Breaking the wall of silence of trees
Mining metabolomics to describe hybridization and predict performance in the *Populus* – *Sphaerulina musiva* pathosystem

by

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Abstract

Stem canker diseases in poplars caused by the fungal pathogen *Sphaerulina musiva* Peck. remain some of the least understood forest diseases despite causing considerable damage, particularly in hybrid poplar plantations. *S. musiva* is endemic to eastern Canada in provinces including the Maritimes, Quebec, and Ontario. Although these provinces contain poplar species such as *Populus deltoides* (Bartr.) Marsh and *P. balsamifera* L., they are purported to be resistant to the Septoria canker in their eastern range. The Northwestern range (Alberta and British Columbia) mainly home to *P. balsamifera* and *P. trichocarpa* Torr. and Gray., the pathogen is found sporadically in shelterbelts, nurseries, and plantations.

Anthropogenic crosses among native species of poplars in North America or with exotic species have been undertaken to produce clones with 'hybrid vigour' which combine high growth rates with ease of propagation and disease resistance. While vigour of hybrid trees can be modeled and predicted, it is still challenging to predict disease performance in anthropogenic crosses. In the present work metabolic phenotyping was successfully used as a way of predicting performance in various cross types in response to *S. musiva.*

In its native range, *P. balsamifera* in Alberta has shown high susceptibility to stem canker, unlike its eastern counterpart. Selected genotypes with variable introgression levels with *P. trichocarpa* and covering the spectrum from *P. trichocarpa* to *P. balsamifera* were inoculated with a fungal spray mixture of *S. musiva* and their phenomics measured both visually (infection estimates) and phytochemically. Susceptibility in *P. balsamifera* was linked to two parameters: (1) the level of admixture with *P. trichocarpa*; and (2) their evolutionary history. Metabolic phenotyping identified metabolites involved in the genotypic responses to *S. musiva* and other metabolites describe hybridization between different species. Using genetic characterization along with phenomic data provides predictive tools to inform tree breeding programs, shape management decisions, and sheds light on the poorly understood *Populus – S. musiva* pathosystem.

Preface

The experimental setup was designed by Ahmed Najar, with the assistance of Associate Professor Dr. Nadir Erbilgin and Associate Professor Dr. Barb Thomas. All data collection and analysis in this thesis is my original work. I performed the data collection and analysis as well as writing the manuscript. Dr. Erbilgin and Dr. Thomas assisted with review, edits and bringing concepts to maturity.

Dedication

To my brothers Mohamed and Mohamed-Anis for being present unconditionally. To their growing families,
To my sister, my first inspiration and my all times role model. To her wonderful family,
To my mother, thank you for reminding me of who I am and grounding me in times of unthinkable tragedy,
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Chapter 1: Thesis Introduction

I. Biology of *Populus* spp.

1. Ecology, Service, and Meaning

From the earliest reports on poplar nomenclature, the genus *Populus* seemed to be predestined to go along with human development: *Arbor populi* (Latin for the People's tree) was the Roman name of poplar trees sheltering public squares (Dickmann 2001). Perhaps the ability of poplars to colonize various habitats, particularly alongside water bodies and riparian lands, which are backbones of human settlement, was the key to this close relationship (Stettler and Bradshaw 1996).

Poplars also colonize naturally a variety of bioclimatic stages and landscapes in the northern hemisphere ranging from the deserts of China, to the Middle East, to the boreal forest (Eckenwalder 1996, Wang et al. 2014a). As an early successional fast growing species, poplars also grow and thrive in disturbed sites and riparian lands (Braatne et al. 1996). They capitalize on major disturbances to push the boundaries of their establishment (Eckenwalder 1996). The variety of poplar reproductive strategies serves them well in colonizing disturbed lands as they can reproduce from seeds, root suckers or propagules. For instance, forest fires and clear cuts pave the way for poplar regeneration through two mechanisms: (1) the removal of the main stem (apical dominance) stimulates a hormonal balance suitable for adventitious growth and (2) the openings provide the proper light levels for root suckers to take-off and grow (Steneker 1974, Gom and Rood 1999). This capacity of asexual reproduction gives rise to forest stands made up of single clones (Adonsou et al. 2016). Some stands are thought to be reproducing clonally after emerging from a single seed millennia ago (Eckenwalder 1996, Dickmann 2001, Ally et al. 2010, Gylander et al. 2012). Riparian poplars in particular have the capacity to colonize water bodies through cladoptosis where portions of fallen branches travel along rivers and root once proper environmental conditions are met (Rood et al. 2000, Galloway and Worrall 1979, Eckenwalder 1996). Perhaps these observations inspired humans to propagate poplars from cuttings, which in part explains their use as forest crops. However species such as *P. deltoides* although resistant to several pathogens, cannot be easily propagated from cuttings (Riemenschneider et al. 2001). Special precautions have to be taken when rooting *P. deltoides* (Zalesny et al. 2005), thus rendering *P. deltoides* unfit for mass production and industrial nurseries. Hence breeding

programs usually aim at combining the high rootability of certain poplar species particularly in the *Tacamahaca* section (*P. balsamifera, P. trichocarpa, P. simonii, P. maximowiczii*, and *P. nigra*) with the resistance of *P. deltoides* to pathogens and insects (Stettler et al. 1996, Singh and Pandey 2002). The same breeding programs take into account industrial considerations for production volumes, rotation time and fiber quality when managing for industrial usage (engineered wood products e.g. oriented strand boards, pulp and paper or energy). Otherwise traits of eco-service make poplar successful in natural ecosystems where they exist in a variety of stand conditions and act as a backbone for the ecosystem by being an umbrella species for a variety of other plant, fungi and animal species (Folke et al. 2004, Eckenwalder 1996, Heller and Zavaleta 2009). Poplars are also amazingly plastic species, highly responsive to their environment. For instance, a recent work has shown that poplar cuttings of the exact same clone propagated in different locations in Canada reacted differently to induced drought in a greenhouse experiment, expressed different transcriptomes and had different DNA methylation patterns (Raj et al. 2011).

From a social perspective, poplars have different meanings to different communities. The needs of industry and cities are usually voiced and defined in terms of volume, rotation time, fiber quality or urban landscaping. However, other than their use as shelterbelts and windbreaks on farm lands, little has been done to understand what poplars actually mean to non-urban communities (Neumann et al. 2007a, b). Even less has been done to identify the needs of First Nations communities. For instance, the Nakoda (aka Stoney) First Nation in Alberta requires juvenile, fire-replaced poplar stands for ceremonies that celebrate light and rebirth (Elder Tom, 2014). The Cree First Nation uses poplar extracts and associated fungi in traditional medicine and smudging ceremonies (Elder Elsie 2014). This is not surprising for a “Pharmacy Tree” with a wide range of extractable compounds (Devappa et al. 2015). It is worth considering the inclusion of how local communities and First Nations perceive poplars in policy decisions and management programs as it could affect management techniques and reforestation.

2. Phylogeny of *Populus* spp.

a. Evolutionary history of *Populus* spp.

The genus *Populus* has evolved mainly in the northern hemisphere where speciation was particularly marked by glacial episodes (Levsen et al. 2012, Wang et al. 2014a). The genus is comprised of six sections:

1. *Abaso*: as a monotypic group with a single tree species, *P. mexicana,* it is a riparian poplar growing between the Pacific and Atlantic coasts of Mexico (Dickmann 2001).

2. *Turanga*: stretches along North and Central Africa as well as western and central Asia. It used to be considered monotypic until recently when the section became home to three species (Dickmann 2001).

3. *Leucoides*: hosts one North American species and two Asian species. The ecological relevance of the species of this section supersedes its commercial importance.

4. *Aigeiros*: includes some of the major riparian poplars in North America and Europe, *P. deltoides* and *P. nigra* respectively, as well as other species. Traditionally this section was the main contributor to early breeding programs in Europe and North America (Dickmann 2001). *Populus deltoides* is resistant to a variety of forest pests and insects (Stettler et al. 1996, Singh and Pandey 2002) and was thus used in breeding programs in both North America where it is a native species and Europe, where it is not native.

5. *Tacamahaca*: also known as the section of balsam poplars and includes species from both sides of the Pacific and across the new world (Dickmann 2001). The species of this section occupy high latitudes and their speciation is thought to have resulted from the last glacial era (Levsen et al. 2012, Wang et al. 2014a). This section includes *P. balsamifera* and *P. trichocarpa*, which are closely related.

6. *Populus*: comprises – among others – aspens (*P. tremuloides, P. tremula, P. davidiana*) and white poplars (*P. alba*) both of which have extensive ranges in the northern hemisphere (Dickmann 2001) as well as a variety of commercial uses including manufacturing and land reclamation (Pinno et al. 2012).

While the classification of sections is relatively agreed upon, the same cannot be said about species classification, which ranges from 22 to 85 species (Riemenschneider et al. 2001). This difference is due to two interrelated factors: (1) hybrids classified as separate species; and (2) different taxonomic cultures between "Lumpers" in North America and "Splitters" in Russia and China (Eckenwalder 1996). Another challenge to classification in species is the amazing, almost unlimited, potential of poplars to hybridization (Dickmann 2001). More will be said in this regard in section c.

b. Evolutionary history of *Populus balsamifera* and *Populus trichocarpa*

Populus balsamifera and *P. trichocarpa* belong to the *Tacamahaca* section based on morphological and geographical cues. However molecular analyses reveal that the classification in the section is less categorized (Hamzeh and Dayanandan 2004, Wang et al. 2014b). Unlike for other sections, members of the *Tacamahaca* show a paraphyletic relatedness to trees of the other sections (Dickmann 2001, Hamzeh and Dayanandan 2004, Wang et al. 2014b). The same studies consistently reveal the close relatedness of *P. balsamifera* and *P. trichocarpa*. As mentioned previously, members of the *Tacamahaca* section occupy higher latitudes which hints to the role of glaciation in shaping their speciation. Indeed, the divergence between *P. balsamifera* and *P. trichocarpa* is thought to have happened during the Pleistocene glacial maxima in North America (Levsen et al. 2012). The same has been observed for *P. euphratica* and *P. purinosa* from the *Turunga* section in China (Wang et al. 2014a) even though species in the *Turunga* section goes as far south as the Middle-East and North Africa.

Populus balsamifera is thought to have spread from two glacial refugia based on chloroplastic DNA: (1) Beringia, the former bridge across the Bering Strait that used to link North America to Asia, and (2) populations at the periphery of the ice sheet particularly in eastern North America (Breen et al. 2012). A study based on nuclear DNA using a larger set of markers showed three demes for *P. balsamifera* across its range (Keller et al. 2010):

- Two peripheral populations one emanating from the ancient Bering Strait and the second from the eastern periphery of the ice sheet. These two populations were characterized by low intraspecific diversity.
- One central population in the prairies with the highest intraspecific diversity.

Today *P. balsamifera* spans from eastern Canada to Alaska illustrating their success in recolonizing after the ice sheets receded and also a large portion the Canadian boreal forest. *Populus trichocarpa*, on the other hand, is also known as western balsam poplar, given it is purported to only grow west of the Rocky Mountains of North America. The common name hints to the close relationship between *P. balsamifera* and *P. trichocarpa*, this closeness is also supported through the use of different molecular markers (Hamzeh and Dayanandan 2004, Wang et al. 2014b). Similarly, the speciation of *P. trichocarpa* was also affected by the Pleistocene glacial era (Levsen et al. 2012). Given its present days pattern of diversity, *P. trichocarpa* is thought to have expanded from multiple glacial refugia along its actual range (Slavov et al. 2012). However, introgression of *P. balsamifera* was also found to contribute to today's pattern of diversity in *P. trichocarpa* populations (Geraldes et al. 2014). Further, a case was made for introgression of *P. balsamifera* into *P. trichocarpa* as a contributing factor for colonizing higher latitudes (Suarez-Gonzalez et al. 2016). Together these finding raise the question: is today's diversity reminiscent enough of the original diversity of the species or has introgression and hybridization made that harder to track down? More about the hybridization of poplars will be discussed in later chapters as it relates to their response to a pathogen. *Populus trichocarpa* was also found to be structured according to environmental factors (McKown et al. 2014a,b). Perhaps this environmental distribution is a side effect of the high responsiveness of poplars to environmental conditions that can affect the genome (Raj et al. 2011).

On the other hand, more is yet to be done regarding the population structure of *P. balsamifera* in terms of hybridization and backcrosses with surrounding species. Indeed, unlike other species with an overlapping range (Godbout et al. 2010), deeper understanding of how *P. balsamifera* is structured across zones of contact and how pathogens perform relative to genotypes from these zones could shed light on ecological questions such as to the potential for host-pathogen range expansion. Recent work has duly shown how admixture and introgression between *P. balsamifera* and *P. trichocarpa* is shaping adaptation to the variety of environments they cover (Suarez-Gonzalez et al. 2016). However, little research has been done to define the role of admixture between *P. balsamifera* and *P. trichocarpa* in shaping the response to pathogens such as the canker causing agent *Sphaerulina musiva* (Peck) Quaedvileig, Verkley and Crous.

c. Hybridization and *Populus*

Poplars have an extraordinary capacity to hybridize both within and across sections particularly in the Tacamahaca, Aigeiros, and Leucoides (DiFazio et al. 2011). Interspecific hybrids form naturally in zones of contact between compatible parental species or are obtained through artificial breeding. Anthropogenic hybridization could involve individuals from the same geographic range (sympatric) or from different geographic ranges (allopatric) (Zasada et al. 2001). For simplicity sake the following three concepts will be described as such: (1) Hybridization happens when two fertile species cross, producing what is referred to in genetics as F_1 offspring; (2) introgression refers to the introduction of genes/alleles from the gene pool of one species into that of another through repeated backcrossing of interspecific hybrids with members of one of the parental species; and (3) admixture describes the population make-up in terms of pure species (parents), F_1 (first generation hybrids) and introgressed individuals (with different percentage of one or the other parental type). In general, with hybridization being so common, it is sometimes hard to distinguish pure species from hybrids (Eckenwalder 2001). This can lead to an overestimation of the range of pure species and an underestimation of hybrid zones.

Hybrids also pose several challenges to taxonomists; the prominence of hybrids in the genus *Populus* is at the root of the differences in taxonomy between Chinese/Russian and North American taxonomic authorities (Dickmann 2001). On the other hand, admixture zones are the boon of evolutionary biologists and one of the choicest platforms for the study of environmental adaptation, reproductive isolation, and speciation (Lexer et al. 2010). Admixture zones are also meaningful ecologically and have been used to test several hypotheses about the ecology and evolution of hybrids. Some hypotheses were constructed around the premise of a gene – ecosystem interaction where the acquisition or loss of certain alleles/genes can shape interactions between communities and structure the ecosystem accordingly (Wymore et al. 2011). Within the gene – ecosystem construct, a model was proposed where hybrids show one of four trends in comparison to their parental lines where hybrids are: (1) more susceptible; (2) more resistant; (3) intermediate between the set of parents; or (4) similar to one of the parents (Fritz et al. 1999). On the other hand, the hybrid sink hypothesis postulates that hybrid zones sustain high biodiversity and are attractive to parasites (Whitham 1989). The phenological sink hypothesis stems from the observation that hybrids have a “staggered” phenology which potentially leads to a higher

exposure to pests and pathogens, though this hypothesis does not necessarily require a hybrid zone (Floate et al. 1993). The last two hypotheses were tested on the *Sphaerulina* - *Populus* pathosystem (LeBoldus et al. 2013). The hybrid bridge hypothesis (Floate and Whitham 1993) supposes that admixture zones are stepping stones for arthropod and pathogen expansion in the search for new hosts. The evolutionary novelty hypothesis, on the other hand, suggests that because of hybrid zones distinctiveness, admixture zones could be the endpoint of range expansion and a venue for specialization or even speciation. The last two hypotheses were supported in gall forming insects in poplars (Floate et al. 2015). The actual place and function of hybrids in ecosystems is subject to rich discussions: Do hybrids function in one of the above hypotheses in a monolithic way, or does a combination of hypotheses describe the response patterns to pests in hybrid populations?

From a host perspective, some authors see in hybridization and introgression a source of diversity and a reservoir for speciation. Others argue, it is an "evolutionary dead end" (Seehausen 2004). Mallet (2005) however contends that introgression as an "invasion of the genome", is part and parcel of the speciation process and that introgression is more common in recently diverged species since they are in a quest for a more defined and "stable" genotype less prone to "invasion" (Baack and Rieseberg 2007). Lagache et al. (2013) challenged Mayr's biological species concept using several criteria. Lagache et al (2013) introduced the notion of a continuum between species where species are presented as an "extreme point" against which individuals can be compared and to which individual trees are related to different degrees. This could mean that, for instance, *P. trichocarpa* is at the extreme of *P. balsamifera.*

Whether in the field or in the greenhouse, most of these hypotheses were tested measuring parameters like growth, abundance of arthropods and pathogens, or quantitative traits of susceptibility such as infected stem and leaf areas, or lesion length (Pilson 1999, LeBoldus et al. 2013, Floate et al. 2015). Measures of secondary metabolites and other metabolic traits in poplars have essentially been used in descriptive studies seeking to establish ecological functions of some of these metabolites as well as their role in shaping interactions between species in the ecosystem, particularly when it comes to insects (Hwang and Lindroth 1997, Osier et al. 2000, Scioneaux et al. 2011). Physiological measurements seem to be more common in studies determining the underlying mechanisms of heterosis, or hybrid vigor, which stipulates that

hybrids accumulate more biomass, show faster growth and achieve higher fertility than both parental lines (Birchler et al. 2010). Studies using phenotypic measurements of secondary metabolites to confirm heterosis are rare. For example, in Eucalyptus which is also a fast growing species, physiological measurements and secondary metabolites have been used as proxies for heterosis (O'Reilly-Wapstra et al. 2014). In poplars, research in this area is ever growing and it seems that the very concept of species is at the verge of a major breakthrough, particularly with the idea that hybridization will be the main driver for speciation in the Anthropocene (Thomas 2013).

Populus balsamifera and *P. trichocarpa* do hybridize naturally in their overlapping ranges of eastern British Columbia and western Alberta. However the extent of that hybridization and its direction are not fully known and the reports in this regard are rather scarce (Geraldes et al. 2014). This could mean that populations, previously thought to be pure *P. balsamifera* or *P. trichocarpa* are only putative pure species. There are practical implications for these observations particularly to the *Sphaerulina – Populus* pathosystem: *P. trichocarpa* is highly susceptible to the canker caused by *Sphaerulina* while other work suggests that *P. balsamifera* is resistant to canker (not leaf spots) in its native range (Bier 1939, Waterman 1954, Sivanesan 1990, Newcombe et al. 2001). However, in its western range, *P. balsamifera* has shown to be susceptible to *S. musiva* (Zalasky 1978, LeBoldus et al. 2009). I propose that the differences in the response to the pathogen within the species range of *P. balsamifera* is ultimately a result of the introgression of *P. trichocarpa* alleles into *P. balsamifera* as the western range is where the hybridization is most likely between these two sister species.

3. Future of the "forest crop"

In the Anthropocene, processes that used to take decades are now accelerated (Lugo 2015) which makes forecasting the sustainability of today's poplar culture and management practices a challenging task. Thus, in this section I will discuss some of the decisions made over the past 50 years in the field of poplar culture that could potentially have an important role in shaping the future poplar management.

The issues pertaining to poplar culture have two interacting facets: economy and ecology. The claim of economy is driven by the ever increasing world demand for wood products (be it pulp,

laminated wood, oriented strand board or biofuels). The economic potential often dictates to forest managers the types of decisions they take, which in turn affects the ecosystem.

As explained previously a typical poplar breeding program combines the selection of clones that are easy to propagate, have a short rotation, are resistant to pests and pathogens while maximizing fiber production. This is a hard task particularly in breeding for resistance to *S. musiva* in North America (Newcombe et al. 2001). The pathogen, as will be detailed later, has indeed been the main limiting factor to plantation programs in the eastern United States and Ontario in Canada (Ostry 1987). Similarly, in Europe and Pacific Northwest of North America, the breeding for resistance against *Melampsora* poplar leaf rust is the focal point. However in both cases, selecting resistant and productive clones have resulted in an introduction of non-native genotypes to both continents, namely the Asian *P. maximowiczii* and the European *P. nigra* to North America and the American *P. trichocarpa* and *P. deltoides* to Europe (Dickmann 2001). In some cases, these "exotic" genotypes have made their way, faster than anticipated, into natural populations in Quebec, particularly in peripheral populations of *P. balsamifera* (Meirmans et al. 2010). The same can be seen in Europe but at lower rates (Jelić et al. 2015). This is to be expected since the range of species used in North America for breeding poplars is larger than that used in Europe (Meirmans et al. 2010).

There seems to be two major approaches to dealing with this matter: interventionist and noninterventionist approaches. The first tries to act when chances of success seem favorable and the other adopts a more lenient attitude (Lugo 2015). Advocates of intervention cite examples where whole populations are wiped out because of lack of anticipation and understanding. Telford et al. (2015) identified several points where intervention is possible and where genetic drift and species extinction can potentially be broken. The noninterventionists believe that natural processes tend to self-regulate, hence intervention is not necessary.

However, in the case of poplars, it seems as if few decisions pertaining to poplar culture have been made with potentially global impact. Aside from the anthropogenic hybridization: (1) we are witnessing an important, anthropogenic, shift of range to the southern hemisphere with Chile and Argentina having intensive plantation programs (Carmona et al. 2015, Senisterra et al. 2012), (2) China has been using transgenic poplars since 2002 and is now the world's top producer of poplar wood (Häggman et al. 2013), and (3) the anticipated latitudinal shifts in both

natural and planted stands due to global warming and climate change. Poplars can be expected to be active players in climate change: their ability to adapt to different growing conditions is duly documented. The astounding capacity for poplars to adapt to changing environments in relatively short time frames will certainly be an asset for the genus (Raj et al. 2011).

II. Biology of *Sphaerulina musiva*

1. Infection biology and life cycle

More than seven decades ago, Thompson (1941) infected poplar leaves with –then named – *Septoria musiva* Peck. This revealed the sexual stage (teleomorph) of the pathogenic fungus and named it *Mycosphaerella populorum*. The same procedure also revealed the sister species *Mycosphaerella populicola* (anamorph *Septoria populicola*). Both species are present in North America even though *S. musiva* was reported as far south as Argentina (Senisterra et al. 2012). *M. populi* is the Eurasian sister species. For decades, the genus was pooled with *Mycosphaerellacae* alongside *Septoria tritici* a wheat pathogen. The ever growing genus became home to fundamentally different species hence a split became necessary (Crous et al. 2011). In recent years, septation and other morphological features ushered *Septoria musiva* and *Septoria populicola* into the realm of *Sphaerulina*, resulting in the renaming to *Sphaerulina musiva* (Peck) Quaedvileig, Verkley and Crous (Quaedvlieg et al. 2013).

Sphaerulina musiva overwinters in fallen leaves infected in the previous season as well as inside the canker of infected trees. By the spring, both conidia and ascospores have the potential of infecting leaves and barks mainly vectored by wind and rain splash (Bier 1939). The spores germinate in appropriate conditions and infect their host through natural openings as well as wounds (Qin and LeBoldus 2014). Leaf spots often precede cankers which develop shortly after in the growing season. The infection can also spread through the petiole to reach the closest stems or branches (Bier 1939).

Very little was known about the fungus and its biology until recently when the development of a non-wounding infection procedure provided better insight to the biology of the pathosystem (LeBoldus et al. 2010). Studies on infection and evolutionary biology of the fungus followed shortly after (Qin and LeBoldus 2014, Dhillon et al. 2015).

Scanning electron micrograph imaging suggested that the pathogen infects trees through natural openings without major differences between moderately resistant and susceptible clones. This supposes that the host resistance mechanisms take place after penetration of the mycelium into the host tissue (Qin and LeBoldus 2014). Once inside the plant, *S. musiva* seems to be involved in plant cells communication mainly mediated by chemotropism (Qin and LeBoldus 2014). The same is suggested through the finding of a battery of genes for secondary metabolite expression in *S. musiva* that could potentially be involved in cross-talk (Dhillon et al. 2015). In both susceptible and moderately resistant clones the penetration of *S. musiva* seems to be challenged by the induction of necrophylactic periderm by the plant tissue and for a still unknown reason the moderately resistant clones keep the fungus contained while the susceptible clones do not (Qin and LeBoldus 2014). Necrophylactic periderm is an organization of plant cells that takes places when the phenylpropanoid pathway is solicited to counteract a foreign agent (Qin and LeBoldus 2014). The traceability of that pathway could shed more light on the features of resistance to *S. musiva* that takes place in resistant clones but not in others. The signaling mechanism is still not fully understood such as for instance in *Agrobacterium tumefaciens*, and several plant and fungus effectors are yet to be discovered. However, important findings have already been made and will pave the way for a better understanding of the infection biology of . *musiva* and perhaps canker causing agents in general (Dhillon et al. 2015).

2. Metabolism and phylogeny

The study of canker causing agents in poplars recaptured interest upon the redeployment of plantations in western Canada in the last couple of decades. The genus *Septoria* is more studied in cereals, particularly in wheat with *S. tritici*.

Little work has been done to conduct a full, comprehensive study on the pathogen until recently (Dhillon et al. 2015). Early on studies identified selective medium for the growth and isolation of *S. musiva* thus making isolation from infected tissue more convenient (Spielman 1986). Later, Captan, a now banned fungicide and pesticide, was found to have a positive effect on mycelial sporulation (Stanosz and Stanosz 2002). The development of non-wounding inoculation methods using a spore suspension (LeBoldus et al. 2010) helped propel more recent studies.

Phylogenetically, the North American sister species of *S. musiva*, i.e. *Sphaerulina populicola*, causes mainly leaf spots on poplars but rarely stem canker in the field (Newcombe et al. 2001).

Leaf spots can affect productivity, though it's the canker that causes mortality, hence breeding programs are focused on minimizing cankers (Newcombe et al. 2002). The comparison between the two species of the fungi has revealed to be very useful in delineating the genes used by *S. musiva* to colonize the woody tissue that were not expressed in *S. populicola* in the same fashion. These genes include two main clusters: (1) an array of Carbohydrate-Active enZymes (acronym CAZy) that are used by *S. musiva* to extract and metabolize sugars from the woody matrix; and (2) a cluster of secondary metabolite genes thought to be used in the process of infection, perhaps in cross-talk with plant tissues or in paving the way for the mycelial growth and expansion in the plant tissue (Dhillon et al. 2015). However while the genes for metabolite production have been identified, the actual compounds are yet to be determined.

3. Host-parasite co-evolution

The initial taxonomy of *Sphaerulina* supposed a strict relationship between poplar hosts and the pathogen (Feau et al. 2006). However, this poses a problem in delineating closely related strains that co-occur on the same host (Feau et al. 2006). *S. musiva* was also found to infect willows as both willows and poplars belong to the Salicaceae (Feau and Bernier 2004) which casts doubts on its strict relationship to poplars. A deeper molecular study using several species of *Mycosphaerallacea* revealed that a model of host pathogen co-evolution was not plausible (Feau et al. 2006). A later study has also shown that *S. musiva* had most likely acquired its battery of CAZy genes through horizontal transfer from wood decaying fungi which explains why it causes canker while *S. populicola* does not (Dhillon et al. 2015).

Another study conducted on certain poplar hybrids and putative pure species clones found that the main source of variation in response to *S. musiva* infection in the tested *Populus* species was clonal variation rather than the difference in isolate provenances, inferring that clone x isolate interactions are superseded by variation in the response of clones (LeBoldus et al. 2008). For other pathogens of poplar, namely, *Melampsora* the causal agent of poplar leaf rust the screening of clones has to be performed against an array of pathotypes rather than clones (Stanton et al. 2010).

