri.001G032500	nodulin MtN21/EamA-like transporter family protein	-5		-4.6
Potri.002G040200	nodulin MtN21/EamA-like transporter family protein	3.8		5.5
Potri.001G337100	nodulin MtN21/EamA-like transporter family protein			4.6
Potri.001G112600	non-yellowing 1			7.7
Potri.001G044900	NRAMP metal ion transporter 6	-5	-4	-3.2
Potri.001G158000	Nucleotide-diphospho-sugar transferases superfamily protein	-6.5		
Potri.001G400900	Nucleotide-diphospho-sugar transferases superfamily protein	3.9	5.6	3.5
Potri.001G112000	nucleotide-rhamnose synthase/epimerase-reductase			-3
Potri.001G451100	O-methyltransferase 1		-8	
Potri.002G097800	Outer arm dynein light chain 1 protein	3	4	6.9
Potri.001G239900	outer plastid envelope protein 16-1			-3
Potri.001G190700	oxidative stress 3	5	3	
Potri.001G013000	peroxidase CA	-5.6	-20	-17
Potri.001G011200	peroxidase CB		-7	
Potri.001G113000	PGR5-LIKE A		3.9	
Potri.001G050100	phloem protein 2-B1	-5		
Potri.002G098600	Phosphate-responsive 1 family protein	-9		-24.7
Potri.002G098800	Phosphate-responsive 1 family protein	-37.7		-12
Potri.001G347300	phosphoenolpyruvate (pep)/phosphate translocator 2	4.9	9.5	29
Potri.002G108600	Phosphoglycerate mutase family protein	5.7	14.8	13
Potri.001G134000	phosphoribulokinase	4	7	
Potri.002G040000	Photosystem I, PsaA/PsaB protein	12	23	
Potri.002G057400	phytoene desaturation 1			3.8
Potri.002G116300	phytosulfokine 4 precursor		-3.9	-3

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

Gene ID	Gene Annotation	Fold Change		
		1 wk	2 wk	3 wk
Potri.001G133200	pinoresinol reductase 1	-7.5		
Potri.001G299500	Plant basic secretory protein (BSP) family protein	3	9	8
Potri.001G046000	Plant protein of unknown function, (DUF247)	-27	-7	
Potri.002G009700	Plant regulator RWP-RK family protein	6		4
Potri.002G070500	plant U-box 29	-7	-4	-16
Potri.001G073900	plasma-membrane associated cation-binding protein 1			8.5
Potri.001G360700	PLC-like phosphodiesterase family protein			-5
Potri.001G048800	pleiotropic drug resistance 12	4.5	14	11
Potri.002G093100	Pollen Ole e 1 allergen and extensin family protein			-3
Potri.001G239300	poltergeist-like 4	-5		
Potri.002G055300	polyamine oxidase 3			3
Potri.001G263000	polyubiquitin 10	3	3	
Potri.002G105000	Predicted AT-hook DNA-binding family protein	-4.8	-17	-4
Potri.002G045100	Primosome PriB/single-strand DNA-binding			-5
Potri.002G014000	Prolyl oligopeptidase family protein			-25.5
Potri.001G438400	Protein kinase superfamily protein	-4.8		
Potri.002G004900	Protein kinase superfamily protein	-40.6	-2.9	
Potri.002G065400	Protein kinase superfamily protein	-4.9		
Potri.002G188600	Protein kinase superfamily protein	-5		
Potri.002G019300	Protein kinase superfamily protein		-7.5	7.9
Potri.001G260800	Protein kinase superfamily protein			4
Potri.001G393200	Protein kinase superfamily protein			5
Potri.002G147100	Protein of unknown function, (DUF1442)	-5		
Potri.001G003800	Protein of unknown function, (DUF239)		4.5	
Potri.002G125700	Protein of unknown function, (DUF3223)	-4	-4	-4
Potri.002G065200	Protein of unknown function, (DUF3511)			5

