UNDERSTANDING THE ROLES OF PHENOLICS AND TERPENOIDS IN PINE DEFENSE AGAINST FUNGAL PATHOGENS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

the Degree Doctor of Philosophy in the Graduate

School of the Ohio State University

By

Christopher Michael Wallis, M.S.

* * * * *

The Ohio State University
2007

Dissertation Committee

Professor Pierluigi Bonello, Adviser

Professor Terrence Graham

Professor Daniel Herms

Professor Dennis Lewandowski

Professor Donald Cipollini

Approved by:

_____

Adviser
Plant Pathology Graduate Program

ABSTRACT

Pines are important because they are major components of many forest ecosystems, but also because, more generally, they have great environmental, economic, and aesthetic value. Pathogen attack greatly reduces the value of these trees. Pines have evolved constitutive and induced defenses to combat pathogens. Constitutive defenses are produced all the time and function to prevent infections from establishing in the first place. Induced defenses form after pathogen recognition and involve both local and systemic responses. Around an infection, trees mount additional defenses to compartmentalize, inhibit, or otherwise eliminate the pathogen as part of a localized induced resistance (LIR) response. An increase in defense-associated compounds also occurs systemically following infection and is often associated with a systemic induced resistance (SIR) response. The major aim of this research was to utilize a system comprising Austrian pine (*Pinus nigra*), a common landscape tree, and two fungal pathogens belonging to the genera *Diplodia*, to explore different aspects of how pines defend themselves against pathogens. The major objectives of this research were to: 1) discover biochemical changes that occur as part of the induced resistance response, with an emphasis on the defense-associated phenolics and terpenes; 2) discover the effects of fertility on defense compounds; and 3) identify potential systemic signaling molecules of induced defense responses in pine. This research showed that: 1) phenolic compounds increase both locally and systemically (in the phloem and xylem) following pathogen

infection; 2) terpenes are induced around an infection site but not systemically; 3) phenolics positively associated with SIR; 4) terpenes appear linked only to LIR; 5) soil fertility affects the accumulation of phenolic compounds in induced defense only, while terpene compound production is affected only in non-infected trees; and 6) no phenolic compounds could be linked with systemic signaling in the context of this study. Knowledge about how pines deploy defense responses will be useful in developing new strategies that could more effectively manage pine diseases as well as better preserve the ecological, environmental, economical, and aesthetical values of pines.

Dedicated to my parents and my family: Michelle, Sarah, Rosemary, and Timothy

ACKNOWLEDGMENTS

I especially wish to thank my adviser, Enrico Bonello, whose great advice, intellectual support, and enthusiastic encouragement made this dissertation possible. I am grateful for his monumental efforts in reviewing this text for both stylistic and scientific errors, as well as for helping me to constantly improve as a scientific writer.

I thank the additional members who served on my advisory committee, Dan Herms, Terry Graham, Sophien Kamoun, Don Cipollini, and Dennis Lewandowski, for their useful input, advice, and constant help in the carrying out of this research and preparation of this dissertation. I am also grateful to Brian McSpadden Gardner, whose statistical advice helped me with the many analyses that were performed in this work, and Alieta Eyles, whose identification work was crucial in forming my conclusions. I also wish to acknowledge my former advisors from Penn State, Fred Gildow and Bill Schneider, as well as the Department of Plant Pathology at Ohio State for giving me the firm foundation that I needed to succeed in plant pathology.

I also am grateful to Robert Hansen, Nathan Kleczewski, Duan Wang, Anuprit Kaur, Justin Whitehill, Nick Weidenbenner, and the members of the Dan Herms lab for their frank advice and technical assistance in all this research.

The research was supported by USDA National Research Initiative Competitive Grants, an Interdisciplinary OARDC SEEDS Research Grant, The Tree Fund Duling Grant Competitive Grants Program, and by funds appropriated to the OARDC. Trees and media used in this study were graciously provided by Willoway Nursery, Avon, OH, Smith Evergreen Nursery, Inc, Magnolia, OH, and Kurtz Brothers, Inc.

VITA

Christopher Michael Wallis

September 19th, 1979……..	Born – Baltimore, MD
2001……	B.S. Biology and Biochemistry, Mt. St. Mary's University, Emmitsburg, MD
2004…….	M.S. Plant Pathology, The Pennsylvania State University, University Park, PA
2004-2007……	Graduate Research Associate, The Ohio State University, Columbus, OH

PUBLICATIONS

Eyles, A., Chorbadjian, R., Wallis, C., Hansen, R., Cipollini, D., Herms, D., Bonello, P. 2007. Cross-induction of systemic induced resistance between an insect and a fungal pathogen in Austrian pine over a fertility gradient. Oecologia 153: 365-374

Wallis, C., Stone, A., Sherman, D., Damsteegt, V., Gildow, F., Schneider, W.L. 2007. Impacts of Host Shifting and Mode of Transmission on PPV Microevolultion. Journal of General Virology 88: 2839 – 2845

Wallis, C., Fleischer, S., Luster, D. Gildow, F.E. 2005. Potential Aphid Vectors of Plum Pox in Pennsylvanian Peach Orchards. Journal of Economic Entomology 98: 1441-1450

FIELDS OF STUDY

Major Field: Plant Pathology

Specialization: Plant Molecular Biology and Biotechnology

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

GENERAL INTRODUCTION

The Importance of Pine to Ecosystems and the Environment

Pine trees are arguably among the most important organisms on Earth. They are the dominant species in the boreal forests in the Northern Hemisphere, some of the largest ecosystems on Earth (Richardson and Rundel 1998). In these ecosystems, pines provide important habitat for a myriad of organisms, as well as serve as valuable carbon sinks. Pines have also become very important landscape and plantation trees in countries throughout the Southern Hemisphere. Different pines grow in a multitude of environments ranging from arid climates (e.g. Colorado pinyon, *Pinus edulis* Engelmann, and western bristlecone pine, *Pinus longaeva* Bailey), temperate climates (e.g. Eastern white pine, *Pinus strobus* L.), wet tropical climates (e.g. Aztec pine, *Pinus teocote* Schiede ex Schlechtendal et Chamisso), montane climates (e.g. ponderosa pine, *Pinus ponderosa* Douglas ex C. Lawson), Mediterranean climates (e.g. Austrian pine, *Pinus nigra* Arnold), and boreal forest climates (e.g. Scots pine, *Pinus sylvestris* L.) (Richardson and Rundel 1998). Within the aforementioned environments, pines fill crucial ecological roles.

Unlike the other dominant conifer genera (*Abies*, *Picea*, and *Pseudotsuga*), *Pinus* spp. are characterized as pioneer species. As such, the life history of most pines is that of a rapid colonizer, that take advantage of open conditions created by disturbances (such as fire), or occupy areas where harsh, severe environmental conditions limit the growth of other trees (Keeley and Zedler 1998). Contrary to other pioneer species, pines are extremely stress-tolerant and have evolved mechanisms to survive fires, extreme cold, and/or severe arid conditions. As a further testament to the resiliency of pines, some species, such as western bristlecone pine (*P. longaeva*) have lived for thousands of years (Richardson and Rundel 1998).

Pines provide crucial habitats for many forest dwelling animals. They are especially important to animals that live where pines are the dominant species because environmental conditions might be too harsh for other trees to thrive year-round. Certain birds have evolved to depend on particular pines for their survival, with the best example being the dependence of the endangered Kirtland's warbler (*Dendroica kirtlandii*) on jack pine (*Pinus banksiana*) (Bull and Farrand Jr. 2003). Other species of birds that depend on pines called crossbills have evolved specialized bills to remove pine seeds from cones (Bull and Farrand Jr. 2003). Mammals also depend on pines for food and habitat, such as squirrels that eat the pine seeds and moose and deer that may browse on pine needles during winter months. Certain insects, some of which are considered pests on pines, have evolved to depend exclusively on pines for survival including bark beetles and pine sawflies (e.g. de Groot and Turgeon 1998).

Pines also provide niches for microorganisms such as fungi. Many species of mycorrhizal fungi form obligate symbiotic relationships with pines on their roots. These fungi are often classified as ectomycorrhizal (in the phyla Ascomycetes and Basidiomycetes), and often form common mushrooms including those of the *Amanita* and *Boletus* genera (Read 1998). The mycorrhizae are dependant on their host for respiratory carbon. In exchange, the fungus provides benefits to the pine through improved tolerance to nutrient and drought stress, and protection against soil-borne pathogens and toxic metals (Read 1998; Schoenholtz et al. 1987).

As colonizers in disturbed areas, pines are responsible for alterations in soil chemistry and shade that are necessary for forest succession (e.g. Ashton et al. 1997; Newmaster et al. 2006; Vallauri et al. 2002). Furthermore, in environments characterized by excessive heat or cold, pine litter insulates the forest floor, providing insulation necessary for the survival of myriad herbaceous species (e.g. Ashton et al. 1997; Newmaster et al. 2006).

Besides the value to individual ecosystems, pines also have great value to the global environment. Pine forests reduce the reflectance of snow in the Northern hemisphere, leading to milder winters in North America and Europe (Richardson and Rundel 1998). In an age where global warming caused by released greenhouse gases, pines may provide some relief by utilizing CO_2 in photosynthesis given their prevalence in many ecosystems (e.g. Dewar and Cannell 1992; Goodale et al. 2002; Nosetto et al. 2006). Pines also deter erosion by growing on poor, nutrient-deprived areas, which

ultimately improve stream water quality (Rey and Berger 2006). Finally, pines are important in the worldwide environment as they can allow for reclamation of certain highly disturbed sites such as strip mines (e.g. Grossnickle and Reid 1982; Newmaster et al. 2006; Sullivan 1969).

In Ohio, pines are utilized to regenerate forests and woodlands. Arborists routinely plant *Pinus strobus* to gain impetus in forest regeneration from farmland. Pines are common in the urban forest of Ohio, and can be observed in most metropolitan areas as street plantings, in parks, and in the yards of homeowners. Lastly, pines are utilized by Ohioans as natural wind-breaks and for erosion control.

The Economic Importance of Pine

Pines are among the most valuable timber species. Estimates of worldwide pine timber may reach into the billions of dollars, but the actual economic value is difficult to gauge due to regional fluctuations in supply and demand.

In the United States, there was an estimated 5 billion cubic board feet of pine round-wood products in 1996 from four pine species: longleaf (*Pinus palustris* Miller), slash (*Pinus elliottii* Engelmann), loblolly (*Pinus taeda* L.), and shortleaf (*Pinus echinata* Miller) pines, which accounted for nearly 50% of softwood product in the U.S. (Johnson et al. 2001). Almost 35% of the saw-log production and pulp-wood production was from these pine species (Johnson et al. 2001).

An estimated value of up to $250 per thousand board feet of standing pine lumber was calculated for 2007 [Ohio Timber Price Report 2007 (http://www.oardc.ohio-state.edu/ohiowood)]. If 5 billion cubic board feet are sold at close to this price, which is an underestimate as the wood is still standing, the potential income easily enters into the billions of dollars.

Pines are also extremely valuable in the billion-dollar nursery and landscape industry in the United States, with 30 billion pines propagated annually with the estimated value of $108,840,000 (USDA 1998). Within the landscape the value of pines can increase to $200 per tree, a value which would result in $6 billion worth of trees in the landscape (Bonello et al. 2001a). The intrinsic value of landscape trees, including pines, to the homeowner is the ability to increase land value up to 7%, reduced home cooling costs by 30 to 50%, and reduced heating costs (by acting as wind breaks) by up to 22% (Ciesla 1995; Harris 1976; Sampson et al. 1992).

In addition to the lumber and the nursery industries, pines can be sustainably planted and harvested for a variety of uses: a) cones can be harvested for seeds (pine nuts); b) needles and bark can be used for fibers and basket-making; c) resin can be used for tanning, dyes, varnishes, waxes, turpentine, and waterproofing; d) solid wood can be used in construction of houses, boats, and poles; e) wood fiber can be used to make paper; f) live trees can be sold as bonsai trees; and g) both cut and live trees can be used as Christmas trees (Le Maitre 1998).

Aesthetic Values of Pines

Over the years, mankind has come to rely on *Pinus* not only for its products, but also for its beauty. Pines have had a long association with people, as humans have lived within the natural range of *Pinus* for about 1.4 million years (Le Maitre 1998). Pines were regarded as sacred trees for the ancient Greeks and Romans, the Aztecs, and many indigenous tribes living in boreal forests (Mirov and Hasbrouck 1976). Later, a large variety of artworks have addressed the beauty of pines. This includes Roman, Renascence, and Victorian artworks, with the latter using pines frequently as a backdrop in paintings (Mirov and Hasbrouck 1976). Pines have also been used as political symbols, including their use on the state flag of Maine (the "pine tree" state) and the state seal of Vermont. These trees have also inspired the naming of a variety of places, from streets to larger geographic areas. Many yards also have planted pines, as they provide a nice contrast to deciduous trees in temperate climates as well as retain their pleasant green foliage year-round.

Pests as the Bane of Pines' Existence: True Threats to Thriving Pines

Pests, including both pathogens and insects, constantly threaten the ecological, environmental, economic, and aesthetic value of pines.

Insects perhaps are the greatest pest threat to the majority of pine species, and they attack all tree tissues. In North America alone, pines are hosts to at least 1,111 species of insects, with the majority either Lepidoptera or Coleoptera (together 78% of

species) (de Groot and Turgeon 1998). It is estimated that half of these insect pests attack the foliage, one quarter affect small branches, and one quarter affect the bole or large branches (de Groot and Turgeon 1998).

Damage and impact of insect pests on their pine hosts is quite variable. Most pines and insect pests have evolved to remain relatively harmonious under natural conditions. Thus, the majority of insect attacks perhaps do not diminish their host's value in natural ecosystems, at least when examining an entire pine forest.

However, certain insect pests can erupt into massive, highly visible outbreaks that destroy millions of acres of trees (de Groot and Turgeon 1998). An example of such a pest is the bark beetle *Dendroctonus ponderosae* Hopkins, which is currently killing millions of lodgepole pine trees (*Pinus contorta* Douglas ex Loudon) in the United States and Canada. This insect pest is predicted to kill 80% of the lodgepole pines in British Columbia by 2013 (Anon. 2006). The result of this outbreak is that certain pine forest ecosystems could take decades to recover, and the timber industry is losing millions of dollars in potential profit from the loss of standing timber (Anon. 2006).

Although most insect pest attacks are not as destructive as those by *D. ponderosae*, which have greatly affected the ecological, economic, and aesthetic value of the lodgepole pine forests, insects can still degrade the economic and aesthetic value of pines. Economically, insects such as bark beetles may introduce blue-stain fungi reducing the quality of the wood. Likewise, particular folivores can cause needle yellowing and dropping, decreasing the value of live trees in the ornamental industry.

Pathogens also may seriously affect the value of pines. Pines are affected by many different pathogenic organisms, including: parasitic plants (dwarf mistletoes), nematodes (pine wood nematode), fungi, and oomycetes. The majority of pathogens on pine are fungi, which can infect all of the tissues within a tree.

Pathogens may lead to the extinction of certain pine species in particular locales, particularly when the pathogens are introduced exotics. Although these cases are rare, local extinction of pine species can have enormous ecological and environmental impacts. An example of a pathogen threatening extinction of pines is the pine wood nematode (*Bursaphelenchus xylophilus* Nickle) which, following its introduction into Japan, has devastated populations of *P. luchuensis* Mayr and *P. thunbergii* Parlatore and threatens to eliminate these species from the landscape (Harrington and Wingfield 1998). Likewise, the fungal pathogen *Cronartium ribicola* Fisch. threatens to destroy populations and forests of *P. flexilis* James and *P. albicaulis* Engelmann where they are naturally dominant tree species (Harrington and Wingfield 1998). In these examples, it appears that the pine-dependent forest ecosystems may never fully recover from the devastation of these pathogens.

Most pathogens do not pose as serious a threat as those described above. However, pathogens still can adversely affect the economic and/or aesthetic values of pines in the forestry, nursery, and landscaping industries.

The decay fungi probably affect the economic potential of pines the greatest by rotting the wood of standing trees. As a result, the value and amount of quality, sound wood within a pine stand can become greatly diminished. This has led to the formulation of the concept of “pathological rotation”, whereby managed stands are harvested well

before a point in time where loss to decay fungi surpasses annual growth (Harrington and Wingfield 1998). Ultimately, decay pathogens lead to tree decline and death (Manion 1996).

Other pathogens adversely affect the economic and aesthetic value of pines in nurseries, Christmas tree farms, and other plantations. Certain pathogens affect the regeneration and establishment of pines before they can reach a marketable size. Examples of such pathogens are those belonging to the genera *Phytophthora* and *Pythium*, among the causal agents of damping-off diseases. Still other pathogens infect older, more mature pines hampering their economic and aesthetic values by discoloring foliage and causing needlecasts, tip blights, branch diebacks, and cankers, resulting in declining, sick-looking trees. Frequent chemical controls are necessary to prevent these diseases and stop the pathogens under managed conditions, such as in nurseries and urban landscapes. Diseased trees can remain unsightly for years, and often prompt expensive controls or eventual removal. Declining trees lose their aesthetic value quickly. An example of a persistent pine disease that can cause decline and death in the landscape is *Diplodia* tip blight, caused by *Diplodia pinea* (Desm.) Kickx. Infected pines can be unsightly for years with this highly-visible tip blight and canker disease. Often the only option to maintain a pristine looking landscape would be to remove and no longer plant pines that are susceptible to *Diplodia* as other management measures such as fungicidal sprays can be cost-prohibitive.

Defenses against Pine Pathogens: Shared Defensive Strategies Common to Most Plants

Plants rely on four basic defensive strategies to combat pathogens: 1) expressing constitutive defenses to repel or inhibit invasion; 2) killing or sealing off the invaders; 3) repairing damage from the pathogen and prevent opportunistic infections; and 4) utilizing acquired (systemic) resistance to more easily defend against future attack (Franceschi et al. 2005). These divisions might be somewhat artificial as plants express these strategies simultaneously and there is much overlap in these responses. However, using these artificial divisions is an effective way to discuss various aspects of plant defenses against pathogens.

Much is unknown about how pines defend themselves from pathogens. Because many of the defenses against plant pathogens are held in common for most plants, knowledge gleamed from work within herbaceous models is relevant in the understanding of how pines resist pathogens. Model systems using rice, soybean, *Arabidopsis*, and other species were developed to advance knowledge about generalized plant responses to pathogens. The advantages using herbaceous model systems in the study of plant defenses against pathogens are many as model plants: 1) have a wealth of genomic information known about them; 2) have short lifespans; and 3) are easy to propagate. This is in contrast to studying pine model systems which prove challenging because: 1) less genomic information is known about pines; 2) pines have very long lifespans; and 3) pines are much more difficult to propagate (due to the size and resources needed to grow each individual to maturity).

As a first line of defense against pathogens, most plants utilize constitutive defenses such as thickened epidermal layers (covered with waxes, trichomes, and other structures), and suberized or lignified tissues. Constitutive defenses are produced prior to an attack, and by definition are always present. They are a “fixed cost” or “insurance” that all plants have to prevent devastating pest attacks (Burdon and Thrall 2003; Franceschi et al. 2005). A significant proportion of total resources used allocated to growth is involved in the production of constitutive defenses. Once constitutive defenses are formed, the resources used to produce them are unable be re-attained for primary metabolism (Burdon and Thrall 2003; Christiansen et al. 1987). However, without these constitutive defenses, plants become extremely susceptible to pathogen attack, and death might occur prior to the induction of other defenses (Burdon and Thrall 2003; Franceschi et al. 2005).

The second defense strategy that plants utilize in combating pathogens involves the induction of additional defenses at the infection site in an attempt to kill, inhibit, and/or otherwise mitigate the effects of the pathogen. Following pathogen recognition, most plants are capable of producing a hypersensitive response (HR) as a first attempt to stall an infection. HR is a form of programmed cell death whereby cells die in an attempt to compartmentalize the pathogen. This keeps the pathogen away from the nutrition that is found within living tissues. Furthermore, as part of HR a series of toxic free radical compounds are produced (often in an “oxidative burst”) (Ferreira et al. 2006). Likewise, local and systemic signaling occurs to elicit a wider defense response (Ferreira et al. 2006).

The ability of plants to recognize pathogen attack, an event that leads to HR, has been extensively studied since Flor first defined his gene-for-gene hypothesis (Flor 1971). The gene-for-gene hypothesis states that when plant resistance (R) gene products recognize pathogen avirulence (Avr) gene products, downstream events are triggered that ultimately lead to the HR response (Flor 1971). The processes by which plants recognize pathogen attack is now known to be much more complicated than what is predicted by the gene-to-gene hypothesis (Ferreira et al. 2006; Park and Paek 2007). It is now thought that general elicitors, i.e. pathogen components known as pathogen associated molecular patterns or PAMPs (e.g. fungal cell wall components), can elicit a type of "non-host" HR where specific gene-for-gene interactions do not occur (Ferreira et al. 2006). Likewise, many defined R genes actually encode products that seem to be more involved in detecting disruptions of normal cellular activities rather than directly recognizing avirulence gene products (e.g. Mackey et al. 2002).

A variety of compounds within the infected tissues are produced following the recognition of pathogen attack. Among the most described induced defense associated compounds are the PR proteins, which are thought to carry out a variety of functions against pathogens (Ferreira et al. 2006; van Loon and van Strien 1999; van Loon et al. 1994). Comprising 17 "families", these proteins include enzymes that degrade cells walls [chitanases (families PR-3, -4, -8, and -11) and β-1,3-glucanases (PR-2)], proteins that inhibit the digestive enzymes of the invader [e.g. protease inhibitors (PR-6)], and proteins that might be directly toxic to pathogen [e.g. thaumatin-like proteins (PR-5) or defensins (PR-12)] (van Loon and van Strien 1999; van Loon et al. 1994).

In addition to PR proteins, a variety of low molecular weight compounds are produced. These are often called phytoalexins, which are defined as antimicrobial compounds produced *de novo* following pathogen attack. These low molecular weight compounds are represented largely by phenolics and terpenes. The identities of these compounds varies widely from plant to plant (Baker et al. 2005). The phytoalexins, as well as compounds called phytoanticipans (defined as antimicrobial compounds produced prior to attack), are known to inhibit pathogen growth within and around the infected tissue.