In North America, throughout both the eastern provinces of Canada, the prairies, and in the northeastern and central USA, *S. musiva* has become the limiting factor driving plantation development and screening for a mix of resistant genotypes to be used in a plantation is

mandatory. For example, despite its high productivity, the cross between *P. deltoides* x *P. maximowiczii* has shown to be highly susceptible to *S. musiva* and was discarded from further plantation programs in the north central USA (Riemenschneider et al. 2001). Also, anthropogenic hybrids deployed in plantations seem to be overall more prone to *S. musiva* attacks than in their natural stands in native range (Riemenschneider et al. 2001, Newcombe et al. 2001).

In every pathosystem there is an interaction between host, pathogen and the environment (disease triangle). Newcombe et al. (2001) highlighted the existence of disease escapes in areas where *S. musiva* is present and yet susceptible clones are not infected. It is still not clear as to why that is happening. Until clones from these escapes are artificially infected, it will be hard to pinpoint their mechanisms of resistance: environmental, genetic, epigenetic or an interaction of two or more factors. For instance, the populations of *P. trichocarpa* and *P. deltoides* grown in wet environments, more conducive to pathogen establishment showed more resistance to *Melampsora* leaf rust than those grown in dry environments where it is harder for the fungus to establish (Stanton et al. 2010).

Sphaerulina musiva was historically reported to infect poplars with noticeable damage in north eastern US, Quebec and Ontario (Ostry and McNabb 1985, Strobl and Fraser 1989, Mottet et al. 1991, Newcombe et al. 2001). In contrast, the northwest was thought to be free from *S. musiva* eruptions in both hybrid plantations and nurseries. Now, the fungus has become a problem also in the northwest, with recent eruptions in a nursery in British Columbia (Callan et al. 2007) and a plantation in Alberta (LeBoldus et al. 2009). *S. musiva* was present in the northwest in shelterbelts, however the more recent eruptions have raises questions about the nature and the cause of it moving from its historic eruption zones in the east to the west coast of North America.

III. Defense features in poplars

The genus *Populus* deploys a variety of mechanisms to ensure survival over its lifetime (Chen et al. 2009). Aside from the capacity for regenerations of poplars that was explained earlier, the tree also has a potential for tolerance (Erbilgin et al. 2014) and for chemical defense (Hwang and Lindroth 1997, Chen et al. 2009). Most of the body of work done on poplar chemical defenses

was mostly conducted on aspen with a focus on herbivores rather than pathogens. This helped tremendously in elucidating the ecological function of aspen defensive chemicals. Those defensive chemicals have been shown to be a reliable tracer of gene x environment interactions (Najar et al. 2014), and also found to be responsive to several features of climate change such as temperature, ozone and carbon dioxide (Koch et al. 2000, Couture et al. 2015). Secondary metabolites are also found to be dependable indicators of plant response to herbivores and pathogens, a feature of resistance and a biomarker for species (Nagle et al. 2011). However, as mentioned earlier, most of the studies addressing host-pathogen interactions measured simple and visible phenotypic signs of diseases more than the underpinning physiological and chemical changes (Caseys et al. 2012). Most of the research in this regard focused on the phenylpropanoids and salicinoids, both of which are carbon based defense compounds from the shikimic acid pathway. Salicinoids include phenolic glycosides known to be deterrents of herbivores, while phenylpropanoids include lignins and lignans units and polymers thought to act as a first line of defense against pathogens (Hwang and Lindroth 1997). The necrophylactic periderm formed upon the penetration of *S. musiva* is indeed linked to the phenylpropanoid metabolism (Qin and LeBoldus 2014).

Poplars however, have a wider array of defensive chemical compounds including both primary and secondary compounds, in particular terpenes, phenolic glycosides, volatile organic compounds and fatty acids (Chen et al. 2009). Phenolic glycosides are perhaps the most studied amongst all compounds initially for pharmaceutical properties and then for ecological functions (Boeckler et al. 2011). These compounds have the potential to defend poplars against a large variety of attackers (mammals and arthropods) since it targets the widely conserved enzyme β-glucosidase. The enzyme unleashes the phenolic moiety from the glycoside. The phenolic portion can be toxic to herbivores (Boeckler et al. 2011).

Aside from their defensive function, secondary metabolites have been used to distinguish between hybrids long before isozymes were used. Simple techniques like thin layer and paper chromatography were first used to fingerprint pure and hybrid poplars in southern Alberta (Rood and Mahoney 1991). This double function of defensive chemicals could be used to trace the direction of hybridization and predict hybrid features in terms of resistance. In the same line of thought, two research studies addressed divergent poplar species: *P. alba* and *P. tremula* and

their hybrids and tried to overlay genomic and metabolomics data to test – among other goals – if secondary chemistry could be a predictor of speciation and if hybrids have different chemical profiles (quantitatively or qualitatively) than their parents (Caseys et al. 2012, 2015). Using a metabolomic untargeted approach (both quantifiable and non-quantifiable compounds) mediated by Upper High Liquid Chromatography Mass Spectroscopy (UPLC-MS), the authors were able to find several patterns particularly in salicinoids and phenylpropanoids. The authors distinguished three main trends: (1) dominance of one of the parents; (2) hybrids being intermediate between parental phenotypes; or (3) hybrid dominance over both parents (Caseys et al. 2015). These studies tested interesting hypotheses and used solid methodologies, however the results could have been different if they had used a metabolite profiling approach (more like in chemical ecology), where only identified and quantifiable compounds are utilized as response variables and where collinear compounds are rigorously accounted for. Furthermore, a combination of both primary and secondary compounds has the potential to be more insightful since some primary compounds are precursors to a variety of secondary compounds.

1. Gaps between available genomics and metabolomics data in the *Populus* model

Indeed, "the promise of the metabolomics approach is to bridge the gap" between the genotype and the expressed phenotype in poplars, thus accounting for the ever growing list of regulatory mechanisms in between (Robinson and Mansfield 2011).

Recent works (Chedgy et al. 2015) used a functional genomic approach helped to uncover two acetyltransferases from *P. trichocarpa* with a potential role in the synthesis of benzoic acid derivatives. Also benzoic acid derivatives were found to be at the intersection of the phenylpropanoid and phenolic glycosides pathways, both of which are the backbone of defense in poplars as we know them (Babst et al. 2010, Whidalm and Dudareva 2015). However their use as a biomarker for plant response to *S. musiva* is yet to be investigated.

2. Mitigating resistance, market value and ecology: what is compromised and at what price?

As described earlier, the focus of managers and breeders is to combine volume and productivity with a short rotation time and disease resistance. One case study is the breeding and in some cases the genetic transformation to reduce lignins in poplars, as lignins are recalcitrant and energetically consuming to the industrial processes using poplar biomass in the production of

bioethanol or biodiesel. In fact, a modest down regulation of genes involved in lignin synthesis has been shown to increase production of ethanol by more than a third (Fu et al. 2011). The potential in hybrid poplar trees is substantially more important given the higher volumes of biomass they can produce, particularly in short rotations (Dwivedi et al. 1994, Shi et al. 2010).

This industrial approach (i.e. fast growing short rotation low lignin trees) aims at satisfying market demand, yet ignores the ecological meaning of lignins and the consequences of their removal (Mottiar et al. 2016). The biopolymer plays two major roles in the evolutionary history of land plants and the planet as a whole: (1) lignins have allowed plants to attain considerable sizes while sequestering substantial amounts of carbon dioxide; and (2) lignins are involved in a variety of defensive pathways (including the phenylpropanoid pathway) which are important for plant defense against pests and pathogens (Weng and Chapple 2010, Harding et al. 2014). It is thought that it took fungi the whole Carboniferous Period in evolutionary time scale (60 million years) to acquire the ability to overcome and degrade lignins, unlock carbon from trees and bring the levels of oxygen down to the current level of 20% (Floudas et al. 2012).

In this line of research, I am building upon previous findings in Dr. Erbilgin's lab, where sizeable amounts of levulinic acid were detected in several conifers (Ishangulyyeva et al. 2016), all species of poplars profiled so far (*P. tremuloides, P. balsamifera*, *P. trichocarpa*, *P. maximowiczii*) as well as their crosses. Levulinic acid is a key molecule in the conversion of biomass to biodiesel, however the actual engineering process leading to its production is not cost effective as it requires high temperatures and expensive catalyzers (Rackemann and Doherty 2011). Aside from its ecological meaning which was recently investigated (Ishangulyyeva et al. 2016), establishing the mechanism through which trees produce levulinic acid could open new opportunities in terms of breeding for biodiesel production and could have repercussions on downstream bioprocessing.

IV. Current objectives and research questions

The present work stems from the observation that there is an apparent difference in resistance of *P. balsamifera* infected with the canker causing agent *S. musiva* between its eastern and western range. From this observation, I hypothesize that the putative *P. balsamifera* showing susceptibility to *S. musiva* in the west (Alberta), are hybridized with the susceptible *P. trichocarpa*. This hypothesis is being tested in two steps: (1) by identifying how susceptibility to

S. musiva is transferred through controlled crosses; and (2) by sampling genotypes representative of the ranges from western to eastern Canada, of *P. balsamifera* and *P. trichocarpa* and infecting them with a representative mix of *S. musiva* isolates (from Alberta, Ontario and Quebec) in order to study the trend of the measured phenotypic traits (e.g. phenolic glycosides, fatty acids and benzoic acid derivatives, growth, stem and leaf infected areas).

The following questions are being investigated:

What information could be derived from controlled crosses in terms of the resistance of parents and their hybrids?

Is Alberta's *P. balsamifera*, a pure species?

How do different clones of pure *P. trichocarpa* and pure *P. balsamifera* respond to inoculation by *S. musiva*?

Is the increase in susceptibility of *P. balsamifera* observed from east to west caused by the introgression of *P. trichocarpa* into *P. balsamifera* or is it a more intricate process?

Can differences in composition of plant secondary compounds between pure *P. balsamifera* and pure *P. trichocarpa* explain the differences in tree resistance to *S. musiva* if observed?

Can introgression be predicted by plant secondary metabolite profiling?

V. Conclusion

Introgression zones offer a unique opportunity to study population dynamics, host-pathogen interactions and evolutionary biology. In my dissertation, I am working towards combining phenotypic data, including phytochemistry, with genotypic data in order to determine new players in the host-pathogen interactions between *S. musiva* and species and hybrids of *Populus* while gaining more understanding of the evolutionary biology of *Populus*.

Chapter 2: Breaking the wall of silence in trees, could plant chemistry foretell resistance in anthropogenic crosses?

I. Introduction

Given their fast growth, often short rotation and adaptation to a variety of site conditions, poplar (*Populus*) species and their hybrids have been used for wood products and eco-services over the last century in North America (Stettler et al. 1996). The ease of clonal propagation and the limited number of mating barriers made poplars the prime target for plantations and breeding programs (Riemenschneider et al 2001, DiFazio et al 2011).

In North America, native species such as *Populus trichocarpa* Torr. and A. Gray ex Hook, *P. deltoides* W. Bartram ex Marshall and *P. balsamifera* and exotic species such as *P. maximowiczii* A. Henry (*P. suaveolens*, aka: Japanese poplar) have been used as parents in breeding programs in a variety of combination of crosses. Such crosses have made it possible to harness advantageous characteristics from each species and result in hybrid vigour (Riemenschneider et al. 2001, Newcombe et al. 2001). For instance, *P. trichocarpa*, *P. maximowiczii* and *P. balsamifera* from the Tacamahaca section are coveted for high productivity and rooting success (Riemenschneider et al. 2001). Likewise, *P. deltoides* (section Aigeiros) cuttings are well adapted to flood disturbed sites with poor soil (Dickmann 2001) and sought-after for their resistance to pathogens, particularly *Sphaerulina musiva* (Teleomorph *Mycosphaerella populorum*), and the causal agent of Septoria stem canker and leaf spots in poplars. However, some breeding programs that deployed hybrids such as *P. deltoides* x *P. trichocarpa* for their coveted productivity, unpredictably faced poor resistance to *S. musiva* (Newcombe et al. 2001). The mechanism underlying this often poor pathogen resistance in hybrid poplars is unknown. To address this issue, the following questions are being investigated: how is resistance carried on in hybrids from half resistant and susceptible crosses? What tools and metrics could be used to foretell the performance of such hybrids?

Canker caused by *S. musiva* where conducive conditions are met is a major threat to plantations in North America as the pathogen can cause significant damage on poplars, leading to plantation failure (Ostry 1987). Native to the north east of the United States and Canada, *S. musiva* has

recently erupted in the western provinces (Callan et al. 2007, LeBoldus et al. 2009) with important levels of damage in the populations of poplars (Sakalidis et al. 2016) . For instance, in Alberta, *S. musiva* can infect putative *P. balsamifera* and hybrids in plantations and in controlled greenhouse experiments (LeBoldus et al. 2009). Additionally, in Quebec and British Columbia, where *P. maximowiczii* has been commonly used in breeding programs, both parents and hybrids are highly susceptibility to *S. musiva* canker (Dickmann 2001, Herath et al. 2016). *P. deltoides* is the only North American species known to be resistant to *S. musiva* and only shows symptoms of leaf spots. Whereas *P. balsamifera* was thought to be resistant to *S. musiva* canker in the pathogen's native range (Bier 1939, Waterman 1954, Sivanesan 1990), in Alberta it showed variable susceptibility to Septoria stem canker in plantation and greenhouse (LeBoldus et al. 2009).

Complete resistance to the pathogen designates genotypes that do not develop stem canker even when they may show leaf spots (Sniezko et al. 2016). Only *P. deltoides* and eastern *P. balsamifera* meet this definition. Other parents and hybrids show features of "partial resistance" or "quantitative disease resistance" where stem cankers can develop with different levels of the fungal spread (Sniezko et al. 2014, Sniezko et al. 2016), resulting in a visual susceptibility that can be graded and that reflects the interaction between the pathogen and the host tree. Although *P. deltoides* has been used in breeding for its resistance to *S. musiva,* its crosses with less resistant genotypes may not show full resistance to the pathogen. Breeders will have to mitigate partial resistance in order to meet demands of wood volume, site suitability, and disease resistance. This justifies the need for the development of predictive tools that could foretell performance without the tedious task of traditional screening which can be a time and resource exhaustive process.

Poplar's Salicinoids have established functions in describing and responding to the interaction between trees and their environment (biotic and abiotic) and have the potential to inform about poplar susceptibility or resistance to pests, insects and diseases (Hwang and Lindroth 1997, Constabel and Lindroth 2010, Najar et al. 2014, Couture et al. 2015). Defensive metabolites in poplars encompass both primary (fatty acids) and secondary (terpenes, salicinoids and phenylpropanoids) compounds (Chen et al. 2009). Fatty acids have shown to negatively affect the growth of certain tree pathogens and fatty acids metabolism is linked to the jasmonic acid

pathway, a central plant defense pathway (Turner et al. 2002, Ishangulyyeva et al. 2016). Establishing the role of plant chemicals in predicting performance against specific pathogens is the focus of this chapter. I hypothesize that by using genotypes ranging in response to the pathogen from complete resistance to partial resistance to *S. musiva* I would be able to reveal differences in selected metabolites that will have a predictive power in determining the performance of the hosts against the pathogen.

First, parents and offspring of three species, *P. deltoides, P. balsamifera* and *P. maximowiczii*, were infected with isolates of *S. musiva* from eastern and western Canada. Second, target compounds of the metabolic profile of all plants were quantified and investigated for the correlative and regressive relationships with the visual metrics of infection (e.g. infected stem and leaf areas and number of cankers along the stem). Finally, by establishing these relationships, I describe the potential for the of plant constitutive chemistry in predicting the pathogen's performance upon infection and also show how induced chemistry (after infection) complements that information.

II. Materials and methods

1. Plant material

Four parents corresponding to three species (*P. balsamifera*, *P. maximowiczii* and *P. deltoides*) and their offspring were used to describe how the plant response to the pathogen differs between parents and their hybrid progeny. Parental crosses and progeny genotypes are summarized in Table 1. The 13 genotypes represent the three parental species, five progenies from the *P. balsamifera* x *P. maximowiczii* cross, and four progeny from the *P. deltoides* x *P. balsamifera* cross. Out of the three parents, only *P. deltoides* is resistant to the pathogen while the other two are known to be susceptible to *S. musiva*. These parents provide a spectrum of anticipated responses to the pathogen ranging from complete resistance to high susceptibility with partial resistant individuals between these two extremes. In addition, the parents and their hybrids are particularly important as they have been used in breeding programs particularly in Quebec (Riemenschneider et al. 2001).

2. Plant growth

Cuttings (10 cm long) from 13 genotypes (Table 1) were collected from the Alberta-Pacific Forest Industries Inc. (Al-Pac) mill site in northern Alberta (latitude 52.92N, longitude -112.84W, elevation 560 m) in late March 2013. The cuttings were soaked in distilled water at 4°C for 24 hrs and then planted in 20 cm Rootrainers® trays (Spencer-Lemaire Industries, Edmonton, Alberta) filled with Metromix 290 soil (WR Grace and Co., Ajax, Ontario). Only the top bud remained exposed above the soil surface after planting. The trays were then placed in controlled atmosphere growth chambers with a day:night temperature of 23°C:10°C and a photoperiod of 16:8 hrs, 70% relative humidity with artificial lights at 600 μmol $m^{-2}s^{-1}$ photosynthetic active radiation. Upon leaves and stem expansion (two weeks after planting), the clones were fertilized only once with 500 ppm solution of 15-30-15 (N-P-K) fertilizer. When the plants reached 10-15 cm height, each plant was transferred separately to a 4 L pot filled with the same soil substrate amended with slow release fertilizer Nutricote (Sungro, MA, USA) 100 Days at 3 g L^{-1}. After transfer, plants were left to grow inside a greenhouse maintained at 25°C:15°C (day:night) supplemented with artificial light (≈ 400 μmol m^{-2} s^{-1}). All plants were taken outside to harden and undergo winter dormancy starting late August of the following year (2014).

In mid-February 2014 the plants were placed back into the greenhouse to break buds and flush leaves. Fertilization with 500 ppm solution of 20-20-20 (N-P-K) continued biweekly for five weeks. All plants had over a 90% survival rate except for the *P. maximowiczii* parent where only 11 ramets out of 16 survived. Trees were inoculated on March 2014, 40 days after returning to the greenhouse which was considered Day 1 of the experiment.

Cuttings were arranged in a block design randomized by both clones and isolates with a total of four separate blocks, each with 13 genotypes, three isolates and one control treatment. Each block contained 52 cuttings (13 genotypes x 4 trees (3 treatments + 1 control)). Cuttings in each block were moved on a weekly basis in order to reduce location effects. A total of 41 control and 122 infected trees survived until harvest on Day 110.

3. Inoculum preparation

Three isolates of *S. musiva*, namely APB4 from Alberta (LeBoldus et al. 2009), ONT0902 from Ontario, and SO424 from Quebec (LeBoldus et al. 2008), were selected. Cryogenic vials with pure isolates were taken from -80°C storage and thawed at room temperature then each isolate

was propagated on Petri-dishes containing KV-8 growth medium, which consists of 180 mL V-8 juice (Campbell Soup Company, Camden, NJ, USA), 2 g calcium carbonate (Anachemia Canada Inc. Montreal, QC), 20 g agar (BD Bacto™ Agar, Becton, Dickinson and Company, Sparks, MD, USA), and 820 mL distilled deionized water, amended with streptomycin at 25 $mg.L^{-1}$ and chloramphenicol at 300 $mg.L^{-1}$ (Sigma Aldrich). The isolates were then left to grow under full spectrum light bulbs (Gro-Lux®, Sylavania®, Osram Gmbh, Munich, Ger) for 3-4 days until the surface of the plate was covered with dark fungal mycelium. A plug of agar (1 cm^2) was transferred to fresh KV-8 agar petri dishes. All plates were kept under the same conditions described earlier. For each isolate, up to 50 plates were prepared in order to provide enough inoculum for the experiment.

4. Inoculation

On Day 60, inoculations were carried on using a non-wounding technique (LeBoldus et al. 2010). Briefly, the fungal spores were recovered from the plates by swirling about 1 mL of distilled water while scrapping the mycelium with a glass rod. The concentration of the recovered spore suspension was then measured with a haemocytometer (Bright-Line™, Reichert, Buffalo, NY, USA). The final concentration was standardized to 10^6 spores mL^{-1}. Each plant was profusely sprayed with a spray bottle until the spore solution covered the whole leaf and stem area. Different spray bottles were used for each isolate. Control plants were mock inoculated with a solution of water. Each individual plant was then wrapped in a black six inches plastic bag and moved to a dark room at 20°C for 48 hours to promote spore germination. Plants were then transferred back to the greenhouse. At approximately Day 81, three weeks after inoculation, the first leaf spot symptoms appeared and five to six weeks after inoculations cankers became visible. Re-isolations were only performed on plants showing no visible signs of the disease (i.e. pycnidia observed under microscope for spores) and were performed on KV-8 selective medium in order to confirm infection according to Koch postulates. The majority of the plants did show signs of the disease easily validated with microscopic observation.

5. Disease severity assessment

I used three different metrics to estimate disease severity. Infected stem area (metric one) was estimated by tracing and coloring the infected area on a transparent sheet. On each sheet, the infected area was estimated using image processing software Assess 2.0 (American

Phytopathological Society). The infected area was then adjusted to the total area of the main stem. By approximating stems and branches to a conical trapezoid shape, the total area of the plants' woody tissue was calculated using the base (b) and top (t) collar diameters and the length (h) of the stem as per the following formula: $A = \frac{1}{2}\, h\, (b + t)$.

The infected leaf area (metric two) was measured by collecting five to 10 infected leaves per plant from every infected plant with clear symptoms. The leaves were flattened and scanned using a digital scanner. The recorded images were then analyzed with Assess 2.0.

The number of entry points (metric three) was visually recorded wherever a fungus caused wound was observed. When cankers were extensive (a merger between two or more cankers), the entry point was assumed from the shape of the stem (wherever obvious loss of structure and sometimes oozing were observed).

6. Chemical analysis

After measurements of infected leaf area were taken, all leaves per plant were placed in labelled aluminum foil and immediately dipped in liquid nitrogen and then stored at -40°C. The leaves were then freeze dried and ground using a Mini Wiley Mill with a 40 mesh screen (Thomas Scientific, Swedesboro, NJ, USA).

Fatty acids were extracted in a one-step extraction-methylation procedure. The efficiency of the extraction was validated using trinonadecanoin as a recovery standard. The profile for fatty acids was analyzed with by GC-MS followed by a quantification using analytical standard and validated through the use of internal standard (Curtis et al. 2008, Ishangulyyeva et al. 2016). Along with fatty acids the same method revealed benzoic acid derivatives, namely anisic acid, benzoic acid and salicylic acid. The presence of these compounds was also confirmed by analytical standards and quantified by standard curves.

Phenolic glycosides and soluble tannins were extracted following Najar et al. (2014) and their concentrations determined by UPLC-DAD (Agilent 1290, Agilent Tech, Santa Clara, CA, USA). Briefly, a gradient of 0.1% acetate in acetonitrile and 0.1% acetate in water was used on a UPLC column ZORBAX RRHD EclipsePlus C18, 1.8µm 2.1 x 150 mm (Agilent Tech). The method was validated using methyl cinnamate (TCI chemicals, Portland, OR, USA) as internal standard

as it elutes outside of the range of the compounds of interest with little to no interaction with the matrix.

A total of 16 compounds were identified and quantified with authentic standards by both methods. Fatty acids methyl esters standards were acquired from NU-CHEKPREP INC (Elysian, MN 56028, USA), phenolic glycosides and soluble tannins were gratefully supplied by Dr. Rick Lindroth (University of Wisconsin-Madison, USA) and benzoic acid and its derivatives were purchased as analytical standards (Sigma-Aldrich).

7. Experimental design and statistical analysis

The experiment was initially designed to test for the following model,

$$Y_{ijk} = \mu + Blk_i + Isolate_j + Clone_k + Clone_k \text{ x } Isolate_j + \varepsilon_{ijk}$$

Where:

Y_{ijk}: is the measure of the three disease metrics (infected stem area, infected leaf area, number of cankers).

Blk_i: the random effect of the i^{th} block, i = 1– 4.

$Clone_k$: the fixed effect of the k^{th} clone, k = 1– 13.

$Isolate_j$: the fixed effect of the j^{th} isolate, j = 1– 3.

$Clone_k$ x $Isolate_j$: the fixed effect of the interaction between the k^{th} and the j^{th} isolate, l = 1 – 3.

$\boldsymbol{\varepsilon}_{ijk}$: residual error term.

All the statistical tests were computed using R (R core team 2016). ANOVA tests were performed using the nlme package and least significant difference test (LSD test) was performed using the agricolae package. Canonical correspondence analysis (CCA) was also used to establish multivariate relationships among clones and between isolates using the candisc package (Friendly and Fox, 2016) and permutational multivariate ANOVA (perMANOVA) was used to assess the validity of the CCA models using the adonis2 function from the vegan package. Heat maps were used to monitor the quantitative difference between infected and control plants as well as the relationship between clones via corresponding cluster analysis. gplot package was

used to plot the heat maps and their corresponding cluster analysis while RColorBrewer package was used to generate the color spectrum. The cluster analysis used Euclidean distances and the validity of linkage method was performed by correlating the cophenetic matrix to the original distance matrix.

Traditional approaches to prediction from a set of multivariate data tend to opt for the use of multiple regression which requires assumptions of normality and homogeneity of variances along with a strict control for collinearity. Classification and regression trees (CART) do not require these assumptions and preserve information by accounting for the complexities of interactions within data, a process that linear or logistic regressions fail to capture (Hayes et al. 2016). I used Classification And Regression Trees (CART) to describe the relationship between the visuals of disease symptoms and the quantified metabolome (the identified and quantified 16 compounds) at the constitutive level and establish how constitutive chemistry could predict those disease metrics.

More specifically, I used Conditional Inference Trees (a variant of CART) which are non-parametric classification regression trees that help determine how different plant genotypes group together in function of both their symptomatic response (i.e. visual metrics of disease severity) and their chemical signature at the constitutive and induced levels. The output also determines the concentration thresholds of the most important compounds that predict the response and explains the classification. This statistical technique is the most appropriate as it controls for multicollinearity while maximizing information gain by harnessing more meaning from existing associations between compounds in relationship to the response variable (metric of disease severity). RandomForest was used in order to model and validate this relationship. The predictive power of the model was assessed using the Area Under the Curve (AUC) parameter. The different analyses were performed using RandomForest package (Liaw A and Wiener M, 2002) in order to generate the model and the function ctree from the party package (Hothorn et al. 2006) in order to generate the corresponding conditional inference tree.

III. Results

1. Visual assessment of disease symptoms

1.1. Plant response

Overall, differences between genotypes accounted for most of the variation in cankered stem area ($F_{(12,400)} = 8.82$, $p < 0.001$), infected leaf area ($F_{(12,154.4)} = 8.43$, $p < 0.001$) and the number of canker entry points ($F_{(12,141.5)} = 6.27$, $p < 0.001$, Table 2). All three metrics of disease severity showed significant variation among parents (Fig. 1). The complete resistance of *P. deltoides* to *S. musiva* was confirmed as it had no infected stem and the lowest infected leaf area. *Populus maximowiczii* sustained significantly lower levels of infected stem area (p=0.05), than both *P. balsamifera* parents (Fig. 1A) and lower infected leaf area than one of *P. balsamifera* parents (Fig. 1B). However there was no significant difference in the infected leaf area (Fig. 1B) or the number of canker entry points (Fig. 1C) between *P. maximowiczii* and *P. balsamifera*. Though different in sex, both *P. balsamifera* parents responded similarly to the disease.

The responses of the nine offspring in terms of cankered stem area were highly variable: partially resistant clones show more relatedness to their most resistant parent. For instance, AP-4576 was statistically similar to its resistant parent *P. deltoides*. In addition, relatively more susceptible clones sustained levels of infection metrics comparable to those of their most susceptible parent i.e. *P. balsamifera* (Figs. 1A and 1C). For example, AP-4578 showed a similar response to its susceptible *P. balsamifera* parent. Two genotypes (AP-4577 and AP-4579) from the *P. deltoides* x *P. balsamifera* cross were intermediately susceptible between their two parents. In the *P. balsamifera* x *P. maximowiczii* cross, we can distinguish two main groups in terms of the spread of stem infection: AP-4147 and AP-4149 were as susceptible as their most susceptible parent, *P. balsamifera,* while the rest of the clones (AP-4146, AP-4148 and AP-4150) were intermediate between the two parents. However, for the infected leaf area, most of the F_1 offspring of *P. deltoides* x *P. balsamifera* showed more similarity to the resistant parent (*P. deltoides*) and had less variability than the hybrids from the susceptible cross (*P. balsamifera* x *P. maximowiczii*). It is also noteworthy in this context that there was a high correlation between the infected leaf area and the number of canker entry points (Spearman coefficient of correlation = 0.75, $p < 0.01$).