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.002G092900	Protein of unknown function, (DUF581)	3	3.8	
Potri.002G140300	Protein of unknown function (DUF581)	3		3
Potri.002G050800	Protein of unknown function, (DUF581)			5.5
Potri.002G067300	Protein of unknown function, (DUF677)	71.9	103.8	∞
Potri.001G008400	Protein of unknown function, (DUF538)	3		
Potri.002G128800	Protein of unknown function, (DUF617)	5	5	9
Potri.001G278500	Protein phosphatase 2C family protein	-4		-2.6
Potri.001G297200	Protein phosphatase 2C family protein	-18		
Potri.001G381000	Protein phosphatase 2C family protein	-32.8		-15.9
Potri.002G007500	Protein phosphatase 2C family protein			4
Potri.001G403300	protochlorophyllide oxidoreductase A	-3		-10.7
Potri.001G423700	purple acid phosphatase 18			3.7
Potri.001G018200	Putative glycosyl hydrolase of unknown function, (DUF1680)		3	
Potri.001G111600	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein			-5.7
Potri.002G188100	RAD-like 6		6	5.8
Potri.001G413400	receptor kinase 3	33.7	9	40.8
Potri.001G064800	receptor-like protein 13		11.9	15
Potri.001G064500	receptor-like protein 14	10	10	5.8
Potri.001G063700	receptor-like protein 56	8.7	9.7	7
Potri.001G168200	RELA/SPOT homolog 3			4.9
Potri.002G094200	related to AP2 4	-3		
Potri.002G157700	Remorin family protein	-3	-3.5	
Potri.001G137500	Ribosomal protein L14p/L23e family protein	-14	-32	-33.9
Potri.001G453900	Ribosomal protein L6 family			-3

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G364100	Ribosomal protein S10p/S20e family protein		-3	-7
Potri.001G057300	Ribosome associated membrane protein RAMP4	-4.9		
Potri.002G052400	ribosome recycling factor, chloroplast precursor			-3
Potri.002G196600	RING/FYVE/PHD zinc finger superfamily protein		21	26.5
Potri.001G297000	RING/U-box superfamily protein	-4.8		
Potri.001G371900	root FNR 1			-3
Potri.001G131400	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	-2.9	-4	-2.9
Potri.001G405200	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein			-5.7
Potri.002G189200	S-adenosylmethionine synthetase 2		4.5	7.6
Potri.002G028200	salt tolerance homolog2			5.9
Potri.001G295500	salt tolerance zinc finger	-12.5		
Potri.002G119300	salt tolerance zinc finger	-24		-6
Potri.001G241800	SCARECROW-like 14	-4		
Potri.001G242000	SCARECROW-like 14	-7	-3.6	-4.5
Potri.001G409500	SCARECROW-like 5	-13		-4
Potri.002G014700	SEC14 cytosolic factor family protein/phosphoglyceride transfer family protein	3	4	
Potri.001G068000	Sec14p-like phosphatidylinositol transfer family protein	3.5		
Potri.002G203500	senescence-associated gene 21	-8.5		
Potri.001G355100	senescence-related gene 1	-6	-22	
Potri.001G382400	senescence-related gene 1		3	9
Potri.001G364000	serine acetyltransferase 2;2	3		
Potri.001G010500	seven in absentia of Arabidopsis 2			3
Potri.001G029700	sigma factor binding protein 1	-5	-3	
Potri.001G316300	SIN3-like 1	-49		
Potri.001G441800	S-locus lectin protein kinase family protein			6
Potri.001G299700	S-methyl-5-thioribose kinase	16.7	16	16.9

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G140200	SNARE associated Golgi protein family	-4		
Potri.001G375300	sodium/calcium exchanger family protein/calcium-binding EF hand family protein	6	3	
Potri.001G398200	squamosa promoter binding protein-like 5		-13.5	-67
Potri.002G092400	STAS domain/Sulfate transporter family	5	6.6	
Potri.001G455800	Subtilase family protein			8.7
Potri.001G177700	Tetratricopeptide repeat (TPR)-containing protein	18.7	14	15
Potri.001G250100	Tetratricopeptide repeat (TPR)-like superfamily protein	4	6.7	
Potri.001G140500	Tetratricopeptide repeat (TPR)-like superfamily protein		-4	-5
Potri.002G017500	Thioredoxin superfamily protein	5.6	3.8	8
Potri.001G440900	Transcription factor jumonji (jmj) family protein/zinc finger (C5HC2 type) family protein	5	5.6	12
Potri.001G241600	TRF-like 2			3.5
Potri.001G034200	Ubiquitin-conjugating enzyme/RWD-like protein	-4		
Potri.002G144600	Ubiquitin-like superfamily protein			3.5
Potri.001G239100	U-box domain-containing protein kinase family protein			5
Potri.001G320000	UDP-D-glucuronate 4-epimerase 6			-3.5
Potri.001G302400	UDP-glycosyltransferase 73B4	6	4.5	26.7
Potri.001G449500	UDP-Glycosyltransferase superfamily protein	3	3	
Potri.002G162300	UDP-Glycosyltransferase superfamily protein	17.5	119.8	38.9
Potri.001G281900	UDP-Glycosyltransferase superfamily protein		-8.8	-3
Potri.001G282100	UDP-Glycosyltransferase superfamily protein		-3	
Potri.002G168600	UDP-Glycosyltransferase superfamily protein		5	
Potri.002G064800	Uncharacterised conserved protein UCP009193			3