An effective wound repair process to prevent secondary attack can perhaps be defined as a third strategy that plants utilize to defend themselves against pathogens. However, much overlap occurs with wound repair processes and the induced defenses produced within infected tissues. Following wounding of the outer bark, callose and cellulose production is triggered to quickly repair the damaged tissue and secure the tissues surrounding the wound against potential pathogen attack (e.g. Conrath et al. 2001; Schneider 1980; Thomas and Hall 1979; Valluri and Soltes 1990). Within cells surrounding the wound, additional cell wall modifications such as lignification and suberization occur to further fortify the wound site against secondary pathogens (Schneider 1980).

As a fourth defense strategy, plants exhibit “acquired” resistance, which can be both local and systemic, whereby they become more capable responding to future attacks. It is thought that acquired resistance allows for phenotypic flexibility in plant defenses against pests by allowing plants to escape inherent genotype-defined limitations imposed upon their ability to form constitutive defenses (Cipollini 1998).

In the model systems, two forms of acquired resistance have been described: systemic acquired resistance (SAR) and induced systemic resistance (ISR). Tuzun (2007) suggests that both the terms SAR and ISR should be used synonymously, and the mediating compounds or the inducers should become added to distinguish the separate types. Regardless of the name, both involve plants becoming systemically more resistant to pathogen (pest) attack, i.e. subsequent pathogen infections are less successful in "induced" versus non-induced plants. The mechanisms behind these responses differentiate these two types of resistance.

SAR commonly forms in plants that are or were infected by biotrophic pathogens (Durrant and Dong 2004; Mauch-Mani and Metraux 1998; Metraux 2001; Metraux et al. 2002; Sticher et al. 1997). SAR involves systemic increases in defense-associated compounds (often phytoalexins) (Baker et al. 2005; Sticher et al. 1997), but can also lead to quicker recognition of future pathogen attack. It appears to be most often mediated by salicylic acid (SA) as the application of this compound to uninfected plants will often trigger this type of induced resistance (Beckers and Spoel 2006; Durrant and Dong 2004; Mauch-Mani and Metraux 1998; Metraux 2001; Sticher et al. 1997). As a result of SAR being induced by salicylate, it might be more appropriate to call this type of induced resistance SA-ISR as suggested by Tuzun (2007). It is important to point out that some evidence suggests that salicylic acid is not the actual signaling component of SAR despite its ability to mediate the response; instead, evidence points to a lipid molecule as the systemic signaling agent (Durrant and Dong 2004). Furthermore, many plants cannot produce SA and/or do not respond to exogenous applications of that compound.

ISR is also characterized as an increase in systemic resistance as well, but is induced by associations of plants with plant-growth promoting rhizobacteria (PGPR) (Bakker et al. 2007; Kloepper et al. 2004; Pieterse et al. 2001; Pieterse et al. 2003; van Loon et al. 1998). It is mediated by jasmonic acid and ethylene (Pieterse et al. 2001; van Loon et al. 1998), and therefore might be more appropriately be called JA-ISR (Tuzun 2007). There is some thought that ISR is a more generic response against all pests than SAR (Bakker et al. 2007; Kloepper et al. 2004; Pieterse et al. 2001; Pieterse et al. 2003; van Loon et al. 1998).

Both SAR and ISR responses can be metabolically costly for plants to produce and sustain (Cipollini et al. 2003), as key work by Baldwin (1998) revealed fitness losses associated with artificial induction of defenses in plants against insects (using methyl jasmonate to induce the defense responses). However, it is thought that the alternative of not being able to defend against the pest is much more costly in the long run than producing an induced defense response (Baldwin 1998). Because the costs associated with SAR and ISR are high, it appears that plants have developed cross-talk between the pathways is order to make responses more cost-effective and specific to the particular type of pest (e.g. Beckers and Spoel 2006; Biere et al. 2004; Bostock 2005; Bostock et al. 2001; Cipollini et al. 2004; Felton et al. 1999; Heil 2001; Ton et al. 2002).

Pine specific defenses against pathogens

Pine trees are thought to share most of these common components of defense that were discovered in model systems. Research specifically working on pine pathosystems

has confirmed many similarities between model system plants and pine defenses, but such work has also revealed intrinsically different mechanisms that pines utilize to defend themselves.

Over millennia, pines have evolved a multitude of defense responses to combat pests (Franceschi et al. 2005). Just as with the plant model systems, pines utilize four layers of defense to protect themselves including: 1) preformed (constitutive) defenses, 2) defenses produced following an attack to kill/compartmentalize the pathogens, 3) healing mechanisms to repair damage/prevent opportunistic invaders, and 4) induction of acquired resistance. Together, both constitutive and induced defenses are quite effective at repelling invaders allowing pines to complete long lifecycles and remain successful despite insect and pathogen invaders evolving to become better and better at thwarting their defenses (Blanchette and Biggs 1992; Christiansen et al. 1987; Franceschi et al. 2005).

The focus of the next sections in this introduction is predominantly on defense mechanisms found in pines, but includes work on related gymnosperms as fir and spruce are more phylogenetically similar to pines (they are all in the family Pinaceae) than to herbaceous model system plants and are known to deploy defenses similar to pines. The majority of work examining defenses in pine against pathogens has focused on those within the stem; however, similar responses are likely to occur in the roots. Needle defenses against pathogens are less studied perhaps because pines can shed infected needles to prevent further infection, but the stem must be preserved or the entire tree would perish.

Constitutive Defenses Particular to Pines and Conifers

Constitutive defenses in pine, much like in herbaceous systems, involve both mechanical and chemical components that are broad enough to combat a multitude of invaders, as the trees must prepare for any possible form of attacker, whether pathogen, insect, or other herbivore (Blanchette and Biggs 1992; Franceschi et al. 2005).

Constitutive mechanical defenses in pines include thick outer bark composed of dead cells for roots and stems, waxes and thick epidermal cell walls for leaves, and lignification and suberization of certain tissues throughout the tree (Franceschi et al. 2005). Conifers may also have schlerenchyma layers within the bark composed of massive, irregularly-shaped stone cells (schlereids) (Franceschi et al. 2005; Wainhouse et al. 1997). The main role of these stone cells is to deter grazing or bark-boring organisms, but it is unknown how these might affect pathogens (Franceschi et al. 2005; Wainhouse et al. 1997). Layers of sieve cells through which photosynthate flows could also limit pathogen penetration as these often have thickened, hard to penetrate cell walls (Franceschi et al. 2005). Altogether, mechanical defenses limit penetration of more susceptible tissues by physical repulsion of the invaders, as most tree pathogens require wounds to invade the host.

Chemical components of conifer constitutive defenses work in unison with mechanical defenses to repel pathogen attack. Anti-herbivory and toxic compounds, such as calcium oxalate crystals (Franceschi 2001; Franceschi and Nakata 2005), tannins (Kraus et al. 2003), phenolics (Franceschi et al. 2000; Franceschi et al. 1998; Krekling et al. 2004), and terpenes (Franceschi et al. 2005) are often produced by specialized cells to inhibit or kill invaders that have bypassed the mechanical defenses.

Calcium oxalate crystals are formed as waste products of certain metabolic pathways, but are preserved within the vacuoles of certain cells to act as toxins against particular pests such as bark beetles (Franceschi and Nakata 2005). Tannins are also thought to act as anti-herbivory agents within pine tissues (Kraus et al. 2003).

Phenolics, which may be both anti-herbivory and antifungal, are for the most part produced in specialized cells within the bark called polyphenolic parenchyma (PP) cells (Beckman 2000; Franceschi et al. 2005; Franceschi et al. 1998; Krekling et al. 2004). PP cells seem to be designed for rapid cell communication due to the presence of enlarged plasmodesmata, and therefore could be important signaling relays of induced (acquired) resistance in the stems of pines (Krekling et al. 2004).

Finally, pines produce specialized resin-bearing cells as part of the constitutive defense repertoire. Resin cells and resin duct-lining cells produce large amounts of terpenoids which are then secreted into the gaps between cells or in resin ducts (Franceschi et al. 2005). Such a high volume of terpenoid compounds accumulates in those spaces that the system becomes pressurized, and upon wounding or damage, the resin is flushed out providing an excellent repelling mechanism (Langenheim 1994; Wu and Hu 1997). Furthermore, the terpenoid components in the resin can be directly toxic to the invader, and some organisms can be encased in the resin and die (Franceschi et al. 2005; Keeling and Bohlmann 2006). Finally, the exuded resin diterpenes crystallize over wounds once volatile components evaporate, effectively sealing the tissue from other invaders (Keeling and Bohlmann 2006).

Armed with these constitutive defenses, pines and other conifers are usually able to fend off pathogen and herbivore attack successfully throughout a long lifespan.

Induced Defenses: the Next Line of Defense

Despite their complex constitutive defenses, most pines are bound to encounter a pathogen that manages to penetrate their primary defenses during their lifetimes. Induced defenses are the second line of defense once constitutive defenses fail to repel invading pests, working in unison with repair mechanisms and acquired resistance (Berrymann 1969; Berrymann 1972; Blanchette and Biggs 1992).

Induced defenses include a suite of mechanical and chemical components, produced following pathogen or other elicitation, that work together to limit the pest's damage and contain or eliminate the threat. Induced mechanical defenses include increased production of stone cells (Hudgins and Franceschi 2004) as well as thickening, suberization, and/or lignification of cell walls in infected tissues (Bonello and Blodgett 2003; Franceschi et al. 2005). Cell wall appositions are also known to be induced by pathogen infection (Bonello et al. 1991). Induced mechanical defenses are closely associated with wound repair responses, which are discussed in the next section of this introduction.

Conifers have evolved a variety of induced chemical defenses including the production of phenolics and terpenes within unique structures to combat both insects and pathogens (Franceschi et al. 2005). Conifers also have been shown to produce PR proteins, similar to those in model systems, to apparently combat fungal infection (e.g. Liu et al. 2005; Sharma et al. 1993).

PP cells are induced to produce greater amounts of phenolic compounds following pathogen attack (e.g. Franceschi et al. 2000; Hudgins et al. 2005; Klepzig et al. 1995). In addition, new PP cells are formed to further increase phenolic compound

production (Krekling et al. 2004). Because of potential antifungal (antibiotic) activity (as discussed below), increased production of phenolics is hypothesized to be a potent defense against fungal pathogens (Bois and Lieutier 1997; Bonello and Blodgett 2003; Brignolas et al. 1995; Cvikrova et al. 2006; Lieutier et al. 1996; Lieutier et al. 1991; Viiri et al. 2001).

Constitutive resin-producing cells are also induced to produce greater quantities of terpenes following defense induction (Hudgins and Franceschi 2004; Ruel et al. 1998). However, many conifers, including pines, also produce traumatic resin ducts (TRDs) following fungal infection that are capable of producing much larger quantities of resin (Cheniclet 1987; Klepzig et al. 2005; Krekling et al. 2004; Luchi et al. 2005). Since the resin produced in TRDs is of different chemical composition than constitutively produced resin (Martin et al. 2002; Nagy et al. 2000), it is possible that it is modified to become more toxic to pathogens (Nagy et al. 2000).

Wound Repair: Acting to Prevent Pathogen Entry in Conifers

Following infection, pines initiate repair mechanisms to limit pathogen damage, defend against secondary invaders, and repair destroyed/wounded tissues. This often coincides with the onset of induced defenses, especially when wounds are created because of the infection process. Wound repair begins with the increased production of oleoresin to coat the wound(s) (Keeling and Bohlmann 2006), followed by the proliferation of certain cells to form a wound periderm and phellogen, which produces a phellem (cork layer) (Franceschi et al. 2000; Oven and Torelli 1994). This phellem

consists of thick-walled lignified cells and thin-walled suberized cells that greatly resist fungal penetration (Franceschi et al. 2000). Increased production of callose and cellulose further fortify tissues surrounding wounds to aid in resisting secondary pathogen attack (Schneider 1980; Thomas and Hall 1979). Finally, PP cells just outside the tissues under repair deposit phenolics into their cells walls as an additional line of defense (Franceschi et al. 2000).

Acquired Defenses: The Final Phase of an Effective Defense

Systemic induction of defenses, especially chemical defenses, occurs in conifers infected by pathogens. This includes increased formation of PP cells and phenolics away from the infection site (Bonello and Blodgett 2003; Cvikrova et al. 2006; Evensen et al. 2000; Franceschi et al. 2000; Krekling et al. 2004; Viiri et al. 2001). The increased production of terpenes (and thus oleoresin) and the systemic production of new traumatic resin ducts is also observed as part of the systemic induced defense response in conifers under pathogen attack (Christiansen et al. 1999; Krekling et al. 2004; Krokene et al. 2003; Luchi et al. 2005; Nagy et al. 2000; Viiri et al. 2001). Systemic induction of protein accumulation also occurs as a systemic induced defense response, but work examining this phenomenon has only just begun in conifers. So far no differentially or systemically expressed PR proteins have been detected in fungal induced pines, but systemic induction of small heat-shock proteins was observed (Wang et al. 2006).

Systemic induced defense responses have been observed one week after fungal infection (Bonello and Blodgett 2003; Krekling et al. 2004), and could be fairly long-term, lasting perhaps years (Bonello et al. 2006; Krekling et al. 2004).

The induction of certain defenses systemically could lead to a phenomenon known as systemic induced resistance (SIR), characterized by a reduction in pathogen (or insect pest) success in trees previously induced by pest attack. SIR is a more generic term than SAR or ISR, as it makes no assumptions on the signaling or chemical mediation that lead to its onset. However, if one is using SAR and ISR synonymously, then SIR becomes redundant with these terms (Tuzun 2007). Regardless, SIR is commonly utilized for all types of induced responses in conifers [for examples see Bonello et al. (2006)], and thus will be used throughout this work instead of SAR or ISR.

SIR leading to reduced pathogen success has been observed in pines and other conifers that were infected previously by pathogens (e.g. Blodgett et al. 2007; Bonello et al. 2001b; Christiansen et al. 1999; Eyles et al. 2007a; Krokene et al. 1999; Krokene et al. 2001; Swedjemark et al. 2007) or induced by plant-growth promoting rhizobacteria (PGPR) (Enebak and Carey 2006), and it is thought to work in part by induction of the systemic defenses described above. There does appear to be some organ dependency of SIR, as in one study stems that were induced at the base with a pathogen had increased systemic resistance to a second fungal infection, but the shoots had increased susceptibly to pathogen attack in a phenomenon classified as systemic induced susceptibility (SIS) (Blodgett et al. 2007).

Little is known about what signals or mediates SIR in conifers. However, induction of SIR against certain pests can be induced by applications of 5-clorosalicylic acid (e.g. Reglinski et al. 1998) as well as methyl jasmonate (e.g. Erbilgin et al. 2006; Franceschi et al. 2002; Hudgins et al. 2003; Hudgins et al. 2004; Martin et al. 2002; Miller et al. 2005). Ethylene is also involved along with methyl jasmonate in mediating the SIR response (Hudgins and Franceschi 2004; Hudgins et al. 2006).

Cross-Induction of Defenses in Conifers and Other Plants

It has been hypothesized that one organism can influence the expression of plant defense responses to other organisms, although not too many studies have overtly examined this (Hatcher et al. 2004; Moran and Schultz 1998; Mumm and Hilker 2006; Padgett et al. 1994; Rostas et al. 2003; Stout et al. 1999). Prior fungal infection was shown to increase (e.g. Moran and Schultz 1998), have no effect (e.g. Moran 1998), or decrease (e.g. Friedli and Bacher 2001) the ability of the host to resist an insect pest. Likewise, prior insect pest activity either increased (e.g. Hatcher et al. 1994), had no effect (e.g. Rostas and Hilker 2002), or decreased (e.g. Padgett et al. 1994) the ability of a host to resist fungal pathogen attack. The ability of one pest to increase or decrease resistance to another is thus hard to predict as studies have varying results. Without thorough investigation it might be difficult to establish the link between how one pest affects host defense and the subsequent formation of host resistance to another organism. Therefore, without direct investigation it might prove impossible to determine how host resistance is modulated for each pathogen/insect combination.

In pines (and trees in general) the influence of one type of pest on resistance to another has been rarely studied. However, it could be hypothesized that the increases in both phenolic and terpene production caused by one pest can influence another, as both types of pests (insects and fungal pathogens) are adversely affected by these compounds.

Many studies in conifers have observed the modulation of defense that bark beetles infestation causes and its impact on resistance to bark-beetled vectored fungi, with both increases and decreases in resistance observed (Klepzig et al. 1996; Raffa et al. 1985; Raffa and Smalley 1995). The opposite situation has also been studied whereby fungal infection was examined for its ability to modulate resistance to bark beetle infestation, and once again both positive and negative affects on resistance have been observed (Klepzig et al. 1996; Raffa et al. 1985; Raffa and Smalley 1995). In these systems it appears that the partner fungi rely on bark beetles for vectoring, and in exchange the fungal colonization of the host apparently increases bark beetle success, although it is less clear if this is simply a matter of reducing host resistance to the insect (Klepzig et al. 1996).

Increased resistance to a folivore (*Neodiprion sertifer*) was observed in *P. nigra* infected with the canker pathogen *Diplodia pinea* (Eyles et al. 2007a). Defoliation by the insect also led to the expression of resistance to the fungal pathogen (Eyles et al. 2007a). However, these responses varied between years (Eyles et al. 2007a). This appears to be the first study whereby two non-associated pests (an insect folivore and canker pathogen) have systemically affected the success of each other in pine or any other tree.

In another case, it was determined that canker fungal infection resulted in increased inhibition of bark beetle attack by altering the structure and composition of host tissues (Storer et al. 2002).

Despite these documented types of resistance, if the damage caused by a pest (e.g. a persistently infecting pathogen) becomes substantial enough to deplete resources available for defenses it might lead to the increased susceptibility to a subsequent pest, a phenomenon normally associated with symptomatic plants (Bonello et al. 2006).

Environmental Influences on Tree and Conifer Defenses

Many environmental factors are detrimental to the plant resistance to pathogens. As a result of this, the effect of environment and physiological stress on defense development has been a crucial area of research. Earlier reviews had investigated the role of environmental stress in predisposing plants to pathogenic infection and damage (e.g. Ayres 1984; Schoeneweiss 1975). However, recently great strides have been made in understanding how physiological changes within trees brought on by abiotic stress affect their defenses against insect pests (e.g. Herms 2002; Herms and Mattson 1992), and hypotheses to explain the effects of environmental factors on resistance have been formed to comprise what is termed “plant defense theory” (Stamp 2003).

Among the hypotheses that comprise plant defense theory are the Optimal Defense (OD), Carbon:Nitogen Balance (CNB), Growth Rate (GR), and Growth-Differention Balance (GDB) hypotheses (Stamp 2003). According to the OD hypothesis, plants evolved defenses to maximize their fitness, and defenses are costly as the divert

resources from other needs (Rhoades 1979). The CNB hypothesis predicts that the carbon to nutrient ratio controls how the plant allocates its resources, which in turn affects defense against pests (Bryant et al. 1983). The GR hypothesis concludes that if the maximum growth rate (measured at optimal conditions) decreases, than constitutive defense increases (Coley et al. 1985). Finally, the GDB hypothesis predicts that if environmental factors negatively affect growth more than photosynthesis, than more resources would be allocated to secondary metabolite production, which might include defense-associated compounds such as phenolics and terpenes (Herms and Mattson 1992). Each of these hypotheses focus on different aspects on environment affecting defense, ranging from evolution of defenses (the OD hypothesis) to nutrient availability (the CNB hypothesis) to growth affects on defense (the GR and GDB hypothesis). Of all of these hypotheses, the GDB hypothesis is perhaps the most mature and as a result likely the most useful in testing environmental affects on defense (Stamp 2003).

The majority of research examining the role of environment on host defense development in conifers has focused on soil fertility affects on host resistance. Anecdotal evidence, sometimes by arborists and landscape professionals, has lead to the common notion that vigorously growing trees, often spurred to grow faster by fertilizer applications, are less susceptible to insect and pathogen attack than slower-growing trees. However, the GDB and GR hypotheses predict the opposite case: decreases in secondary metabolites (and thus host resistance to pests) will be observed when fertilizer applications result in increased relative growth rates of a host (Stamp 2003). This prediction has been confirmed in many different studies that have been conducted to test

these hypotheses (Herms 2002; Stamp 2003). Therefore, fertilization beyond initial soil conditions is rarely warranted to increase a conifer's ability to combat pests, and may actually cause more harm than good.

Beyond this practical application of plant defense theory which could directly aid landscaping professions in their management decisions, these hypotheses allow for scientists to understand the competition for resources that occurs between host primary and secondary metabolism. Because of its importance on plant growth, the majority of research on plant defense theory has focused on nutrient availability on constitutive secondary metabolite production. The GDB predicts that as nutrient availability is reduced in plants living in a "source-limiting" state (brought on by poor environmental conditions), the production of resources is lowered and both relative growth rate and constitutive secondary metabolism production of those plants are reduced (Herms and Mattson 1992). However, for plants living in a "sink-limited" state (i.e. the state that plants exist when they are in environments with adequate nutrition), increases in nutrient availability increase relative growth rates, but decrease secondary metabolite production (Herms and Mattson 1992). The resulting response of constitutive secondary metabolisms to nutrient availability is thus quadratic. In most studies the latter case is observed, i.e. increasing soil fertility results in decreased resistance as in most cases plants already have enough nutrients to be in a sink-limited state (Herms 2002). Furthermore, plants tend to adapt physiologically to source-limited states and over time adjust their growth and photosynthetic capacities to once again enter into a situation where a negative relationship exists between relative growth rate and secondary metabolite production (Glynn et al. 2007). However, studying the effects of soil fertility and fertilizer on

defenses and testing the GDB hypothesis has been tricky, and results have been found to vary (Herms 2002; Koricheva et al. 1998; Koricheva et al. 2004; Kytö et al. 1998; Kytö et al. 1999; Lambert 1986; Stamp 2003; Tomova et al. 2005; van Akker et al. 2004). The majority of studies focused on testing just predictions of the GDB hypothesis, and did not include measurements of net assimilation rate (NAR) and relative growth rate (RGR), both of which are crucial to the hypothesis (Stamp 2004). Furthermore, there are temporal aspects involved in plant responses to nutrient availability that are often overlooked (Glynn et al. 2007). Because of these issues, often erroneous conclusions are made (Stamp 2003). Only a few studies adequately addressed this hypothesis to date (e.g. Glynn et al. 2007), and more are definitely warranted to fully test the GDB hypothesis and all of its predications.