1.2. Isolate performance

Isolates from different provenances differentially affected stem cankers ($F_{(2,15.34)} = 4.06$, p = 0.02), leaf spots ($F_{(2,10.12)} = 6.63$, $p = 0.002$), and the number of canker entry points ($F_{(2,9.26)}$ = 4.93, $p = 0.009$). Overall SO424, isolated from the native range of *S. musiva* in eastern Canada, was the most aggressive pathogen although the other two isolates (APB4 and ONT0902) caused

similar responses (Fig. 2). There also was a significant interaction between isolates and clones for infected leaf area and the number of canker entry points (Table 2). These interactions were visible in certain clones. For instance, the *P. balsamifera* parent crossed with *P. maximowiczii* was highly susceptible to both ONT0902 and SO424 and less susceptible to APB4. In contrast, the other *P. balsamifera* parent was highly susceptible to SO424 but not the other two isolates (Fig. 3). The performance of SO424 is confirmed by the integrated area under the curve representing the amount of damage the isolate sustained across all clones.

2. Effects of clonal variability and pathogen infection on plant chemistry

Constitutive clonal variability

Mock inoculated plants showed variability in their leaf chemistry. The identified chemicals explained about two thirds of the difference (62.3%) between *P. deltoides* and *P. maximowiczii* (Fig. 4A and 5A). *Populus deltoides* was characterized by high levels of levulinic acid and tremulacin and *P. maximowiczii* with high levels of benzoic acid derivatives (anisic acid, salicylic acid, benzoic acid, and tremuloidin). Furthermore, the differences in chemistry between both *P. balsamifera* parents and the remaining parents explained about 20% of the variation (Fig. 4A). Two *P. balsamifera* parents were characterized by higher oleic acid and salicortin levels compared to *P. maximowiczii* and *P. deltoides* parents (Figs. 4A and 5A).

The offspring from both crosses were intermediate between their parents. The F_1 offspring from both susceptible parents (*P. balsamifera* x *P. maximowiczii*) were similar as shown by the overlap of their spread in the canonical analysis; whereas F_1 offspring from one resistant and one susceptible parent (*P. deltoides* x *P. balsamifera*) were slightly different (Fig. 4A). The permutational MANOVA reveals that clonal differences are the main drivers of differences in chemistry (Table 3A).

At the constitutive level, two groups of chemical compounds shape the differentiation between the clusters of plant genotypes as revealed by the cluster analyses corresponding to the heatmap (C1 and C2 Fig. 5A). The North American parent species (*P. deltoides* and *P. balsamifera*) along with the F_1 offspring of the *P. deltoides* parent cross cluster together and show relatively high levels of the group C1 (α-linoleic acid, γ-linolenic acid, stearic acid, palmitic acid, behenic acid, soluble tannins, salicin, tremulacin, and levulinic acid). Whereas *P. maximowiczii* along with its offspring formed a separate group characterized by high levels of chemicals in the group C2

(salicortin, oleic acid, salicylic acid, pentadecanoic acid, tremuloidin, benzoic acid, and anisic acid).

3. Induced chemistry

The constitutive patterns between chemical compounds and clones were mostly maintained in infected plants. Comparisons between Figures 4A and 4B show that *P. deltoides* and *P. maximowiczii* maintained their positions in the canonical projection while they differed from other clones. There was however a shift in the associations of certain chemical compounds; γ-linolenic acid, for instance became associated with *P. deltoides* only after infection. Differences in chemistry between *P. balsamifera* parents and the other two parents explained 24% of the overall differences between clones. Both *P. balsamifera* parents show similar chemical signatures when subjected to *S. musiva* infections as shown by the overlap of their spread in the canonical projection (Fig. 4B).

However, a comparative look at the heatmaps and cluster analyses (Figs. 5A and 5B) shows that quantitative variations among chemical compounds reorganized the clones differently at the induced level. The two clusters that characterized the constitutive level are no longer present at the induced level. It is notable that *P. maximowiczii*'s response to *S. musiva* was drastically different from that of all other clones including its own offspring with a unique chemotype characterized by high levels of pentadecanoate, tremuloidin, benzoic acid, anisic acid, and salicylate. The chemotype of *P. maximowiczii* was similar in both its constitutive and induced levels.

Overall, at the induced level, genotypes structured mainly according to quantitative variations of individual chemicals rather than groups of chemicals such as at the constitutive level. The F_1 offspring responded to the infection similarly to one or both of their parents (for certain chemical compounds) or uniquely by showing individual responses (for other chemical compounds). The chemotypes of the F_1 offspring from the resistant cross (*P. deltoids* x *P. balsamifera*) showed similarities at the induced level and varied slightly from their chemotype at the constitutive level (Fig. 4B). Although their responses to canker were different, hybrids from the *P. balsamifera* x *P. maximowiczii* cross showed more similar chemistry at the induced level than that at the constitutive level. It is also noteworthy that progeny AP-4576, the most resistant hybrid to stem canker, clustered separately due to high levels of soluble tannins, salicin, and tremulacin and low

levels of levulinic acid (Fig. 4B). *Populus deltoides* clustered with AP-4577 and AP-4578 mainly due to high levels of γ-linolenic acid, tremulacin and levulinic acids. On the other hand, AP-4579 had more similarity to its *P. balsamifera* parent mainly because of the high levels of salicylate, behenate as well as the similar low levels of tremuloidin, benzoic acid, anisic acid and γ-linolenate.

Similar to the constitutive level, differences between individual clones were the main source of variation in plant induced chemistry ($F_{(12,7668)} = 8.82$, $p < 0.001$; Table 3B). The provenance of isolates had no effect on plant chemistry ($F_{(2,165.1)} = 4.06$, $p = 0.43$). However, the interaction of clone and isolate was significant ($F_{(24,2921)} = 1.44$ $p < 0.01$). For instance, SO424 is associated with higher levels of anisic acid in AP-4576 but lower levels of the same chemical in *P. deltoides*. Likewise, levulinic acid was higher in AP-4577 and *P. deltoides* parent inoculated with ONT0902 (Appendix 2). Also mock inoculated and infected plants were significantly different in terms of metabolic profile ($F_{(119,1)} = 2.63$, $p < 0.01$, ANOVA table not shown). In brief, the chemistry of infected and non-infected- plants was different not because of specific chemicals, rather by changes in the overall chemical profile of the various genotypes used in this experiment.

4. Predicting tree responses to *S. musiva* using their chemistry

Since all three metrics of disease severity were highly correlated, we chose one metric as a response variable that describes most of the biological effect of the fungus on the plants. The relative infected stem area was selected because it translates the damage potentially leading to the death of the tree and captures both the performance of the fungus and the response of the tree.

In order to assess the ability of constitutive chemistry of mock inoculated plants in predicting tree responses to *S. musiva*, the infected stem area for each genotype was averaged and assigned to each individual tree. I then used 16 quantified chemical compounds as explanatory variables for the infected stem area. Then, the tree response was modeled using RandomForest since such models control for multicollinearity and non-linear distributions. All genotypes including parents and hybrids were pooled together in order to capture the full spectrum of tree response (in terms of visual and chemical metrics) to *S. musiva*. The model shows that tremulacin, tremuloidin and benzoic acid are the three most important compounds in predicting the response across all

genotypes despite their differences to *S. musiva* infection (Fig. 6A). The model was validated by using the Area Under the Curve (AUC) parameter of the Receiver Operating Curve (ROC). I considered all genotypes (except *P. deltoides* which is the only genotype with complete resistance to the pathogen) as susceptible regardless of the level of resistance they showed to stem infection and used RandomForest model for validation. Thus, *P. deltoides* ramets were assigned to 0 (resistant) and the rest of the genotypes to 1 (susceptible). The ROC analysis yielded an AUC = 0.772, which portrays a fair to good model prediction (0.7 represents a fair model and 0.8 represents a good model). Hence, poplar constitutive chemistry is a reasonable predictor for the response of poplar trees tested against *S. musiva.*

Further, regression trees were used to define classes of responses from all 13 genotypes against *S. musiva* based on concentration thresholds of key chemicals validated by the RandomForest model. Regression trees grouped plants based on their response to infection (e.g. high, low or moderate resistance to stem canker) as a function of the concentrations of certain chemicals (i.e. explanatory variables). The compounds found to be predictive of tree response were consistent with the RandomForest output. At the constitutive level, only tremuloidin and tremulacin were predictors for the extent of disease symptoms. The resulting output classifies all genotypes into three groups: (1) a highly susceptible group which sustained the highest levels of canker spread and included both *P. balsamifera* parents; (2) a quantitative resistant group which sustained no or low damage and included *P. deltoides* and two of its most resistant offspring AP-4576 and AP-4577; and (3) a moderately susceptible group which were average between (2) and (3) and included *P. maximowiczii* parent and all other remaining offspring (Fig. 7A). The classification tree correctly predicted the levels of infection for all clones except for *P. maximowiczii*, which showed low levels of infection comparable to that of AP-4576 (Fig. 1A). Overall, clones with high levels of tremulacin and tremuloidin at the constitutive level showed more resistance to *S. musiva* canker.

Similarly, RandomForest and regression trees were used to determine how induced chemistry explains the observed disease severity (infected stem area). Parallel to the constitutive level, the RandomForest model revealed that tremulacin, tremuloidin and benzoic acid were the main contributors in explaining the infected steam area (Fig. 7B). The model was validated with AUC=0.78 which indicates a fairly good predictive power of chemistry. However, the regression

tree analysis showed a different classification: unlike for mock inoculated plants, the level of benzoic acid separated the resistant genotype (*P. deltoides*) from the partially resistant clone AP-4576. Likewise, tremuloidin levels separate different classes of genotypes susceptible to *S. musiva*; for instance, low levels of tremuloidin were a determinant factor in pooling *P. balsamifera* parents together and distinguishing them from the rest of their hybrids. Overall, higher tremuloidin level was associated with higher partial resistance to canker spread (Fig. 7B).

Furthermore, correlation graphs were established between the constitutive and induced levels of the three chemicals (tremulacin, tremuloidin, and benzoic acid) to assess their reliability as predictors of tree resistance (Appendix 1). The constitutive and induced levels of all three compounds were highly correlated. Additionally, benzoic acid, a key compound in predicting the infected stem area as revealed by RandomForest, did not affect CART classification of the different genotypes at its constitutive level. Its concentration however seems to be indicative of pathogen recognition as shown by CART of infected trees since it was the compound that distinguished resistant and partially resistant genotypes. Given the seeming importance of benzoic acid, I plotted the constitutive concentrations of benzoic acid against infected stem area averaged by genotype (Appendix 3). The graph in appendix 3 shows that the predictive trend of benzoic acid depends on the cross: in the resistant cross, higher concentrations of the chemical compound were associated with higher susceptibility while the opposite is true for the susceptible cross [*P. balsamifera* x *P. maximowiczii*].

IV. Discussion

1. Difference in susceptibility across poplar genotypes

Differences between genotypes accounted for half of the variation (50%) in infected stem area. The response to *S. musiva* ranged from the resistant parent (*P. deltoides*) to the highly susceptible genotype AP-4147. Both *P. balsamifera* parents and their F_1 offspring sustained consistently higher levels of pathogen infection than the other parents or offspring, demonstrating that the susceptibility trait passed from parent to F_1 offspring through crosses. These results are consistent with the reports about high susceptibility of *P. balsamifera* to *S. musiva* in a combined field and greenhouse study in Alberta (LeBoldus et al. 2009).

According to Fritz et al. (1999), hybrids tend to follow one of the following four trends in relation to their parents: (1) more susceptible than parents, (2) more resistant than parents, (3) intermediate between the parents, or (4) similar to one of the parents. When all isolates are averaged, we only observe the last two trends (3 and 4) as none of the F_1 offspring surpassed the parents in their susceptibility or resistance. However, even though the clone-isolate interaction was not significant for infected stem area and the isolate effect was modestly significant, for certain isolates, some F_1 offspring surpassed susceptibility of their *P. balsamifera* parents. For instance, AP-4149 was more susceptible for the Albertan isolate APB4 than its corresponding *P. balsamifera* parent. Similarly, both *P. balsamifera* parents were less susceptible to APB4 but were most susceptible to the SO424 isolate from Quebec.

2. Difference in isolate performance

Isolate effect accounted for 23% of the variation in the percent infected area and the clone by isolate interaction was not significant. These results are in disagreement with earlier studies conducted using some of the same isolates on different poplar genotypes (LeBoldus et al. 2008, 2009, 2010). Earlier studies reported that isolate effects were not significant while clone by isolate interactions were significant. It is noteworthy that the SO424 isolate from Quebec caused more damage (all three disease metrics) than the other two isolates (APB4 and ONT0902) and all three susceptible parents were relatively more susceptible to SO424 than APB4. These results suggest that plant-pathogen interactions as poplar clones bred for resistance to local isolates of in one location can be susceptible to different isolates of the same pathogen in another location. This phenomenon was reported in poplars as hybrids of *P. alba* x *P. grandidentata* are usually bred for resistance against the rust pathogen *Melampsora populnea* in the Midwest in North America, but when planted in the Pacific Northwest they showed unexpected susceptibility (Newcombe et al. 2001).

Early reports describe *P. balsamifera* as resistant to canker development in the pathogen's native range in the north and central part of eastern North America where balsam only sustains leaf spots (Bier 1939, Waterman 1954). In Alberta *P. balsamifera* has been shown to be highly susceptible to *S. musiva* canker (LeBoldus et al. 2009). I found that both *P. balsamifera* parents, originating from Alberta, showed relatively more resistance to APB4 and more susceptibility to SO424, suggesting that they are more adapted to the Albertan isolate than the Quebec isolate.

Likewise, *P. maximowiczii* showed stem cankers and leaf spots after infection only by SO424, further validating the importance of co-evolutionary relationship between the pathogen and the host.

Populus maximowiczii is commonly used in breeding and plantation programs in eastern North America and known to be prone to canker in the native range of the pathogen (Newcombe et al. 2001) and yet mildly susceptible to *S. musiva* in the field in Alberta (Barb Thomas, personal communication). The response of *P. maximowiczii* in the present work corroborates with the literature as it was mostly susceptible to the isolate from Quebec (SO424) but not to APB4 and ONT0902 both of which are not from the native range of the pathogen (Newcombe et al. 2001).

Finally the correlation between all disease metrics suggests that the use of one could make up for the use of the others particularly in the field where leaf spots are more present and easier to survey (LeBoldus et al. 2009).

3. Plant chemistry differs between species

From a chemistry viewpoint, most of the differences observed among clones was driven by the difference between *P. deltoides* and *P. maximowiczii*. These two species belong to distant sections in the genus *Populus*, respectively Aigeiros and Tacamahaca. These species also cover two distinct geographic ranges as *P. deltoides* stretches in north central and eastern North America all the way to the eastern slopes of the Rockies, while *P. maximowiczii* is an East Asian species. *Populus balsamifera* belongs to the Tacamahaca section and populates most of the boreal region in North America. *Populus balsamifera* showed a unique chemical profile and is at equal canonical distance from *P. deltoides* and *P. maximowiczii*. I expected members of the Tacamahaca section to show similar chemical profiles as they are more genetically similar (Isabel et al. 2013, Cervera et al. 2005). However, heatmap and cluster analysis show that *P. maximowiczii* tends to either group with its own offspring at the constitutive level or form a separate cluster when infected with *S. musiva*. Likewise, the parents from North America tend to group in relatively closer clusters and show more relatedness compared to the East Asian parent. For instance, *P. maximowiczii* had 50 times more benzoic acid than *P. deltoides* and about nine times more than *P. balsamifera*. It is not clear why geographic proximity would transcend genetic proximity as *P. balsamifera* shows more chemotypic similarity to *P. deltoides* upon infection by *S. musiva* rather than its genetically closer *P. maximowiczii*. One possible

explanation is that environmental factors play a critical role in transcriptome and metabolite expression of poplars as shown in earlier studies on hybrid poplars (Raj et al. 2011). It is worth mentioning that *P. balsamifera* and *P. deltoides* parents are from central and southern Alberta respectively.

Once infected with *S. musiva*, *P. maximowiczii* was the only clone that maintained the same chemical profile. Though *P. maximowiczii* sustained very low levels of pathogen infection, it is hard to associate the low levels of cankered area to the high level of benzoic acid derivatives because *P. deltoides* and AP-4576 had respectively no or equally low levels of infection when compared to *P. maximowiczii*, but they also had significantly less amount of the benzoic acid.

4. Role of chemistry in predicting susceptibility disease

CART and RandomForest analyses provided consistent results about which chemicals can be used to predict poplar susceptibility. Tremulacin and tremuloidin at the constitutive level and benzoic acid at the induced level were the most important predictors to explain the extent of pathogen infection. The validation method using AUC suggests the model has a fairly good predictive power.

At the induced level, a threshold concentration of benzoic acid was a determining factor in separating resistant from partially resistant poplars to stem canker. These results suggest that benzoic acid can potentially be indicative of host – pathogen recognition as its low concentration (such is the case for *P. deltoides*) seems to be indicative of complete resistance to the pathogen. This information can be useful for breeders and molecular biologists in modulating the expression of the gene(s) controlling the production of benzoic acid and testing the implications of change in its level on resistance to *S. musiva*.

Since both *P. balsamifera* x *P. maximowiczii* and *P. deltoides* x *P. balsamifera* hybrids had on average similar disease responses. However, the relationship between benzoic acid and canker spread (measured by the infected stem area metric) was mostly cross-dependent, i.e. it depends more on the parents used in the crosses. My results show that at low concentration an increase in the concentration of benzoic acid was linearly associated with an increase in canker spread in *P. deltoides* x *P. balsamifera* cross whereas in the *P. balsamifera* x *P. maximowiczii* an increase of the concentration of benzoic acid was associated with a decrease in canker spread (Appendix 3).

This result supports the idea that benzoic acid could potentially be a proxy for quantitative resistance as observed in the present study. However, some genotypes with significantly different levels of benzoic acid show similar response to *S. musiva* infection (e.g. AP-4576 and *P. maximowiczii*). This further suggests that the predictive use of benzoic acid should be cross specific.

The levels of benzoic acid, tremuloidin and tremulacin remained stable across all 13 genotypes regardless of infection, suggesting that these compounds are most likely indicators or biomarkers of resistance/susceptibility of *P. balsamifera*, *P. deltoides*, *P. maximowiczii* and their hybrids that are not affected by infection. Indeed, benzoic acid derivatives such as temulacinin and tremuloidin have been widely investigated in *P. tremuloides* and *P. tremula* as deterrents and toxic to herbivores. This class of chemicals was found to be relatively stable across environments, highly heritable and more responsive to clonal variations than inductions by herbivory or damage (Hwang and Lindroth 1997, Osier et al. 2000, Keefover-Ring et al. 2014). All these studies suggest that these compounds can be used as a biomarker of resistance/susceptibility for several poplar species.

In conclusion, leaf chemistry was used as an accessible predictive tool of the resistance/susceptibility potential of poplars within the framework of our parents and hybrid selection. Machine learning and data mining techniques are fairly new and their use in predictive biology for fields other than the medical field is yet to be popularized (Li et al. 2016, Demertzis et al. 2015).

Tables and Figures Chapter 2

Table 1: Summary of information about *Poplar* species used in the experiment. Clones starting with AP prefix in their collection ID come from the Alberta Pacific Inc. Industries Mill collection. *Populus maximowiczii* (77594) was supplied from Pierre Perinet's collection in Quebec (Canada).

	Cross 1		**Cross 2**	
Putative genotype	*P. deltoides*	*P. balsamifera*	*P. balsamifera*	*P. maximowiczii*
Provenance (native to)	Southern Alberta	North east Alberta	North east Alberta	Japan
Sex	♀	♂	♀	♂
Susceptible/Resistant	Resistant	Susceptible	Susceptible	Susceptible
Collection ID	AP-2440	AP-2878	AP-2884	77594
Progeny clones (F_1)	AP-4576 AP-4577 AP-4578 AP-4579		AP-4146 AP-4147 AP-4148 AP-4149 AP-4150	

Table 2: Interaction Analysis of variance (ANOVA) of all 13 clones (parents and F_1 offspring), isolate and clone isolate interaction for three measured disease metrics. **A**: Relative infected stem area; **B:** Relative infected leaf area; **C:** Number of cankers' entry points

Table 2.A.

Source of variation	Df	Sum of Squares	Mean Sq	F value	Pr
Block	3	26.587	8.862	2.345	0.077
Clone	12	400.007	33.334	8.819	<0.001
Isolate	2	30.683	15.342	4.059	0.020
Clone x Isolate	24	130.574	5.441	1.439	0.106
Residuals	108	408.200	3.780		

Table 2.B.

Source of variation	Df	Sum of Squares	Mean Square	F value	Pr
Block	3	10.268	3.423	2.243	0.087
Clone	12	154.383	12.865	8.431	<0.001
Isolate	2	20.243	10.121	6.633	0.002
Clone x Isolate	24	85.855	3.577	2.344	0.002
Residuals	108	164.797	1.526		

Table 2.C.

Source of variation	Df	Sum of Squares	Mean Square	F value	Pr
Block	3	8.384	2.795	1.488	0.222
Clone	12	141.466	11.789	6.277	<0.001
Isolate	2	18.530	9.265	4.933	0.009
Clone x Isolate	24	83.752	3.490	1.858	0.017
Residuals	108	202.827	1.878		

Table 3: Permutational Multivariate Analysis of Variance of plant chemistry response .
A: At the constitutive level across the 13 genotypes of poplars (parents and their F_1 hybrids); **B.** at the induced level across the 13 genotypes of poplars (parents and their F_1 hybrids), the 3 isolates from different geographic provenances and the interaction between clones and isolates.

Table 3.A.

Source of variation	Df	Sum of Squares	F value	Pr(>F)
Block	3	489.6	2.6	0.02
Clone	12	3568.1	4.7	<0.001
Residuals	25	1590.0		

Table 3.B.

Source of variation	Df	Sum of Squares	F value	Pr(>F)
Block	3	282.9	1.17	0.3
Clone	12	7668.6	8.819	<0.001
Isolate	2	165.1	4.059	0.43
Clone x Isolate	24	2921.4	1.439	<0.01
Residuals	80	6449.7		

Figure 1: Assessment of disease severity with three disease metrics for each poplar genotype. Parents are in red, hybrids of *Populus deltoides* and *P. balsamifera* cross are coded AP-45xx. The code for hybrids from the *P. balsamifera* and *P. maximowiczii* cross starts with AP-41xx. Metrics include:

(**A)** Infected stem area relative to the total stem area. (**B)** Infected leaf Area. (**C)** Number of entry points for the canker. Least significant difference test was used in order to assign letters to each genotype. All 13 genotypes were compared and those sharing one letter or more are not statistically significant at $p \leq 0.05$. Bar graphs with the same color belong to the same cross. Statistical values are shown in Table 2.

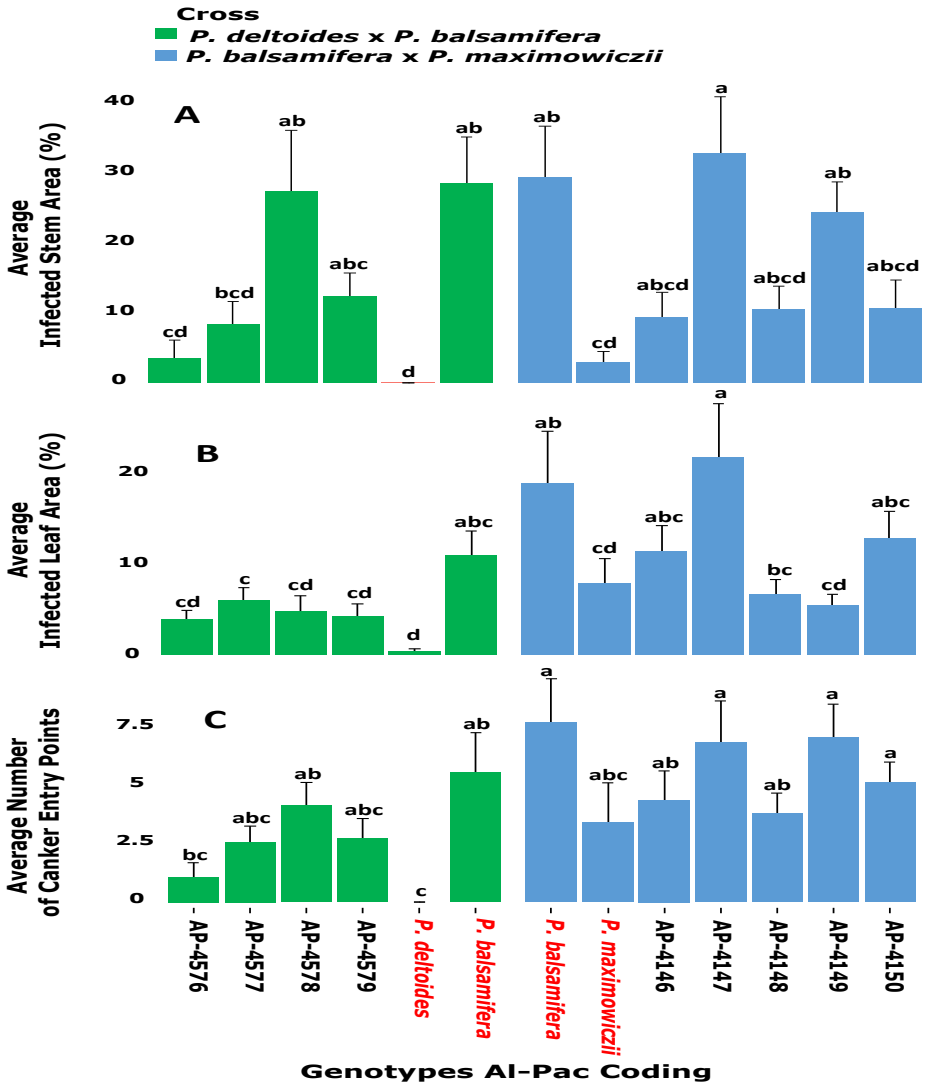


Figure 2: Assessment of isolate performance across poplar (*Populus*) genotypes.
Three disease metrics were used as proxies of isolate performance in eliciting a specific response in trees. (**A)** Infected stem area relative to the total stem area. (**B)** Infected leaf Area. (**C)** Number of entry points for the canker. Least significant difference test was used in order to assign letters to each isolate. Isolates and crosses sharing the same letter are not statistically significant at $p \leq 0.05$. Statistical values are shown in Table 2.