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.001G315400	Uncharacterised protein family SERF	-4.6		
Potri.002G104400	uncoupling protein 5	-4		
Potri.002G123100	VIER F-box protein 1			3.5
Potri.001G294100	voltage dependent anion channel 2	19.5	24.9	36
Potri.001G230800	VQ motif-containing protein		4.9	12.5
Potri.001G399100	VQ motif-containing protein			-7
Potri.002G075900	wall-associated kinase 2			3
Potri.001G312200	white-brown complex homolog protein 11	-4	-6.8	-67.5
Potri.001G311300	white-brown complex homolog protein 11			-11
Potri.001G206900	xylem NAC domain 1	102	23.7	∞
Potri.001G071000	xyloglucan endotransglucosylase/hydrolase 5		-6.5	-47.5
Potri.001G243300	zinc finger (C3HC4-type RING finger) family protein	-11		
Potri.001G336800	zinc finger (C3HC4-type RING finger) family protein			5.7
Potri.001G364400	zinc transporter 11 precursor	73	534	47.3
Potri.002G140100	Zinc-binding ribosomal protein family protein	-2.9		-3
Potri.006G159900	nudix hydrolase chloroplastic-like isoform X1			6.3
Potri.013G028200	autophagy-related 18h-like			10
Potri.T130000	disease resistance RPM1-like			5.9
Potri.001G449800	Avr9 Cf-9 rapidly elicited	-34.9		
Potri.013G097300	TIR-NBS-LRR-TIR type disease resistance	-102	-390	-250
Potri.015G086800	leucine-rich repeat receptor-like serine threonine tyrosine-kinase SOBIR1	-6.8		-3
Potri.010G044600	disease resistance RPM1-like	56.8	40	27.9
Potri.014G031100	BRI1 kinase inhibitor 1-like	9	6.9	7.5
Potri.013G069200	DETOXIFICATION 24	8	14	
Potri.015G002800	DOWNY MILDEW RESISTANCE 6-like	3.9		
Potri.008G220200	TMV resistance N-like	-4		

Table B1. List of differentially expressed genes in moderately resistant and susceptible *Populus* genotype during interaction with *Sphaerulina musiva* (continued).

		Fold Change		
Gene ID	**Gene Annotation**	**1 wk**	**2 wk**	**3 wk**
Potri.006G274300	NBS-LRR resistance RGH2	-5		
Potri.018G104800	shikimate O-hydroxycinnamoyltransferase-like	-34	-33.7	-18
Potri.002G102100	DETOXIFICATION 27-like	3		
Potri.T008700	receptor kinase RLPK1	-11.7	-7.4	-5.5
Potri.003G021000	SUPPRESSOR OF GENE SILENCING 3-like isoform X1	5.8	3	9.7
Potri.001G134700	NBS-LRR resistance gene ARGH35	8.5	6	27
Potri.004G182000	endochitinase 2-like		-34	
Potri.014G031300	BRI1 kinase inhibitor 1-like		-3	-4
Potri.012G006300	DOWNY MILDEW RESISTANCE 6-like			-6
Potri.004G073000	late blight resistance homolog R1B-19	-8.9		
Potri.001G092900	WRKY family transcription factor	-14		-4
Potri.002G168700	WRKY family transcription factor	-44		-10
Potri.002G186600	WRKY family transcription factor		4	3