In addition to soil fertility, the GDB hypothesis predicts that other site conditions might lead to nitrogen and carbon resource constraints that limit the ability of trees to allocate resources optimally between primary metabolism and secondary (including defense-related) metabolism, as thus might lead to increased susceptibility to pest attack (Herms and Mattson 1992). Such constraints can be brought on by poor site quality (such as soil texture (Mattila et al. 2001)), toxic levels of certain minerals in the soil (Kytö et al. 1998), water pollution (e.g. acid rain) (Cates et al. 1987; Saikkonen et al. 1995), drought stress (e.g. Blodgett et al. 1997; Stanosz et al. 2001), airborne pollution (especially ground-level ozone) (e.g. Bonello et al. 1993), increased CO_2 in the atmosphere (e.g. Heyworth et al. 1998), and exposure to fire (e.g. Lombardero et al. 2006). All of these stresses are predicted to affect relative growth rate and therefore impact the availability of resources for constitutive defense production (Stamp 2003). Another stress that plants can

have that affects defense production is the availability of light (e.g. Klepzig et al. 1995). Unlike the other stresses, light availability would affect the production of resources (photosynthesis) greater than relative growth rate, and therefore the GDB predicts a different impact of this stress than other environmental factors regarding the formation of secondary metabolites (Stamp 2003). In this case, increasing light would be predicted to result in increases of both relative growth rate and secondary metabolism production (Herms and Mattson 1992), as increases in light would increase total resource availability for both growth and defense.

Phenolics Compounds in Defense

Phenolic compounds, derived from the pheylpropanoid pathway, have a diverse array of possible functions. The shared precursor for all phenolic compounds is phenylalanine. A key regulatory enzyme is phenylalanine-ammonia lyase (PAL), which converts phenylalanine into a phenolic precursor by removing the amino group. Other enzymes are then required for the production of specific phenolics and these might be co-regulated through the activity of PAL (Ralph et al. 2006; Schmidt et al. 2005).

Individual phenolics have been implicated in defense in the following ways: 1) local and systemic induction in conifers following pathogen infection (e.g. Bonello and Blodgett 2003; Bonello et al. 2003; Brignolas et al. 1995; Cvikrova et al. 2006; Evensen et al. 2000; Krekling et al. 2004; Lieutier et al. 1991; Shain 1971; Viiri et al. 2001); 2) they possess antifungal (antibiotic) activity (e.g. Blodgett and Stanosz 1997a; Bois et al. 1999; Klepzig et al. 1995; Klepzig et al. 1996; Lindberg et al. 1992; Shain 1967;

Shrimpton and Whitney 1968; Woodward and Pearce 1988; Zou and Cates 1997); and 3) systemic induction following pathogen attack is associated with SIR (Blodgett et al. 2007; Bonello and Blodgett 2003; Bonello et al. 2003; Cvikrova et al. 2006; Evensen et al. 2000; Viiri et al. 2001). At least one study had observed a positive correlation between phenolics (lignin) and resistance in pine (Blodgett et al. 2007). Phenolics also bind to amino acids and proteins in disrupted tissue, making the tissue less nutritious to the invading organism (Franceschi et al. 2005). Induction of these compounds implies that the trees utilize them to specifically fight off pathogen (or insect) attack, although they are also produced constitutively at lower concentrations.

Phenolics as a group as are actively involved in a multitude of other plant functions beyond defense to ensure the plant remains healthy, the most important of which are free radical scavenging and UV protection (Waters 2003). Lignin in particular is an important phenolic due to its formation as part of the free radical scavenging process as well as its role in plant growth (i.e. secondary cell wall formation) and defense (Franceschi et al. 2005).

Terpenoid Compounds in Defense

Terpenoid compounds have also been implicated as important players in pine chemical defenses against pathogens (Christiansen et al. 1999; Hudgins et al. 2005; Krekling et al. 2004; Luchi et al. 2005; Zeneli et al. 2006), and their roles in defense has been reviewed by many, including Francheschi et al. (2005), Keeling and Bohlmann (2006), Tholl (2006) and Phillips and Croteau (1999). Terpenoids are derived from two

different pathways. The acetate-mevalonate pathway, which occurs in the cytoplasm, utilizes acetyl co-A as an initial precursor and ultimately leads to, among others compounds, the production of sesquiterpenes (Dubey et al. 2003). The non-mevalonate pathway, or GAP-pyruvate pathway, occurs in the plastids and utilizes glyceraldehyde-3-phosphate and pyruvate as precursors (Dubey et al. 2003). This non-mevalonate pathway is what predominately produces monoterpenes and diterpenes (Dubey et al. 2003).

In general, oleoresin contains roughly half monoterpenes, half diterpenes, and a low percentage of sesquiterpenes, with other compounds, such as phenolic stilbenes, in lower amounts (Keeling and Bohlmann 2006; Kopper et al. 2005; Trapp and Croteau 2001). Produced by resin cells and stored in ducts, oleoresin is both physically inhibitory and directly toxic to fungal pathogens (Bridges 1987; Zeneli et al. 2006). Oleoresin composition undergoes changes following induction to potentially become more toxic (Martin et al. 2002; Nagy et al. 2000).

The individual terpene classes have been examined for their role in pine defenses and monoterpenes have been the most extensively studied. Individual monoterpenes are induced in response to pathogens infection (e.g. Croteau et al. 1987; Klepzig et al. 1995; Raffa et al. 1985; Viiri et al. 2001; Wallin and Raffa 1999) and have shown some antifungal activity *in vitro*, albeit oftentimes at unnaturally high concentrations (Blodgett and Stanosz 1997a; Jurc et al. 1999; Klepzig et al. 1996).

Much less work has been conducted on individual diterpenes and sesquiterpenes. Diterpenes can be assumed to increase following elicitation by fungal infection because of the induction of oleoresin, which is mainly composed of diterpenes and monoterpenes

(Keeling and Bohlmann 2006). Diterpenes have also shown direct inhibitory effects on fungal pathogens, including inhibiting spore germination and fungal growth (e.g. Kopper et al. 2005).

Sesquiterpenes, in particular germacrene D, have also been shown to be induced by fungal infection (e.g. Viiri et al. 2001), although a role in defense has yet to be established.

More recent work has focused on the genetic regulation of enzymes that produce terpenes (Bohlmann et al. 1998; Byun-McKay et al. 2006; Faldt et al. 2003; Funk et al. 1994; Huber et al. 2004; Keeling and Bohlmann 2006; Savage et al. 1994). It is hoped that studying these pathways could result in a better understanding of the genetic regulation behind induced defense responses (Bohlmann et al. 1998; Huber et al. 2004; Keeling and Bohlmann 2006). Furthermore, even in different populations of the same species, it was observed that terpene content can vary and this might lead to different levels of susceptibility to various pests (Bojovic et al. 2005; Latta et al. 2000; Lewinsohn et al. 1991; Rosner and Hannrup 2004). By examining different alleles of genes associated with terpene synthesis, it may be possible to breed pines (and conifers) that are more resistant to particular pests.

Studying Pine Defenses against Pathogens: The Austrian Pine-*Diplodia* spp. Pathosystem

The work presented here explores different aspects of pine defense by utilizing a model pathosystem of Austrian pine infected with the fungal pathogens *Diplodia pinea* and *Diplodia scrobiculata* (Blodgett et al. 2007).

Austrian pine (*Pinus nigra*) has a native range in the Mediterranean basin, particularly at lowered elevations. It is found from Spain to Asia Minor, with small populations in the Atlas Mountains in Africa (Richardson and Rundel 1998). It grows well in both Mediterranean and wetter temperate climates (Richardson and Rundel 1998). Due to its ability to tolerate salt sprayed on roadways, it has become an important roadside tree in the Midwestern United States (van Haverbeke 1990). Furthermore, its dark green foliage, pleasant shape, rapid growth, tolerance of pollution and dust, and drought tolerance has made it a popular landscape tree in the United States (Little 2001; van Haverbeke 1990).

Austrian pine is most impacted by the fungal pathogen *Diplodia pinea* (Maresi et al. 2001). This fungus causes the disease *Diplodia* tip blight, whereby new shoots infected with the fungus whither, become necrotic, and die. The disease then can progress down the infected branch, which may result in the death of the entire branch. Severe cases of *Diplodia* tip blight can cause widespread crown blight, leading to unsightly and hazardous trees. The pathogen occasionally infects the stem, perhaps through introduction by pine engraver beetles (Whitehill et al. 2007), leading to cankers and tree death (Stanosz and Cummings Carlson 1996). The fungus overwinters in dead host tissue, including both dropped needles and second-year cones (Sinclair et al. 1987). On those tissues it produces the asexual fruiting structure known as a pycnidium, from which conidia are produced in wet weather and are spread by wind and rain-splash to new, uninfected tissues. *Diplodia pinea* has never been found to reproduce sexually, and

therefore is classified as a Dueteromycete, or “imperfect fungus”. Because of the large numbers of spores it produces, it is difficult to prevent this fungal pathogen from infecting any susceptible two-needle pine, including Austrian pine (Sinclair et al. 1987).

The only effective means to control *Diplodia* tip blight involves frequent and costly fungicide sprays throughout the growing season (Sinclair et al. 1987). However, society is moving towards greatly reducing chemical management of plant diseases and insect pests, and this may no longer be a viable option for controlling *Diplodia* tip blight.

In previous work, the Austrian pine / *Diplodia pinea* pathosystem has been used to investigate local and systemic induction of phenolic compounds, as well as SIR (Blodgett et al. 2007; Bonello and Blodgett 2003). As part of this pathosystem, the less aggressive but genetically similar pathogen *Diplodia scrobiculata* de Wet (de Wet et al. 2003) has also been used (Blodgett et al. 2007). Inclusion of the less aggressive pathogen *D. scrobiculata* can yield a better understanding of the disease if trees infected with this pathogen have a different intensity of defense induction than trees infected with *D. pinea* (Blodgett et al. 2007). This might provide complementary information to research using tree varieties with different levels of susceptibility to garner a gradient of defense responses (Franceschi et al. 1998).

Aims and Objectives

The main aim of this work is to further explore the phenomenon of induced resistance in pine, and to examine how defenses are integrated to combat pathogens. The three major objectives are to: 1) examine the role of soil fertility and previous insect

defoliation on defense against *D. pinea*; 2) determine the effects of fungal induction on phenolic and terpenoid metabolism and the role of induced metabolic changes on disease resistance; and 3) elucidate the signaling mechanisms of SIR in pine.

Accomplishment of the first objective would lead to a better understanding of the role played by soil fertility on both constitutive and inducible defenses. It is expected that such knowledge could ultimately lead to improved soil management strategies. New knowledge obtained from studying interactions between insects and *Diplodia*, mediated by the host, could reveal potential cross-talk in induction of defenses against either pest. This cross-talk could either increase or decrease the ability of the pines to successfully defend themselves against a variety of pests following induction of acquired resistance by a particular pathogen or insect. This scenario perhaps occurs quite frequently in nature as pines are constantly exposed to a variety of pathogen and insect attacks.

Accomplishment of the second objective will complement previous work examining phenolic induction using the same pathosystem (Bonello and Blodgett 2003). In other words, it will lead to a fuller understanding of how arguably the two most important chemical classes produced in a resistance response change following fungal infection by the same fungus. Comparisons then can be made between the induction of phenolics and terpenes to understand if either one or both are important in resistance to *Diplodia* infection.

Finally, elucidation of signaling mechanisms would fill a large gap in our knowledge of intra-plant communication. It could also lead to the development of novel

products, based on the same chemistry as the signaling compound(s), which could be applied to trees in an eco-friendly way to increase resistance even prior to a fungal infection.

Taken together, the sum of this work will greatly expand the current knowledge of pine defensive mechanisms, and could eventually lead to better ways to protect the ecological, environmental, economical, and aesthetic values of all pines.

CHAPTER 2

SYSTEMIC INDUCTION OF PHLOEM SECONDARY METABOLISM AND ITS RELATIONSHIP TO RESISTANCE TO A CANKER PATHOGEN IN AUSTRIAN PINE

Introduction

Conifers have evolved both constitutive and inducible defenses to ward off attack from pathogens (Franceschi et al. 2005; Schmidt et al. 2005). Induced plant defense responses consist of two major types: those associated with local induced resistance (LIR), i.e. in tissues surrounding an infection site; and those associated with systemic induced resistance (SIR), which is a phenotype characterized by increased resistance to subsequent attacks on distal parts of a plant (Bonello et al. 2006).

While SIR phenotypes in conifers are known to occur (Christiansen et al. 1999; Enebak & Carey 2000; Bonello et al. 2001; Zeneli et al. 2006; Blodgett et al. 2007; Swedjemark et al. 2007), the chemistry that mediates this systemic response is poorly understood (Bonello et al. 2006). As part of the SIR phenomenon, it is assumed that co-regulation of plant defenses occurs, and this co-regulation is important to mounting defenses against multiple threats (Stout et al. 2006).

Plants are often attacked simultaneously by a variety of pests throughout any tissue in any growing season so there is increasing interest in studying host-mediated

cross-effects between insects and pathogens (Franceschi et al. 2005). Reviews that focus primarily on interactions between pathogens and insects mediated by herbaceous and woody angiosperms found that pathogen infection had varied effects on the behavior or performance of foliage feeding insects, and insect attack had varied effects on the growth of fungal pathogens (Rostas et al. 2003; Stout et al. 2006). Insect herbivory on the same part of the plant (i.e. leaves) that was later infected by a fungal pathogen had negative effects (e.g. Hatcher et al. 1994; Russo et al. 1997; Moran & Schultz 1998; Stout et al. 1999; Hatcher & Paul 2000), no effects (e. g. Russo et al. 1997; Moran & Schultz 1998), or positive effects (e. g. Padgett et al. 1994; Simon & Hilker 2003) on fungal growth or disease development.

An additional and often overlooked factor in the development and deployment of host defenses is nutrient availability to the host. Many studies have examined the effects of soil fertility on constitutive defenses, especially against insects, and results are mixed (Kytö et al. 1999; Herms 2002; Koricheva et al. 2004; van Akker et al. 2004), in part because nutrient availability may have nonlinear effects on these responses (Herms & Mattson 1992; Herms 2002; Glynn et al. 2007). The nutrient status of a plant determines the amount of resources that the plant can allocate to constitutive defenses (Franceschi et al. 2005). The effects of nutrient availability on constitutive defense chemistry in conifers are little understood, but even less studied are the effects of nutrient availability on induced defense responses, both locally and systemically (Bonello et al. 2006). The few studies that have addressed this question in conifers have found that increased fertilization often leads to increased severity of fungal diseases (Entry et al. 1991; Blodgett et al. 2005).

In a companion paper, Eyles et al. (2007a) showed that a fungal pathogen can affect insect growth and survival indirectly through their shared pine host and *vice versa*. To test that hypothesis they used the Austrian pine (*Pinus nigra*)/ *Diplodia pinea* model system for SIR in conifers as described in Bonello and Blodgett (2003) and Blodgett et al. (2007). To this system we added the European pine sawfly [*Neodiprion sertifer* (Geoff.)], a defoliator of *P. nigra*. This tripartite system was subjected to variable nutrient availability. Included in the findings by Eyles et al. (2007a), two SIR-related phenomena were apparent: (1) fungal induction led to a reduction in subsequent fungal lesion development; and (2) fertilization did not affect the expression of induced resistance to the pathogen (Eyles et al. 2007a).

The objective of this study was to profile systemic chemical changes in phloem tissues of induced plants and examine the extent to which they relate to fungal performance. We hypothesized that (1) conifers rely predominantly on systemically inducible, co-regulated chemical defenses (both phenolic and terpenoid compounds) to limit the growth of fungal pathogens and (2) the development of the chemical components of inducible resistance are influenced by the nutrient status of the trees. We evaluated the effects of previous fungal infection and insect defoliation on levels of phloem phenolic and terpenoid metabolism in relation to the development of a subsequent *D. pinea* infection across three fertilization regimes. We also evaluated potential trade-offs between different metabolic pools such as those associated with the terpenoid-producing and phenolic-producing pathways.

Materials and Methods

Location of the study, plant material, experimental design, fertilization schemes, pathogen and insect induction treatments, and pathogen challenge bioassays are described in detail in Eyles et al. (2007a). This experiment was conducted at the Landscape Nursery Crop Engineering Research Laboratory of The Ohio Agricultural Research and Development Center (OARDC), Wooster, Ohio (40º81' N and 81º94' W). A total of 240 open-pollinated four-year-old Austrian pine saplings [from Ridge Manor Nursery (Madison, OH)] were potted in 21-liter containers using a commercial substrate (KB container mix, Kurtz Bros. Central Ohio, LLC). These trees were placed into 5 blocks, positioned on a gravel bed, in May 2004. Throughout the experiment the trees were exposed to ambient weather conditions.

This experiment used a randomized complete block design with three nutrient levels applied in a factorial combination with a total of four induction treatments. Each of 240 four-year-old trees was given one of three different fertility treatments (40 trees for each treatment) applied with irrigation (fertigation): 30 ppm N, 75 ppm N, or 150 ppm N fertilization level, using the following ingredients: $Ca(NO_3)_2$, NH_4, $H_2 PO_4$, KNO_3. These were applied in a 3:1:2 NPK ratio, with all sources of nitrogen adding up to the required ppm. Irrigation was set to trigger when calculated levels of evapotranspiration were reached, as described by Lee et al. (2000). The trees were pretreated under these conditions for the 2004 growing season, and this regime continued through the 2005 season.

In the Spring of 2005, the following induction treatments were applied in equal replication (n = 60 per induction treatment, assigned to 5 blocks) across the fertilizer-treated trees (n = 20 per induction and fertilizer treatment combination): (1) fungal induction, conducted by making a wound with a 10 mm diameter cork borer in the main stem 5 cm above the soil line and placing an 8 mm diameter *D. pinea*-colonized agar plug into the hole and securing with duct tape; (2) mock-inoculation, where the fungal induction was mimicked using a non-colonized agar plug; (3) insect defoliation, conducted by introducing approximately 150 *N. sertifer* larvae to each tree, which resulted in 75% defoliation (with 25% of the foliage protected by bagging branches); and (4) non-wounded untreated control trees. This resulted in a total of 4 replicates for each fertilizer by induction by block factorial combination.

After a 16-day incubation period, the fungal challenge (whereby a branch about 15 cm from the soil line was inoculated using an infected agar plug similar to the fungal induction described above) was applied to half the trees of each induction treatment. At the same time, another branch was removed from every tree for phytochemical analysis. An exception was the insect defoliated trees, for which only one third of the trees were assayed for phytochemical analysis, and another third of the trees underwent the fungal challenge. This was a result of the trees having only four total branches, with three defoliated to obtain the target of 75% defoliation. The remaining, protected branch (25% of the foliage) was then utilized for the phytochemical analysis or the fungal challenge. Following a period of 10 days, the fungal challenged branch was removed and the lesion length just under the outer bark was recorded as a measure of resistance, according to the procedure described by Blodgett et al. (2007).

Phytochemical Analyses

Phloem samples were obtained from an excised untreated branch at the same time the pathogen challenge was initiated and flash-frozen in liquid nitrogen. This tissue was ground to a powder in liquid nitrogen, and 100 mg (fresh weight) of ground tissue was used for extraction of phenolics and lignin, following the procedures described by Bonello and Blodgett (2003) and Blodgett et al. (2007), with slight modifications. In brief, methanol-soluble phenolics were obtained by extracting the ground phloem tissue twice in 500 μL of HPLC grade methanol (Fisher, Pittsburgh) for 24 h at 4^{o} C. The supernatants were combined and stored at -20^{o} C until they were analyzed by high-pressure liquid chromatography (HPLC), as described below. Total phenolic content was estimated using a modification of the Folin method of Bonello and Pearce (1993). 1.875 μL of methanol extract were added to 73.1 μL of methanol and 675 μL of water and mixed. To this, 37.5 μL of Folin's Phenol Reagent (Sigma, St. Louis) was added, followed three minutes later by 37.5 μL of 1 M $NaHCO_3$ and thorough mixing. After one hour incubation at room temperature, the samples were analyzed spectrophotometrically against a standard curve of gallic acid at 725 nm.

HPLC analysis was conducted using a Waters 2690 separations module (Waters, Milford, MA) equipped with a Waters Xterra^{TM} RP18, 5 μm, 4.6 X 150 mm column and a 996 photodiode array detector. The Waters Millennium HPLC software was utilized for data acquisition and managing the separation gradient. A binary solvent system was used, with solvent A consisting of 2% glacial acetic acid (Fisher) in HPLC grade water, and solvent B consisting of 2% glacial acetic acid in HPLC grade methanol. The linear

gradient was the same used by Bonello and Blodgett (2003), with the following run time (min), flow rate (mL / min), and % solvent A: 2.0, 1.0, 100.0; 4.0, 1.0, 90.0; 20.0, 1.0, 52.0; 38.0, 1.75, 0.0; 39.0, 1.75, 0.0; 41.0, 1.75, 100.0; 43.8, 1.75, 100.0; and 44.0, 0.5, 100.0 (total run time 44 min). The photodiode array detector was set to scan wavelengths between 237 nm and 400 nm, with two channels selected for data processing at 280 nm and 308 nm.

To measure lignin concentration, the pellets from the crude phenolic extraction were washed with 1 mL of water and 0.9 mL of *tert*-butyl methyl ether (Sigma). The pellets were left to dry overnight, and then processed according to the methods of Bonello et al. (1993). In brief, 200 μL of 1 N NaOH (Fisher) was added to each pellet and the mixture was left 21h at 40 ^{o}C on a shaker. A total of 200 μL of 1.5 M formic acid (Fluka Chemie, Buchs, Switzerland) and 400 μL of methanol were added to the extract, and centrifuged at 16,100 rcf for 5 min. The supernatant was then removed, and the remaining pellet was washed once in 1 mL of water. The pellets were resuspended in 1 mL of 2 N HCl (Fisher), and to the mixture 250 μL of thioglycolic acid (Sigma) was added. The solutions were incubated at 86 ^{o}C for 4h, and then the supernatants were discarded. The pellets were rinsed twice with 1.5 mL of water, and then resuspended in 1 mL of 0.5 M NaOH. The solutions were then incubated at room temperature on a shaker for at least 18 h. The supernatants were kept, and the pellets were re-extracted in 0.5 mL of 0.5 M NaOH on a shaker for 18 h. The supernatant was combined with those from the previous step. To the samples, 0.3 mL of 12 N HCl was added, and the solutions were incubated at room temperature for 4 h. The supernatants were discarded, and the pellets

were allowed to dry overnight. Finally, the pellets were resuspended in 1 mL of 0.5 M NaOH. Quantification was made using a spectrophotometer with a standard of spruce lignin (Sigma) used to derive the concentration of each sample.