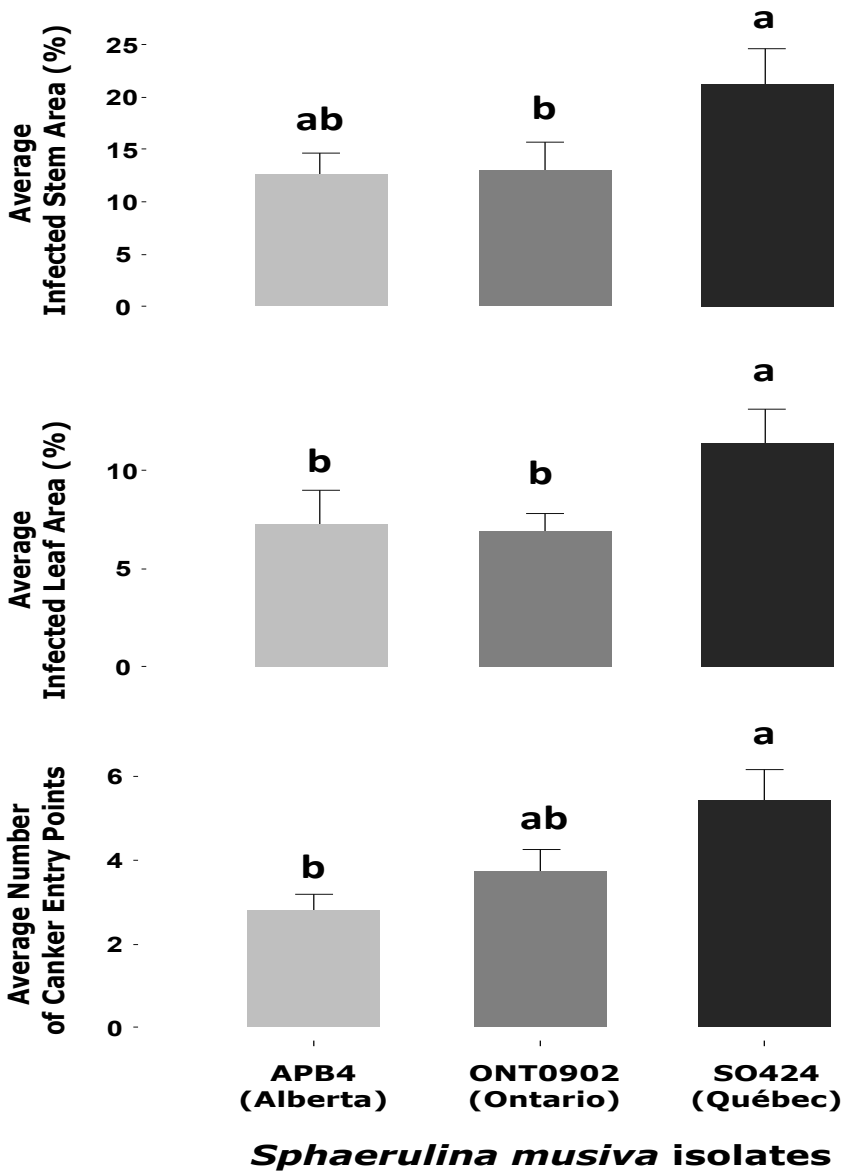


Figure 3: Detailed interactions between clones and isolates portraying the performance of each isolate across the 13 poplar genotypes for the disease metric of infected stem area.
Each hybrid clone was labelled with its coding and the parents with their species, P. balsamiferaxM is AP-2884 the balsam poplar crossed with *P. maximowiczii* and P.balsamiferaxD is AP-2878 the balsam poplar crossed with *P. deltoides*. APB4 is the isolate of *S. musiva* from Alberta, SO424 is the isolate from Quebec and ONT0902 is the isolate from Ontario. Clones were arranged counterclockwise from the least susceptible to the most susceptible to SO424 since this isolate revealed to be the most aggressive.

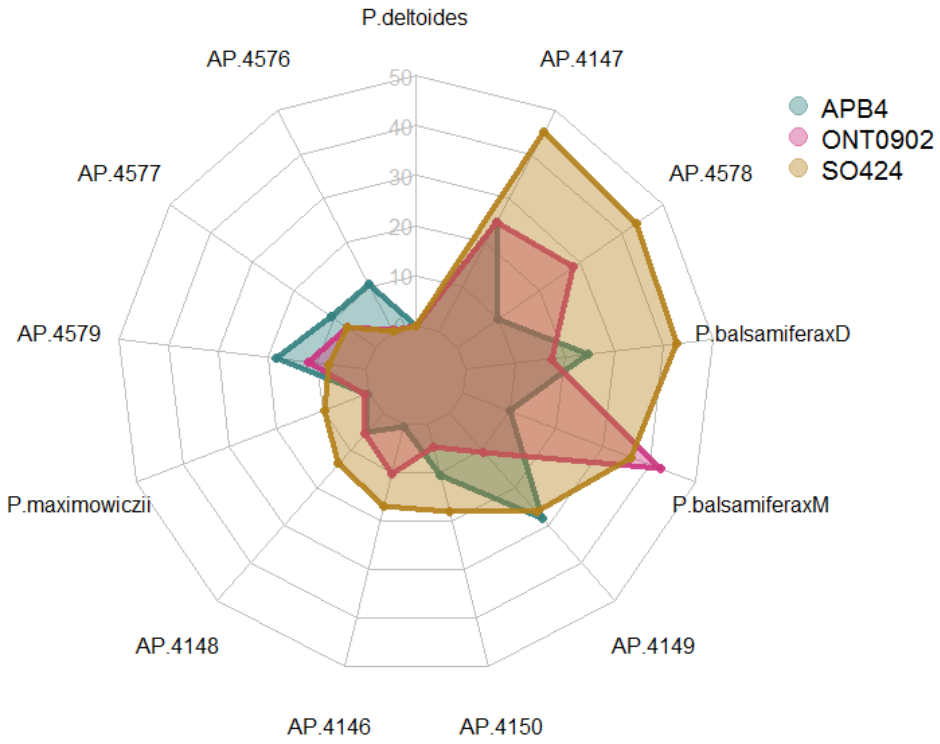
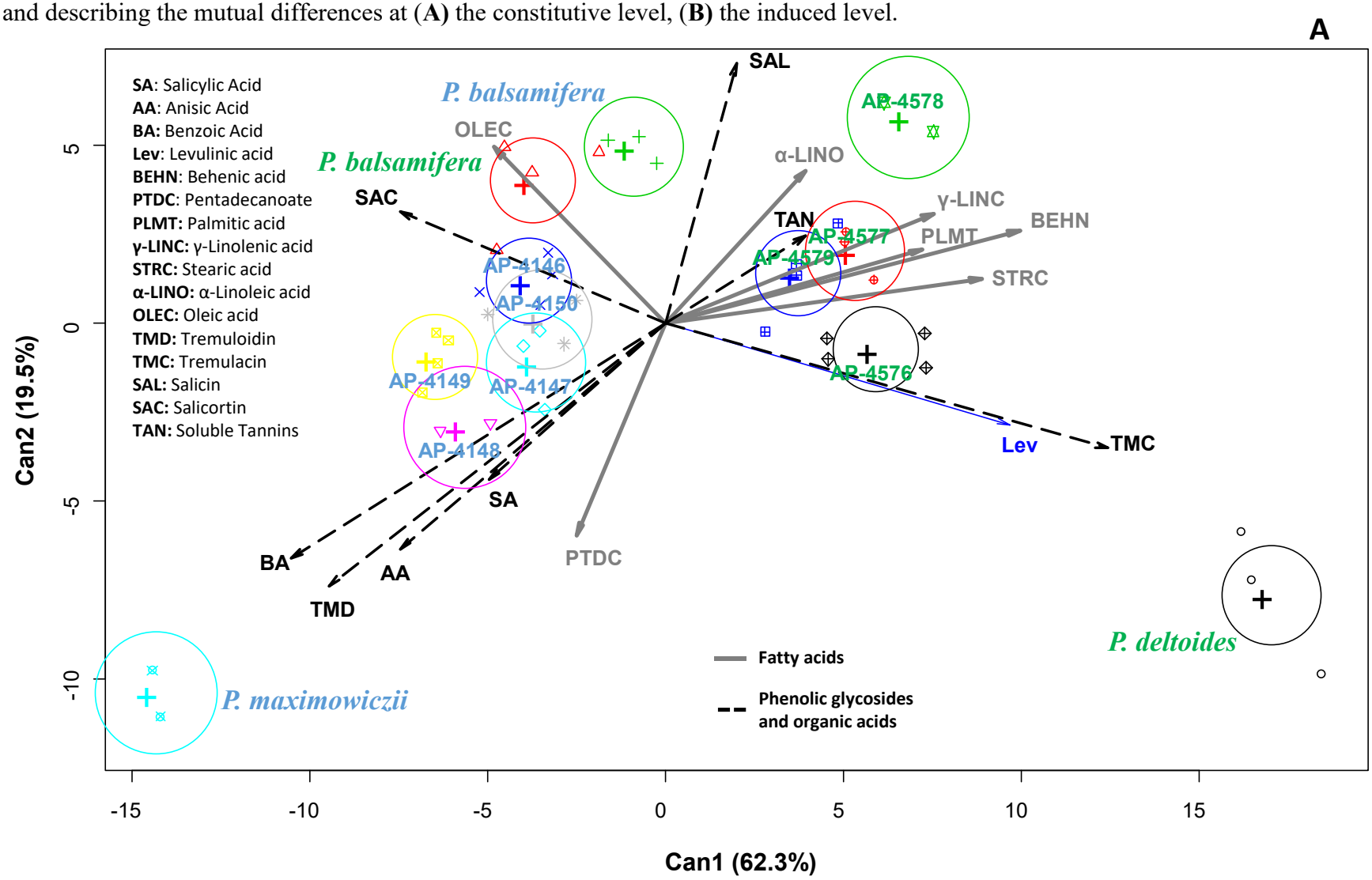


Figure 4: Canonical Correspondence Analysis establishing associations between leaf chemistry and the 13 different poplar genotypes and describing the mutual differences at (**A)** the constitutive level, (**B)** the induced level.



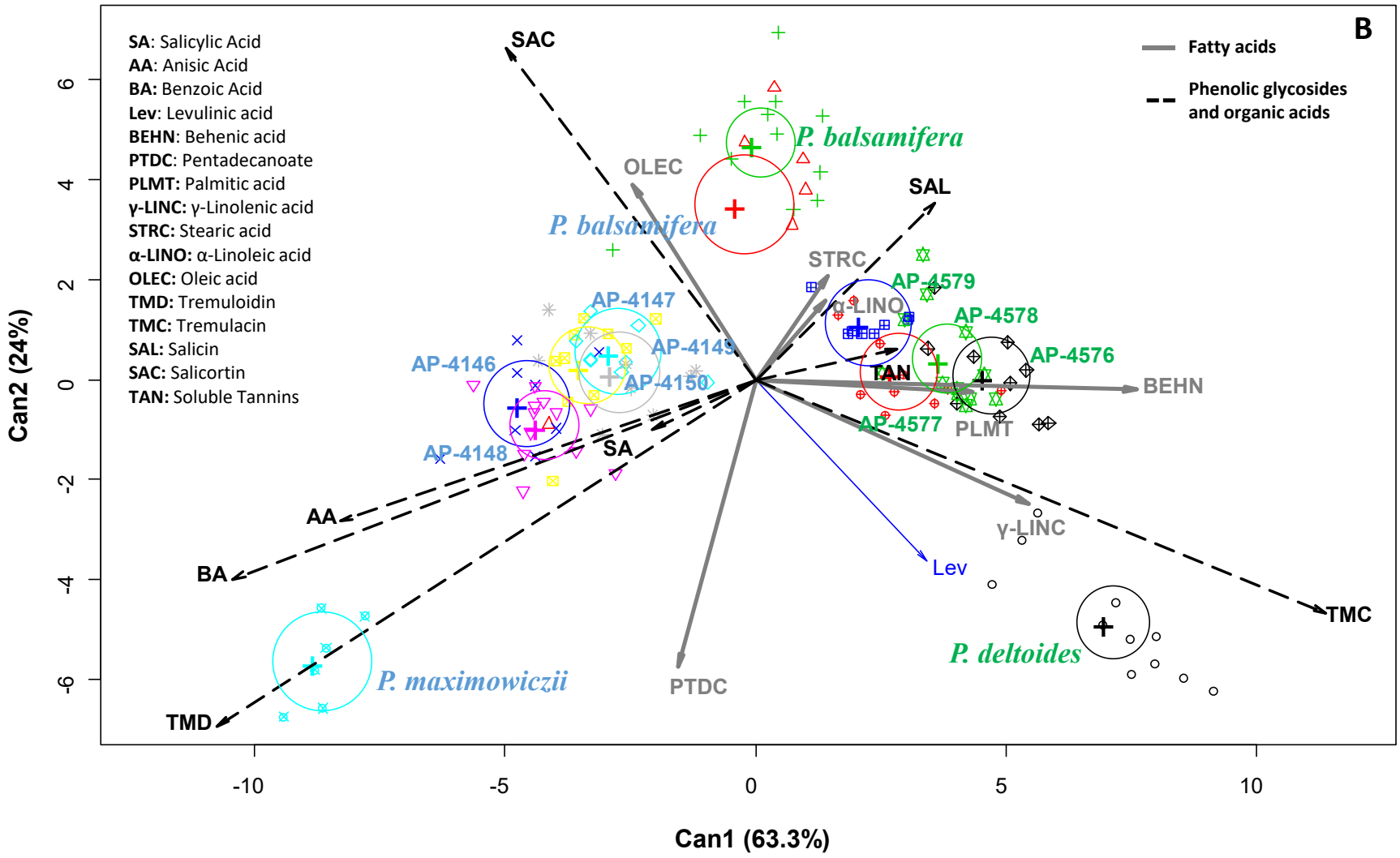

B
SA: Salicylic Acid
AA: Anisic Acid
BA: Benzoic Acid
Lev: Levulinic acid
BEHN: Behenic acid
PTDC: Pentadecanoate
PLMT: Palmitic acid
γ-LINC: γ-Linolenic acid
STRC: Stearic acid
α-LINO: α-Linoleic acid
OLEC: Oleic acid
TMD: Tremuloidin
TMC: Tremulacin
SAL: Salicin
SAC: Salicortin
TAN: Soluble Tannins
Fatty acids
Phenolic glycosides and organic acids
SAC
OLEC
SAL
STRC
α-LINO
BEHN
PLMT
γ-LINC
Lev
PTDC
TMC
AA
BA
SA
TMD
TAN
P. balsamifera
P. balsamifera
P. maximowiczii
P. deltoides
AP-4147
AP-4149
AP-4150
AP-4146
AP-4148
AP-4579
AP-4578
AP-4576
AP-4577
Can2 (24%)
Can1 (63.3%)
6
4
2
0
-2
-4
-6
-10
-5
0
5
10

Figure 5: Scaled Heatmaps and corresponding cluster analysis for (**A)** control plants and (**B)** Infected plants.
Data is scaled where the white color represents the average of the compound across genotypes, red represents an increase, and blue represents a decrease from the average of the compound across genotypes. Genotypes coding written with the same color belong to the same cross. C1 and C2 are the two clusters of chemical compound shaping the two major clusters of poplar genotypes at the constitutive level.

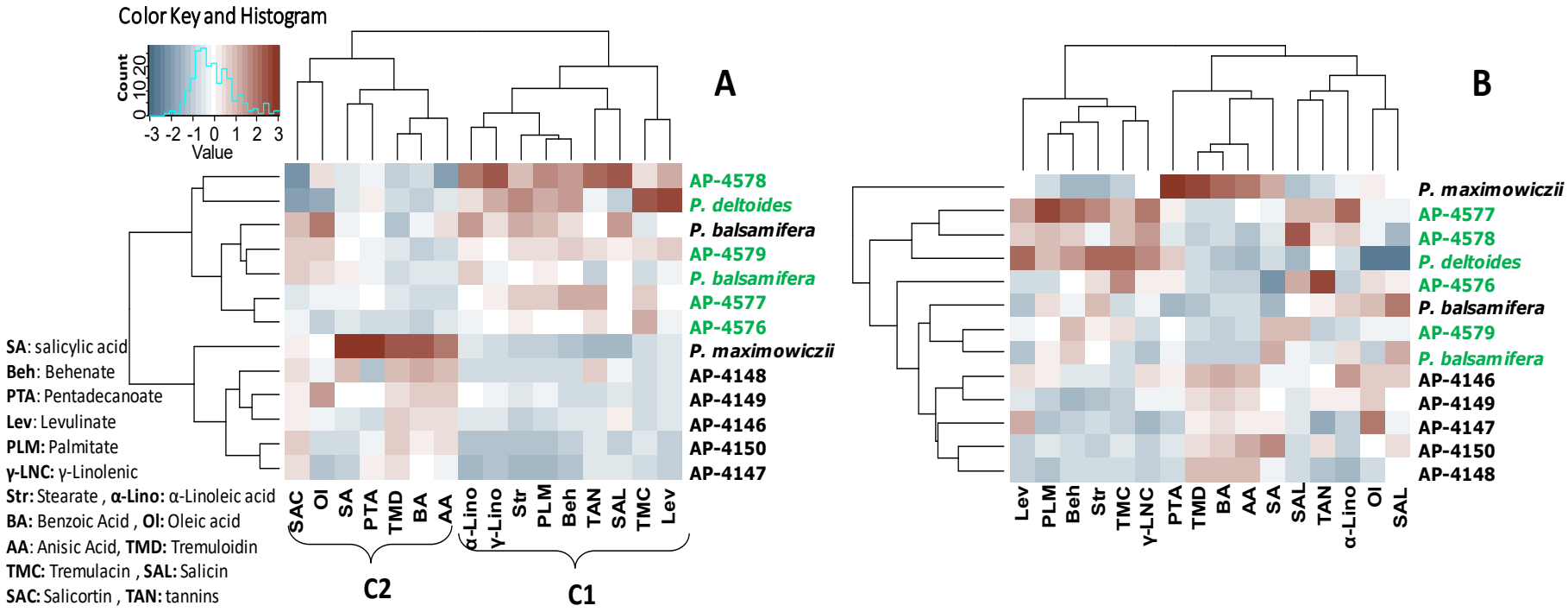


Figure 6: Plot of importance of the model deriving form randomForest analysis and based on the Gini index. Chemical compounds are classified in the order of the importance of their contribution to the model. **A.** Control plants, **B.** Infected plants.

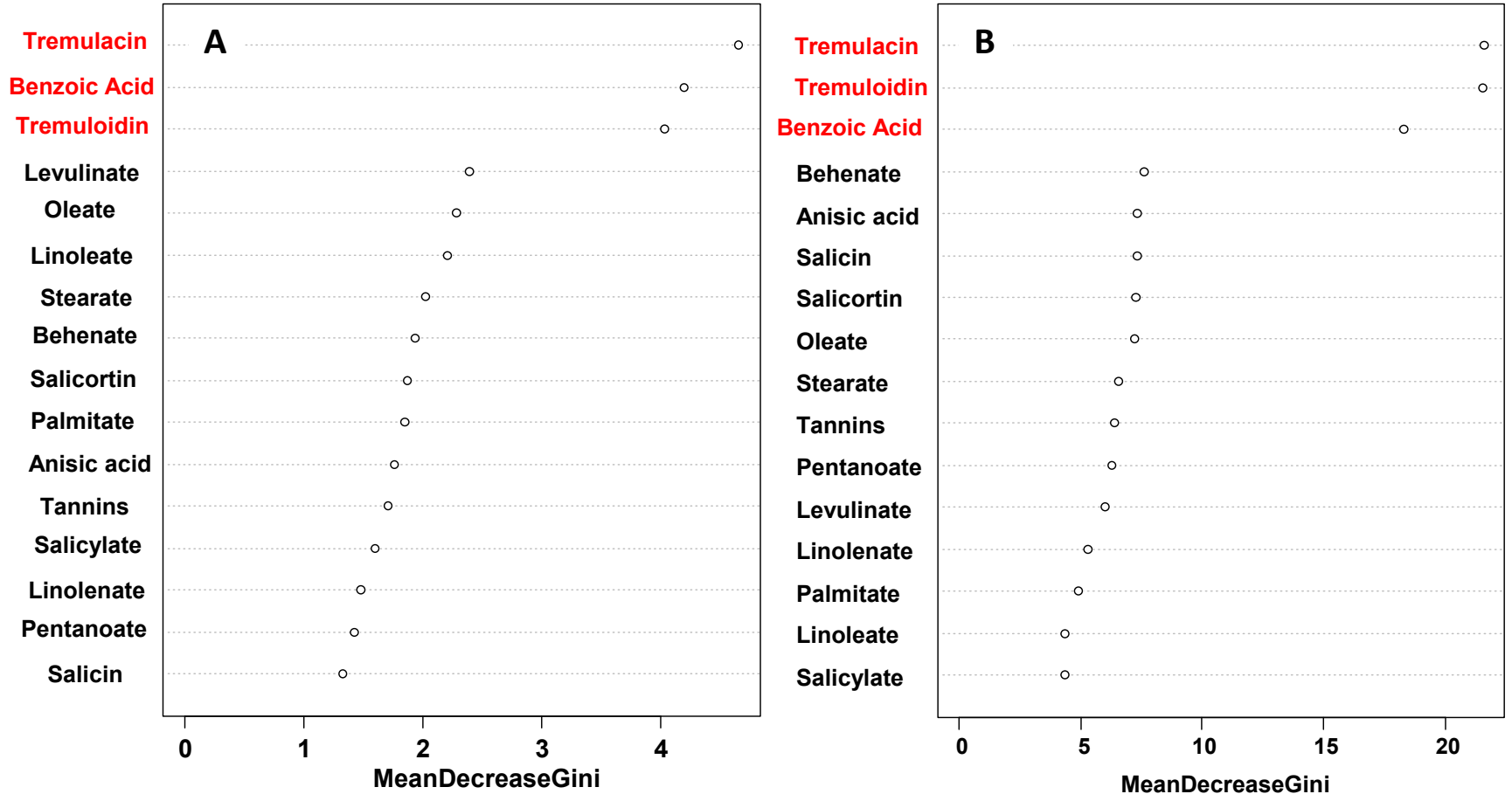
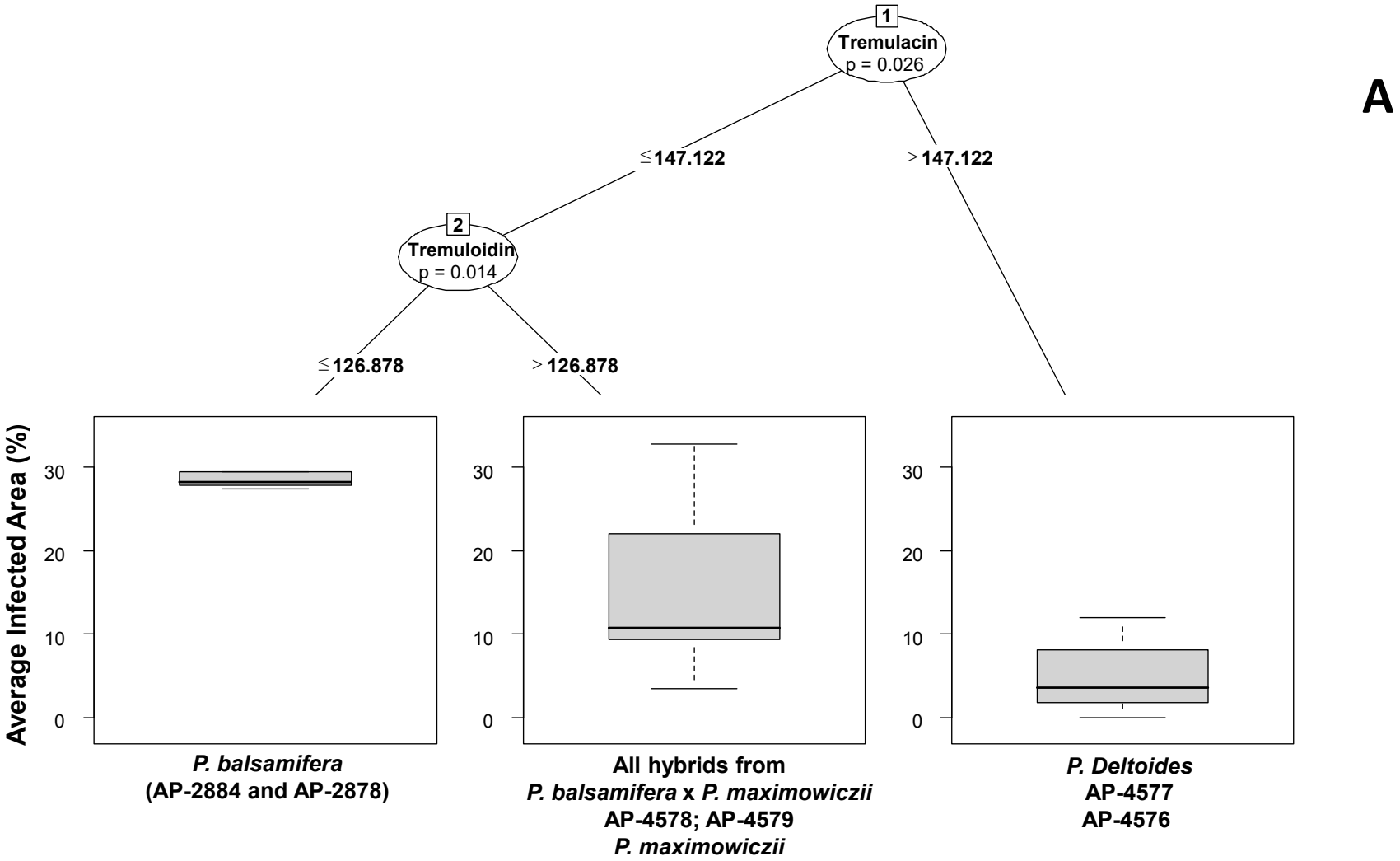
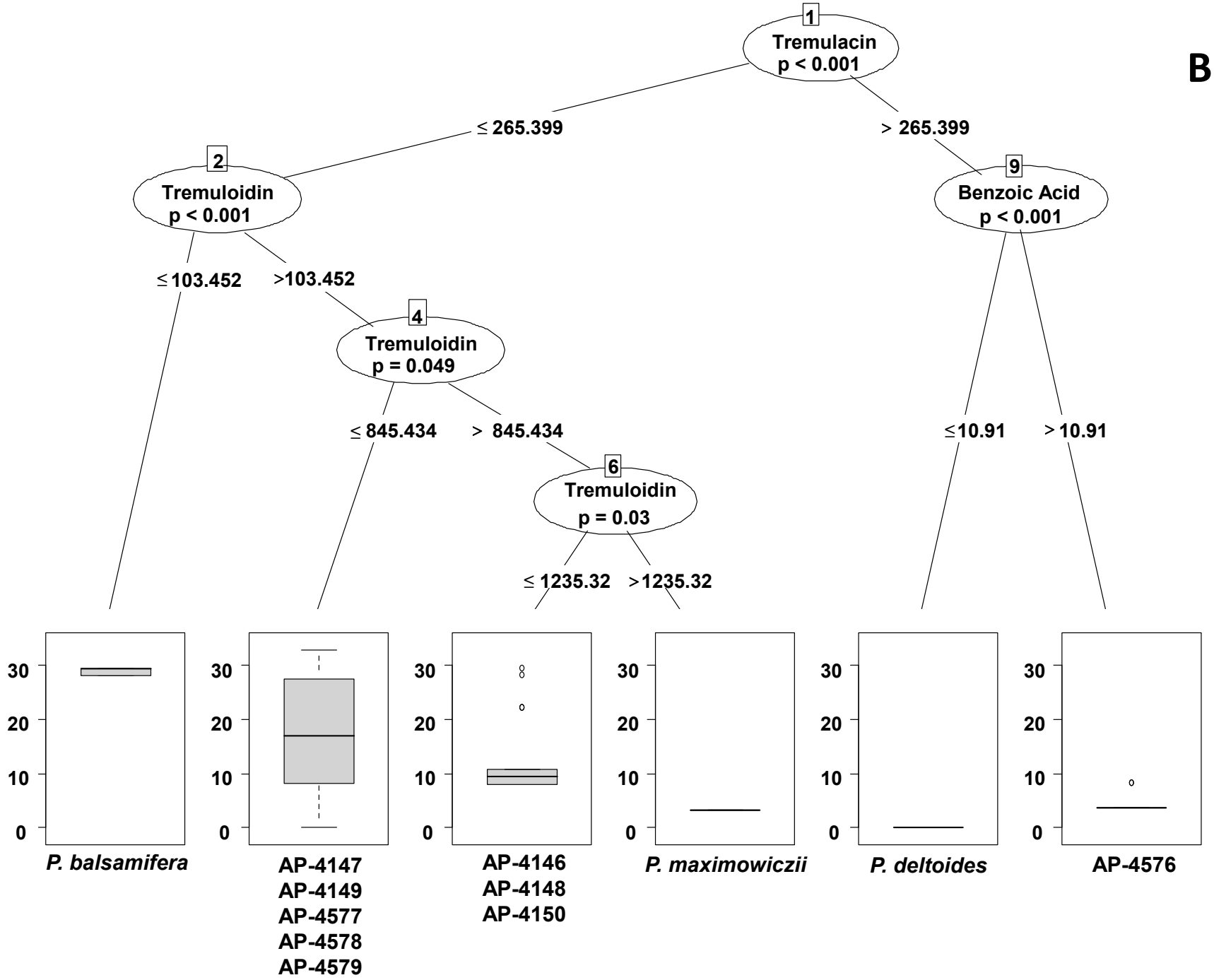


Figure 7: Conditional Inference Trees classifying clones based on the relationships between the measured chemical compounds across the 13 genotypes of parents and their offspring and their corresponding infected stem area by *S. musiva*. **A.** constitutive response with corresponding infected area, **B.** induced response with observed infected area.




B
1
Tremulacin
p < 0.001
≤ 265.399
> 265.399
2
Tremuloidin
p < 0.001
9
Benzoic Acid
p < 0.001
≤ 103.452
>103.452
4
Tremuloidin
p = 0.049
≤ 845.434
> 845.434
6
Tremuloidin
p = 0.03
≤ 1235.32
>1235.32
≤10.91
> 10.91
30
20
10
0
P. balsamifera
AP-4147
AP-4149
AP-4577
AP-4578
AP-4579
AP-4146
AP-4148
AP-4150
P. maximowiczii
P. deltoides
AP-4576

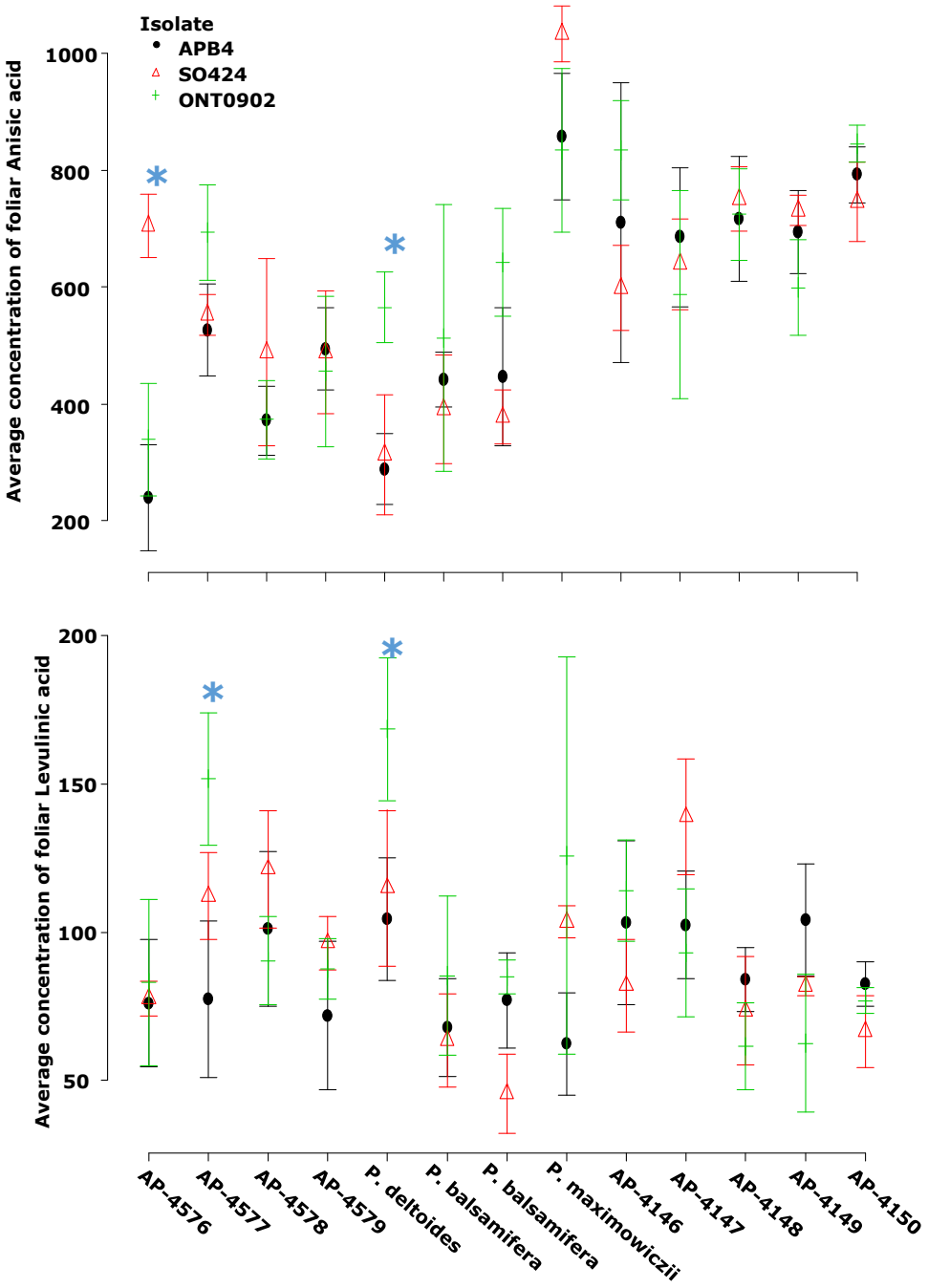
Appendices Chapter 2

Appendix Table 1 Correlation matrix between the different metrics of disease symptoms. The white background cells contain the Spearman rank correlation factor and the shaded cells represent the corresponding *p* value.

Trait	Infected stem area	Infected leaf area	Number of entry points
Infected stem area	1	0.56	0.82
Infected leaf area	0.049	1	0.75
Number of entry points	0.001	0.005	1

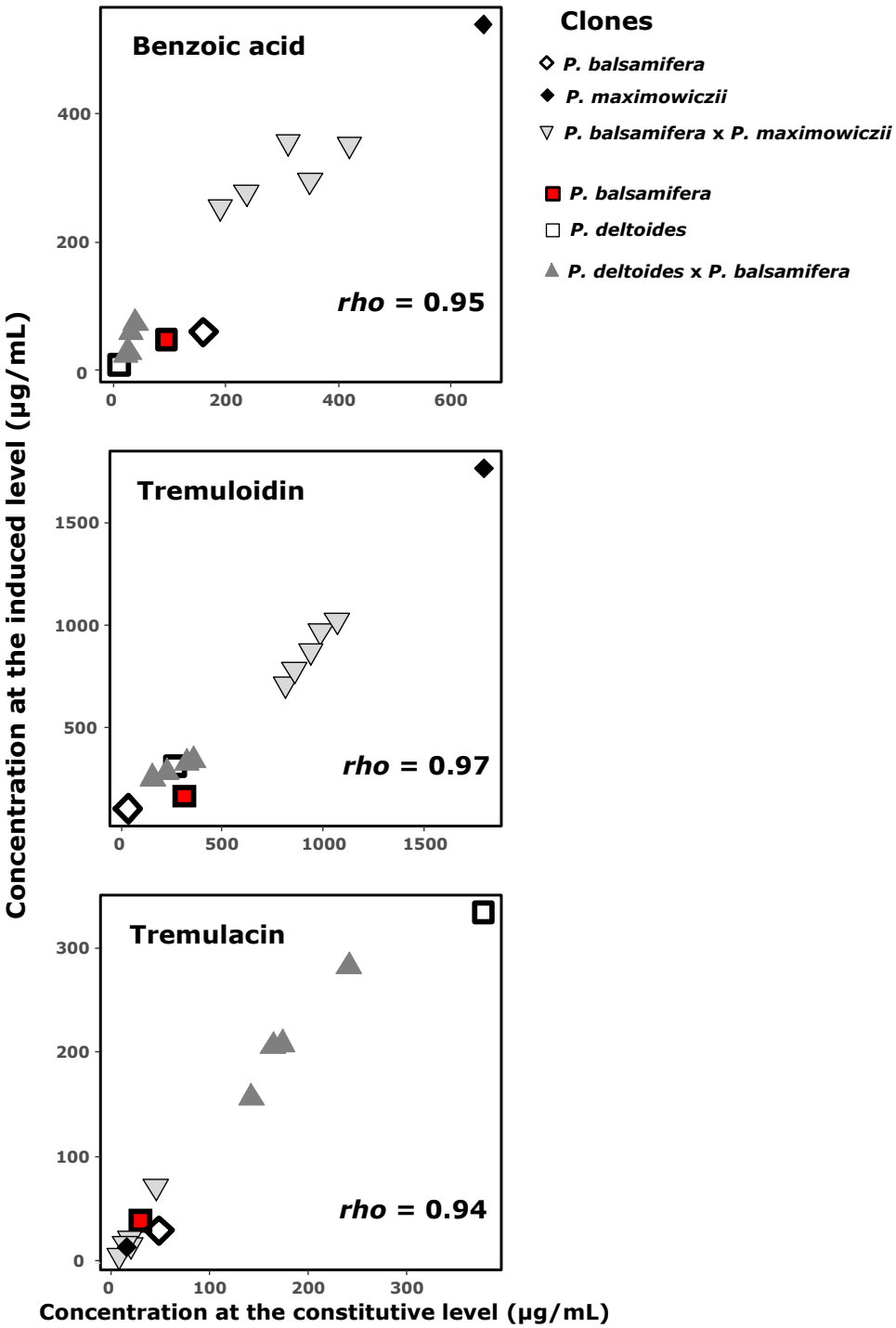
Appendix Figure 1 Interaction plots showing relationships between clones and isolates for select chemical compounds.

(A) Anisic acid and **(B).** Levulinic acid. The stars represent significant difference at $p = 0.05$ within the clone between the different isolates. The difference between isolates was determined using the least square means applied to the overall model of the analysis.

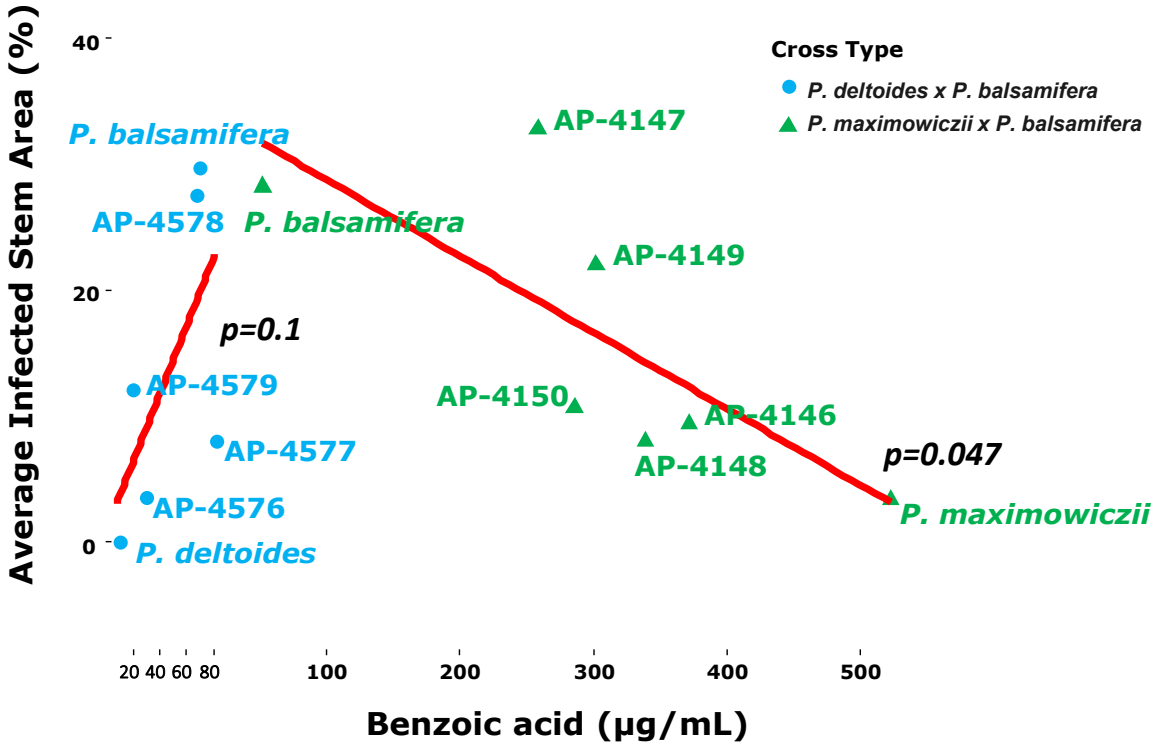


Appendix figure 2 Relationships between the constitutive and induced levels of the three most predictive chemical compounds.

Rho represents the Spearman correlation coefficient. All coefficients are significant at p=0.01.



Appendix Figure 3 Modelled relationships between the average constitutive level of Benzoic acid for each clone and their corresponding infected stem area.



Chapter 3: Can introgression of *Populus* trichocarpa into *Populus balsamifera* explain the observed susceptibility of *P. balsamifera* to *S. musiva* in Alberta?

I. Introduction

Populus balsamifera and *P. trichocarpa* are recently diverged North American poplar species growing in a sympatric setting with no postzygotic reproductive barriers (Levsen et al. 2012). *Populus balsamifera* stretches from the Atlantic to the Pacific coasts (Little 1976) and is adapted to higher latitudes, low temperatures, and dry sites in the boreal forests (Dickmann et al. 2001). A recent investigation of the population structure of *P. balsamifera* by Keller et al. (2010) has shown that the present day range of the species originated from three relict populations, following the receding ice sheets of the last glacial maximum. Two of these populations were located at the western and eastern extremes and one at the center of the current range, notably in Alberta where the intra-population diversity is the highest (Keller et al. 2010).

Likewise, *P. trichocarpa* stretches from northern California to Alaska and is confined between the western slopes of the Rockies and the Pacific Ocean (Little 1976). Its present days distribution and pattern of diversity today suggest that the species originate from ancient populations at the periphery of the ice sheet of the last glacial maximum (Slavov et al. 2012). More recent studies have shed light on the role of introgression with *P. balsamifera* in shaping the genetic diversity and environmental adaptation in populations of *P. trichocarpa* across its natural range (Geraldes et al. 2014). In fact, such introgression with *P. balsamifera* has apparently allowed *P. trichocarpa* to grow beyond its natural range (Suarez-Gonzalez et al. 2016). As the tree line seems to be moving north due to global warming, the understanding of species' introgression is key in speculating about the future of landscapes as well as the fate of admixed individuals and parental species.

Management of poplar stands can be natural or anthropogenic through breeding programs the purpose of which is to match the growing world demand for wood and fiber. Given their properties for site adaptation and high growth, poplar species in the Tacamahaca section are prime parental sources for such breeding programs in North America (Newcombe et al. 2001). Unfortunately, the same species are highly susceptible to *Sphaerulina musiva*, which is the causal agent of leaf spots and stem canker in poplars (exception made for aspen) and one of the most limiting factors of poplar plantation programs in North America (Newcombe et al. 2001).

Thus, it is equally important to track down the movement of natural enemies along with potential genetic movements of the host plant species and hybrids.

Sphaerulina musiva was originally thought to be confined to northeastern North America where it only causes leaf spots in *Populus deltoides* and *P. balsamifera* in the field (Bier 1939, Newcombe et al. 2001). However, *S. musiva* canker has become problematic upon the deployment of anthropogenic hybrid poplars in plantation programs in Quebec and northeastern USA (Newcombe et al. 2001). It wasn't until recently that parental species started to gain attention as *P. balsamifera* was previously thought to be resistant (Bier 1939, Waterman 1954) were revealed to be susceptible to *S. musiva* (Sivanesan 1990, LeBoldus et al. 2009). More concern was raised as the pathogen erupted in British Columbia and Alberta outside of its native range in the north eastern states and provinces of the US and Canada (Callan et al. 2007, LeBoldus et al. 2009). Although possible scenarios of *S. musiva* introduction to the Pacific Northwest were recently laid out (Sakalidis et al. 2016) and features of the pathogen aggressiveness have been investigated (Dhillon et al. 2015), mechanisms underlying susceptibility of specific poplar species particularly *P. trichocarpa* and *P. balsamifera* in western North America are not clear yet.

The present work investigates the susceptibility of *P. trichocarpa* and *P. balsamifera* to *S. musiva* as these two sister species have shown different degrees of susceptibility (Newcombe et al. 2001). *P. trichocarpa* is highly susceptible to the pathogen, while the resistance or susceptibility of *P. balsamifera* is controversial. Early research using eastern genotypes (Ontario and Quebec) claimed *P. balsamifera* as resistant (Bier 1939, Waterman 1954) while later research using genotypes from Manitoba and Alberta showed *P. balsamifera* can be susceptible to *S. musiva* (Zalasky 1978, LeBoldus et al. 2009). In addition, since the two species hybridize frequently where their natural ranges overlaps, their admixture plays an important role in their adaptation to a variety of environments (Geraldes et al. 2014, Suarez-Gonzalez et al. 2016), and therefore it is worth investigating how admixture shapes the disease response against the same pathogen.

In this context I hypothesize that the introgression of *P. trichocarpa* into *P. balsamifera* is one of the mechanisms explaining the observed susceptibility of *P. balsamifera* in Alberta since the province is the closest to the natural range of *P. trichocarpa*.

In order to test the validity of this hypothesis, genomics data describing a gradual levels of admixture between the two species was overlaid with phenomic data, including both visual and metabolomics metrics describing both the plants genotypic class and their response to the pathogen. Poplars have rich primary and secondary compounds so much so that they are likened to a "bio-refinery" (Devappa et al. 2015). Some of these chemical compounds have established ecological functions in poplar defense against biotic and abiotic agents (Chen et al. 2009). Whether these compounds can also be used for understanding other biological phenomena such as speciation and hybridization of poplar species, is unknown.

In Europe, studies have reported that flavonoids were controlled by both genetic and geographic structure while salicinoids were more genetically controlled in two highly divergent species, *Populus alba* and *Populus tremula* as well as their hybrids (Caseys et al. 2015). Similarly, benzoic acid and its derivatives such as hydroxyl-benzoic acid are involved in shaping geographic patterns for *P. tremula* (Bernhardsson et al. 2013). Both studies also sought to reestablish phylogenetics liaisons based on phenomics. This approach of using metabolites as an end product of genetic and cellular mechanisms is gaining traction as it establishes relationships between species not only in light of the rigid frame of DNA markers but also takes notice of the expression of the DNA in a particular environment or provenance (Burleigh et al. 2013).

In this chapter I used a similar approach to investigate the role of chemicals in speciation of poplars while linking plant chemical responses to the infection by *S. musiva*. The pathogen was used to elicit defense responses in poplars, both pure species and different classes of hybrids. Ultimately the present work seeks to shed light on the strategies of poplars as a model tree in responding to pathogens based on their genetic and environmental legacies (provenance).

II. Materials and Methods

1. Sources of genetic material

The genetic material of poplar trees used in experiments originated from three separate sources: (1) the collection of putative *P. balsamifera* from Alberta-Pacific Forest Industries Inc. (Al-Pac, Alberta, Canada), (2) the IUFRO collection of putative *P. trichocarpa* provided by Pierre Perinet (Forêts, Faune et Parcs, QC, Canada), the collection is in Quebec though the plant material is

from British Columbia, and (3) the AgCanBaP collection of putative *P. balsamifera* from Agriculture and Agri-Food Canada (Soolanayakanahally et al. 2009).

The Al-Pac collection consists of mainly genotypes from central Alberta (boreal forest) and some from north eastern British Columbia (between tundra and boreal forest). Clones from the Al-Pac collection are part of their *P. balsamifera* Controlled Parentage Program and I only selected 180 genotypes from about 50 different provenance included in the program. Samples do not represent hybridization other than *P. balsamifera* and *P. trichocarpa* (sampled from latitudes so to avoid hybridization with *P. deltoides* in the southern part of the province). The Al-Pac collection was used to provide insight into the diversity of the genetic make-up of putative *P. balsamifera* populations in Alberta as well as to determine if there was any natural hybridization between *P. balsamifera* and *P. trichocarpa* in the boreal forest region of Alberta. The AgCanBaP genotypes represent putative *P. balsamifera* from across the whole range of the species with majority of the genotypes from eastern Canada (Maritimes and Quebec) and some from Ontario, Manitoba, Saskatchewan, Alberta and Alaska. The IUFRO collection harbors genotypes from British Columbia, Washington State and Oregon along the west coast and at the western slopes of the Canadian Rockies.

My work involves two interlinked phases. In the first phase, the purity of putative *P. balsamifera* in Alberta was determined through SNP based genetic analysis. In the second phase, based on the results of the first phase, clones of selected genotypes of *P. trichocarpa* and *P. balsamifera* and their hybrids were used to evaluate their responses to the inoculations with *S. musiva* pathogen in a greenhouse experiment (phenotyping).

2. Preparation of genetic material for genotyping and inoculation experiments

In early March 2015, dormant branch cuttings of 10 cm from over 200 genotypes of all three sources were collected and shipped to the University of Alberta from three different locations: Indian Head, SK for the genotypes from the AgCanBaP collection, Boyle, AB for the genotypes from the Al-Pac collection and Laval, QC for the genotypes from the IUFRO collections. Once received, the cuttings were soaked for 48 hours in distilled water at 4°C and then planted in 20 cm Rootrainers® (Spencer-Lemaire Industries, Edmonton, AB, Canada) filled with Metromix 290 soil (WR Grace and Co., Ajax, ON, Canada). Only the top most bud remained exposed. The

containers were placed in a controlled greenhouse at 23°C:10°C (day:night), 70% relative humidity with artificial lights at 600 $\mu mol.m^{-2}.s^{-1}$.

Upon leaves and stem expansion, the clones were fertilized once with 500 ppm solution of 15-30-15 NPK fertilizer (Plant Prod, Hamilton, ON, Canada). Leaves were collected from all clones for genotyping. Only the genotypes selected for the nationwide genetic and geographic gradient were propagated twice in order to control environmental preconditioning (Riemenschneider and McMahon 1993) as they are used for genotyping (year 1) and inoculation experiment (year 2) as described below. On September 1 2015, the cuttings were moved outside the greenhouse to slowly achieve cold hardiness. Upon leaf senescence and prior to the first snow fall, the cuttings were covered with a layer of straw for better frost insulation. On January 30 2016, the cuttings were placed back in the greenhouse at 22°C to thaw for 48 hours before taking uniform cuttings (2 to 3 buds per cutting). The cuttings were planted and fertilized as described above. Once they reached about 20 cm height, the cuttings were transferred to 5 L pots (Treepot® Stuewe and Sons, OR, USA) containing the same soil mixture and fertilization regime as described above. The cuttings were then left to grow for 55 days until they reached on average 40-50 cm in height before being infected with *S. musiva*.

3. Genotyping of genetic material

All cuttings were grown in the greenhouse until producing fully expanded leaves (about 10 to 15 cm in size). Three juvenile leaves were selected using the Leaf Plastochron Index and selecting leaf numbers 3, 4, 5 and left to dry in small coin envelopes at room temperature for 2 weeks. DNA extraction and subsequent genotyping were conducted at the Laurentian Forestry Center (Laval, QC, Canada). All cutting were genotyped with a panel of markers developed specifically to distinguish *P. balsamifera* and *P. trichocarpa* (Isabel et al. 2013). A total of 31 SNP markers were analyzed for the separation among *P. trichocarpa*, *P. balsamifera*, and their various hybrid classes (I-V). The STRUCTURE software was used to determine pure species and hybrids (Pritchard et al. 2000, Hubisz et al. 2009) (Table 1). The analyses were initially run using tentatively a K (number of populations) of 10, a burnin period of 5×10^5 and a number of MCMC after burnin period of 10^6. The analysis was run for 10 times and the best K was inferred using the Delta K method (Evanno et al. 2005), where the K matched with the highest ad hoc

quantity (ΔK) is considered the 'true' number of populations. In our case the analysis reveals K=2 for both collections: the Al-Pac collection as well as nationwide collection.

4. Selection of a gradient of hybrids between *P. trichocarpa* and *P. balsamifera*

Based on genotyping and provenance, clones were selected in order to reflect both the genetic and geographic diversity of the populations of *P. trichocarpa* and *P. balsamifera* and their hybrids across Canada (Fig. 2). In other words, based on the SNPs analysis genotypes from close provenances with similar sequences were reduced to only genotypes descriptive of the provenance and genotypes with different sequences within a provenance were selected in a way that portrays the diversity of that specific provenance. The proportion of each genotype for each clone was computed with the STRUCTURE software for population genetics generating an estimate of the relative admixture level for each clone (Fig. 3).

5. Preparation of *S. musiva* inoculum

Four isolates of *S. musiva* were selected based on their performance on different clones of poplars (unpublished data) as well as their geographic range. I used two isolates from Alberta (APB3, APB4), one from Ontario (ONT0902), and one from Quebec (SO424). These isolates were grown from spore stock solutions from a local collection kept at -80°C. Briefly, vials with spores stored in 50% glycerol solution were thawed at room temperature and spread on 3 to 5 plates of KV-8 growth medium amended with streptomycin and chloramphenicol (Sigma Aldrich, Oakville, ON, Canada). The plates were then grown under full spectrum light bulbs (Gro-Lux®, Sylavania®, Osram Gmbh, Munich, Germany) for 3 to 4 days at room temperature until the surface of the plate was covered with dark mycelium. Agar plug squares (1 cm^2) were then transferred to fresh KV-8 agar plates to prepare enough inoculum to infect the rooted cuttings. Each plug was pressed 5 to 6 times on the surface of the new agar plate and then placed in the center of the plate. For every isolate, 40 to 50 plates were prepared and similarly the fungi were left to grow continuously under the same light intensity for about 7 to 10 days at room temperature until maximum sporulation was reached and before secondary growth takes place. The inoculum was prepared to coincide with optimal receptive phase of infection (about 40 to 50 cm in size) in the rooted cuttings.

The spores were scrapped from the plates using a glass rod and 1 to 2 mL distilled water and a stock solution of spore suspension was prepared for each isolate. The stock solution was then

diluted to prepare 4 L of spore suspension at 10^6 spores mL^{-1} for each isolate. The total volume was mixed in a 20 L bucket and 10 mL of tween 20 (Sigma Aldrich) at a concentration of 0.5 $mL.L^{-1}$ were added to the mixture in order to enhance spores adsorption to stem and leaves upon spray. Spore suspensions were then restocked and stored at -80°C until use.

6. Inoculations of genetic material with *S. musiva* spores

Five rooted cuttings from each of the 120 clones selected were assigned for the fungal inoculations, and three rooted cuttings were assigned as controls (mock inoculated) and sprayed with 1 $mL.L^{-1}$ tween 20 solution in distilled water. The cuttings were sprayed with a controlled stream of compressed air using a paint spray gun (SP-33000 LVLP Gravity Feed Spray Gun, Air Tools, CA, USA) to provide a homogenous and uniform mist. All rooted cuttings (mock inoculated and infected) were then wrapped in plastic bags lined with wet paper towels for 48 hours in a dark room in order to provide a level of humidity conducive to spore germination and subsequently successful plant infection.

7. Validation of the infection

The spore solution used for the infection trials above was also sprayed on ten water-agar plates. The plates were prepared by mixing 20 g of agar (BD Bacto™ Agar, Becton, Dickinson and Company, Sparks, MD, USA) in 1 L distilled deionized water. The agar solution was then autoclaved and poured into petri dishes in thin layer for optimal light penetration when counting germinating spores under compound microscope. The plates were left 24 hours to incubate at room temperature in the dark and the germination rates were estimated by randomly scanning five different spots of each plate and calculating the percentage of germinated spores (spores showing germination tubes). The germination rate was 99.4%, proving the viability of the spores before infection.