Terpenoids were quantified by extracting ground phloem tissue (100 mg) twice in 500 μL dichloromethane (Fisher) containing 0.1% (v/v) *p*-cymene (Sigma) (internal standard) for 24 h at 4^{o} C. The supernatants were combined, and analyzed using gas chromatography (GC). GC analysis was conducted on a Hewlett Packard 6890 GC (Hewlett Packard, Palo Alto, CA), equipped with an Agilent 19091B-102 Ultra 2 5% phenylmethylsiloxane column (Agilent, Palo Alto, CA), and a flame ionization detector. The gradient and data acquisition were managed by the HP Chemstation software. The oven gradient was based on the method of Martin et al. (2002) and was as follows: initial temperature of 40^{o} C for 2 min; ramping at 10^{o} C/min to 240^{o} C; ramping at 50^{o} C/min to 300^{o} C, followed by a 5 minutes hold (total run time of 28.20 min). A split injection mode with a split ratio of 12:1 and an initial temperature of 220^{o} C were used. Hydrogen was used as the carrier at a constant flow of 2.1 mL/min. The front detector was set at 220^{o} C.

Compound Identification

Phenolic peaks were identified by a combination of matching UV spectra and retention times with standards or through the use of liquid chromatography-mass spectrometry (LC-MS). This process is described in full in Chapter 3, page 73. Only three compounds that were quantified by HPLC remained unidentified, and these were labeled

unknown 1 (uk1), unknown 2 (uk2), and unknown 3 (uk3). An additional peak, labeled unknown 4 (uk4), was subsequently discovered to be a co-elution of levopimaric and abietic acids, which only on occasion separated into distinct peaks.

Monoterpenes were identified by retention time against the following standards: β-pinene, α-pinene, camphene, myrcene, limonene, and bornyl acetate (all from Sigma). Other terpenoids were identified using gas chromatography-mass spectrometry (GC-MS) and matching to the Wiley Registry of Mass Spectral Data and the NIST Library, using RSI values of at least 850 as a threshold for identification. Confirmation of the sesquiterpenes trans-caryophyllene and α-humulene were made by comparison with retention times of commercial standards (Sigma). Identification of the terpenes in described in full in Chapter 3, pages 73 and 74.

Statistical Analyses

Statistical analyses were conducted using SPSS 14.0 for Windows (SPSS Inc., Chicago). Treatment effects on lesion length (which has been used as a measure of resistance – Blodgett et al. 2007; Eyles et al. 2007a) and the relative level of each individual phytochemical were analyzed by three-way univariate ANOVA. The model included 3 fertility levels, 4 induction levels, 5 blocks, and all interactions. Fisher's LSD multiple range tests were then used to separate treatment means ($\alpha < 0.05$) if main effects or interactions were significant.

Natural groupings of the analyzed compounds were identified through a combination of cluster analysis and principal component analysis (PCA), as suggested by McSpadden Gardener (2006). Such groupings may be used to infer coordinated regulation when their levels change in concert (McSpadden Gardener 2006). This methodology to group variables in response to multiple treatments has been utilized before (Benitez et al. 2007). For these analyses, the HPLC-derived peak areas were scaled into quintiles. A hierarchical cluster analysis was performed using furthest-neighbor joining based on squared Euclidean distance for each of the separate induction treatments (four dendrograms). Compounds that grouped together in at least three of the four dendrograms were considered a natural group and combined into a cluster. Additionally, PCA was conducted on the data from all the treatments by defining components based on Eigen values > 1. Natural groupings were assessed by evaluation of relative placement on ordination plots composed of the first three principal components. The compounds were grouped together by calculating a quintile mean of the clustered compounds for each tree. These groups were inferred to be co-regulated, when their rescaled cluster distance from each other was 17 or less, a distance chosen based on its ability to resolve compounds in part to what was known *a priori* about their chemical classification (phenolics or terpenoids), as well as correspondence with the PCA analysis.

Univariate ANOVAs were carried out on the defined clusters to measure the effects of induction treatment on each group of apparently co-regulated compounds and Fisher's LSD mean separation tests were used to separate effects of induction treatment on each of the cluster groups ($\alpha < 0.05$).

Spearman's rho correlations were conducted between lesion size (resistance phenotype) and the scaled abundance of each of the individual compounds, and between lesion size and the scaled abundance of apparently co-regulated groups defined by the multivariate analyses described above. Likewise, the relative abundances of the various natural groups were correlated with one another. Significant correlations were defined as having a $P < 0.05$.

Results

Analysis of Individual Compounds

Total phenolics, lignin, coumaric acid hexoside, dihydroconiferin II, ferulic acid glucoside, taxifolin hexoside, uk1, taxifolin, pinosylvin, and pinosylvin monomethyl ether were all characterized by significantly higher amounts in the fungal induction treatment compared to non-wounded and/or wounded (mock-inoculated) control trees (Table 2.1). Fungal induction increased the levels of α-pinene and β-pinene over that of the non-wounded and insect defoliation treatments. β-pinene occurred at greater levels in due to the fungal induction treatment when compared to the mock-inoculated treatment as well (Table 2.1). Insect defilation reduced limonene levels compared to mock-inoculated and fungal induced trees (Table 2.1). (It should be noted that we did not conduct stereoisomer analysis and therefore these compounds were combinations of their stereoisomers.) Finally, germacrene D was present in significantly higher concentrations in insect defoliated trees than in all of the other treatments (Table 2.1).

Fertilization had no significant effects on the concentrations of any of the phloem terpenoids (data not shown). Total phenolics measured by the Folin method, coumaric acid hexoside, hydroxypropiovanillone hexoside, ferulic acid glucoside, and taxifolin hexoside were characterized by as occurring at lowered levels in the 150 ppm N treatment compared to the other treatments (Table 2.2). The level of uk3 was significantly higher at 150 ppm N compared to the other treatments (Table 2.2).

A significant induction x fertilizer interaction ($F_{6, 166} = 3.612$, $P = 0.003$) was found only with the total phenolics, with greater total phenolic levels occurring in both fungal and insect defoliator induced trees than controls in the 30 and 75 ppm treatments (although for 30 ppm the difference in levels were not statistically significant), but lowered phenolic levels from insect defoliation in the 150 ppm treatment (with no increase/decrease for fungal induction in that treatment). Other significant interactions were found between fertilizer x block for total phenolics, dihydroconiferin I, and neoabietic acid; and induction x fertilizer x block for neoabietic acid (data not shown). The interactions with blocks did not change the patterns of the main effects.

Correlations between Individual Compounds and Lesion Size

Hydroxypropiovanillone hexoside, ferulic acid glucoside, and uk1 were negatively correlated with lesion length, whereas uk3 and the labdadiene isomer were positively correlated with lesion length (Table 2.3). Lignin and β-pinene were marginally ($P < 0.10$) negatively correlated with lesion length (Table 2.3).

Co-Accumulation of Secondary Metabolites

Cluster analyses revealed seven consistent groupings whereby compounds occurred together in at least 3 of the 4 induction treatments (Fig. 2.1). Cluster 1 consisted mainly of phenolic hexosides, taxifolin, and lignin. Cluster 2 consisted of the monoterpenes except β-pinene. Clusters 3, 4, and 6 consisted of the remaining terpenoids. Cluster 5 consisted of uk1, pinosylvin, and pinosylvin monomethyl ether, and cluster 7 consisted of uk2, uk3, and uk4.

The PCA on the complete dataset yielded similar groupings, with four components together explaining 49% of the total variance. Compounds with component matrix scores > 0.50 for each component were grouped together. Principal component 1 (PC1) contained the monoterpenes α-pinene, camphene, myrcene, and bornyl acetate with positive component matrix scores, and contained dihydroconiferin II, coumaric acid hexoside, hydroxypropiovanillone hexoside, ferulic acid glucoside, taxifolin hexoside, and taxifolin with negative component matrix scores. Thus, PC1 delineated groups of compounds very similar with clusters 1 and 2, and indicated that those clusters were negatively correlated with one another. Uk1, uk2, and uk4 were associated with PC2 with positive component matrix scores. Germacrene D, α-muurolene, and neoabietic acid (which compose cluster group 4) were associated with PC3 with negative component matrix scores, while α-pinene was associated with PC3 with positive component matrix scores. PC4 only had trans-caryophyllene and α-humulene (which made up cluster 3) associated positively with it. Component score plots of PC1 vs. PC2 and PC1 vs. PC3 (Fig. 2.2) clearly revealed groupings of compounds classified together in cluster 1, 2, 4,

and arguably cluster 5. Because such groupings appeared independently of the multivariate classification applied, they were considered natural groupings of compounds, a result that suggests co-regulation in the phloem of Austrian pine.

ANOVA of Metabolite Groups

Cluster 1, which consisted primarily of phenolics, increased significantly in the fungal treatment over both control treatments, while insect defoliation resulted in a significant increase only over the non-wounded control. Cluster 2, which consists mostly of the monoterpenes, decreased significantly in the insect defoliation treatment compared to the other induction treatments. Cluster 4, which includes germacrene D, α-muurolene, and neoabietic acid, increased significantly in the insect defoliation treatment over controls. Cluster 5, which includes the stilbenes, increased significantly in the fungal induction treatment over the other treatments. Only cluster 1 was affected significantly by fertility level, with lower concentration at 150 ppm N than the other fertility groups ($F_{2, 165} = 6.974$, p=0.001). In contrast, the relative abundance of compounds defined in clusters 3, 6, and 7 did not change significantly in response to any of the treatments. For the most part, interactions among the main effects variables (i.e. pathogen, insect, and fertility) were not observed for any of these clusters of compounds, with the exception of significant ($P < 0.05$) interactions occurring for fertilizer x block for clusters 5 and 7.

Correlations of Compound Clusters with Lesion Size and Each Other

The scaled concentrations of compounds present in clusters 1 and 5 were negatively correlated with lesion length, while cluster 6 was positively correlated with lesion length (Table 2.4). The combination of clusters 1 and 5 led to a stronger negative correlation with lesion length (Table 2.4). All other correlations with lesion size were not significant.

Correlations between compound clusters were computed to examine any potential resource trade-offs that might occur when Austrian pine mounts an induced defense response. Clusters 1 and 2 were significantly negatively correlated with each other, and cluster 1 was positively correlated with cluster 5 (Table 2.5). Cluster 2 was positively correlated with clusters 3, 6, and 7 (Table 2.5). Finally, clusters 3 and 4 were positively correlated, as well as clusters 3 and 6, 4 and 7, and 5 and 7 (Table 2.5).

Discussion

In this study, we profiled systemic chemical changes in phloem tissues of Austrian pine in response to fungal infection and insect defoliation. We also examined the extent to which chemical changes related to fungal performance and whether responses varied with fertility level. Fertility was previously found to have a negative effect on resistance to *Diplodia pinea* infection (Blodgett et al. 2005). However, in the context of these experiments fertility did not influence resistance expression to *Diplodia* (Eyles et al. 2007a). Although this was the case, fertility did influence the accumulation of four phenolic glycosides in this study (coumaric acid hexoside,

hydroxypropiovanillone hexoside, ferulic acid glucoside, taxifolin hexoside), with the lowest level observed in the 150 ppm N treatment. There were no significant effects of fertility on the levels of any of the terpenes across all of the induction treatments.

Because fertility did not alter the expression of resistance against the pathogen, but influenced levels of some compounds, it suggests that phenolic glycosides or terpenes are not involved in resistance. However, we found that the systemic levels of eight phenolic compounds, two monoterpenes, and one unknown compound increased significantly in trees that were induced by the fungal pathogen over non-wounded and/or mock-inoculated trees (Table 2.1). A significant interaction between fertility and induction treatment on total phenolic levels further confounded the relationship between fertility treatment, phenolics, and resistance.

It should be noted that three of the monoterpenes monitored in this study, α-pinene, β-pinene, and limonene represent combinations of stereoisomers. Further study may be warranted to determine if the individual stereoisomers differentially accumulate in response to various induction treatments (Faldt et al. 2006).

Germacrene D was the only terpenoid that increased systemically following induction by *N. sertifer* defoliation, compared to the controls. This sesquiterpene was described previously as having no influence on the growth of *D. pinea* (Jurc et al. 1999). However, it was described as increasing in the stem of methyl jasmonate-treated Douglas-fir (*Pseudotsuga menziesii* Carrière) (Huber et al. 2005). This is of biological interest because methyl jasmonate is known to mimic the induction of terpenoid metabolism that is often observed in several conifers following insect attack (Franceschi et al. 2002; Martin et al. 2002; Miller et al. 2005; Erbilgin et al. 2006).

While positive correlations between individual compounds and lesion length may be of some biological interest, negative correlations are of higher significance in SIR studies in which the basis of the observed resistance is investigated (Blodgett et al. 2007). Among the individual compounds analyzed in this study, only ferulic acid glucoside, hydroxypropiovanillone hexoside, and uk1 were negatively correlated with lesion size (Table 2.3). Of these, only ferulic acid glucoside increased significantly in response to pathogen induction, and this association suggests a role in resistance to *D. pinea*. It should also be noted that, while only marginally significant ($P < 0.10$), the correlations between lignin and lesion length, and β-pinene and lesion length, were also negative. Thus, it is possible that these compounds play a role in SIR observed here, as suggested by previous studies (Blodgett & Stanosz 1997a; Bonello et al. 2003; Blodgett et al. 2007).

Support for the interpretation that clusters 1 and 5 may be involved in resistance derives from the known in vitro antifungal activity of many of the compounds contained therein, some of which were significantly affected by treatment. For example, some of the best examples of the involvement of phenolics in conifer defense involve the stilbenes, i.e. pinosylvin and pinosylvin monomethyl ether, which have been found to increase in concentration, both locally and systemically, in response to pathogen attack in several pathosystems (Shain 1967; Hart and Shrimpton 1979; Lindberg et al. 1992; Lieutier et al. 1996; Bois et al. 1999; Chiron et al. 2000; Bonello & Blodgett 2003). Pinosylvin and pinosylvin monomethyl ether have strong anti-fungal activity in in vitro assays against fungi, including D. pinea (e.g. Bonello et al. 1993; Blodgett & Stanosz 1997a; Celimene et al. 2001; Seppanen et al. 2004). The role of stilbenes in disease resistance has been questioned because of a lack of individual correlations with resistance

(e.g. Bonello & Blodgett 2003; Blodgett et al. 2007). However, additive, but minor, effects of individual stilbenes or their interactive effects can lead to the negative association of this group with fungal lesion lengths that was observed here. A similar argument could be made for the phenolic glycosides in cluster 1.

Phenolic glycosides may accumulate as part of the pathogen-induced, systemically resistant phenotype in anticipation of additional fungal attack. In some cases it is known that fungal pathogen attack results in cleavage of the sugar moiety of phenolic glycosides and that the released aglycones have fungistatic/fungicidal activities (e.g. Woodward & Pearce 1988). For example, coumaric acid released by pathogen hydrolases from its glycoside and coumaric acid has been shown to be toxic against *Heterobasidium annosum in vitro* (Tomova et al. 2005). Ferulic acid, which is known to have antifungal activity and may be implicated in resistance of some plants to fungal infection (e.g. Sarma & Singh 2003), may be similarly released from its glucoside.

While only a few individual phenolics were negatively correlated with lesion length, we found that several secondary metabolites were negatively correlated with lesion length as discrete groups. Clusters 1 (phenolic glycosides and lignin) and 5 (stilbenes) were negatively correlated with lesion length, suggesting that these groups of compounds act or are regulated in concert to contribute to disease resistance. In fact, the combination of clusters 1 and 5 resulted in a stronger correlation than either group by itself. Such additive effects are an often overlooked aspect in studies of resistance mechanisms, although it is well known that regulation of the induced defense response occurs well upstream of individual components, so that whole groups of co-regulated compounds may be involved (Metraux et al. 2002; Koricheva et al. 2004; Hale et al.

2005; Schmidt et al. 2005; Ralph et al. 2006). The clusters of compounds correspond well with natural secondary metabolite groups: cluster 1 consisted mainly of phenolics, particularly phenolic glycosides; cluster 2 of monoterpenes; clusters 3 and 4 of the sesquiterpenes; cluster 5 of the stilbenes; and cluster 6 of the diterpenes. These groupings were consistent for at least 3 of the 4 induction treatment dendrograms, indicating that the relationships derived between the compounds were robust and these compounds were indeed co-regulated.

A role for terpenoids in resistance of Austrian pine to *D. pinea* is also supported by our analysis, because the fungal induction treatment resulted in significantly greater concentrations of α-pinene and β-pinene over the non-wounded control treatment and there was a negative correlation between β-pinene and lesion length. Blodgett and Stanosz (1997a) found that α-pinene and β-pinene had toxic effects on *D. pinea in vitro*, and these and other monoterpenes have previously been implicated in defense against complexes of bark beetles and associated fungi (Klepzig et al. 1995; Raffa & Smalley 1995; Klepzig et al. 1996; Phillips & Croteau 1999), as well as other fungi (Ennos & Swales 1991; Himejima et al. 1992) due to accumulation following a local attack. However, ours may be one of the first studies in which a negative correlation between experimentally manipulated *in planta* levels of a monoterpene (β-pinene) and extent of pathogen-induced tissue damage has been documented.

Cluster 1 (phenolics) and cluster 2 (monoterpenes) were negatively correlated with each other (Table 2.5). This finding suggests a trade-off between the shikimic acid

(phenolic) and the GAP-pyruvate (monoterpene) pathways and has intriguing implications for the way that trees defend themselves against multiple threats from taxa as diverse as microbes and insects.

In summary, our work suggests that the systemic accumulation of "anticipatory" compounds (phytoanticipins), in particular phenolic glycosides, in response to the induction by a fungal infection could be one of the bases of SIR in pine branches. We have also documented a trade-off between terpenoid and phenolic metabolism that may have important implications in our understanding of complex, multitrophic and multi-partite systems. Furthermore, the only consistent fertility effect found in this study was lowered phenolic glycoside levels at the highest N addition, and this was not sufficient to affect lesion length in that treatment (the interaction between induction treatment and fertilizer was also not significant for cluster 1, which contained those phenolics). Trees may have been able to respond to the challenge attack by up-regulating local defense responses in the branches, thus overcoming any shortfalls in phytoanticipins caused by the high fertility treatment. The hypothesis that phenolic glycosides play an anticipatory and perhaps essential role in SIR could be mathematically modeled. These compounds would be used as predictors of relative resistance in naïve trees, coupled with studies aimed at determining the levels of antifungal activity that these phenolic glycosides and their aglycones possess.

	Non-wounded		Mock-Inoculated		Fungal-Inoculated		Insect-Defoliated		ANOVA Stats $F^{\dagger}_{3, 121\text{-}181}$ (*P*)
Lesion Length (mm)[‡]	21.62	(1.37) a	19.86	(1.32) a	13.39	(1.20) b	20.57	(1.12) a	9.415 (<0.001)
Lignin (mg g^{-1} FW)	11.50	(0.492) b	10.59	(0.530) b	14.75	(0.505) a	12.63	(0.852) b	12.231(<0.001)
Total Phenolics[1] (mg g^{-1} FW)	2.57	(0.226) ab	2.33	(0.225) b	3.16	(0.217) a	3.03	(0.370) ab	ns[3]
Phenolics (AU, unless indicated)									
dihydroconiferin I	$1.52X10^5$	($1.14X10^4$)	$1.25X10^5$	($1.12X10^4$)	$1.41X10^5$	($1.12X10^4$)	$1.28X10^5$	($1.96X10^4$)	3.359 (0.021)
coumaric acid hexoside	$3.14X10^5$	($3.96X10^4$) b	$3.42X10^5$	($3.79X10^4$) b	$5.03X10^5$	($5.87X10^4$) a	$3.96X10^5$	($8.51X10^4$) ab	ns
hydroxypropiovanillone hexoside	$5.20X10^5$	($5.04X10^4$)	$5.13X10^5$	($4.48X10^4$)	$6.05X10^5$	($5.28X10^4$)	$5.21X10^5$	($6.92X10^4$)	2.787 (0.044)
dihydroconiferin II	$1.34X10^5$	($1.44X10^4$) b	$1.57X10^5$	($1.44X10^4$)ab	$1.93X10^5$	($1.47X10^4$) a	$1.58X10^5$	($2.55X10^4$) ab	4.377 (0.006)
ferulic acid glucoside, μg g^{-1} FW	251	(27.7) b	319	(28.3) ab	393	(33.2) a	377	(48.7) a	3.913 (0.010)
taxifolin hexoside	$4.13X10^5$	($3.85X10^4$) b	$3.99X10^5$	($3.18X10^4$) b	$5.50X10^5$	($4.65X10^4$) a	$4.87X10^5$	($6.03X10^4$) ab	7.452 (<0.001)
unknown 1	$3.88X10^4$	($2.08X10^4$) b	$3.10X10^4$	($1.72X10^4$) b	$1.32X10^5$	($1.65X10^4$) a	$4.68X10^4$	($2.95X10^4$) b	5.078 (0.003)
taxifolin, μg g^{-1} FW	42.0	(3.04) b	40.1	(2.84) b	63.3	(5.97) a	43.8	(4.64) b	4.323 (0.006)
pinosylvin, μg g^{-1} FW	24.2	(2.92) b	22.1	(2.07) b	57.6	(14.6) a	19.6	(1.42) b	4.844 (0.003)
pinosylvin monomethyl ether, μg g^{-1} FW	4.49	(0.696) b	3.74	(0.198) b	19.6	(5.83) a	6.21	(2.37) b	9.415(<0.001)

Continued

Table **2.1:** Effects of the pathogen infection or insect defoliation treatments on phloem chemistry. Means (standard errors) for lesion length and concentration of each compound in branch phloem, with different letters indicating separation of means by LSD within row ($P < 0.05$). F and *P* values for significant ANOVA are also presented.