After the symptoms of infection appeared on leaves, the success of the infection was further validated by re-isolating *S. musiva* in two ways: (1) microscopic observation of the spores from fruiting bodies on leaf spots and stem cankers; and (2) culturing a portion of plant tissue at the junction between the necrotic and healthy tissue on the *S. musiva* selective medium (KV-8) amended with streptomycin and chloramphenicol.

8. Disease assessment

About 80 days after inoculation, three metrics were developed and used to assess disease severity: (1) **infected stem area**: the percentage of canker area relative to the total area of the main stem, (2) **infected leaf area**: the percentage of necrotic leaf area relative to the total leaf area, and (3) **the number of canker entry points:** number of individual entry points on stems and branches.

9. Chemical Analysis

Systemic responses to pathogens are common for several plant species, though they were not clearly demonstrated in poplars (Duplessis et al. 2009). Systemic response to herbivory in poplars is well documented (Babst et al. 2009, Philippe et al. 2010). Poplar response in this chapter was measured through leaf chemistry because leaves can easily be harvested without inducing much trauma to the tree. Also, a subsample of stems was analyzed and the profile of stem and foliage was similar to that of the leaves (data not shown), and thus I will only report the result of foliage chemistry, not stems. All leaves (petioles and fully expanded leaves) of each rooted cuttings were harvested around 120 days after planting, pooled together in labelled aluminum foil, and frozen in liquid nitrogen before being taken to cold storage or freeze drying. Leaves were weighed and ground in a Wiley mill equipped with a 40 mesh screen (Thomas Scientific, Swedesboro, NJ, USA). The resulting powder was stored in glass vials at -40°C before being used for chemical analysis.

Methylated organic acids were measured as the method provided insight into fatty acids (linoleic acid is a precursor of the jasmonic acid defensive pathway), as well as other organic acid which role in defense begs to be elucidated. The metabolic profile was studied by extracting leaf powder metabolic organic carboxylic acids (fatty acids, benzoic acid derivatives and reaction intermediates) using a one-step trans-esterification of the carboxylic group of the organic leaf extracts following a standardized and validated AOAC method (Curtis et al. 2008). The resulting extract was analyzed by Gas Chromatograph-Mass Spectrometry (GC-MS: Agilent Technologies 7890A GC and 5975MSD with Chemstation software, Santa Clara, CA, USA). The details of the method can be found in Ishangulyyeva et al. (2016). The stability and validity of both the extraction and the quantification method was confirmed with the use of one recovery standard and one internal standard. Though the method is designed for fatty acids, the GC-MS method

revealed a variety of secondary metabolites with carboxylic acid groups as well as other compounds.

Except for one unknown, the following standards were used to validate the presence of compounds deduced from the MS NIST library and quantify their concentrations: dimethyl succinate, methyl 3-phenylpropionate, benzyl alcohol, methyl cinnamate, lignocerate, dibenzofuran, methyl myristate, methyl elaidate and trimethyl citrate (TCI chemicals, Portland, OR, USA), methyl esters methyl linoleate, methyl linolenate, methyl oleate, methyl palmitate, methyl stearate and methyl behenate (Nu-chekprep Elysian, MN, USA), methyl anisate, methyl levulinate, methyl benzoate and methyl salicylate (Sigma Aldrich). All standards were analytical grade ($\geq$ 99% purity). The unknown compound is thought to be an alkaloid as it has the typical mass spectrum of a nitrogen heterocycle. This compound was also present in stems and petioles but not in the leaves without petioles.

10. Statistical analysis

The map for the distribution of the clones in Alberta and the nationwide distribution map were generated using the R package ggmap (Kahle and Wickham 2013).

The plants in the greenhouse experiment were arranged in a completely randomized design with five replicates for infected plants (treated plants) and 3 replicates for the corresponding mock inoculated plants.

The studied model is as follow:

$Y_{ijkl} = \mu + T_i + G_j + Cl_k(G) + \varepsilon_l(T_i\ G_j\ Cl_k)$

Where,

T_i: Treatment (inoculation or mock inoculation), $i = 1 – 2$

G_j: Genotypic class (Table 1). $j = 1 – 7$

$Cl_k(G)$: Clone nested within Genotypic class. $k = 1 – 120$

$\varepsilon_l(T_i\ R_j\ Cl_k)$: random error of replicate within Treatment, Genotypic class and Clone.

Given the large number of compounds analyzed and their correlations, and the large number of clones; Orthogonal Partial Least Square Discrimination (OPLS-DA) Analysis was performed using the ropls package in R (Thévenot et al. 2015). The use of OPLS-DA is the most adequate in determining the markers characterizing plant response to the pathogen. The OPLS-DA model aims at determining the validity of segregating inoculated and mock inoculated poplars based on their physiological response measured through the panel of metabolites analyzed.

Network analysis was performed in order to establish differences in relationships between potentially resistant and potentially susceptible clones. The correlation matrix was calculated using the function EBICglasso from the qgraph package in R (Epskamp et al. 2012) where a graphical sparse correlation is computed from a correlation matrix and tuned using the extended Bayesian information criterion (EBIC) with a Bonferroni correction in order to control for multiple comparisons and where only significant correlations were kept. Dunnett test was used as a non-parametric test for the study of the metabolic profile of balsam poplars only as transformations did not allow for reaching satisfactory normality and homogeneity of variance at the same time. Dunnett test and corresponding pairwise comparisons were performed and plotted using the multcomp package in R (Hothorn et al. 2008).

III. Results

1. Genetic composition of *P. balsamifera* in Alberta

The results of genotyping in Alberta show an unexpected presence of *P. trichocarpa* in central Alberta and a presence of different classes of hybrids throughout the area sampled. *Populus trichocarpa* and its closest introgressants were sparse and spread across the sampled area. *Populus trichocarpa* populations, though small in numbers, were mainly located between 112.6ºW and -113.6ºW in central Alberta. However, two thirds of the populations surveyed were either pure *P. balsamifera* or its closest introgressants HybClassI defined as 90% to 98% *P. balsamifera* (Fig. 1B). The sampled location at the north eastern corner of British Columbia at the eastern slopes of the Rockies showed similar distribution with *P. trichocarpa*-rich genotypes (i.e. genotypes with higher proportion of *P. trichocarpa*) facing the mountains and *P. balsamifera* rich genotypes away from them.

2. Symptomatic profile of *S. musiva* infection of poplar genotypes

Leaf and stem infection between *P. balsamifera* and *P. trichocarpa* were inversely proportional. The average infected leaf area decreased from *P. balsamifera* to *P. trichocarpa*, associated with the increased proportions of *P. trichocarpa* (Fig. 4A). In contrast, the average infected stem area and the average number of cankers on the stem increased from *P. balsamifera* to *P. trichocarpa*, associated with the increased proportion of *P. trichocarpa* (Fig. 4B and 4C). It is also noteworthy that HybClassV which harbors 2 to 10% of the *P. balsamifera* genome showed more stem cankers than *P. trichocarpa* though the size of the stem canker was similar between *P. trichocarpa* and HybClassV.

3. Chemical profile of the infection

3.1. Chemical responses of poplar genotypes to *S. musiva* infection

The OPLS-DA model yielded one predictive and three orthogonal components with a cross-validated predictive ability of the model $Q^2Y = 61.5\%$ and a total explained variance $R^2X = 53.1\%$ while the variance for class separation $R^2t1 = 7\%$. The model is fairly reliable since the predictive ability was fairly close to the computed percentage of response variance $R^2Y = 68.3\%$. The OPLS-DA analysis showed a clear separation between the chemical profiles of infected and mock inoculated poplars (Fig. 5). The chemicals contributing to such difference are determined by extracting the loadings from the output of the OPLS-DA analysis and plotting their corresponding correlation and covariance factors in an S-plot (Fig. 6). S-plots uses both correlations and covariance since the use of correlations alone could result in high risk of false positives given the disparity in the range of concentration between highly and less abundant compounds (Wiklund et al. 2007).

The analysis showed that four compounds were responsive to *S. musiva* infection across the genetic gradient from *P. trichocarpa* to *P. balsamifera* (Fig. 6): three of these compounds decrease after infection (anisate, levulinate, and linolenate). In contrast, an unidentified compound increased by 30% upon infection in *P. balsamifera,* HybClassI, HybClassII, and HybClassIII. It is noteworthy that this unknown compound – most likely an alkaloid as it shows a typical fragmentation of a pyridine heterocycle – is present in the petioles and stems of the plant but not in the leaves (data not shown).

3.2. Chemical compounds indicative of plant speciation

The analysis yielded two compounds in *P. balsamifera* and *P. trichocarpa*, as well as their hybrids that did not respond to *S. musiva* infection, but appeared to vary with the level of introgression (Fig. 7). *Populus trichocarpa* had six times more benzoic acid than *P. balsamifera* while *P. balsamifera* contained 3 times more benzyl alcohol than *P. trichocarpa*. These two compounds varied gradually among genotypic classes and are relatively stable between infected and mock inoculated plants. Apparently they are strongly controlled by genetics as their ratio is highly correlated to the proportion of *P. balsamifera* (Spearman correlation coefficient $r = 0.63$, $p < 0.0001$). Regression lines also showed that the increase in the ratio between the two compounds holds at both constitutive and induced levels (respectively mock inoculated and infected plants) with the same level of significance (Fig. 8).

3.3. Interactions among poplar metabolites

I performed different network analyses on potentially resistant (Fig. 9A) and susceptible (Fig. 9B) genotypes as well as their corresponding mock-inoculated control genotypes (Figs. 9C and 9D). I considered genotypes that did not show any symptoms of stem canker as resistant and those that showed obvious symptoms of infection on stems were considered as susceptible. Analyses indicated significant differences on interactions among different metabolites between infected and mock-inoculated genotypes at the constitutive and induced levels. Most of the resistant genotypes belonged to *P. balsamifera* and HybClassI but susceptible genotypes covered all of the seven classes with the majority being *P. trichocarpa* and its most genetically similar hybrids (HybClassIV and HybClassV).

Few interactions standing out in the comparison between constitutive and induced defenses at each group were detailed below: (1) For resistant genotypes, the strong positive correlation between cinnamic acid and hydroxycinnamate at the constitutive level disappears once they were infected by *S. musiva*. In contrast, the link between elaidic and oleic acids, which were absent at the constitutive level, became stronger and positive at the induced level (Figs. 9A and 9C); (2) susceptible genotypes showed a strong negative correlation between benzoic acid and benzyl alcohol both at the constitutive and induced levels (Figs. 9B and 9D); and (3) resistant and susceptible genotypes seemed to modulate the interactions between their metabolites in different ways when infected with *S. musiva*: the network between metabolites of the resistant genotypes

appears more intricate and sophisticated than that of the susceptible genotypes once they were infected. The resistant genotypes seemed to essentially lose the interactions between compounds from different classes in favor of interactions between compounds of the same class.

4. Predictive ability of metabolites for plant suitability to *S. musiva* infection

I used benzoic acid as a predictive variable since it remained stable at both the constitutive and induced levels and is also a surrogate for differentiating species (*P. trichocarpa* had six times more benzoic acid than *P. balsamifera*). Thus, concentrations of benzoic acid and the area of infection in the stems were averaged by genotypic class and plotted against the infected stem area (Fig. 10). Based on Akaike's Information Criterion (AIC = 82.3, R^2=0.91), the best descriptive model fit was the polynomial model and the second best model was the linear model (AIC = 80.1, R^2=0.85). Both models had positive slopes, which suggested that as the concentrations of benzoic acid increases, the infected stem area also increases from *P. balsamifera* to *P. trichocarpa*, and their hybrids ranged between these two species.

5. Chemotypic characteristics of resistance and susceptibility of *P. balsamifera*

Different *P. balsamifera* clones had differential responses. Some showed no signs of stem canker while others were highly susceptible and sustained cankers, similar to the most susceptible hybrids and *P. trichocarpa*. All susceptible *P. balsamifera* cuttings originated from Alberta (Fig. 11). I found a strong correlation of infection with longitude, i.e. susceptibility decreases from western to eastern Alberta (data not shown).

These observations were matched with selected chemicals. Further, resistant and susceptible *P. balsamifera* were compared between mock inoculated (providing constitutive chemistry) and infected (providing induced chemistry) clones. Three organic acids (salicylic acid, anisic acid, and cinnamic acid) and one fatty acid (myristic acid) showed variation depending on the classes defined earlier. At the constitutive level, resistant *P. balsamifera* genotypes showed significantly higher levels of all organic acids than susceptible genotypes. However, once infected with *S. musiva*, susceptible *P. balsamifera* seemed to increase the levels of organic acids to the level comparable to those of resistant *P. balsamifera* genotypes. The latter genotypes maintained similar levels of organic acids as at the constitutive level (Fig. 12). Inversely, myristic acid is induced upon infection only in potentially resistant genotypes and decreased in potentially susceptible plants after infection (Fig. 12).

IV. Discussion

1. *Populus balsamifera* showed a strong genetic variation in Alberta

Surprisingly, Alberta contained all seven classes of genotypes ranging from *P. balsamifera* to *P. trichocarpa* in an area conventionally reported as a stronghold of pure *P. balsamifera* (Keller et al. 2010, Breen et al. 2012) and far from the hybrid zone of *P. balsamifera* with *P. trichocarpa*. Nevertheless, the majority of trees genotyped were pure *P. balsamifera* or its closest hybrid class (>90% *P. balsamifera*). The presence of pure *P. trichocarpa* in central Alberta as well as the level of introgression through the sampled area is intriguing. The diversity of genetic composition of the poplar trees in Alberta could be linked to an earlier study that defined Alberta as a deme of *P. balsamifera* with the highest intraspecific diversity across the species range (Keller et al. 2010). Although the earlier study accounted hybridization between *P. balsamifera* and *P. trichocarapa*, it only used *P. balsamifera* specific SNP markers developed using samples collected across the range of *P. balsamifera* in North America, including Alberta. Based on species morphology, it is hard to distinguish *P. balsamifera* and *P. trichocarapa* before they reach maturity particularly in their hybrid zone in the Canadian Rockies. Two lobes in *P. balsamifera* capsules versus three for *P. trichocarpa* is the only distinguishing feature between the two species at sexual maturity (Dickman 2001). It is not clear if the presence of *P. trichocarpa* in Alberta is natural or anthropogenic.

2. Disease measurements showed variations across poplar species sampled

Leaf and stem infections showed an inverse relationship across the genetic gradient from *P. balsamifera* to *P. trichocarpa*. Similar observations were reported in the field the Midwest states in the US where *P. balsamifera* sustained more leaf damage than canker damage and such differences were attributed to the higher occurrence of *S. populi* (causes more leaf spots than canker in the field) in *P. balsamifera* compared with *S. musiva* (Zalasky 1978, Newcombe et al. 2001). In fact, *S. populi* can cause damage amounting to full defoliation in *P. balsamifera* (Foster et al. 2015). The present study showed that *S. musiva* can inflict the same amount of damage on both *P. balsamifera* and *P. trichocarpa* and that leaf spots are not necessarily indicative of high levels of stem canker development. In fact, as discussed below, the most eastern *P. balsamifera* genotypes showed absolute resistance while Albertan *P. balsamifera* genotypes showed a full

range of susceptibility (sometimes higher than *P. trichocarpa*) even though leaf infections levels were similarly high in both locations.

Likewise, hybrids of *P. trichocarpa* and *P. deltoides* thought to acquire resistance from their *P. deltoides* parent as it is completely resistant to *S. musiva*, turned to be highly susceptible to the pathogen (Newcombe et al. 2001). The recent shift of the pathogen to the Pacific Northwest has shown that it can cause considerable damage to *P. trichocarpa* hybrids in British Columbia (Callan et al. 2007). To the best of my knowledge, the present study is the first account of successful infection of pure *P. trichocarpa* to *S. musiva* in a greenhouse experiment. Previous studies showed the infection of only in hybrids of *P. trichocarpa* by the pathogen (Callan et al. 2007, Liang et al. 2014, Qin et al. 2014).

Hybrids were overall in an intermediate position between the two pure species though HybClassV showed slightly higher susceptibility to the pathogen than *P. trichocarpa*. The gradual response of hybrids to the pathogen from *P. balsamifera* to *P. trichocarpa* seems to corroborate with the hybrid bridge hypothesis (Floate and Whitham 1993). The hypothesis stipulates that hybrids act as intermediate hosts (stepping stone) for natural enemies or mutualists that seek to expand their range and shift from one host to another. Recently, Lusebrink et al. (2013) speculated that the recent host range expansion of mountain pine beetle from pure lodgepole pine (*Pinus contorta*) to the pure jack pine (*P. banksiana*) is facilitated by the hybrid zone of both pine species prior to the invasion of the jack pine forests in Alberta. Such hypothesis for the poplar-*S. musiva* pathosystem should to be tested in the field (Sakalidis et al. 2016) as the pathogen is becoming more noticeable in the Pacific Northwest (*P. trichocarpa* and deployed hybrids in British Columbia) outside of its native range in the north east. Even though it is not clear why we observed such gradual susceptibility from *P. balsamifera* to *P. trichocarpa*, my study has provided a possible explanation for the increasing pathogen spread and suggested that increasing proportion of *P. trichocarpa* in the poplar genome may lead to the increasing susceptibility of poplars to *S. musiva*.

3. Metabolomics based phenotypic screening: how successful?

The OPLS-DA analysis showed a clear distinction between the metabolome of infected and mock inoculated poplars, suggesting the role of pathogen infection in induction of chemicals

defenses in poplars. Though it is used for descriptive purposes in the present study, OPLS-DA is traditionally used as a tool for predicting resistant or susceptible genotypes. However, such predictive tools require additional OPLS-DA analysis to distinguish specific metabolomes of genotypic classes as it may be misleading to assume that all resistant poplars have a similar metabolome as the mock inoculated poplars while resistant poplars belong to different genotypic classes. The purpose of this analysis was to demonstrate a clear distinction between infected and mock inoculated genotypes across the genetic gradient and to use this information to determine the compounds that may be responsible for such differences between resistant and susceptible genotypes. I found that anisate, levulinate, linolenate and an unknown compound differentiated resistant and susceptible genotypes. . Anisate is a benzoic acid derivative related to salicylate. Salicylate is thought to contribute to the activation of plant defense response against pathogens (Turner et al. 2002). The functions of anisate are yet to be determined and to the best of my knowledge this is the first report of anisate in poplars. α-Linolenic acid is a precursor to jasmonic acid, a plant hormone involved of the response to herbivory and wounds (Turner et al. 2002). Levulinate is a keto acid with established technological and economic values (Bond et al. 2013) relatively unknown ecological functions. A recent work suggests that levulinate potentially could affect phytopathogenic fungal associated of an invasive bark beetle species (Ishangulyyeva et al. 2016). The fourth compound is an unknown and induced only in *P. balsamifera* upon infection (data not shown). It is also present in petioles in the foliage as well as the stem but not in foliage without petioles (data not shown).

4. Levels of benzoic acid and benzyl alcohol can distinguish *P. trichocarpa* from *P. balsamifera* and predict susceptibility

Two chemical compounds stood out as descriptive of the genetic gradient between the two pure species, inversely proportional to each other and both based-off a benzene ring namely benzoic acid and benzyl alcohol. Relative to *P. balsamifera, P. trichocarpa* had higher levels of benzoic acid and lower levels of benzyl alcohol. Both compounds increased gradually across the genotypic classes (I-V) and did not differ between infected and mock-inoculated poplars, suggesting that their levels could be reliable indicators of the species and their hybrids. Further, there was a strong correlation between the two compounds and the percentage of *P. balsamifera* in the genome of the tested poplars. Since the percentage of *P. trichocarpa* is complementary to

that of *P. balsamifera*, the same correlations are true with the percentage *P. trichocarpa*. Benzoic acid and benzyl alcohol have a particular importance in poplar defenses as they are thought to be precursors for salicinoids, a class of defense compounds in poplars and Salicaceae in general (Chedgy et al. 2015). Salicinoids are also found to be strongly genetically controlled in *P. tremula, P. alba*, and their hybrids (Caseys et al. 2015). Furthermore, hydroxyl benzoate, a compound belonging to the same class is found to play a role in segregating populations of *P. tremula* (Bernhardsson et al. 2013). In addition, our results suggest that these precursors could potentially be a stable and conserved indicator of speciation as (1) they significantly differ between pure species, (2) they gradually vary between genotypic classes and (3) they are not affected by infection as constitutive and induced levels were similar. Even though benzoic acid derivatives seemed to respond more to species composition rather than infection, it is important to point out that the compound's derivatives play an important defensive role in poplars (Tsai et al 2006). It has also been established that benzoic acid and its conjugates play a defensive role in other higher plants as well (Chong et al 2001). Benzoic acid and its derivatives and conjugates have also shown antimicrobial properties more relevant to the present work (Nascimento et al., 2000).

In relationship with the visual assessment of disease symptoms, benzoic acid levels vary across the different genotypic classes, similar to the metrics used to estimate the pathogen performance and plant response. Benzyl alcohol varies in the opposite direction across the different genotypic classes. Combined with the stability of the compound earlier discussed, benzoic acid can be considered a reliable predictor of the response of *P. balsamifera*, *P. trichocarpa* and their hybrids (Fig. 10). Though the potential role of benzoic acid affecting *S. musiva* is yet to be established, based on its correlative power with the disease symptoms, the concentration of the compound can be considered an indicator of the degree of susceptibility.

5. Relationships between metabolites in potentially resistant and susceptible genotypes

By comparing potentially resistant and susceptible genotypes at the constitutive and induced levels through network analysis, I tried to breakdown different genotypes into more meaningful categories in response to *S. musiva*. In other words, some genotypic classes have genotypes that show resistance and genotypes that show canker damage, thus the classification in this analysis is

rather based on the response to *S. musiva*. It also provides insight into the chemotype of each category in a dynamic setting that accounts for the mutual relationships among different metabolites. The positive correlation between lignocerate and behenic acid is present in all categories suggesting that it could potentially be a conserved pathway across genotype and potentially irresponsive to infections by *S. musiva*. Likewise, the negative relationship between benzoic acid and benzyl alcohol can easily be deduced from Figure 7 and seems to be valid for all genotypic classes. However, when I looked at the plant material used from an angle of phenotypic response to *S. musiva*, the negative correlation is only characteristic of susceptible plants at the constitutive and induced levels. This particular relationship could be monitored in order to determine the percentage of susceptible genotypes when doing *en masse* screening of sizable numbers of genotypes. Along with concentrations, there is a variety of sophisticated models such as neural networks that could be solicited to predict resistance or susceptibility based on metabolic profile.

6. The conundrum of pure *P. balsamifera*: resistant or not?

The phenotypic response within pure *P. balsamifera* includes the whole spectrum of responses observed across the gradient from *P. trichocarpa* to *P. balsamifera*. This means that some *P. balsamifera* genotypes are functionally similar to *P. trichocarpa*, in terms of their response to the pathogen. All susceptible *P. balsamifera* are from Alberta while resistant *P. balsamifera* are either from the eastern provinces or from Alaska. This observation corresponds to the structure of *P. balsamifera* as the species shows essentially three demes based on the relic species that colonized the receding ice sheet after the last glacial maximum (Keller et al. 2010). Alberta and the prairies are indeed home to a separate deme of *P. balsamifera* characterized by the highest intrapopulation diversity (Keller et al. 2010).

The chemotype of susceptible *P. balsamifera* differs from that of the resistant *P. balsamifera* genotypes at the constitutive level for anisic, salicylic and cinnamic acids. Susceptible *P. balsamifera* genotypes show significantly lower levels of these three acids than resistant clones. However, once infected, their levels tend to become similar between resistant and susceptible *P. balsamifera* genotypes. This suggests that these compounds could potentially be involved in an early response to the pathogen and even perhaps in the pathogen recognition process. Hence,

these three metabolites should be screened to determine resistant or susceptible *P. balsamifera* genotypes.

At the induced level, resistant and susceptible *P. balsamifera* genotypes differ by the level of myristic acid (fatty acid) as it was significantly lower in susceptible genotypes upon infection than in resistant genotypes, suggesting its eventual role in fighting the pathogen as its levels are slightly increased only in resistant genotypes. All these findings hopefully put to rest the disputed resistance of *P. balsamifera* to *S. musiva*. Earlier research on eastern genotypes claims resistance (Bier 1939, Waterman 1954), while later research using genotypes from the prairies pleaded for susceptibility (Zalasky 1978, LeBoldus et al. 2009).

V. Conclusion

The initial hypothesis of this chapter stipulated that the susceptibility of *P. balsamifera* in Alberta is due to introgression of *P. trichocarpa*. The results show that there is some truth to this hypothesis, nevertheless the data on pure *P. balsamifera* show that there is more to its susceptibility than hybridization as the non-monolithic genetic fabric of pure *P. balsamifera* seems to also be responsible for its susceptible response to *S. musiva* in Alberta. Also the use of selective markers highlighted the need of reviewing the maps for the range of both *P. balsamifera* and *P. trichocarpa*, which have not been updated for the last 40 years (Little 1976). Future work should seek to establish associations between phenomics (growth, disease metrics and metabolomics) and the set of SNPs used for genetic mapping purposes.

Tables and Figures Chapter 3

Table 1. Classification of pure species and hybrids according to the percentage of *Populus balsamifera* based on the frequency analysis from the STRUCTURE software. Hybrids were categorized into five classes covering the range of admixture between the two species *P. balsamifera* and *Populus trichocarpa*.

Category	% *P. balsamifera*
Pure *P. balsamifera*	> 98%
*HybClassI	> 90 – 98%
HybClassII	> 60 – 90%
HybClassIII	> 40 – 60%
HybClassIV	> 10 – 40%
HybClassV	≥ 2 – 10%
Pure *P. trichocarpa*	< 2%

*HybClass: hybrid class

Figure 1: Genetic composition of *Populus balsamifera* from the Alberta-Pacific Forest Industries Inc. collection.

On a beige-blue gradient, blue dots represent pure *Populus trichocarpa* and light beige dots represent *P. balsamifera.* (**A)** Map showing the geographic distribution of the genotyped individuals. **(B)** Bar graph representing the number of trees in each genotypic class of the sampled area in Alberta.

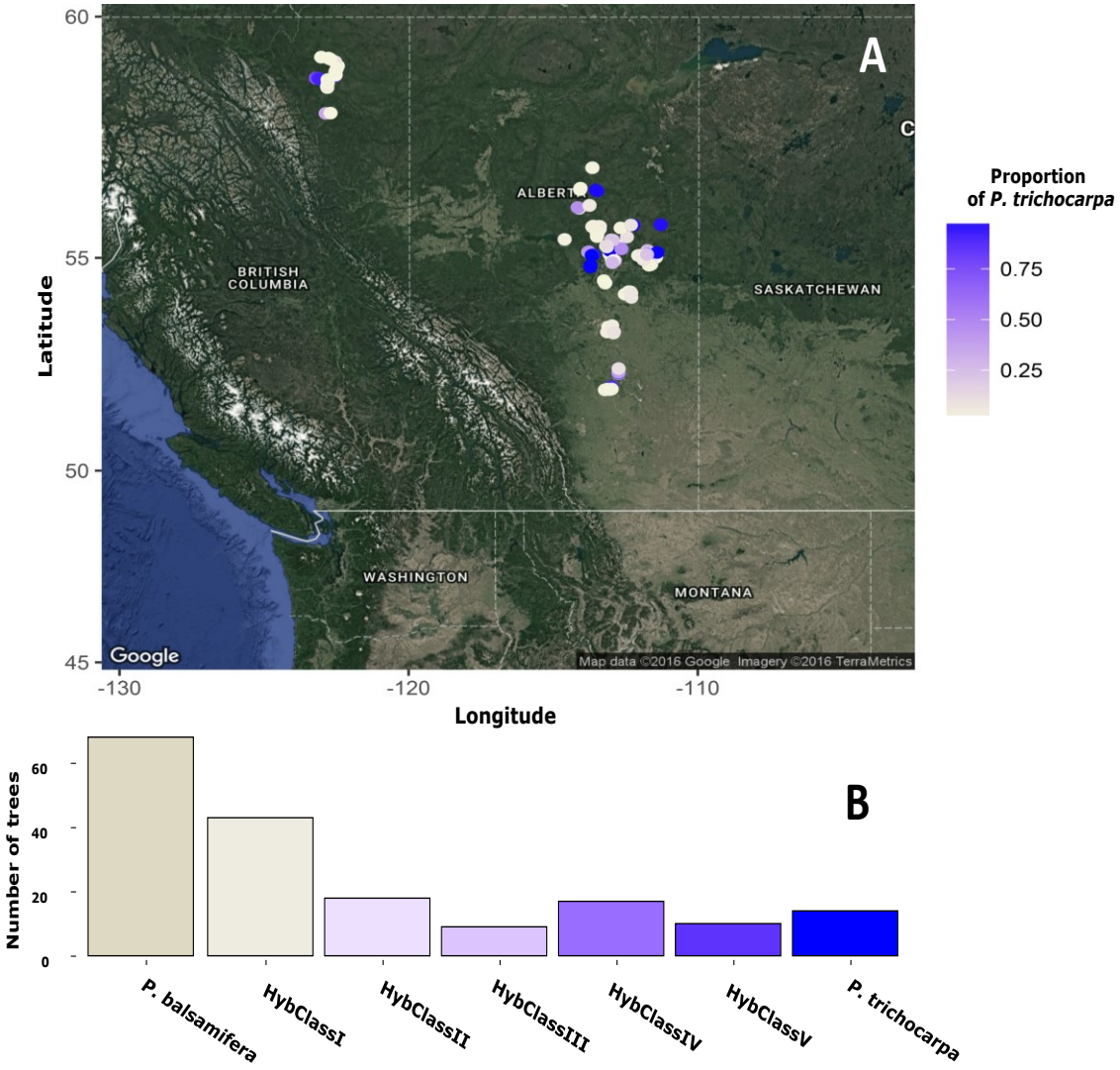


Figure 2: Distribution of clones covering the hybridization spectrum between *Populus balsamifera* and *Populus trichocarpa* across Canada. On a beige-blue gradient, blue dots represent pure *P. trichocarpa* and light beige dots represent *P. balsamifera.* Thresholds for determining pure and hybrid clones are shown in Table 1.

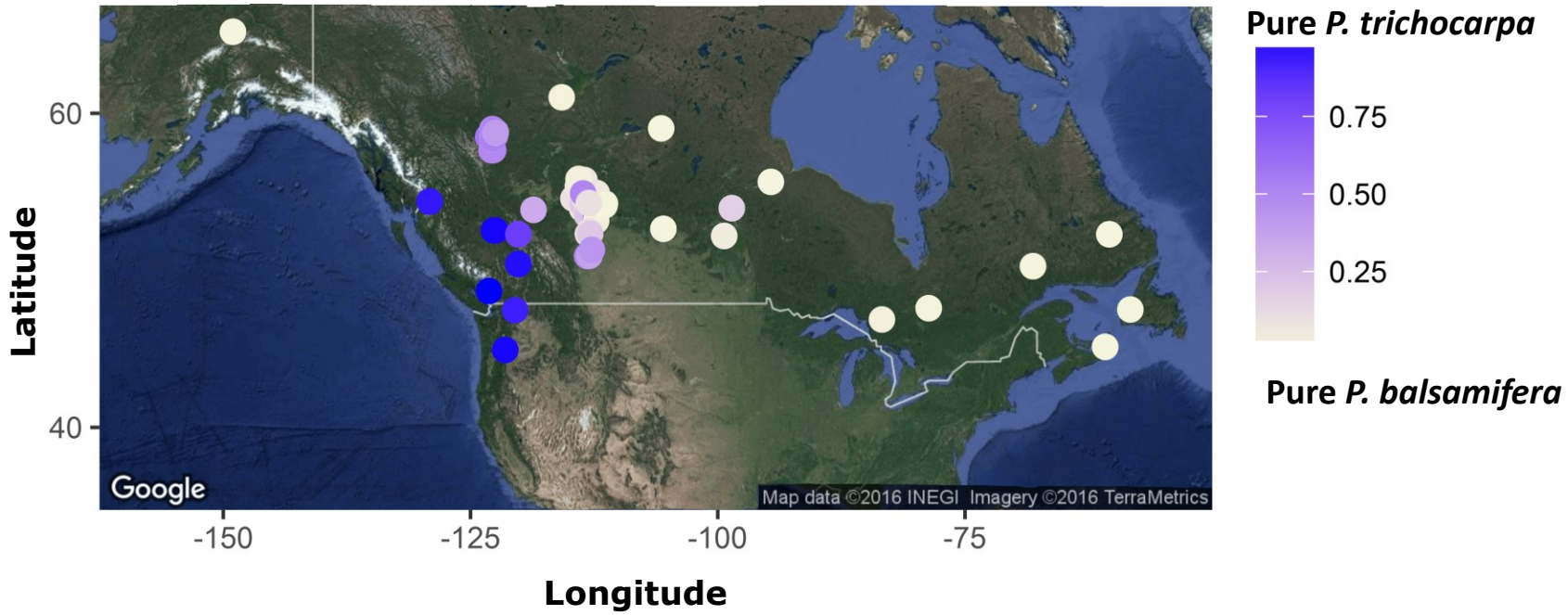


Figure 3: Estimation of admixture levels for a nationwide selection covering a hybridization gradient between *Populus balsamifera* and *Populus trichocarpa*. The values were generated by the software STRUCTURE for population genetics structure produced by the analysis of Single Nucleotide Polymorphisms based on a panel of specific markers for *P. balsamifera* and *P. trichocarpa*. B represents pure *P. balsamifera* and T represents pure *P. trichocarpa*.

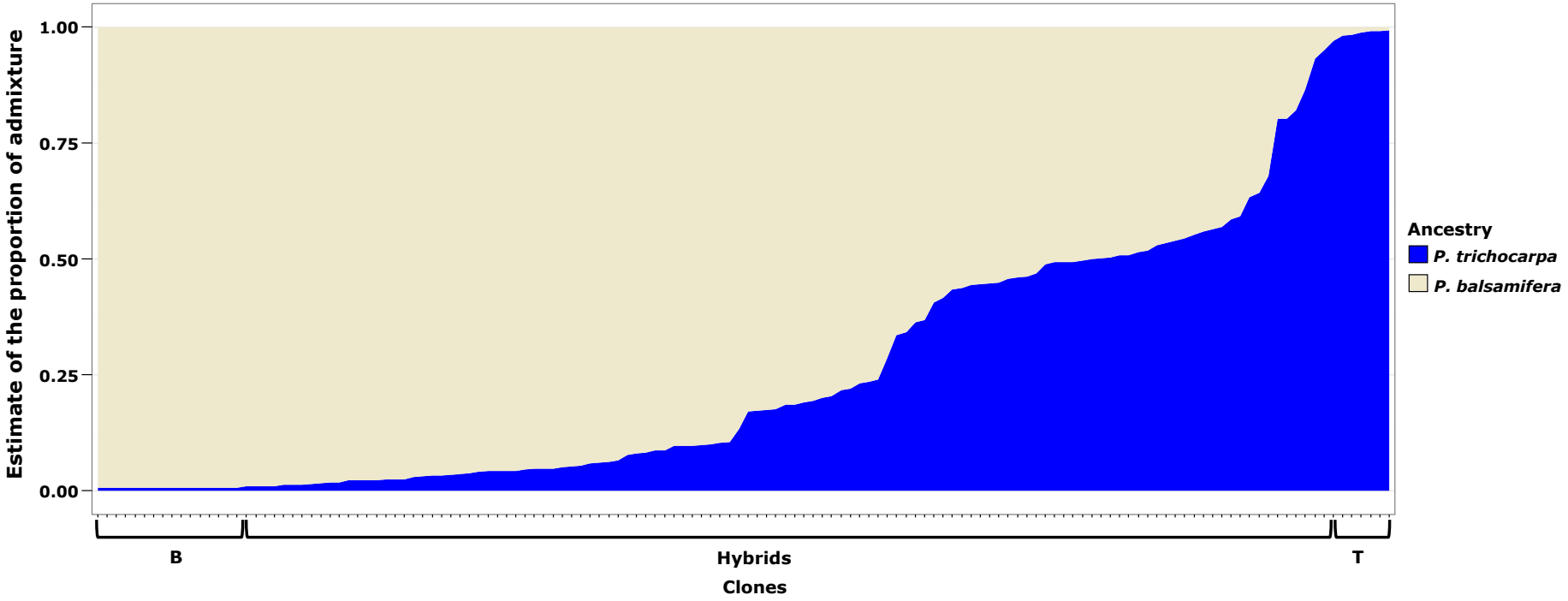


Figure 4: Visual assessment of the effects of the inoculation of the different genotypic classes of poplar clones with a mix of isolates of *Sphaerulina musiva*. Three metrics were used for this assessment: Infected leaf area (A), number of entry points on the stem caused by the pathogen (B), area of cankered tissue in the stem (C). Mock inoculations indicate plants that were sprayed with a solution of 1‰ Tween20 in distilled water and were included to validate that there were no secondary infections from the infected plants. Error bars indicate standard errors relative to each genotypic class on the x-axis. Details of the genotypic classes are shown in Table 1.

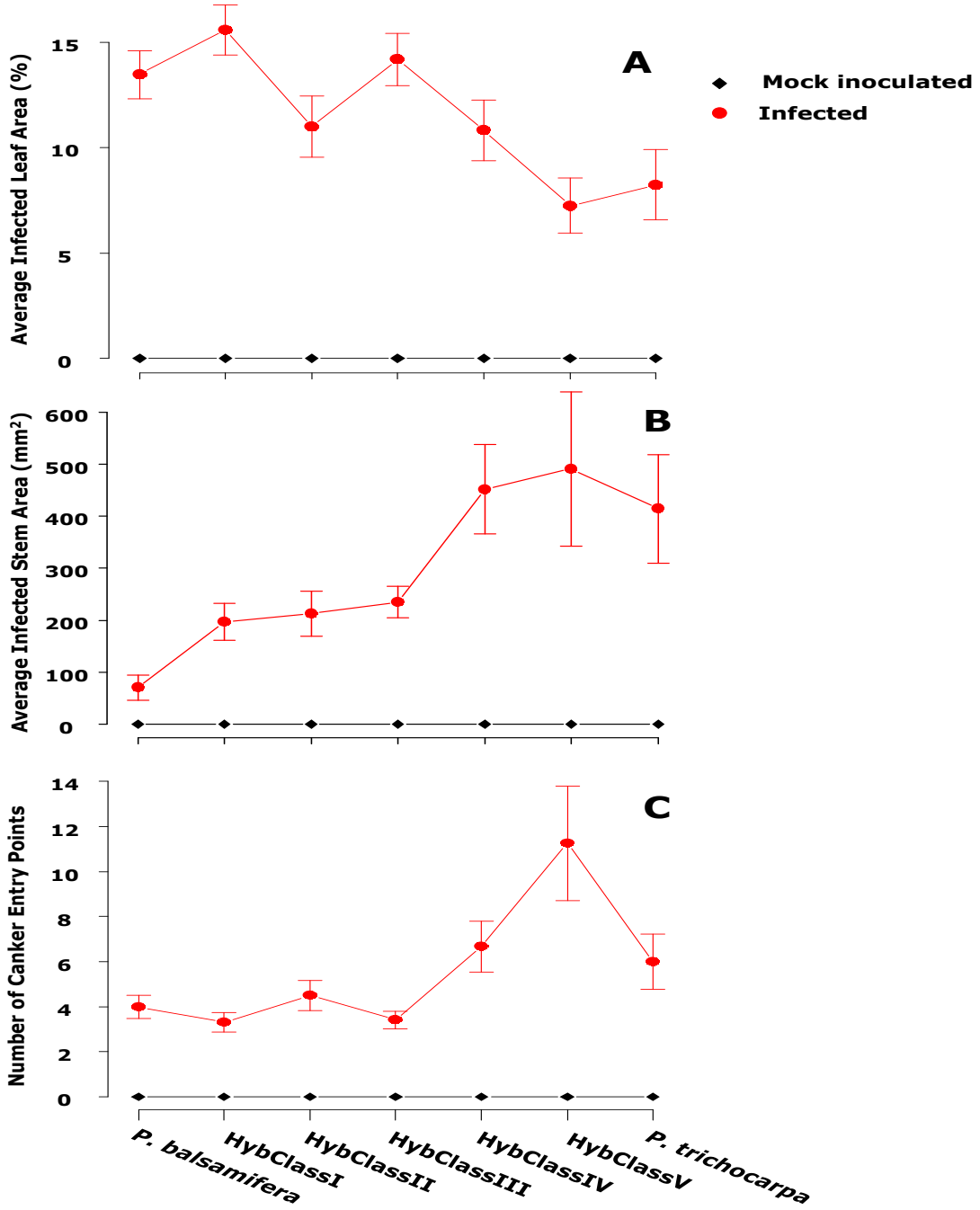


Figure 5: Score plot of the multivariate classification model based on the variance in differences in foliar chemistry between infected and mock inoculated poplars using OPLS-DA. The model was built with one predictive (t1) and one orthogonal component (to1) describing the variation in metabolite quantities as a response. The percentage of 7% attributed to the horizontal separation describes the percentage of response variance explained by the predictive component t1. R2X describes the percentage of predictor variance explained by the model; R2Y describes the percentage of response variance explained by the model; Q2Y describes the predictive power of the model. The OPLS-DA was only used to describe the differences in foliar chemistry between infected and non-infected plant not for predictive purposes as most commonly used.

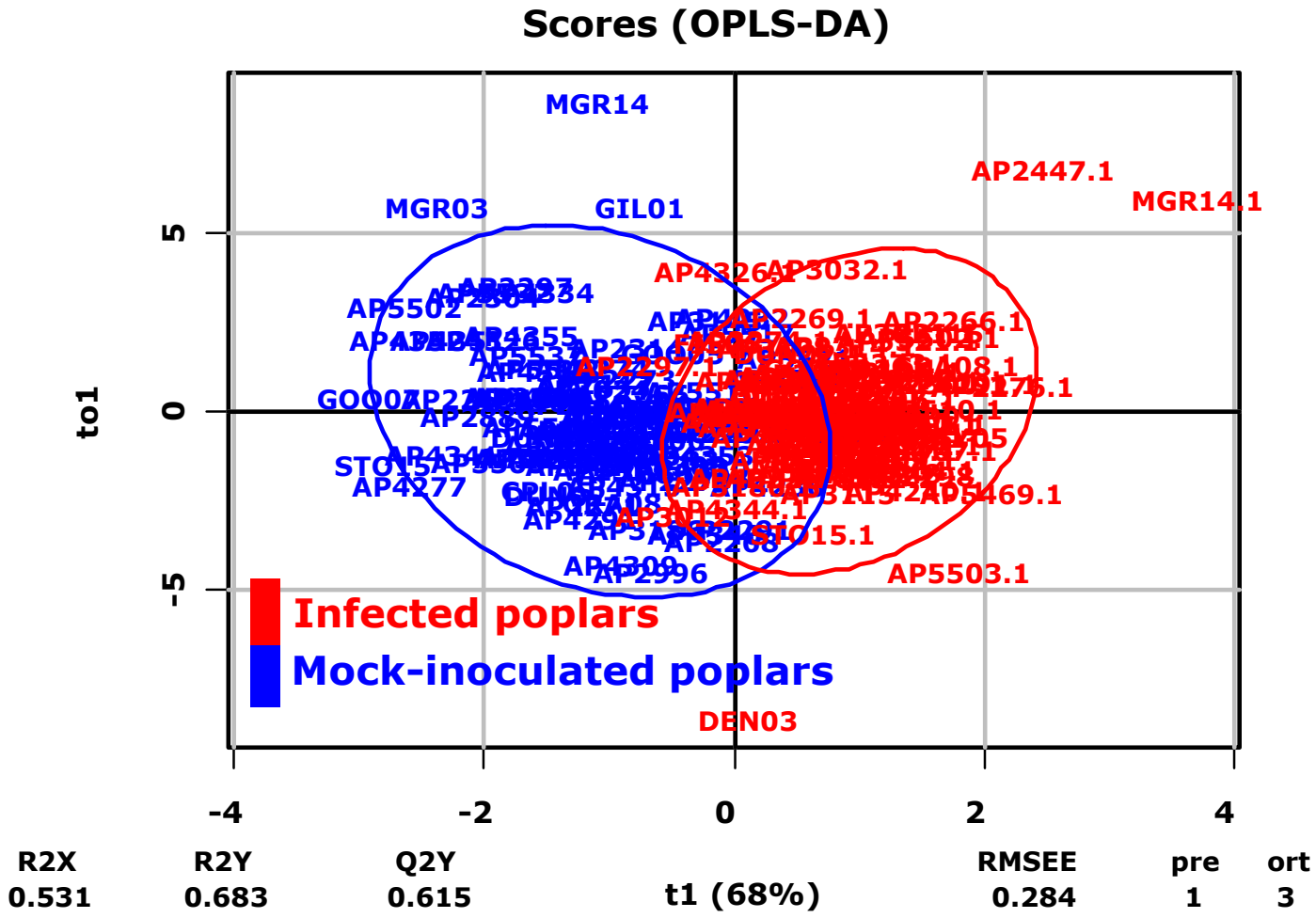


Figure 6: Identification of compounds involved in the response to *Sphaerulina musiva* with S-plot by plotting the covariance and the correlation of the score vector from the OPLS-DA output (***t***) and the data matrix containing the different compounds (***X***). Compounds in red triangles distinguish control and infected poplars.

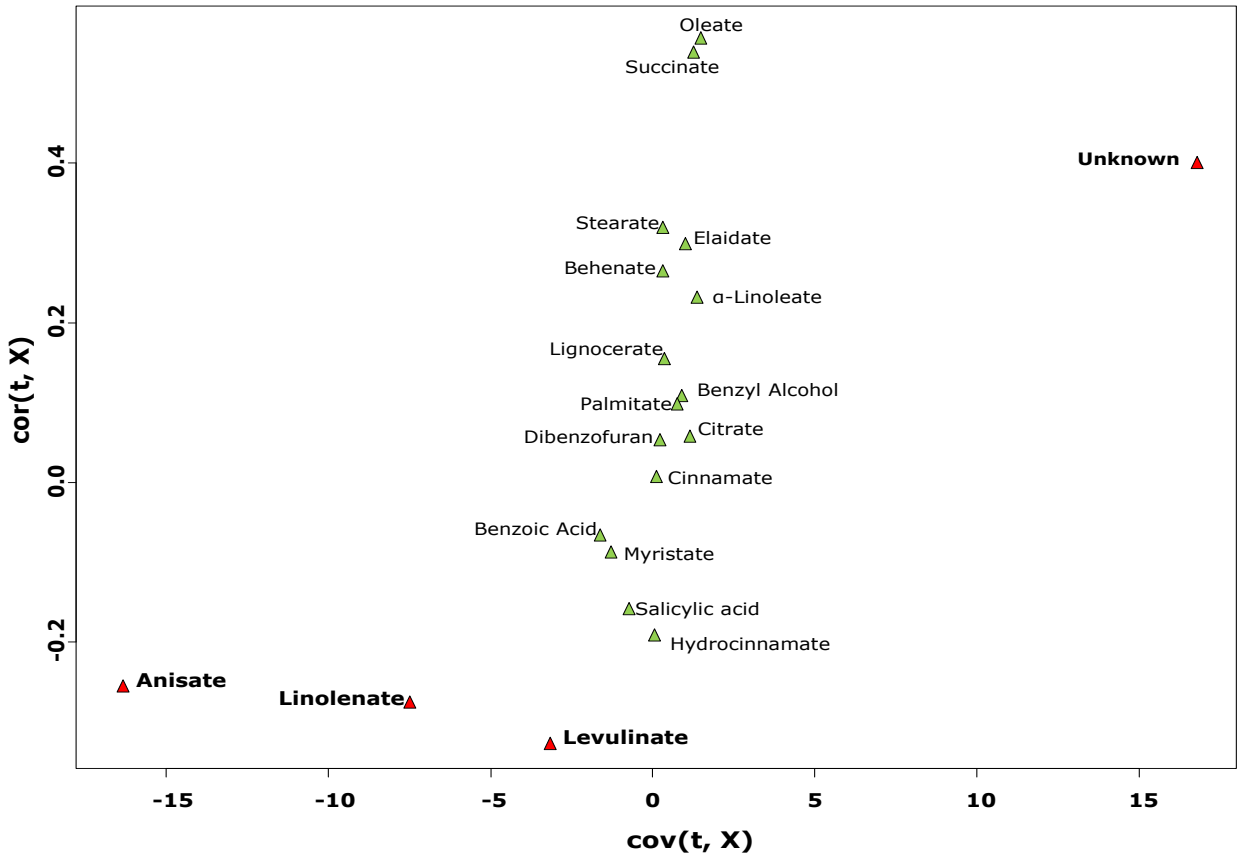


Figure 7: Variation of the concentration of benzoic acid and benzyl alcohol across genotypic classes at the constitutive and induced level. Error bars indicate standard errors relative to each genotypic class on the x-axis. Details of the genotypic classes are shown in Table 1.

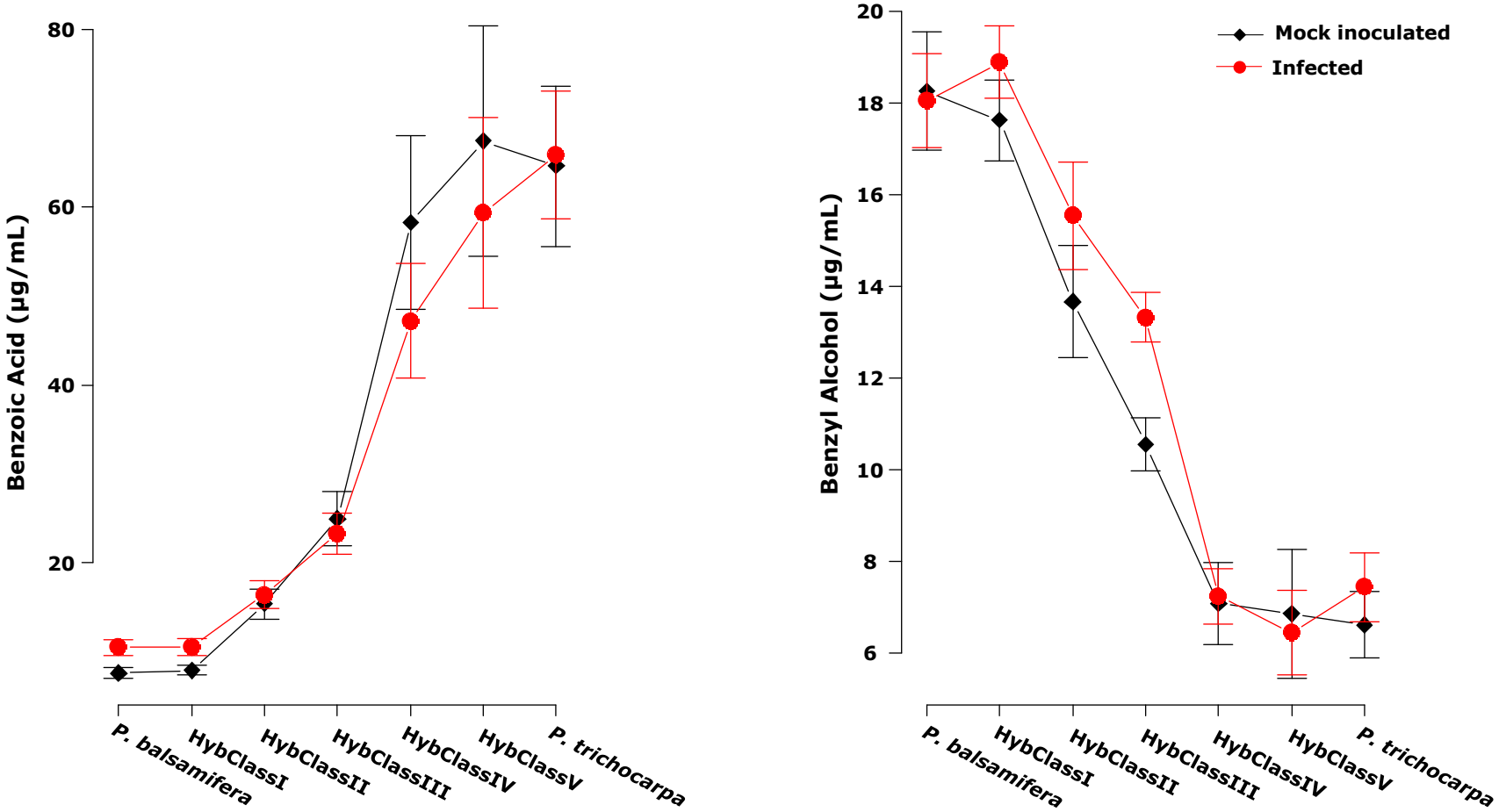


Figure 8: Genetic control of benzoic acid and benzyl alcohol. The ratio of the two compounds was computed and plotted against the proportion of *Populus balsamifera* of the clones used in the experiment.

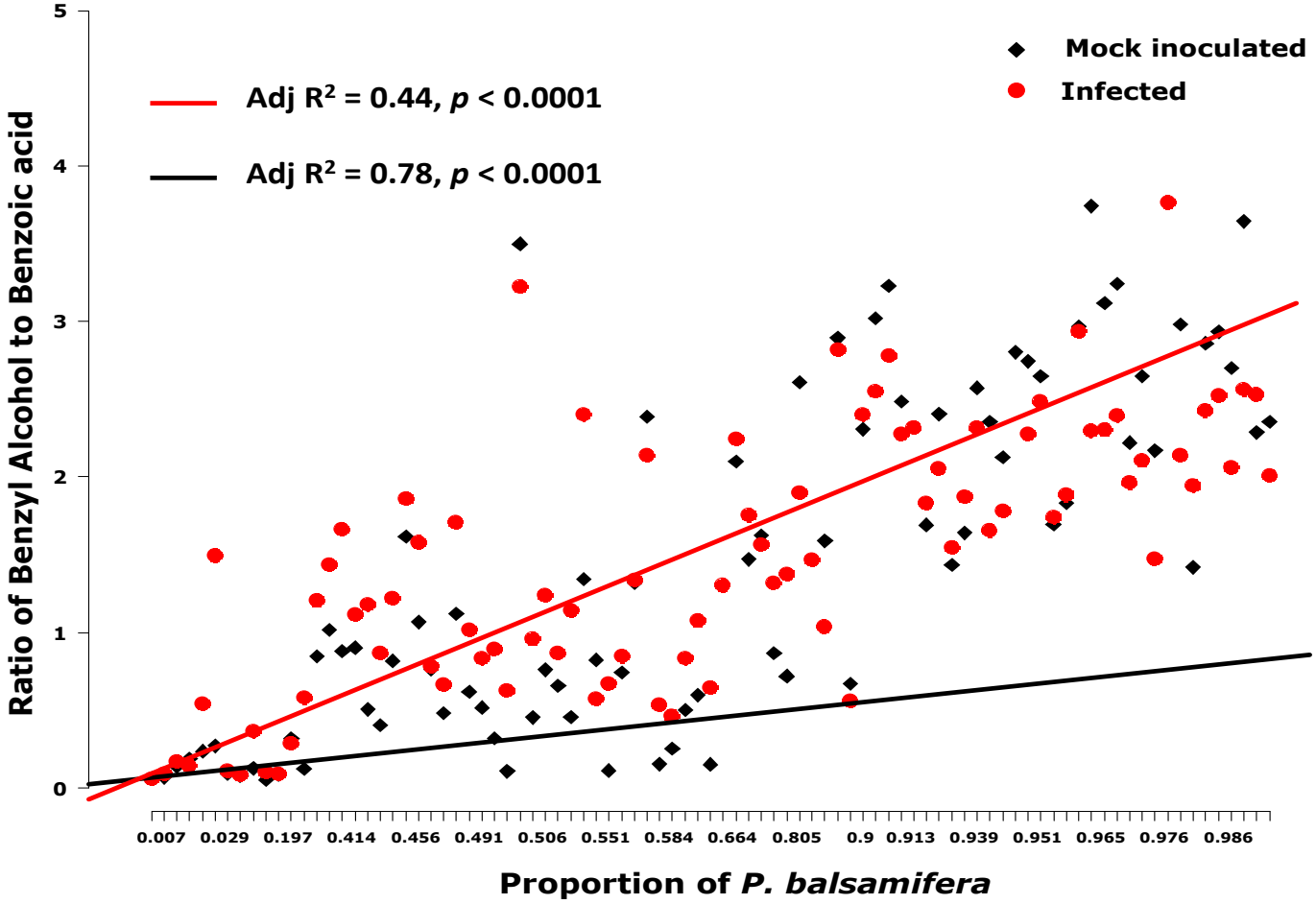


Figure 9: Network plot describing the relationships between metabolites of different plant classes. (**A**) Poplars showing no symptoms of canker upon infection (**B**) Poplars showing canker infection (**C**) Mock inoculated poplars corresponding to poplars in (**A**). (**D**) Mock inoculated poplars corresponding to poplars in (**B**). Metabolites belonging to the same class have the same shape. Green connections portray a positive correlative relationship and red connections portray a negative correlative relationship. The thicker the connection line the stronger the correlation.