Table 2.1 continued

unknown 2	$2.78X10^4$	($1.98X10^3$)	$2.65X10^4$	($2.21X10^3$)	$3.29X10^4$	($3.24X10^3$)	$2.16X10^4$	($1.53X10^3$)	ns
unknown 3	$1.58X10^4$	($1.49X10^3$)	$1.39X\ 0^4$	($1.60X10^3$)	$1.38X10^4$	($1.85X10^3$)	$1.86X10^4$	($4.59X10^3$)	ns
Resin Acids									
unknown 4 (AU)	$2.67X10^4$	($2.71X10^3$)	$2.59X10^4$	($1.92X10^3$)	$2.99X10^4$	($3.11X10^3$)	$2.42X10^4$	($4.42X10^3$)	ns
neoabietic acid (AU)	$1.41X10^5$	($1.92X10^4$)	$1.40X10^5$	($1.80X10^4$)	$1.51X10^5$	($1.99X10^4$)	$2.27X10^5$	($2.35X10^4$)	ns
labdene isomer (μg p-cymene equiv. g^{-1} FW)	319.1	(83.69)	344.6	(80.80)	35.24	(77.73)	212.7	(119.2)	ns
labdadiene isomer (μg p-cymene equiv. g^{-1} FW)	4100	(742.2)	3871	(708.5)	3592	(703.6)	2942	(1073.6)	ns
Monoterpenes (μg p-cymene equiv. g^{-1} FW)									
α-pinene[2]	11670	(891.7) b	12770	(936.7) ab	14150	(907.0) a	9530	(1510) b	2.706 (0.049)
camphene	211.6	(16.72)	216.4	(16.84)	253.2	(16.97)	175.1	(27.90)	ns
β-pinene[2]	670.8	(103.52) bc	921.3	(95.93) b	1287	(89.65) a	523.9	(152.52) c	9.307 (<0.001)
myrcene	309.1	(25.91)	302.7	(27.99)	337.9	(27.19)	211.8	(44.03)	ns
limonene[2]	1929	(241.4) ab	2209	(251.3) a	2627	(249.4) a	997	(431.6) b	3.421 (0.020)
bornyl acetate	416.7	(38.39)	360.5	(40.97)	382.9	(40.64)	268.6	(66.36)	ns
Sesquiterpenes (μg p-cymene equiv. g^{-1} FW)									
trans-caryophyllene	795.1	(116.4)	766.1	(118.2)	1005	(118.2)	1159.8	(180.3)	ns
α-humulene	239.4	(35.51)	222.4	(34.99)	193.6	(34.04)	223.0	(49.49)	ns
germacrene D	4708	(648.6) b	3706	(664.1) b	4017	(638.1) b	7917	(1028.8) a	4.356 (0.006)
α–muurolene	207.6	(43.10)	251.4	(41.84)	211.0	(42.73)	260.7	(54.60)	ns

[1] Measured by Folin method; [2] Exist as a combination of two stereoisomers; [3] ns = non-significant; † df as subscripts; ‡ df = 3, 71

	30 ppm N		75 ppm N		150 ppm N		ANOVA Stats $F^{\dagger}_{2,\ 131\text{-}181}$ (*P*)
Lesion Length (mm)‡	18.25	(1.71)	20.36	(1.20)	18.19	(1.47)	ns[3]
Lignin (mg g^{-1} FW)	12.55	(0.55)	12.51	(0.57)	11.94	(0.52)	ns
Total Phenolics[1] (mg g^{-1} FW)	3.20	(0.23) a	3.15	(0.23) a	1.97	(0.24) b	ns
Phenolics[2] (AU, unless indicated)							
dihydroconiferin I	$1.34X10^6$	($1.17X10^5$)	$1.33X10^6$	($1.18X10^5$)	$1.44X10^6$	($1.19X10^5$)	ns
coumaric acid hexoside	$5.08X10^5$	($5.85X10^4$) a	$3.95X10^5$	($4.03X10^4$) b	$2.61X10^5$	($2.54X10^4$) c	6.435 (0.002)
hydroxypropiovanillone hexoside	$5.92X10^5$	($5.11X10^4$) a	$5.91X10^5$	($4.49X10^4$) a	$4.46X10^5$	($3.91X10^4$) b	3.511 (0.033)
dihydroconiferin II	$1.69X10^6$	($1.47X10^5$)	$1.61X10^6$	($1.50X10^5$)	$1.51X10^6$	($1.52X10^5$)	ns
ferulic acid glucoside (μg g^{-1} FW)	380.2	(32.0) a	359.0	(30.2) a	242.3	(20.2) b	6.870 (0.001)
taxifolin hexoside	$4.79X10^5$	($3.60X10^4$) a	$5.26X10^5$	($4.24X10^4$) a	$3.67X10^5$	($3.09X10^4$) b	6.124 (0.003)
unknown 1	$4.99X10^4$	($1.76X10^4$)	$8.6X10^4$	($1.81X10^4$)	$5.38X10^4$	($1.75X10^4$)	ns
taxifolin (μg g^{-1} FW)	42.23	(3.52)	55.11	(4.31)	45.29	(3.83)	ns
pinosylvin (μg g^{-1} FW)	36.21	(11.00)	29.94	(3.82)	33.66	(8.17)	ns
pinosylvin monomethyl ether (μg g^{-1} FW)	11.41	(4.88)	9.30	(2.03)	6.33	(1.92)	ns
unknown 2	$2.57X10^4$	($2.47X10^3$)	$3.00X10^4$	($2.44X10^3$)	$2.94X10^4$	($2.19X10^3$)	ns
unknown 3	$1.19X10^4$	($9.53X10^3$) b	$1.37X10^4$	($1.21X10^3$) b	$1.94X10^4$	($2.46X10^3$) a	9.009 (<0.001)

[1] Measured by Folin method; [2] Mass provided for compounds quantified with standards; [3] ns = non-significant; † df as subscripts; ‡ df = 3, 71

Table **2.2:** Effects of fertility treatment on phloem chemistry. Means (standard errors) for lesion length and concentrations of phenolic compounds in branch phloem, with different letters indicating mean separation by LSD within row ($P < 0.05$).

	Lesion Length		
	ρ	*P*	N
lignin	-0.209	0.095	65
hydroxypropiovanillone hexoside	-0.242	0.042	71
ferulic acid glucoside	-0.304	0.010	71
unknown 1	-0.297	0.011	72
unknown 3	0.253	0.032	72
β-pinene	-0.231	0.056	69
labdadiene isomer	0.258	0.032	69

Table **2.3:** Spearman correlations between concentrations of individual compounds and lesion length. Only compounds with significant ($P < 0.05$) or marginally significant ($P < 0.10$) correlations are shown.

Groups	Description	Lesion Length	
		ρ	*P*
Cluster Group 1	dihydroconiferin II, coumaric acid hexoside, hydroxypropionvanillone hexoside, ferulic acid glucoside, taxifolin hexoside, taxifolin, lignin[1]	-0.250	0.038
Cluster Group 2	α-pinene, camphene, β-myrcene, limonene, bornyl acetate	0.033	0.790
Cluster Group 3	trans-caryophyllene, α-humulene	0.166	0.343
Cluster Group 4	germacrene D, α-muurolene, neoabietic acid	-0.043	0.728
Cluster Group 5	pinosylvin, pinosylvin monomethyl ether, unknown 1	-0.239	0.043
Cluster Group 6	labdene isomer, labdadiene isomer	0.275	0.022
Cluster Group 7	unknown 2, unknown 3, unknown 4	-0.008	0.946
Cluster Group 1 and 5	dihydroconiferin II, coumaric acid hexoside, hydroxypropionvanillone hexoside, ferulic acid glucoside, taxifolin hexoside, taxifolin, lignin, pinosylvin, pinosylvin monomethyl ether, unknown 1	-0.283	0.016

[1] Lignin weakly grouped in cluster 1 according to the PCA, but met criteria of fitting in 3 of 4 of the dendrograms shown in Fig. 1. Without lignin, cluster 1 compounds are weakly correlated with lesion size ($\rho = -0.171$, $p = 0.150$).

Table **2.4:** Spearman correlations between mean quintile concentrations of each cluster group and lesion length. For all correlations, N = 69.

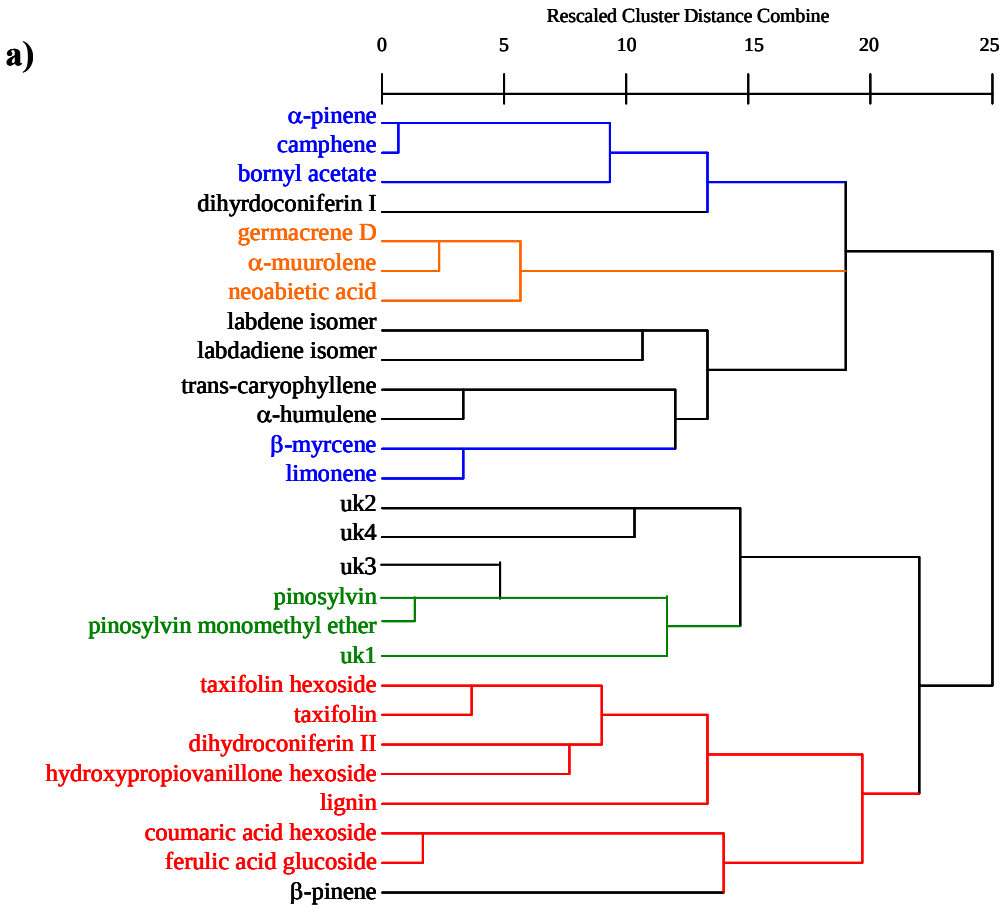
	Cluster 2		Cluster 3		Cluster 4		Cluster 5		Cluster 6		Cluster 7	
	ρ (N)	*P*	ρ (N)	*P*	ρ (N)	*P*	ρ (N)	*P*	ρ (N)	*P*	ρ (N)	*P*
Cluster 1 (phenolics)	-0.276 (166)	<0.001	-0.124 (166)	0.113	-0.122 (165)	0.120	0.275 (166)	<0.001	-0.092 (166)	0.238	0.038 (166)	0.630
Cluster 2 (monoterpenes)	--------	-------	0.262 (166)	0.001	0.114 (165)	0.145	-0.002 (167)	0.980	0.314 (166)	<0.001	0.314 (167)	<0.001
Cluster 3 (caryophyllene)			--------	--------	0.191 (165)	0.014	0.010 (166)	0.901	0.257 (165)	0.001	0.075 (166)	0.337
Cluster 4 (germacrene D)					--------	--------	0.048 (165)	0.539	0.078 (165)	0.317	0.248 (165)	0.001
Cluster 5 (stilbenes)							--------	--------	0.006 (166)	0.938	0.302 (181)	<0.001
Cluster 6 (diterpenes)									--------	--------	0.120 (166)	0.123

Table **2.5:** Pairwise Spearman correlations between mean quintile concentrations of each cluster group.

Figure **2.1:** Cluster analyses revealing the co-regulation of individual compounds in pine defense responses. Dendrograms based on furthest neighbor joining and squared Euclidean distance showing grouping of compounds based on patterns of expression in: (a) the non-wounded treatment, (b) the mock inoculation treatment, (c) the insect defoliation induction treatment, and (d) the fungal induction treatment. Numbers above branches indicate cluster groupings as described in Table 4, determined by consistent grouping in 3 of the 4 dendrograms. Cluster 1 (phenolics) is highlighted in red, cluster 2 (monoterpenes) in blue, cluster 4 in orange, and cluster 5 (stilbenes) in green. All other clusters are in black.

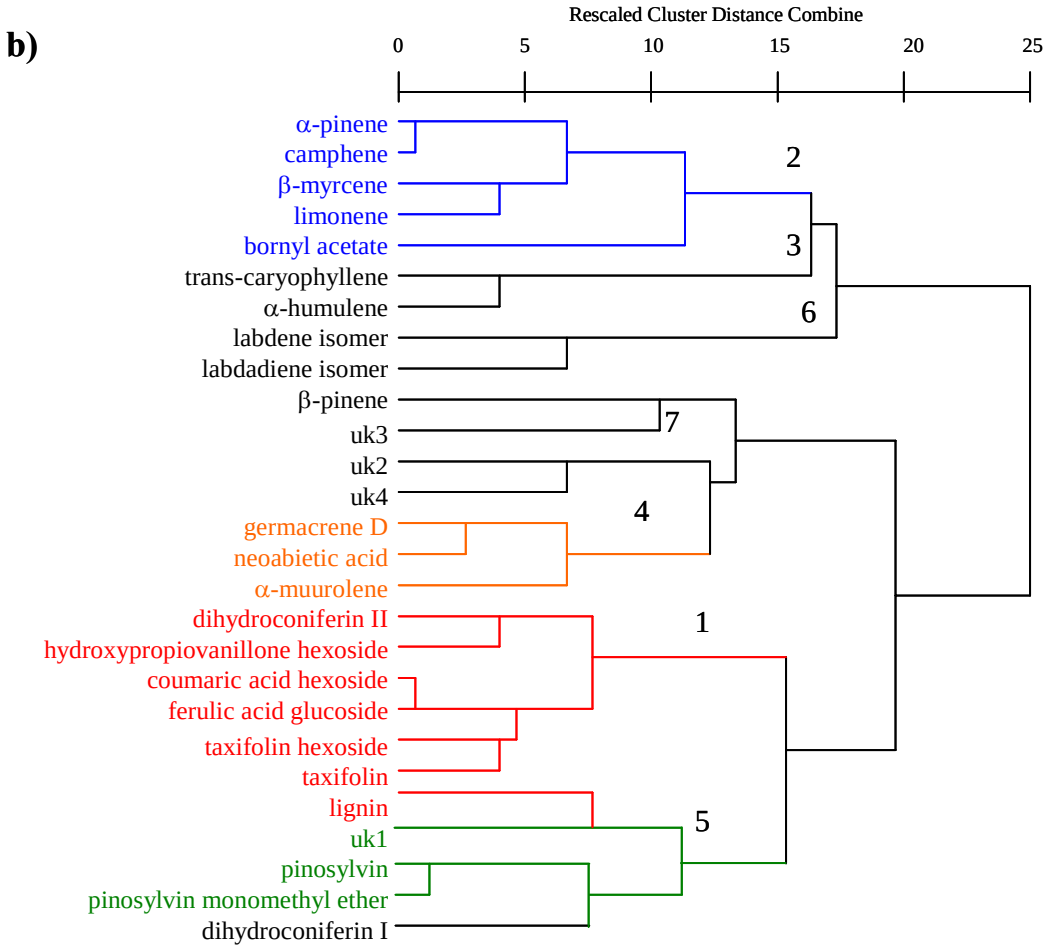
Figure 2.1

a)



Continued

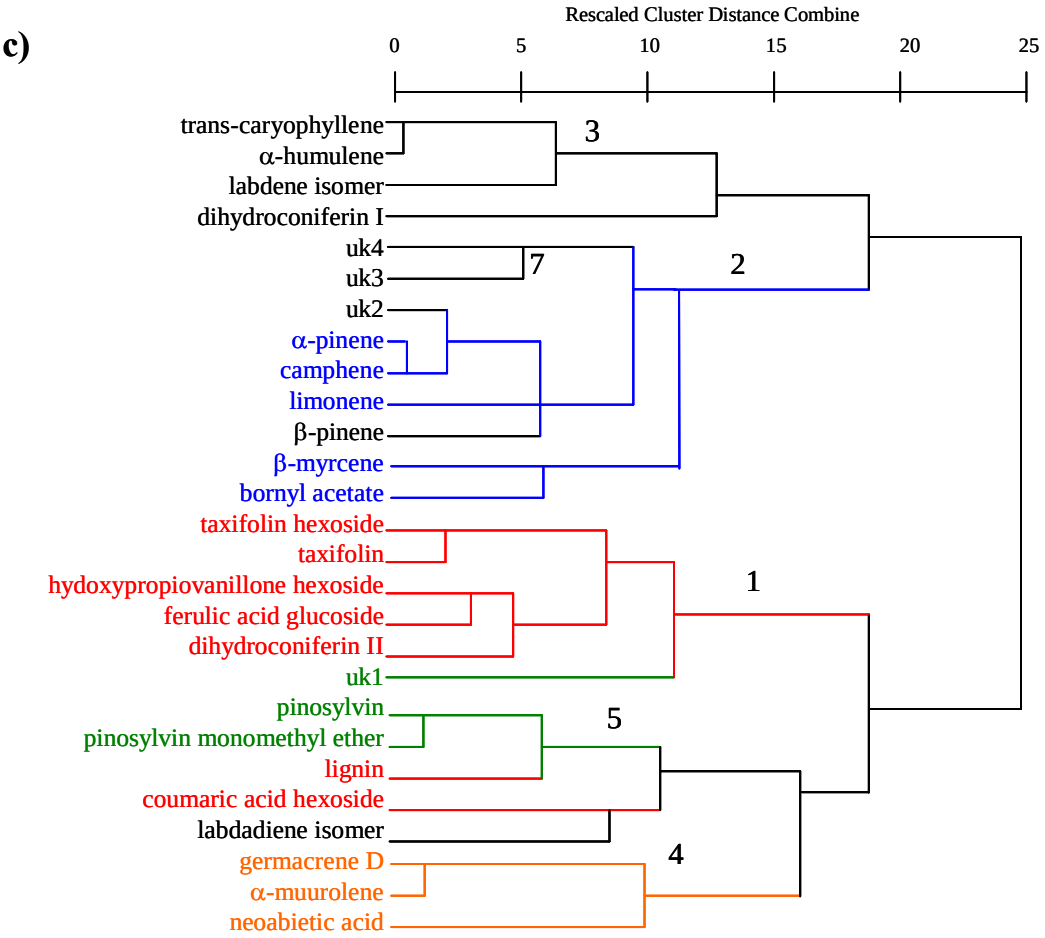
Figure 2.1 continued



Continued

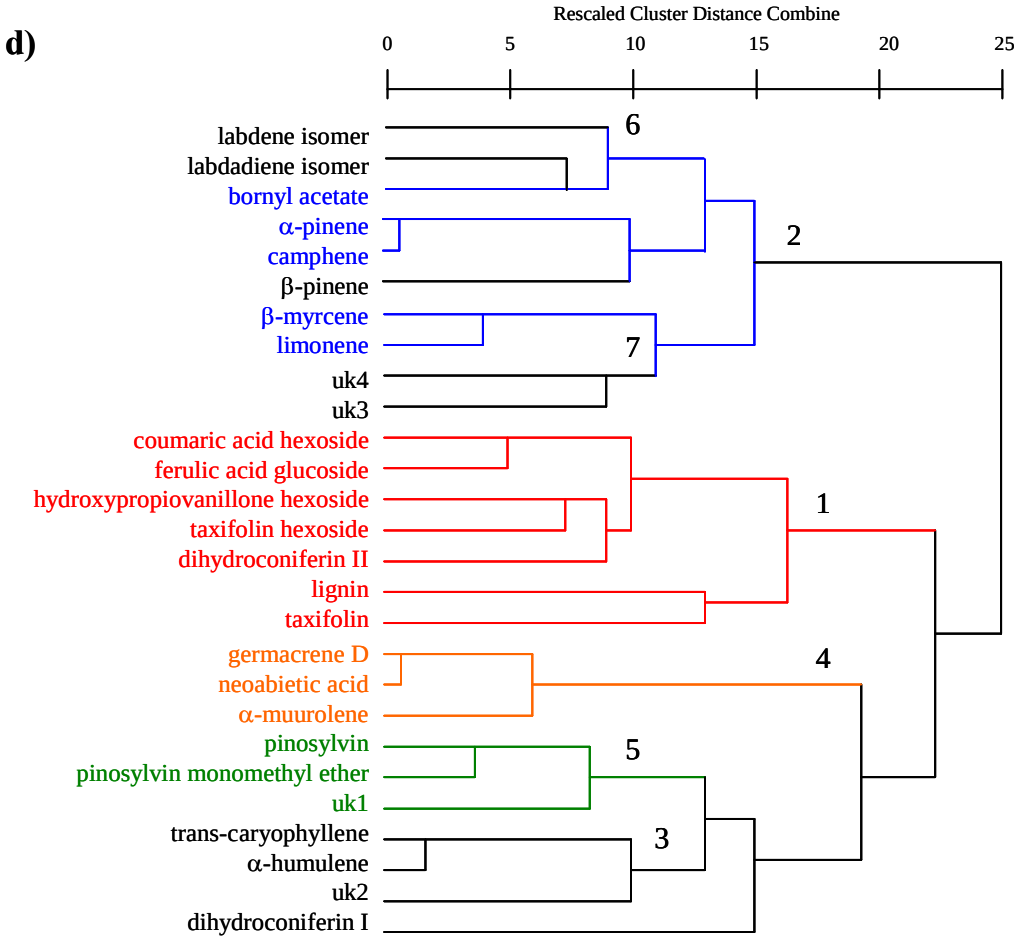
Figure 2.1 continued

c)



Continued

Figure 2.1 continued



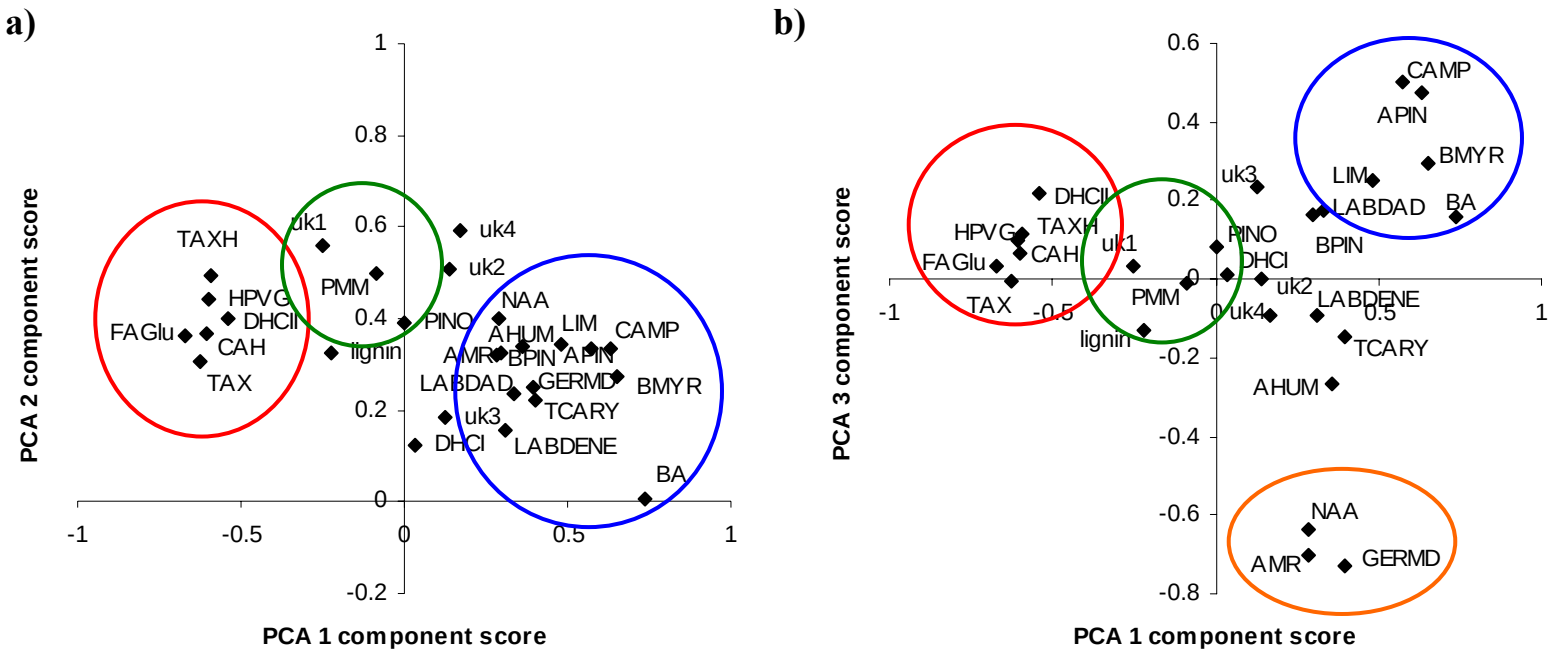


Figure **2.2:** PCA component plots of (a) PCA 1 and PCA 2 and (b) PCA 1 and PCA 3, based on an analysis which included all of the treatments in this study. Circles represent defined, separated groups, and correspond to clusters 1 (red), 2 (blue), 4 (orange), and 5 (green) (identified in Fig. 2.1). The following abbreviations are used: AHUM = α-humulene; AMR = α-muurolene; APIN = α-pinene; BA = bornyl acetate; BMYR = β-myrcene; BPIN = β-pinene; CAH = coumaric acid hexoside; CAMP = camphene; DHCI = dihydroconiferin I; DHCII = dihydroconiferin II; FAGlu = ferulic acid glucoside; GERMD = germacrene D; HPVG = hydroxypropiovanillone hexoside; LABDAD = labdadiene isomer; LABDENE = labdene isomer; LIM = limonene; NAA = neoabietic acid; PINO = pinosylvin; PMM = pinosylvin monomethyl ether; TAX = taxifolin; TAXH = taxifolin hexoside; TCARY = trans-caryophyllene

CHAPTER 3

EFFECTS OF NUTRIENT AVAILABILITY ON THE SECONDARY METABOLISM OF AUSTRIAN PINE (*PINUS NIGRA*) PHLOEM AND IMPACT ON RESISTANCE TO *DIPLODIA PINEA*

Introduction

The causal agents behind forest decline syndromes are known to be important drivers of forest community dynamics and can have significant impacts on forest productivity (Castello et al. 1995). The factors that trigger such declines are often unknown, but a model has been proposed (Manion 1996) whereby a combination of three classes of factors lead to the weakening and death of trees over time: 1) predisposing (i.e. long-term) factors such as nutrient availability (Turtola et al. 2002, Blodgett et al. 2005) and light quality (Klepzig et al. 1995, Klepzig et al. 1996); 2) inciting (i.e. shorter-term) factors such as water stress (Blodgett et al. 1997) or insect defoliation (Raffa et al. 1998); and 3) contributing factors, such as increased attacks by bark beetles (Erbilgin and Raffa 2002) and root pathogens such as *Armillaria* spp. and *Heterobasidion* spp. (Cherubini et al. 2002). Contributing factors are usually those directly responsible for the death of a tree.

Among potential predisposing factors, soil quality and nutrient availability have been shown to affect pathogen invasion (Schoeneweiss 1975, Ayres 1984, Mattila et al. 2001, Moreira and Martins 2005). Thus, understanding the relationship between nutrient availability and constitutive defenses is an important step in furthering our conceptual models of tree decline syndromes and more generally in explaining tree susceptibility to pathogens.

The mechanisms by which nutrient availability affects conifer host defense responses are unclear. Although multiple plant defense hypotheses have been proposed to explain how fertility levels might affect constitutive resistance against insect pests (Stamp 2003), fewer hypotheses have focused on nutrient effects on defenses against pathogens. Plant defense theory generally predicts that increasing fertility above "optimal" levels results in a reduction of secondary metabolites and a corresponding reduction in resistance (Stamp 2003). Tests of plant defense theory have generally confirmed this prediction, with many studies showing that increasing fertility and relative growth rate results in reduced resistance to both insect (Herms 2002 and references therein) and pathogen attack (Blodgett et al. 2005, Lambert 1986, Snoeijiers et al. 2000). However, linking increased susceptibility with a reduction in accumulation of secondary metabolites as fertility and growth increase has been rather difficult (Koricheva et al. 1998; Kytö et al. 1998). Genotypic variability of individual plants (Osier and Lindroth 2001) and complications brought on by environmental factors have confounded the ability to observe how fertility ultimately affects secondary metabolite levels and

resistance (Lambert 1986, Herms 2002). Alternatively, problems in methodology or misinterpretation of results might have resulted in erroneous and possibly conflicting conclusions (Stamp 2003).

The aim of this study was to investigate the effects of different fertility levels on constitutive chemical defense profiles and predisposition of Austrian pine to infection by *D. pinea*. Fertility affects on constitutive levels of phenolics and terpenes was assessed to observe if these different compound classes behaved differently when exposed to varying nutrient availability. An attempt was also made to link this compounds and fertility to the expression of resistance. This study was conducted under highly controlled conditions to limit the complex factors associated with forest or landscape settings.

Materials and Methods

The study involved a subset of the trees utilized in the experiment described in Chapter 2, page 40. In brief, a total of 60 trees were arranged in a complete randomized block design across three fertility levels and in five blocks. Therefore, there were 20 trees per fertility level and 4 trees per fertility and block combination. The fertility levels were applied along with irrigation water at 30 ppm N, 75 ppm N, and 150 ppm N in a 3:1:2 NPK ratio (see page 40 for a more complete description of how these were applied). Plants were irrigated according to a mathematical model of evapotranspiration developed by Lee et al. (2000).

Pathogen Challenge

During the spring of 2005, each tree was challenged with the fungal pathogen *D. pinea* (for more detail, see Chapter 2, page 41). In brief, fungal inoculations involved applying an 8 mm-diameter agar plug colonized by *D. pinea* into a wound made with a 10 mm-diameter cork borer, ~5 cm from the stem on a randomly selected branch. The agar plug was held in place with a small piece of duct tape. After 10 days, the tape was removed and the outer bark was scraped away to reveal the discolored lesion, which was measured lengthwise and utilized as a quantitative measure of pathogen growth (Blodgett et al. 2007).

Phytochemical Analyses

One unchallenged branch was removed from the same whorl on all of the trees in the experiment at the time of the challenges, and ~10 g of phloem tissue was removed from each excised branch and flash-frozen in liquid nitrogen. Tissues were then extracted with methanol to obtain phenolics, as described in Chapter 2, page 42. The supernatants were stored at -20 ^{o}C until they were analyzed by high-pressure liquid chromatography (HPLC), as described in full in Chapter 2, pages 42-43. Chromatographs were obtained by a photodiode array detector and were quantified using absorbance unit values at 308 nm (Bonello and Blodgett 2003). During quantification, levopimaric acid and abietic acid co-eluted as one peak and thus were quantified together and labeled as "resin acids". Lignin was extracted an analyzed as described in Chapter 2, page 43.

An additional 100 mg of ground phloem tissue was extracted twice with 500 μL of dichloromethane (DCM) (Fisher) containing 0.1% (v/v) p–cymene (Sigma) (internal standard), incubated for 24 h at 4 ^{o}C each time, for a total of 1 mL DCM extract. The terpenes were analyzed using gas chromatography (GC) as described in Chapter 2, page 44. It is important to note that α–copanene was detected in only a few samples and only in trace amounts, and therefore was omitted in subsequent analyses.

Compound Identification

Unknown phenolics were characterized by comparison of their UV spectrum and retention times with those of standards (Bonello and Blodgett 2003), or by liquid chromatography/tandem mass spectrometry with electrospray ionization (HPLC- ESI-MS) (Eyles et al. 2003, Eyles et al. 2007b). Standards were: pinosylvin and pinosylvin monomethyl ether (Apin Chemicals, Abingdon, UK), taxifolin (Sigma), ferulic acid glucoside (Bonello and Blodgett 2003), and abietic acid (Alfa Aesar, Ward Hill, MA). Identification of six monoterpenes was based on comparing their retention times with the following standards: β-pinene, α-pinene, camphene, β-myrcene, limonene, and γ-terpinene (all from Sigma). Other terpenes were identified using gas chromatography-mass spectrometry (GC-MS).

HPLC- ESI-MS analyses were conducted on a Hewlett Packard HPLC system model HP 1100 (Hewlett Packard) equipped with a HP DAD G1315A detector coupled to a Q-tof I (Micromass, Cary, NC). The same column and gradient program as described

for the HPLC system were used, except solvent A was water/0.1% acetic acid and solvent B was methanol/0.1% acetic acid. Detailed description of the operating conditions for HPLC- ESI-MS can be found elsewhere in Eyles et al. (2007b).

The DCM extracts were analyzed by GC-MS using a Finnigan trace GC-MS (Thermo Corporation, Waltham, MA). A Rtx®-5 Sil MS fused silica column (30 m x 0.25 mm ID) (Restek, Bellefonte, PA) was used. The program had the same temperature gradient settings as those for the GC analysis conducted on page 43. The carrier gas was helium. Split (20:1) injections of 2 μL, plus 2 μL of air, were made at an injector temperature of 220 °C. Identifications of terpenes were based on the Wiley Registry of Mass Spectral Data (Wiley) and the NIST Library (NIST) and were considered as confirmed when the reverse search index (RSI) values were > 850. In addition, where available, standards were used for unequivocal confirmation.

Statistical Analyses

Compound classes were defined by first using quintile rank normalization of each compound concentration (1 = lowest 20% to 5 = highest 20%) followed by taking the mean across all variables in a compound class (monoterpenes, sesquiterpenes, diterpenes, or phenolics) (McSpadden Gardener 2006).

All statistics were performed using SPSS version 15.0 (SPSS Institute, Chicago), with an $\alpha = 0.05$. Two-way univariate ANOVAs (model: fertility, block, fertility X

block) were performed on concentrations of each variable. Non-parametric tests (Kruskal-Wallis followed by Mann-Whitney U) were utilized when normalization was not possible.

To examine correlations between chemical concentrations and tree and pathogen growth, Pearson's product-moment correlation was conducted between challenge lesion length, tree growth, individual compounds, and compound classes.

Results

Identification of compounds in branch phloem of *Pinus nigra*

From the crude methanol extracts, a total of 25 individual compounds were analyzed in detail. The characterization of unknown compounds was based on comparison of UV, mass spectrometry and mass fragmentation data with standards, where possible, and with published data (Table 3.1). The identities of taxifolin, ferulic acid glucoside, pinosylvin, pinosylvin monomethyl ether, and abietic acid were confirmed with standards. All compounds have been previously reported to be present in the phloem of *P. nigra* and/or another two-needled pine species, *P. sylvestris*, except unknown compounds 1, 2, and 3, a vanillin derivative, and coumaroylquinic acid (Table 3.1). Pinosylvin monomethyl ether was not detected by HPLC-ESI-MS analysis, but was identified independently by matching UV spectra and retention time, as in Bonello and Blodgett (2003).

A total of 13 peaks were identified using GC-MS (Table 3.2) including 6 monoterpenes, 5 sesquiterpenes, and 2 diterpenes (a labdene and labdadiene isomer).

Effects of fertility on pathogen lesion development, terpene concentrations, and phenolic concentrations

There were no significant effects of fertility on fungal lesion length ($F_{2, 25} = 0.586$, $P = 0.565$). Likewise, no significant modulations due to fertility were observed for any of the diterpenes or phenolic compounds (including lignin).

Three monoterpenes (camphene, β-myrcene, and bornyl acetate) increased significantly with increasing fertility (Fig. 3.1). One sesquiterpene (germacrene D) increased in the 75 ppm N treatment over the 150 ppm treatment (Fig. 3.1). Finally, one unknown phenolic (uk2) was significantly higher in the 75 ppm N than the 30 ppm N treatment ($F_{2, 25} = 4.022$, $P = 0.029$ for uk2). Significant block effects were found for camphene, β–myrcene, bornyl acetate, and germacrene D. A significant interaction between fertilizer treatment and block was found only for bornyl acetate ($F_{7, 39} = 4.621$, $P = 0.002$), whereby increases in the 150 ppm N treatment over the other treatments were found in all blocks except block 5, which had equal levels of bornyl acetate across all the fertilizer treatments.

Monoterpenes as a class increased significantly between 30 and 75 ppm N and stayed level at 150 ppm N (Fig. 3.2). Lignin, phenolics, sesquiterpenes, and diterpenes as classes were not affected by fertility.

Correlations between fertility, resistance, and compound classes

Positive correlations with fertility were found for camphene (r = 0.364, *P* = 0.015, N = 44), bornyl acetate (r = 0.316, *P* = 0.047, N = 40), and pinosylvin (r = 0.314, *P* = 0.027, N = 50) concentrations. No other significant correlations of individual compounds with fertility were observed.

Phenolic concentration was negatively correlated (*P* = 0.054) with lesion length (Table 3.3).

Phenolic and monoterpene concentrations were strongly negatively correlated with each other (Table 3.3). Phenolic concentrations were also negatively correlated with sesquiterpene concentrations (Table 3.3).

Discussion

We investigated the effects of three different fertility levels on constitutive chemical defense profiles and predisposition of Austrian pine to infection by *D. pinea*. Preconditioning Austrian pine trees at three different fertility levels for one year did not affect resistance to *D. pinea*. However, concentrations of phloem monoterpenes were increased as a result of increasing nutrient availability. Phenolics were negatively correlated with lesion length, suggesting a role in defense. Phenolics and monoterpenes, as well as phenolics and sesquiterpenes, were negatively correlated with each other.

These findings also suggest that phenolics and terpenes respond differently to nutrient availability because fertility levels influenced only the production of monoterpenes and the compound classes were negatively correlated with one another.

Four terpenes were significantly affected by fertility level: camphene, β-myrcene, bornyl acetate, and germacrene D (Fig. 3.1). Both camphene and bornyl acetate increased linearly in concentration with increased fertility (Fig. 3.1). Conversely, β-myrcene, germacrene D, and the total monoterpenes appeared to follow a quadratic response, albeit not statistically significant, whereby moderate nutrient availability yielded their greatest accumulation (Figs. 3.1 and 3.2). Camphene, β-myrcene, bornyl acetate, and germacrene D have been described as having a potential role in resistance to pathogens or insects, whether by directly toxicity, acting as feeding deterrents, or inhibiting attraction to hosts (Raffa et al. 1985, Cates et al. 1987, Cook and Hain 1988, Klepzig et al. 1995, Raffa and Smalley 1995, Werner 1995, Klepzig et al. 1996, Lindgren 1996, Norlander 1990, Blodgett and Stanosz 1997a; Huber et al. 2005, Keeling and Bohlmann 2006).

No significant correlations between lesion length and levels of any of the monoterpenes were found in this study, suggesting that for this system monoterpenes did not affect the expression of constitutive resistance. This is consistent with a study by Jurc et al. (1999), but inconsistent with a study by Blodgett and Stanosz (1997a). However, the two studies used different sets of monoterpenes and were *in vitro* inhibition studies, whereas our study correlated monoterpenes and resistance *in planta*. A negative correlation was discovered between concentrations of total phenolics and lesion length, suggesting that phenolics, not terpenes, are important in Austrian pine constitutive resistance to *D. pinea*. Previous findings have shown that phenolics increase systemically

following fungal pathogen inoculation, potentially as a mechanism of systemic induced resistance to thwart further fungal attack (Bonello and Blodgett 2003, Blodgett et al. 2007). There were no effects of fertility on the levels of phenolics in this study, and consequently, no effects of fertility on constitutive resistance.

The occurrence of strong negative correlations between phenolics and two terpene classes (monoterpenes and sesquiterpenes) suggests that the two metabolic networks compete for resources. Both terpenoids (Gershenzon 1994) and phenolics (Purrington 2000) are some of the most expensive metabolites to produce, although a negative association between growth and secondary metabolites might only be revealed under certain environmental conditions (Bergelson and Purrington 1996, Purrington 2000, Donaldson et al. 2006, Osier and Lindroth 2001). This study suggests that competition for carbon resources occurs in pines between phenolic and terpene pathways, at least when the total carbon budget might be constrained (e.g. during periods of active growth).

Studies determining the effects of nutrient availability on pine defense have reached varying conclusions because each study had uncontrolled / unconsidered aspects in its experimental design (Koricheva 1998; Stamp 2003). Comprehensive studies have rarely been performed due to limitations in genetic resources, the unavailability of large quantities of host material, gaps in knowledge of host resistance, and the inability to have highly controlled environments (Stamp 2004). Varying fertility levels led to different accumulation patterns of monoterpenes compared with phenolics in this study. This suggests that as many mechanisms of host defense as possible should be considered in any given study before drawing any firm conclusions regarding the physiological basis of the effects of nutrient availability on host resistance to pests.

In summary, we found no significant effects of fertility on resistance to *D. pinea* or accumulation of phenolics. However, the different fertility levels affected terpene concentrations in the phloem. One significant factor in constitutive resistance to *D. pinea* may be the soluble phenolics because they were negatively correlated with lesion length. The fact that neither phenolic levels nor fungal growth responded to fertility strengthens this hypothesis. Finally, a negative correlation was observed between phenolics and terpenes, possibly caused by competition for resources.

Rt (min)	[M-H]-	Main fragments by ESI-MS	UV λ_{max} (nm)	Assigned identity	References
7.81	299	137	252	hydroxybenzoic acid hexoside	Karonen et al. 2004
8.82	329	167, 152, 123, 108	255, sh 285	vanillic acid hexoside	Karonen et al. 2004
9.86	451	289, 245	274	catechin hexoside	Karonen et al. 2004
10.36	343	181, 161	276	dihydroconiferin I	Pan and Lundgren 1996
10.5	325	163, 119	295	coumaric acid hexoside	Pan and Lundgren 1996
11.09	357	195, 136, 151	280, sh 310	vanillin derivative	Karonen et al. 2004
11.56	357	177, 162, 119	277, 310	hydroxypropiovanillone hexoside	Karonen et al. 2004
11.95	343	181, 166, 161	280	dihydroconiferin II	Pan and Lundgren 1996
12.26	355	193	289, 320	ferulic acid hexoside	Pan and Lundgren 1996
12.76	355	193	289, 320	ferulic acid glucoside*	Pan and Lundgren 1996
13.2	289	245, 203, 179, 151, 109	279	epi/catechin	Karonen et al. 2004
13.57	337	163, 191, 119	290, 310	coumaroylquinic acid	Kammerer et al. 2004
14.27	507	315, 327, 345	279	lignan hexoside	Karonen et al. 2004
14.98	495	179, 165, 221, 315, 363, 345, 327, 239, 149	279	lignan xyloside	Karonen et al. 2004
17.52	491	315, 345, 327	280	lignan deoxyhexoside	Karonen et al. 2004
18.28	465	285, 125, 259, 275, 437, 303, 447, 217, 288	287	taxifolin hexoside	Karonen et al. 2004
-^	-	-	315	unknown 1#	
19.33	303	125, 175, 217, 199, 285, 151, 241	285	taxifolin*	Karonen et al. 2004
27. 45	211	169, 167, 152	300	pinosylvin*	
-^	-	-	300	pinosylvin monomethyl ether*#	
31.52	317	299	271	unknown 2	
-^	-	-	239, 328	unknown 3#	
35.57	301	-	270	levoprimaric acid%	Kersten et al. 2006
35.77	301	-	241	abietic acid%	Kersten et al. 2006
36.31	301	-	260	neoabietic acid	Kersten et al. 2006

compound detected by HPLC-UV but not by HPLC-ESI-MS

* verified by standard

^ for these compounds retention times by HPLC-UV (which do not correspond to HPLC-ESI-MS retention times) were: unknown 1, 15.7; pinosylvin monomethyl ether, 27.7; unknown 3, 30.9

% quantified as unknown 4 due to co-elution under HPLC conditions

Table **3.1:** Characteristic ions and UV spectra maxima of compounds from methanol extracts of Austrian pine phloem.