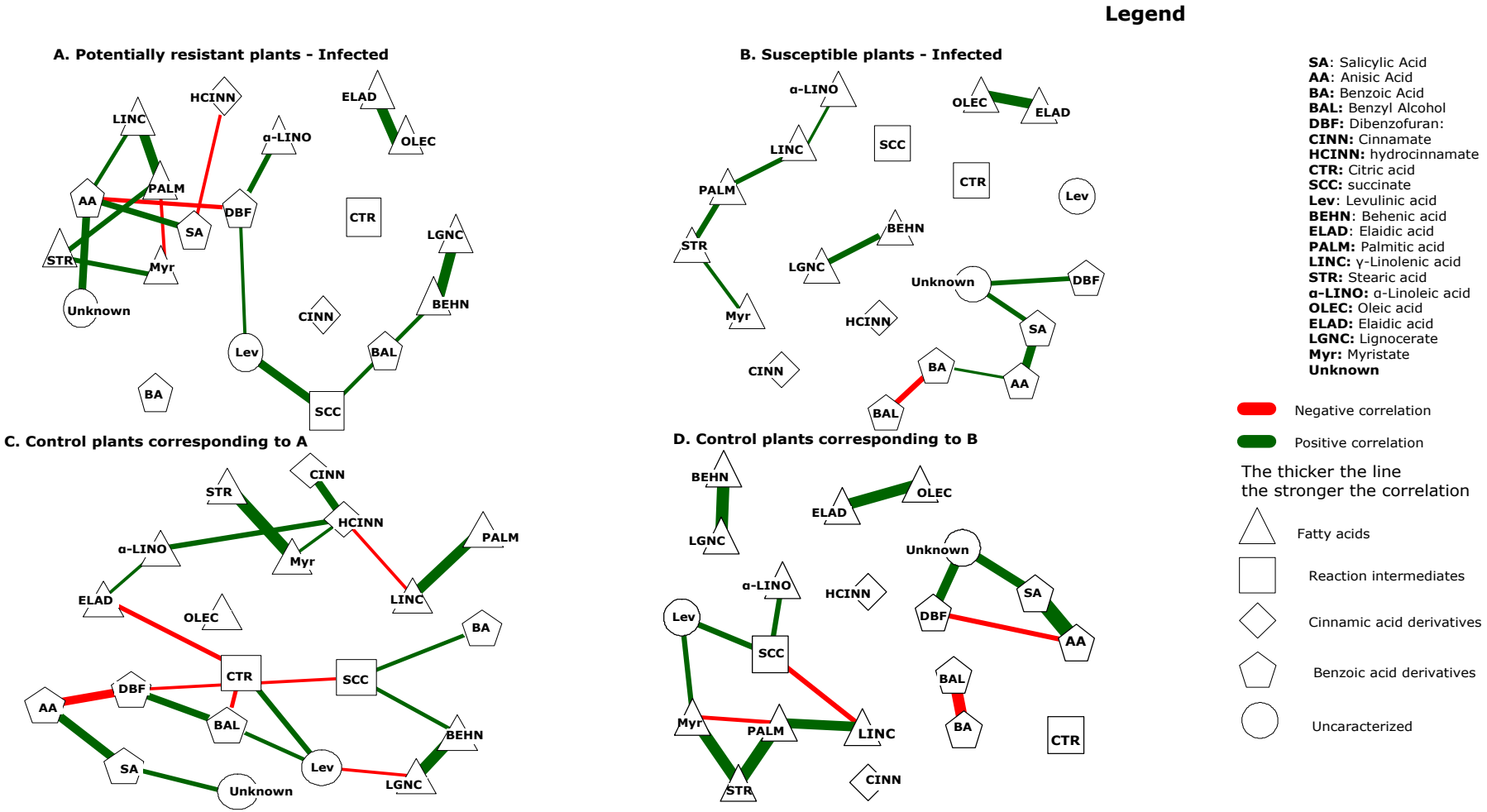


Figure 10: Assessment of the ability of benzoic acid to predict the amount of damage caused by *Sphaerulina musiva* across the seven genotypic classes. Genotypic classes are defined in Table 1 between *Populus trichocarpa* and *Populus balsamifera*.

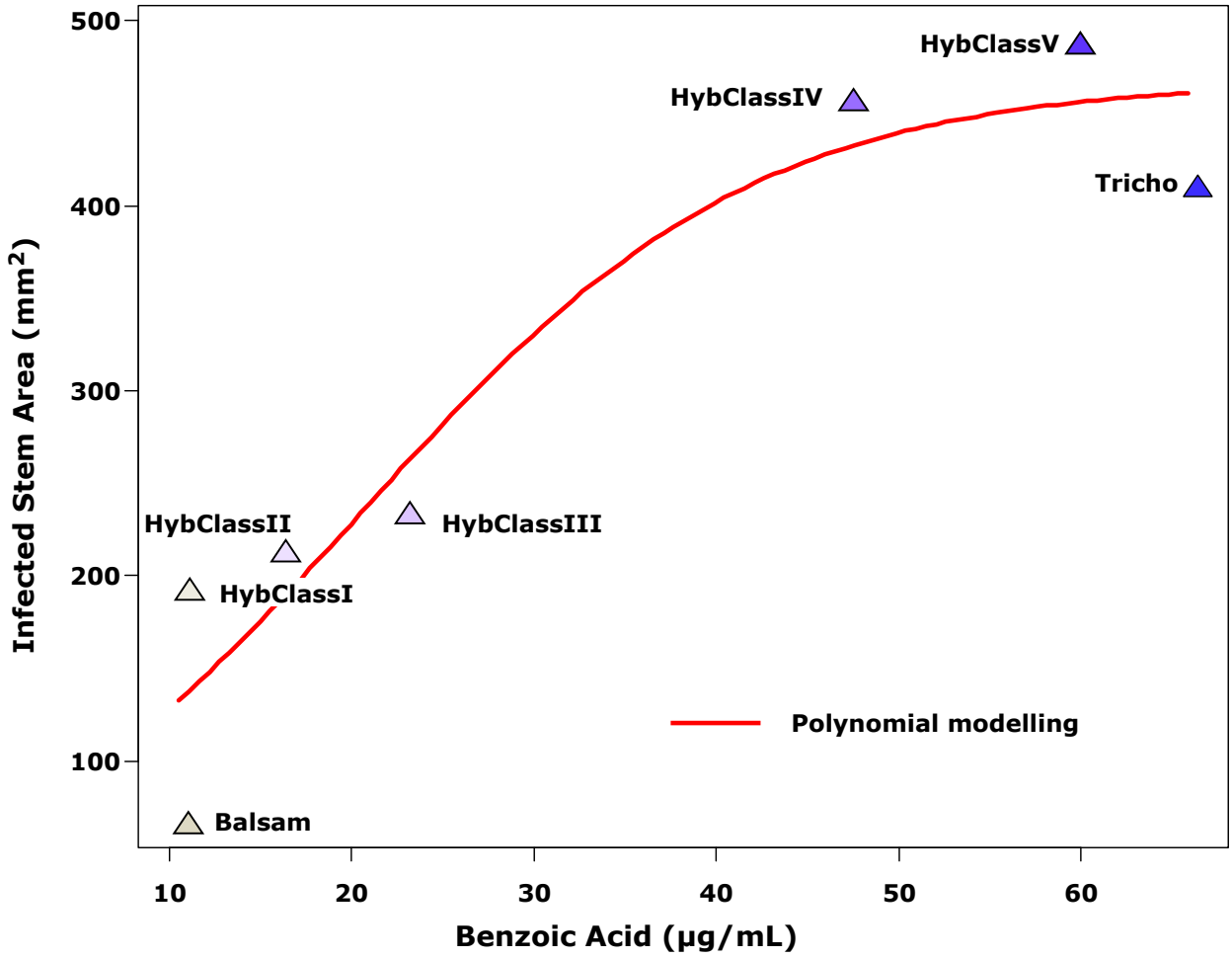


Figure 11: Stem canker response of *Populus balsamifera* from different provenances to infection with *Sphaerulina musiva*. Blue triangles represent balsam poplars from Alberta and dark triangles represent balsam poplars mainly from eastern provenances in Canada, except for Healy which is in the Denali Park in Alaska, USA. Data points were organized from the most susceptible to stem canker to the least susceptible.

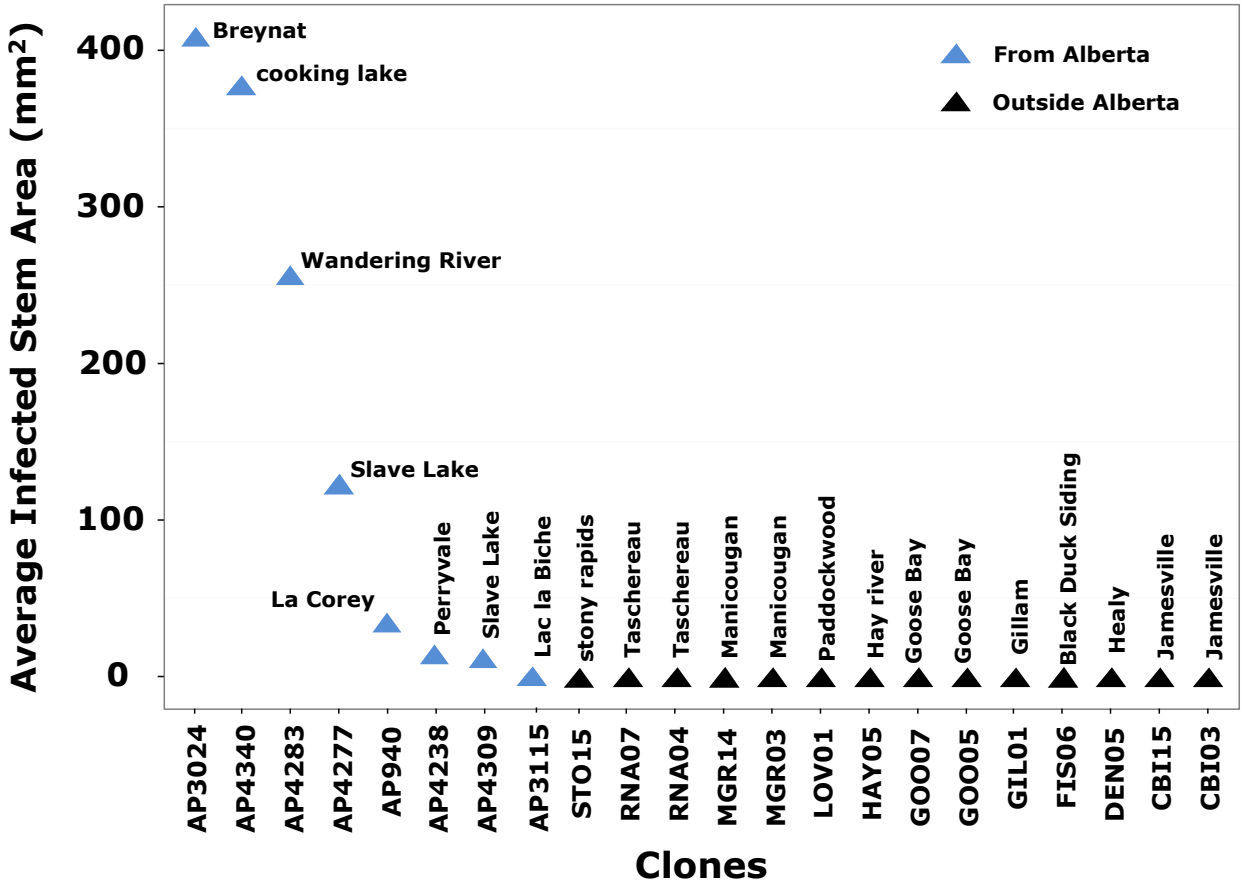
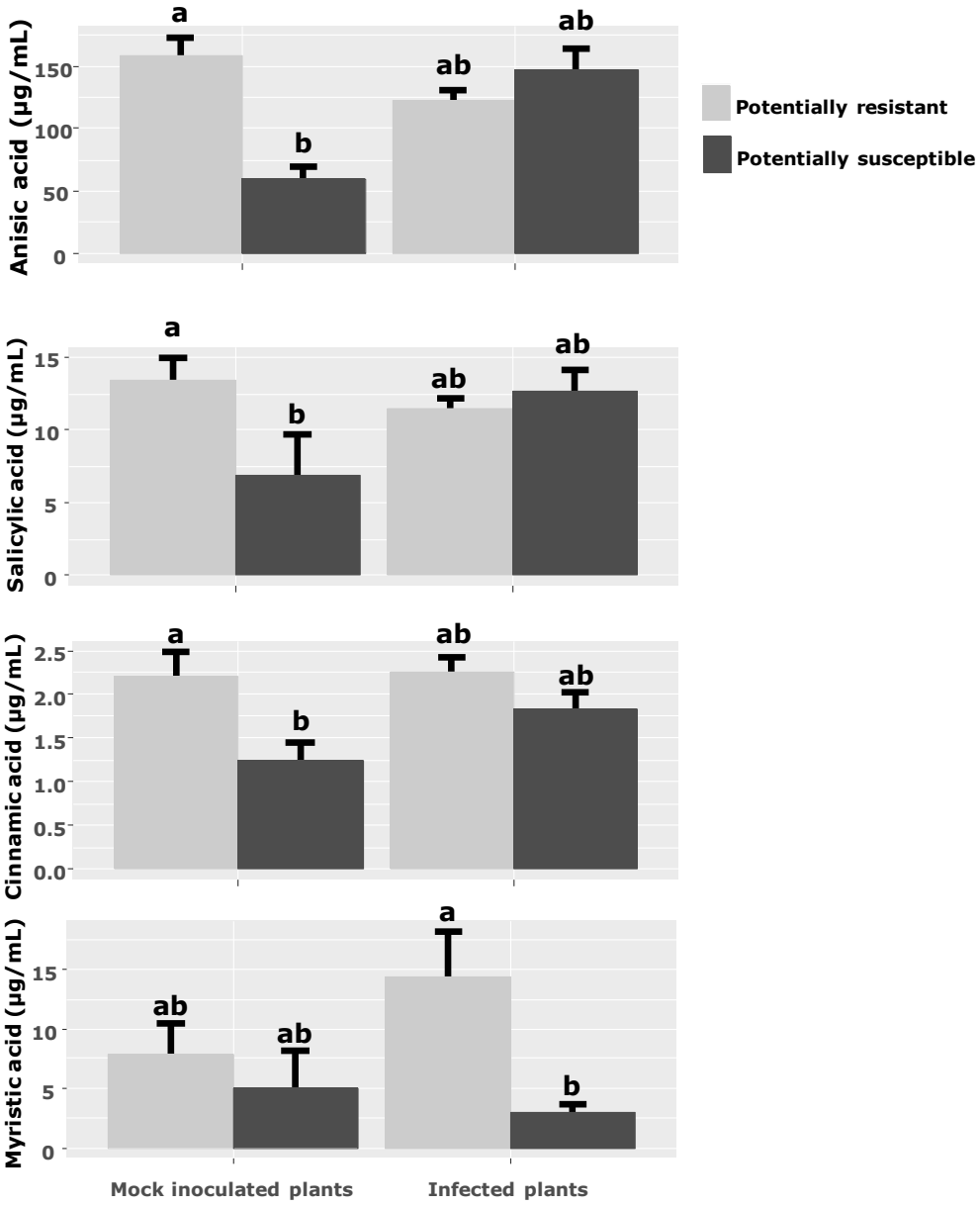


Figure 12: Chemical response of *Populus balsamifera* for select chemicals to infection with *Sphaerulina musiva*. Potentially resistant plants are poplars that had zero stem cankers upon infection. All other poplars are considered potentially susceptible. Mock inoculated plants were not infected with the pathogen and were similarly split into potentially resistant and potentially susceptible similar to the infected plants. Bars with different letters are significantly different at $p \leq 0.05$.



Chapter 4 – Thesis Discussions

I. A new approach to disease prediction models

In the present work I sought to use leaf chemistry in *Populus spp.*: (1) as a proxy for screening resistant and susceptible phenotypes, (2) to gain a mechanistic understanding of the role of plant metabolites in responding to a pathogen, (3) and to establish metabolic signatures for pure species and hybrids that could help identify their performance against the pathogen.

The results from the first chapter establish the potential of plant metabolites in predicting the response of anthropogenic hybrid poplar crosses to *S. musiva*. Classification And Regression Trees (CART) predictions provided metabolite levels that define thresholds for both complete and partial resistance in hybrid poplar crosses. Machine learning algorithms such as CART and neural networks have been successfully used in progeny tests in order to predict wood density in spruce in Canada (Demertzis et al. 2015). These predictive models are slowly making their way into forestry applications to inform forest tree breeders and managers. The present work suggests that similar applications can also be used for predicting hybrid performance in breeding programs.

II. *Sphaerulina musiva* as a potential biotic pressure

The pathogen *S. musiva* is present in both natural and plantation poplar stands but its damage is more noticeable in hybrid plantations where it can cause plantation failure and wipe out a whole stand (Newcombe et al. 2001). Natural stands differ at many levels from plantations and have a different ecology. Indeed, the pathogen has posed its greatest damage following the deployment of plantations with previously unscreened exotic and non-native genotypes in different locations in Canada and the USA (Newcombe et al. 2001). Depending on our level of understanding of the plant-pathogen interactions, plantations may put the host at an advantage (in the case where resistant genotypes are deployed) or jeopardize (in the case where susceptible genotypes are deployed) plantations altogether.

My results from testing clones selected from natural stands show how disease severity increases with the introgression of *Populus trichocarpa* and the higher proportion of its genome in hybrid classes. However, anthropogenic crosses in the first chapter showed variable levels of

susceptibility, suggesting that controlled hybrids have unique disease responses even though the overall response is an average between two parents. Overall, these results suggest that selection in hybrid poplar crosses should be based on individual performance of genotypes rather than cross type (parental types) which is the traditional method of selection (Newcombe et al. 2001). Machine learning predictive modelling is the most suitable technique to reveal individual responses as it can be fine-tuned to detect singular patterns from an overall trend.

III. Chemical compounds as predictors of the plant response to the pathogen infection

Studies on plant-pathogen interactions have focused extensively on cell receptors and their ability in recognizing conserved and specific patterns in fungal infection. When it comes to secondary metabolites effectors (i.e. the end product defensive compound), a pattern transcending species is harder to define. For instance monoterpenes are the specialized defenses of conifers (Erbilgin et al. *in press*), phenolic glycosides for poplars (Constabel and Lindroth 2010), Oleaceae synthesize iridoid glycosides against fungal pathogens (Jensen et al. 2002). The role of effectors in plants is played by defensive chemicals most of which come through pathways activated by cell receptors (Jones and Dangl 2006). Phenolic effectors differ between species, however they are thought to spur from either the salicylate or jasmonate pathways (Robert-Seilaniantz et al. 2011). In angiosperms, most of these effectors have been elicited in the *Arabidopsis* model or in herbaceous, crop species. Different plant species deal with specific pests and pathogens hence must have evolved with different batteries of effectors against their enemies.

In poplars, phenolic glycosides are a major defensive feature against herbivores (Constabel and Lindroth 2010). The present work shows the importance of phenolic glycosides in predicting resistance against *S. musiva* in anthropogenic crosses along with benzoic acid, which is the building block of the aglycone portion of tremulacin and tremuloidin. In natural poplar populations, another benzoic acid derivative, namely anisic acid (4-methoxy benzoic acid) decreases upon infection, suggesting that it could be solicited by other pathways. Benzoic, anisic and salicylic acids are all benzoic acid derivatives yet only salicylic acid is established as a plant hormone involved in resistance to pathogens. The functions for other benzoic acid derivatives are yet to be determined. Highlighting the presence of different compounds without defining a more general pathway or ecological function adds to the challenge of finding an overarching pattern

for plant disease responses that transcends species. I hope the present findings could fill some gaps in our knowledge in the established pathways and initiate new and substantive research. More work is required to determine if the compounds revealed by this study with an impact on resistance to *S. musiva* are specific to poplars only, or common to woody angiosperms.

IV. Constitutive chemistry and stable compounds are reliable predictors of the poplar response to *S. musiva*

In the following discussion, the term "stable compounds" will be used to indicate compounds that are not affected by the pathogen infection. Compounds such as benzoic acid and benzyl alcohol fit this definition as their concentration varied only across genotypes and was not affected by the infection with *S. musiva*. Hence, these two compounds were shown to be reliable predictors of the genotypic class response of *P. balsamifera*, *P. trichocarpa*, and their hybrids Both compounds appeared to be under strong genetic control, suggesting that biochemical pathways producing these compounds are conserved in poplars. It is also possible that by virtue of being precursors for other labile defensive compounds (Chedgy et al. 2015) the two compounds are less responsive to infection and more indicative of the genetic makeup of each genotype.

Benzoic acid was also highly correlated to the infected stem area in natural hybrids and anthropogenic crosses at both its constitutive and induced levels. This suggests that benzoic acid could well be used as a predictor of resistance for the set of genotypes used in the present work and perhaps a wider range of species in the genus *Populus*. I combined both natural and anthropogenic crosses in one graph where I plotted benzoic acid concentrations against the infected stem area (Fig. 1). The graph shows two predictive trends: (trend 1) at concentrations between 10 $\mu g.mL^{-1}$ to 100 $\mu g.mL^{-1}$, where there was a positive correlation between this compound and the percentage of infected stem area for *P. balsamifera, P. trichocarpa*, and their hybrids as well as the *P. deltoides* x *P. balsamifera* cross; and (trend 2) at concentrations between 250 $\mu g.mL^{-1}$ and 550 $\mu g.mL^{-1}$, where there was a negative correlation between level of the chemical compound and the infection area of the stem.

Although the *P. balsamifera* parent used in the *P. deltoides* x *P. balsamifera* cross was not from the hybrid zone area in Alberta, hybrids between these two species occur naturally in the province and are known as *P.* x *jackii*. Thus, trend1 (mentioned above) seems to be characteristic

of natural populations. Otherwise, trend 2 could well be exclusive to crosses of North American species with and the exotic *P. maximowiczii*. This finding potentially suggests that benzoic acid could as well be used to monitor exotic and native crosses.

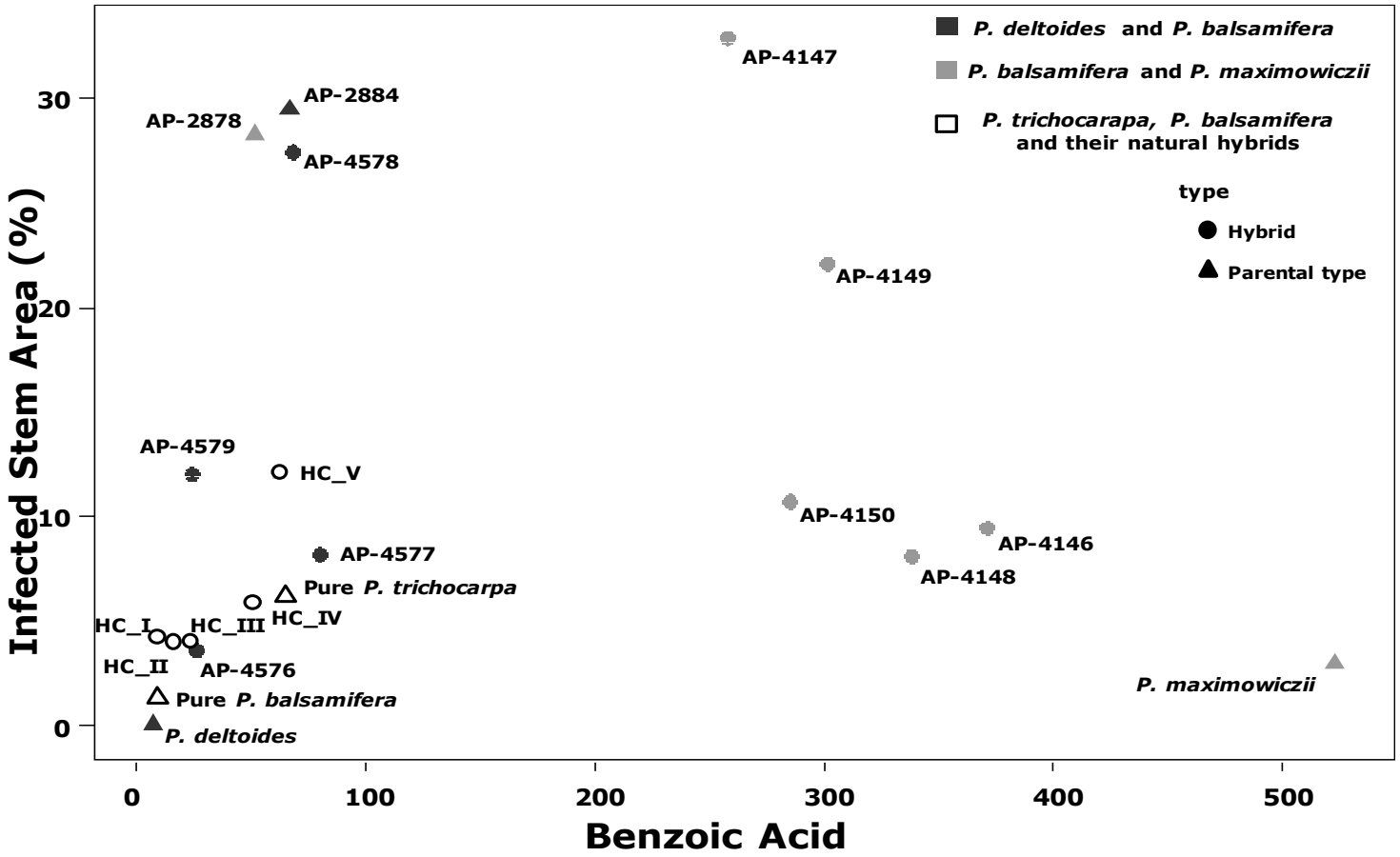
V. Outcomes and conclusions

The present work provides tools that could be used by forest managers and tree breeders to predict resistance without traditional resistance screening since compounds such as benzoic acid and benzyl alcohol are good predictors of performance against *S. musvia* at the constitutive level. The results shed light on the potential of using plant chemistry as a proxy for both disease predication and traceability of hybridization. The role of hybrid zones could also be deduced from these findings as they were found to act as a potential stepping stone for the disease spread between susceptible and resistant genotypes within pure species. It is however needed to (1) establish the effects of the tested chemicals on *S. musiva*'s growth and reproduction through bioassays as well as their relationship to physiological outcomes such as growth and photosynthesis; And (2) establish the stability of the compounds analyzed across phenology as well as between seedlings and mature trees in order to validate that the predictive models established in the present work still hold true.

Figures Chapter 4

Figure 1: Response of poplars from natural populations and anthropogenic crosses as a function of benzoic acid.

* The details about hybrid classes can be found in Chapter 3 – Table 1.



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