Rt (min)	Compound Class	Assigned identity	Library
7.74	monoterpene	α-pinene*	NIST and Wiley
8	monoterpene	camphene*	NIST and Wiley
8.5	monoterpene	β-pinene*	NIST and Wiley
8.75	monoterpene	β-myrcene*	NIST and Wiley
9.37	monoterpene	limonene*	NIST and Wiley
13.38	monoterpene	bornyl acetate*	NIST and Wiley
14.66	sesquiterpene	α-copanene*#	Wiley
15.29	sesquiterpene	trans-caryophyllene*	Wiley
15.73	sesquiterpene	α-humulene*	Wiley
16.08	sesquiterpene	germacrene D	Wiley
16.25	sesquiterpene	α-muurolene	Wiley
22.02	diterpenes	labdene isomer	NIST and Wiley
23.01	diterpenes	labdadiene isomer	Wiley

* confirmed by standard
# compound present in only trace amounts and therefore it is omitted from analyses

Table **3.2:** Retention time and compound class of compounds from DCM extracts of Austrian pine phloem.

	all monoterpenes	all sesquiterpenes	all diterpenes*	all phenolics
fertility				
R				
P	ns	ns	ns	ns
N				
fungal lesion length				
R				-0.382
P	ns	ns	ns	0.054
N				52
all monoterpenes				
R		0.339	0.397	-0.396
P	---	0.020	0.009	0.005
N		47	42	48
all phenolics				
R	-0.396	-0.395		
P	0.005	0.006	ns	---
N	48	47		

ns = non-significant

* excludes resin acids peak from HPLC, with resin acids peak included all correlations were non-significant

Table **3.3:** Pearson correlations between fertilizer level, fungal lesion size, and compound classes.

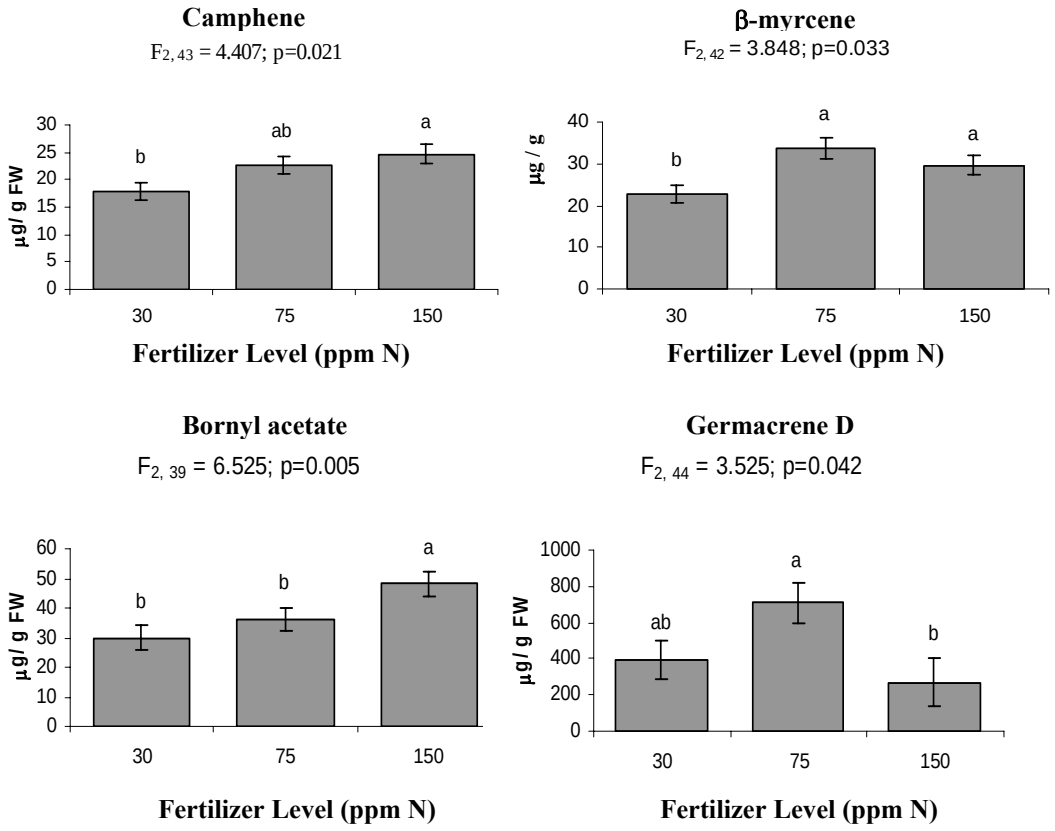


Figure **3.1:** Fertility level effects on individual terpene compounds. Mean concentrations for terpenes for which univariate ANOVAs indicated significant differences, with letters indicating mean separations by LSD tests ($P < 0.05$) and bars representing standard errors.

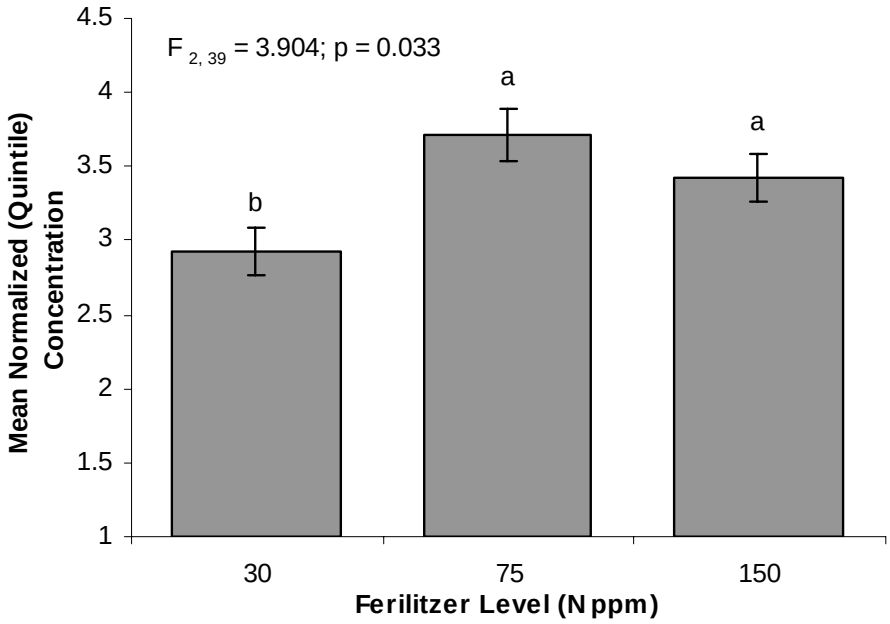


Figure **3.2:** Mean concentration quintiles of total monoterpene concentrations for each fertility level. Letters indicate separations by LSD ($P < 0.05$) and bars representing standard errors.

CHAPTER 4

PATHOGEN-INDUCED TERPENE MODULATION IN AUSTRIAN PINE (*PINUS NIGRA*) AND IMPLICATIONS ON DISEASE RESISTANCE

Introduction

Conifers, which are under constant threat of pathogen and insect attacks, have evolved complex constitutive and inducible defenses to thwart their many pests (Franceschi et al. 2005). Terpenes play crucial roles in both of these types of defenses.

The oleoresin producing structures are perhaps the most elaborate constitutive defense mechanisms that conifers possess. Oleoresin, which consists mainly of monoterpenes and diterpenes, is produced within specialized resin cells and ducts embedded within stem tissue (Franceschi et al. 2005; Keeling and Bohlmann 2006). The major function of oleoresin is to quickly seal wounds and prevent the successful entry of invaders (Keeling and Bohlmann 2006; Langenheim 1994; Wu and Hu 1997).

Terpene production is also extremely important in induced and acquired defense responses. Following pathogen infection, both the localized induction of terpenoid production (e.g. Cheniclet 1987; Hudgins et al. 2005; Klepzig et al. 1995; Krekling et al. 2004; Viiri et al. 2001), and formation of traumatic resin ducts (TRDs) occurs (Cheniclet 1987; Hudgins et al. 2005; Keeling and Bohlmann 2006; Klepzig et al. 1995; Krekling et al. 2004). As a result, a much greater quantity of oleoresin is produced which could aid in

preventing secondary infections (Keeling and Bohlmann 2006). It is also thought that the oleoresin produced within TRDs undergoes composition changes that result in it gaining more toxicity to pathogens and insect pests (e.g. Nagy et al. 2000).

In addition to localized induction, pathogen infection was observed to increase terpene and TRD production systemically (away from the infection site) (Christiansen et al. 1999; Krekling et al. 2004; Krokene et al. 2003; Luchi et al. 2005; Nagy et al. 2000)

It may be hypothesized that terpenes are crucial components in an effective defense against fungal infection. Not only are they induced local and systemically (e.g. Krekling et al. 2004; Krokene et al. 2003; Viiri et al. 2001), but they also have been shown to be toxic to fungal pathogens *in vitro* (e.g. Blodgett and Stanosz 1997a; Jurc et al. 1999; Klepzig et al. 1996; Kopper et al. 2005). These facts, when taken together, suggest that terpenes are required for the formation of local induced resistance (LIR) and systemic induced resistance (SIR) to pathogens.

Diplodia pinea and *D. scrobiculata* were once considered different morphotypes of the same species, *Sphaeropsis sapinea*, but were recently separated based on genetic analyses (de Wet et al. 2003). They differ in their aggressiveness, with *D. pinea* being much more aggressive on Austrian, jack, and red pine (Blodgett and Stanosz 1997b; Blodgett and Bonello 2003). One author notes that *D. scrobiculata* might be considered a relatively harmless endophyte, whereas *D. pinea* can be a severe pathogen (de Wet et al. 2003). It has been hypothesized that the difference in aggressiveness between these two species may be due to differential triggering of defense responses in their common pine hosts, with *D. scrobiculata* limited by a stronger response (Blodgett et al. 2007).

The main objective of this study was to examine how *D. pinea* and *D. scrobiculata* infection of Austrian pine modulates the accumulation of different terpenes and terpene classes both adjacent to infected tissues and systemically throughout the tree. We hypothesize that the two pathogens might trigger different levels of terpene production, with *D. scrobiculata* infection producing greater levels of terpenes than infection by *D. pinea*. We also examined if oleoresin from infected trees became more toxic to *Diplodia* spp., which could account, at least partially, for the expression of SIR (Blodgett et al. 2007).

Materials and Methods

Experimental Design

This study was conducted in the greenhouse on three-year-old potted Austrian pine trees for the phloem experiments, or five-year old potted Austrian pine trees for the oleoresin/xylem experiments. The trees were grown in organic mix with 60% pine bark mulch, 35% peat moss, and 5% hardwood mulch. Trees were watered to field capacity twice daily. The maximum received natural light was 200 W m^{-2}. All experiments occurred between May and August, from 2005 to 2007. Trees were randomly assigned induction treatments as part of a replicated, completely randomized experimental design. The experiments were replicated at different times of the year, and therefore can be considered blocked by time to account for variations in phenology and environment within the greenhouse.

Each experiment involved randomly assigning 5 trees per treatment to each of the four following "induction" treatments (20 trees per experiment): non-wounded control; wounded-only control, and either *D. scrobiculata* or *D. pinea* inoculation. For each induction treatment, except the non-wounded control, a 12 mm wound was made in the stem 5 cm from the soil line. Either a 10 mm diameter sterile potato dextrose agar plug was placed into the wound and secured with duct tape (for the wounded-only control), or a *D. scrobiculata* or *D. pinea* colonized plug was placed, colonized side down, into the wound and secured with duct tape (for the pathogen induction treatments).

Phloem Terpenoid Analysis and Challenge Inoculations

The induction treatments (non-wounded, mock-inoculation, *D. s.* inoculation, or *D. p.* inoculation) were applied as described above in two experiments conducted in May and July 2007. Twenty-one days after inoculation, two 12 mm diameter phloem plugs were removed from all the trees both within 2 cm of the initial treatment site (or equivalent) (the "local" sample), as well as 30 cm from that site (the "distal" sample). In one of the distal wounds, a *D. pinea* colonized agar plug, 10 mm in diameter, was placed as a "challenge" inoculation to test resistance (Blodgett et al. 2007). Ten days later, two more 12 mm diameter phloem plugs (the "challenge" samples) were removed from the reaction zone of the challenge inoculation. A razor blade was then utilized to remove the outer bark around the challenge site of each tree, and the length of the lesion was

measured using a ruler. All plugs were immediately flash-frozen in liquid N_2, and stored at -20^{o} C until further processing. The phloem plugs were processed for phloem terpenoid analysis as described in Chapter 2, pages 44.

Oleoresin and Xylem Terpenoid Acquisition

Trees were treated as described above in three separate experiments conducted in May, July, and August 2005. After 21 days, cork borers with a 17 mm diameter were utilized to create wounds both within 5 cm of the initial treatment site (or equivalent location from soil line for non-wounded trees) (the "local" sample), and 30 cm above that initial treatment site (or equivalent) (the "distal" sample). To these wounds a pre-weighed, labeled 15 mL centrifuge tube was attached using duct tape, and oleoresin (pitch) was allowed to flow into the tubes by gravity for 24 hours. The tubes were then centrifuged at 2,500 X g in a tabletop centrifuge, and the oleoresin weight and approximate volume were recorded. The oleoresin was then diluted 1:10 (w/v) in dichloromethane (Fisher) which contained an internal standard of 0.1% (v/v) p-cymene (Sigma). These solutions were then stored at -20^{o} C until analyzed by gas chromatography (GC) or utilized in the resin plate bioassays described below.

One day after resin collection, a 10 cm segment of stem was obtained 50 to 60 cm from the inoculation sites and debarked. These xylem segments were quartered longitudinally using a wedge, weighed, and each quarter was placed into a 50 mL centrifuge tube. Tissue was vacuum-infiltrated while submerged in dichloromethane with the p-cymene internal standard for 1 hour, removed and placed into new centrifuge tubes,

and centrifuged at 2,500 X g for 15 min to collect the infiltrated solution. The volumes of the collected dichloromethane extracts were recorded, and the extracts were stored at -20^{o} C until analyzed by GC.

Gas Chromatography (GC) Analysis of Terpenes

GC analysis and the identification of terpenoid compounds was conducted according to the procedures described in Chapter 2, page 44. Due to expense and technical limitations, it was not possible to carry out stereoisomer analysis, thus all results refer to individual compounds that were not separated into their isomers.

Pathogen Bioassays

To test the hypothesis that oleoresin from pathogen induced trees might have the ability to inhibit *Diplodia* spp. growth greater than oleoresin collected from uninfected trees we conducted agar plate assays using oleoresin collected from each of the induced trees. In order to ensure sufficient replication, two additional oleoresin collections were made in July and August 2006 for the fungal growth bioassay, in addition to the oleoresin samples collected in 2005. For each experiment, the collected oleoresin was pooled together by induction treatment (non-wounded, mock-inoculated, *D. s.* inoculated, and *D. p.* inoculated) per each experimental replication. The pooled samples were diluted 1:10 (v/v) in dichloromethane. 0.5 mL of diluted pooled sample was pipetted and spread onto each of 10 PDA agar plates (15 cm diameter) for ~1.7 mg / cm^2 of oleoresin per plate surface area, with ~ 300 mg of oleoresin used per plate. Negative controls were prepared

whereby only dichloromethane was spread onto 10 plates, or the plates were non-treated. The spread solutions were allowed to evaporate for at least 4 h to remove the dichloromethane. A sheet of autoclaved dialysis membrane was then applied to each plate to prevent the fungi from penetrating the agar, while allowing for the exchange of nutrients and test compounds.

Five plates were inoculated with *D. pinea* and five plates were inoculated with *D. scrobiculata* by placing a 3 mm colonized agar plug into the center of the plate.

Five days later, photos were taken with a digital camera to enable measurement of colony area. Areas were recorded using the ASSESS software from APS Press (St. Paul, MN). Fungal colonies were then peeled off the dialysis membrane and the fresh weights were recorded. Samples were dried at 40^{o} C overnight, and re-weighed to determine dry weight.

Statistical Analysis

All statistic analyses were performed utilizing SPSS version 15.0 (SPSS Institute, Chicago, IL). In order to analyze the effect of induction treatments on the entire class of monoterpenes, data from individual monoterpenes were quartile ranked transformed (organized 1 to 4 with 4 being equivalent to the highest 25% of the data) by trials using the SPSS version 15.0 RANK function. Means derived from averaging the transformed ranks of each monoterpene were then calculated for every sample. Analysis on this pooled measure was used to determine whether monoterpenes as a whole were modulated by the induction treatments, as performed in Chapter 2, page 44.

Appropriate outlier removal was performed on data before analyses using the EXPLORE feature in SPSS. Significant differences ($P < 0.05$) between the terpene concentrations induced by the different treatments, as well differences in the fungal growth measurements in the bioassays, were determined using univariate ANOVAs with treatment, experiment, and their interactions as main factors. When normality assumptions were not met, data were quartile rank transformed prior to running univariate ANOVA. Mean separation tests were performed using LSD tests, with $\alpha = 0.05$. Equivalent non-parametric tests (Kruskal-Wallis and median tests, followed by Mann-Whitney U tests) were performed on untransformed data when rank-transformation failed to satisfy ANOVA assumptions.

Correlations between terpene compounds and lesion length were calculated using Spearman's correlations. For correlation analysis, α was set to 0.100 in order to account for between-tree variation and the low amount of replicates utilized within this study.

Results

Induction of Monoterpenes within the Phloem around the Pathogen Infection

Around the induction sites, only five monoterpenes (α–pinene, camphene, β–pinene, myrcene, and limonene) accumulated to levels sufficient for quantification. ANOVA assumptions were not met, but significant interactions occurred between induction treatment and experimental trial. This was overcome by ranking data into

quartiles for each experiment separately (May and July), after which ANOVA assumptions were met and no significant treatment X trial interactions were found. Significant increases in the levels of α-pinene ($F_{3, 32} = 12.388$, $P < 0.001$), camphene ($F_{3, 31} = 10.170$, $P < 0.001$), myrcene ($F_{3, 30} = 4.084$, $P = 0.018$), limonene ($F_{3, 33} = 5.693$, $P = 0.004$), and pooled monoterpenes ($F_{3, 33} = 8.087$, $P = 0.001$) were observed in *Diplodia* infected trees relative to controls (Fig. 4.1). These increases were up to 10-fold depending on treatment and trial. No significant differences were observed between *D. pinea* and *D. scrobiculata*, or between the wounded and non-wounded controls.

Modulation of Terpenoid Concentrations within the Distal Phloem

Six monoterpenes (α–pinene, camphene, β–pinene, myrcene, limonene, bornyl acetate) and two sesquiterpenes (trans-caryophyllene and germacrene D) were present in sufficient amounts for analysis in phloem samples taken 30 cm from the inoculation sites. The sesquiterpene levels did not differ between any of the induction treatments. A significant accumulation of limonene was observed in *D. scrobiculata* infected trees over non-wounded and *D. pinea* infected trees ($F_{3, 33} = 3.715$, $P = 0.024$) (Fig. 4.2a). Bornyl acetate ($F_{3, 20} = 4.020$, $P = 0.032$) was also present in significantly ($P < 0.05$) higher levels in *D. s.* infected trees compared with *D. p.* infected trees (Fig. 4.2a). No other significant differences in levels were found for any of the monoterpenes.

Pooling the monoterpenes revealed a significant difference, albeit not in the F-value ($F_{3, 34} = 1.825$, $P = 0.166$), in the phloem levels between *D. scrobiculata* and *D.*

pinea infected trees ($P = 0.036$), but no significant differences were observed between uninfected and infected trees (Fig. 4.2b).

Modulations of Monoterpene Concentrations in the Xylem

In the xylem taken 50 cm from the induction sites, a significant interaction occurred between trial and treatment for each of the 6 monoterpenes quantified (α-pinene, camphene, β-pinene, myrcene, limonene, and bornyl acetate). This interaction was characterized by significant decreases in α-pinene ($F_{3, 19} = 4.487$, $P = 0.002$), camphene ($F_{3, 19} = 4.768$, $P = 0.015$), myrcene ($F_{3, 17} = 5.885$, $P = 0.008$), bornyl acetate ($F_{3, 17} = 4.274$, $P = 0.024$), and the pooled monoterpenes ($F_{3, 19} = 3.490$, $P = 0.004$) in both *D. s.* and *D. p.* inoculated trees compared to non-wounded controls trees in the July 2005 experiment. However, no significant effects of induction treatment occurred in the August and October 2005 trials (Fig. 4.3 for pooled monoterpenes).

Induction of Terpenes around Challenge Inoculation Site

In phloem taken around the challenge inoculation site, only limonene increased significantly ($F_{3, 33} = 3.913$, $P = 0.020$) in response to previous pathogen induction over the mock inoculation controls. No other treatment or trial effects were found for the other five monoterpenes (α–pinene, camphene, β–pinene, myrcene, and bornyl acetate), two sesquiterpenes (trans-caryophyllene and germacrene D), or pooled monoterpenes quantified (data not shown).

Induction of Monoterpenes within Exuded Oleoresin around the Induction Site

Five monoterpenes (α–pinene, camphene, β–pinene, myrcene, and limonene) and two sesquiterpenes (trans-caryophyllene and germacrene D) were analyzed in exuded resin collected near the infection site. Pathogen inoculation significantly increased the level of β–pinene ($F_{3, 38} = 7.532$, $P = 0.001$). For α–pinene ($F_{3, 33} = 2.704$, $P = 0.065$), camphene ($F_{3, 38} = 2.839$, $P = 0.056$), myrcene ($F_{3, 37} = 2.832$, $P = 0.056$), and the pooled monoterpenes ($F_{3, 38} = 2.245$, $P = 0.105$) there were significant increases due to pathogen inoculation according to LSD tests (Fig. 4.4) (although the F-values of the ANOVAs were non-significant). The pattern of induction in the exuded resin was similar to that of the terpenes within the phloem at that location, despite being from separate experiments.

Modulations of Terpenes within Oleoresin Collected Distally

Five monoterpenes (α–pinene, camphene, β–pinene, myrcene, and limonene) and two sesquiterpenes (trans-caryophyllene and germacrene D) were quantified in the oleoresin collected 30 cm from the site of the induction treatments. Sesquiterpene levels were unaffected by induction treatment, and showed no significant interactions of experiment with induction treatment. There were significant interactions between trial and induction treatment for all of the monoterpenes. No significant effects of induction treatment occurred in the July 2005 or October 2005 experiments. Significantly greater oleoresin levels of α-pinene ($F_{3, 15} = 4.210$, $P = 0.030$) and camphene ($F_{3, 15} = 3.972$, $P = 0.035$) occurred in *D. scrobiculata* infected trees compared to all the other induction treatments in the August 2005 trial. β-pinene ($F_{3, 15} = 4.802$, $P = 0.020$) also increased in

D. scrobiculata infected trees over the controls in August 2005. Likewise, the compounds myrcene ($F_{3, 15} = 5.784$, $P = 0.011$) and limonene ($F_{3, 15} = 3.239$, $P = 0.060$) were in higher levels within the oleoresin from both *D. scrobiculata* and *D. pinea* infected trees when compared to the non-wounded controls in the August 2005 trial (although the overall F-values were non-significant). There was also a significant overall pooled monoterpene concentration increase in *D. scrobiculata* infected trees compared to the controls, although the F-values were non-significant ($F_{3, 15} = 3.013$, $P = 0.072$) (Fig. 4.5).

Induction of SIR

A significant decrease ($F_{3, 16} = 4.254$, $P = 0.027$) in lesion length was observed in trees infected with *D. pinea* (lesion length of 28.50 +/- 2.630 mm) compared to the non-wounded control (57.00 +/- 7.342 mm), implying that the phenomenon of SIR occurred, but no means separation was observed between *D. scrobiculata* (38.80 +/- 5.553 mm) inoculated trees and the controls (including the mock control (36.67 +/- 6.360 mm)).

Correlations between monoterpenes in the phloem and challenge lesion length

The only correlations between distal level of individual terpenes and challenge lesion length were a positive correlation between myrcene and lesion length and a negative correlation between trans-caryophyllene and lesion length (Table 4.1). Furthermore, 14 days post-challenge, negative correlations were observed between lesion

length and concentrations of α-pinene, camphene, myrcene, trans-caryophyllene, and the sum of monoterpenes around the challenge inoculation (Table 4.1). This suggests that these monoterpenes are associated with local resistance to *D. pinea* (Blodgett et al. 2007).

Pathogen Growth on Exuded Oleoresin

D. pinea growth was significantly inhibited by oleoresin taken from the infection area (fresh weights $F_{5, 88} = 3.227$, $P = 0.011$; dry weights $F_{5, 89} = 5.391$, $P < 0.001$; colony areas $F_{5, 86} = 11.336$, $P < 0.001$) and away from the induction area (wet weights $F_{5, 91} = 13.186$, $P < 0.001$; dry weights $F_{5, 91} = 11.590$, $P < 0.001$; colony areas $F_{5, 90} = 30.151$, $P < 0.001$) compared to no resin controls, but no significant differences were found between induction treatments (Fig. 4.6), with the exception that distal oleoresin from *D. scrobiculata* infected trees reduced *D. pinea* colony growth less than oleoresin from *D. pinea* infected, mock-inoculated, or non-wounded trees (Fig. 4.6).

D. scrobiculata growth was also inhibited in general by oleoresin, regardless of being taken from near the induction site (wet weights $F_{5, 73} = 7.908$, $P < 0.001$; dry weights $F_{5, 73} = 5.054$, $P = 0.001$; colony areas $F_{5, 67} = 3.459$, $P = 0.009$) or distally (wet weights $F_{5, 63} = 8.535$, $P < 0.001$; dry weights $F_{5, 63} = 3.320$, $P = 0.012$; colony areas $F_{5, 62} = 7.371$, $P < 0.001$) (Fig. 4.7). Local resin from mock-inoculated trees inhibited growth more than resin taken from *D. scrobiculata* or *D. pinea* inoculated trees. Likewise, resin from *D. scrobiculata* inoculated trees collected distally inhibited *D. scrobiculata* growth more than resin taken from *D. pinea* infected trees (especially in relation to fresh weights) (Fig. 4.7).

Discussion

In general, monoterpene levels were more affected by pathogen infection than levels of sesquiterpenes. This suggests that the non-mevalonate terpenoid synthesis pathway is more involved in induced defense responses (as it leads to the production of monoterpenes) than the mevalonate pathway (which produces sesquiterpenes). Pathogen inoculation consistently induced accumulation of monoterpenes in the phloem near the infection site. However, monoterpene induction was more variable at the distal location. *D. scrobiculata* infection increased the production of particular monoterpenes to greater levels in distal phloem than inoculation with *D. pinea*. In distal xylem, infection by either pathogen reduced monoterpene concentrations in one trial, but this was not replicated in the other trials. Post-challenge inoculation, terpene levels around the challenge infection were similar despite prior induction treatment, suggesting that prior fungal infection did not influence terpene accumulation around a subsequent infection. Pathogen infection also led to increases in monoterpene content in local oleoresin. However, in distal oleoresin pathogen infection induced monoterpene production in only one trial. Fungal bioassays with oleoresin obtained under different induction treatments revealed that oleoresin reduces pathogen growth in general, but induction treatments did not seem to enhance the inhibitory capacity of the resin.

Increased accumulation of the monoterpenes α-pinene, camphene, β-pinene, myrcene, and limonene occurred around the initially infected tissues in both the *D. scrobiculata* and *D. pinea* infected trees (Fig. 4.1).

Accumulation of these monoterpenes around an infection occurs in other conifers (e.g. Klepzig et al. 1995; Viiri et al. 2001), and monoterpenes are known to have

inhibitory activity against *Diplodia* spp. *in vitro* (Blodgett and Stanosz 1997a; Jurc et al. 1999). This suggests that increased monoterpene accumulation around an infection site acts to inhibit the growth of the pathogen. It may be possible that infection-localized terpene production results in the phloem becoming more protected against opportunistic pests (e.g. those that could utilize the infection-destroyed tissues to gain entry into the pine host).

The accumulation of terpenes did not differ between the pathogens, suggesting that the mechanism by which *D. scrobiculata* is less aggressive than *D. pinea* does not involve the triggering of greater accumulation of host terpenes by *D. scrobiculata* beyond levels triggered by *D. pinea*.

Monoterpene levels increased within exuded oleoresin collected around the *D. scrobiculata* or *D. pinea* infections, compared with the controls (Fig. 4.4). However, greater monoterpene levels in the oleoresin taken near infections were generally not sufficient to reduce the *in vitro* growth of *D. pinea* or *D. scrobiculata* (Figures 4.6 and 4.7), although the growth of *D. scrobiculata* was significantly reduced by oleoresin from around mock-inoculation wounds than any of the other induction treatments. This latter finding suggests that wounding led to composition changes of oleoresin (although not in the levels of monoterpenes) that limited the growth of *D. scrobiculata*; perhaps because this less aggressive pathogen is less tolerant of composition changes than the more aggressive sister pathogen *D. pinea*. The observation that oleoresin collected around infections by either pathogen did not affect *D. scrobiculata* growth but oleoresin from a wound site did affect growth suggests that both pathogens might interfere with the host's ability to modify the composition of its oleoresin to make it more toxic to pathogens.

In phloem 30 cm from the inoculation sites, neither pathogen modulated terpene levels significantly when compared with the controls. However, phloem levels of myrcene, limonene, bornyl acetate, and overall monoterpenes were at greater levels in trees infected with *D. scrobiculata* than in trees infected with *D. pinea* (Fig. 4.2). Thus, *D. scrobiculata* and *D. pinea* had contrasting effects on their host in terms of monoterpenes accumulating systemically in stem phloem. Perhaps infection by aggressive *D. pinea* destroyed enough phloem tissue at the stem base to interfere with systemic resource allocation to secondary metabolism by the time measurements were made.

Oleoresin collected 30 cm from the inoculation treatments yielded different results depending on the experimental replicate. In July, no modulations were observed between any of the induction treatments (Figure 4.5). In August, *D. scrobiculata* infected trees significantly accumulated greater levels of terpenes than either of the controls (Figure 4.5). Likewise, *D. pinea* infection also led to a slight increase in terpene levels, albeit non-significantly (Figure 4.5). This suggests that trees in August respond differently to infection than those July, perhaps because of different physiological states at the different times of the year. In October the only modulations observed were reduced levels of monoterpenes in trees infected by *D. pinea* when compared with mock-inoculated trees. This supports the hypothesis that *D. pinea* might have inhibited the host's capacity to produce terpenes. It is possible that trees in October had already undergone senescence processes that reduced net assimilation rates and, when coupled with damage from *D. pinea* infection, a reduction in resource availability for terpene production occurred. These findings highlight the need to account for seasonal effects

when examining induced defense responses in pines as well as other trees (Zou and Cates 1998).

The oleoresin collected 30 cm from each of the induction treatments reduced *in vitro* growth of both pathogens when compared to non-resin controls (Figures 4.6 and 4.7). *D. pinea* infection appeared to make oleoresin more suitable for *D. scrobiculata* growth *in vitro,* and *D. scrobiculata* infection made oleoresin more suitable for *D. pinea* growth. Since these effects were not associated with modulation of monoterpene composition we hypothesize that changes in diterpenes (and perhaps phenolics) in the oleoresin might have resulted in this phenomenon.

In xylem 30 cm from the infection, monoterpene levels were significantly reduced in *D. pinea* infected trees in the July experiment only (Figure 4.3). This further suggests *D. pinea* infection reduces the ability of the trees to produce monoterpenes systemically. However, this finding was not observed in the August experiment.

It has been hypothesized that SIR is based on enhanced production of anti-fungal compounds (Blodgett et al. 2007; Wang et al. 2006). In other words, the tissues in systemically induced trees would be "primed" to unleash a stronger response to repel the second invader. This study found that terpenoid production around the challenge lesions was consistent, regardless of previous infection status. The only effect that prior induction had on monoterpene concentrations around the challenge lesion was an increase in limonene accumulation. Other than this observation, it appeared that the tissues around the challenge inoculations produced a similar terpenoid defense response regardless of prior induction, at least in the 14 day timeframe used in this study. It is

possible that any “priming” effects might occur in a much shorter interval than the one used in this study, such as those proposed by Wang et al. (2006).

Bonello and Blodgett (2003) hypothesized that if particular compounds are involved in SIR, their concentrations should be negatively correlated with lesion length. The levels of most terpenes present in the phloem at the challenge site prior to challenge were not correlated with the resulting challenge lesion length, with the exception of myrcene, which was positively correlated, and trans-caryophyllene, which was negatively correlated (Table 4.1). Because the treatments did not have any measurable effects on distal phloem terpenoids (Fig. 4.2), it could be surmised that phloem terpenes are not involved in constitutive or systemic induced resistance to *Diplodia* attack, with the exception of trans-caryophyllene. However, monoterpene and trans-caryophyllene levels in tissues around the challenge lesion, 2 weeks post-inoculation, were negatively correlated with lesion length (i.e. positively correlated with resistance (Blodgett et al. 2007)) (Table 4.1). This suggests a potential role of terpenes in local induced resistance. Austrian pine appears to produce and accumulate phloem terpenes at levels high enough to presumably combat pathogens only around infected tissues and not throughout the tree. Significant phloem terpene production was only associated with LIR, and LIR-associated terpene responses were not affected by previous infections elsewhere in the plant.

In summary, pathogens induced monoterpene production in Austrian pine around the site of infection. It remains unclear if systemic induction of terpenoids by pathogens can occur, because different pathogens, seasonal conditions, and cohorts of trees might influence the systemic modulation of terpenoids. Exuded oleoresin was found to limit pathogen growth, but local and systemic modifications of exuded resin that would make

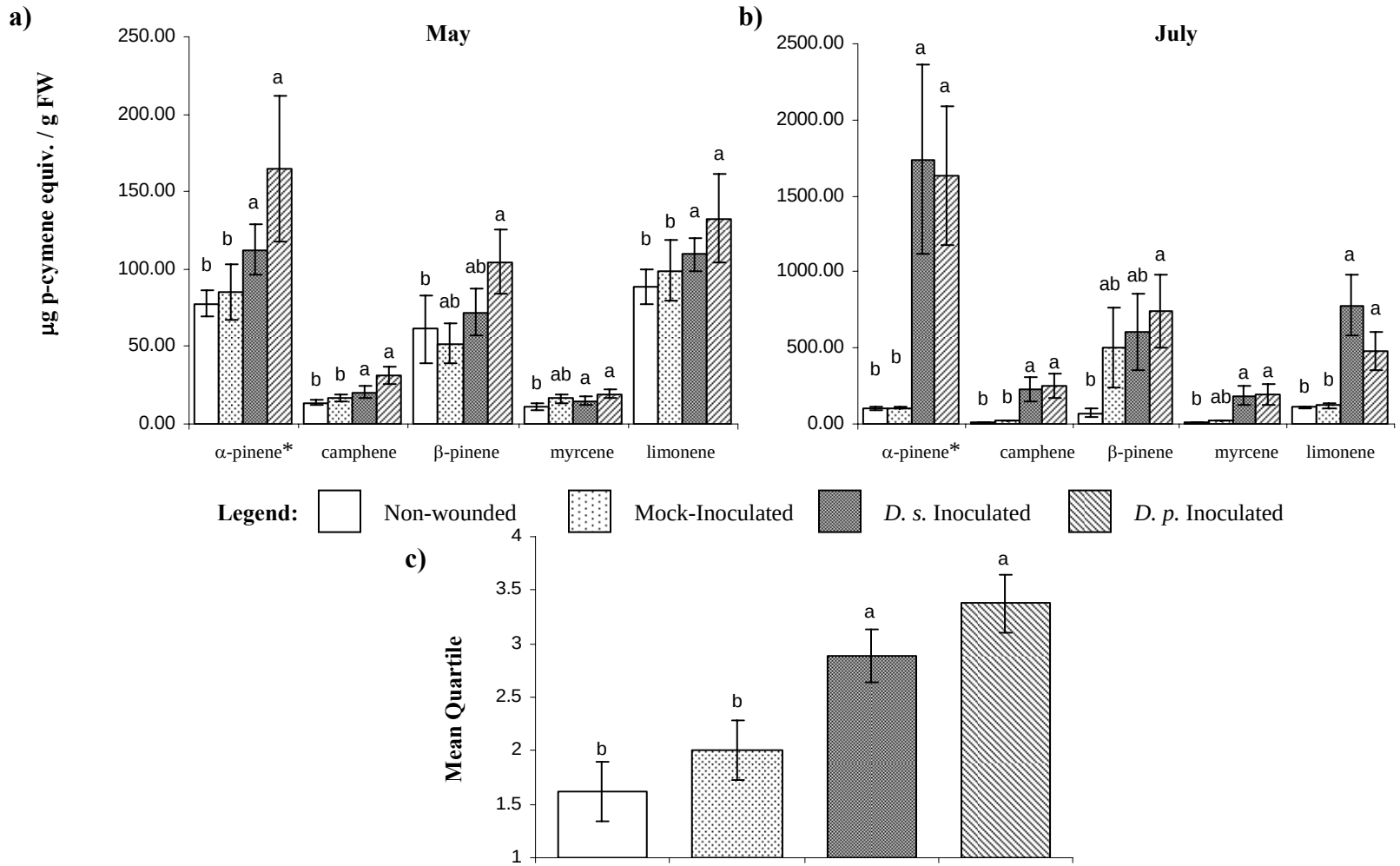
it more anti-fungal were generally not observed. Finally, conifers appear to have mechanisms that manage terpenoid production in the most cost-effective way: phloem terpenes are produced in pathogen-resisting quantities only around active infections.

		pooled monoterpenes	α-pinene	camphene	β-pinene	myrcene	limonene	bornyl acetate	trans-caryophyllene	germacrene D
distal to induction site	ρ	0.362	0.187	0.232	0.158	**0.514**	0.189	0.456	**-0.602**	-0.385
	P	0.154	0.473	0.370	0.560	**0.035**	0.467	0.185	**0.066**	0.217
	n	17	17	17	16	**17**	17	10	**10**	12
challenge area site	ρ	**-0.453**	**-0.511**	**-0.424**	-0.231	**-0.448**	-0.381	-0.405	**-0.585**	0.335
	P	**0.068**	**0.036**	**0.090**	0.389	**0.071**	0.145	0.320	**0.075**	0.242
	n	**17**	**17**	**17**	16	**17**	16	8	**10**	14

Table **4.1:** Spearman correlations between lesion length and pooled or individual terpenoid concentration in phloem samples taken from two different locations. Bold text indicates a significant correlation.

Figure **4.1:** Mean monoterpene concentrations in phloem within 5 cm of inoculation sites or equivalent positions in a) May 2007 and b) July 2007. c) Pooled means of all monoterpenes (normalized by quartile ranks) across both experiments. Bars represent standard error, and letters indicate significant differences by LSD analysis ($P < 0.05$) for each variable. * The actual concentrations of α-pinene are 10 fold greater than the values used here.

Figure 4.1


a)
May
b)
July
µg p-cymene equiv. / g FW
250.00
200.00
150.00
100.00
50.00
0.00
2500.00
2000.00
1500.00
1000.00
500.00
α-pinene*
camphene
β-pinene
myrcene
limonene
Legend:
Non-wounded
Mock-Inoculated
D. s. Inoculated
D. p. Inoculated
c)
Mean Quartile
4
3.5
3
2.5
2
1.5
1

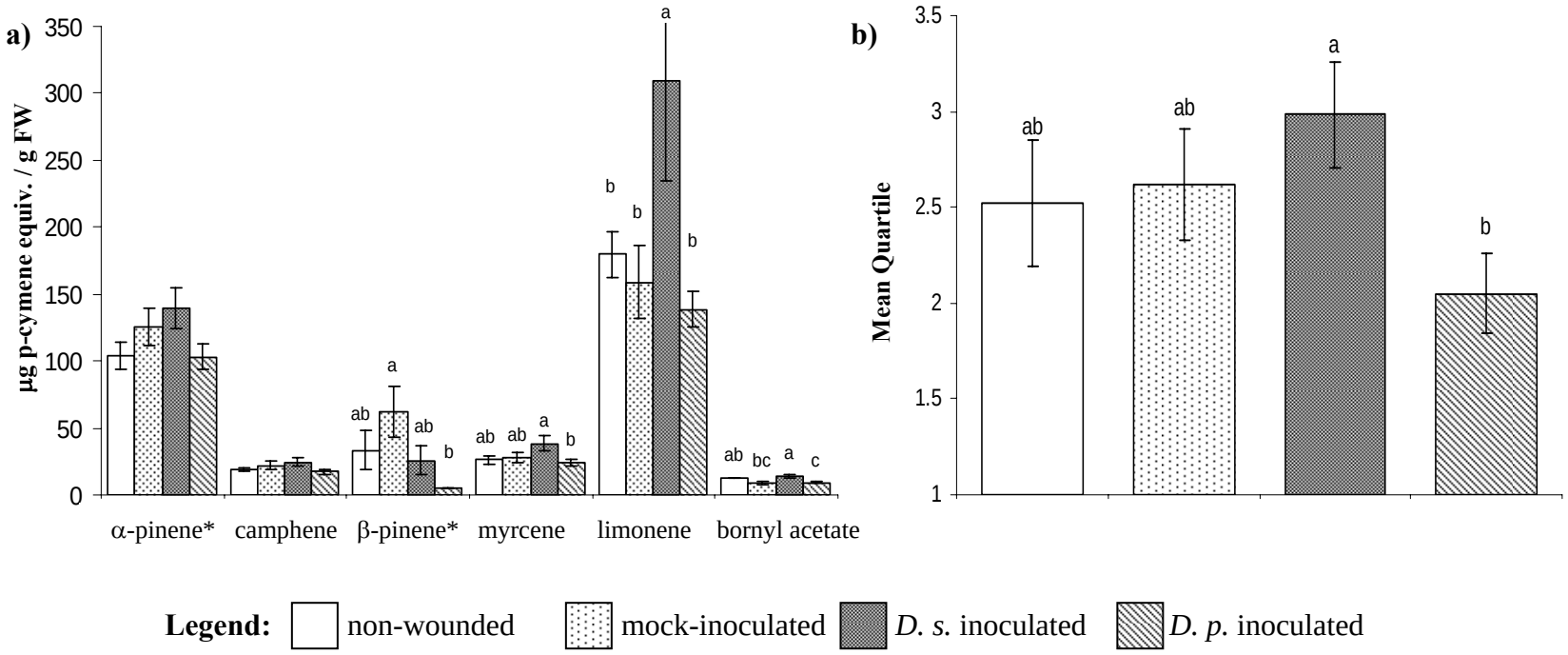


Figure **4.2:** a) Mean monoterpene concentrations in phloem 30 cm from inoculation sites or equivalent positions. b) Pooled means of all monoterpenes (normalized by quartile ranks). Bars represent standard error, and letters indicate significant differences by LSD analysis ($P < 0.05$) for each variable. * The actual concentrations of α-pinene and β-pinene are 10 fold greater than the values used here.

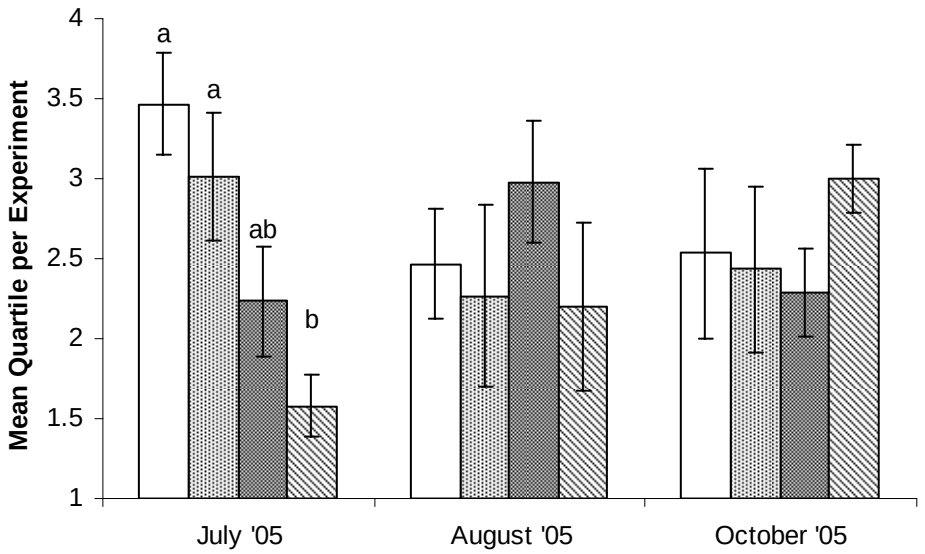


Figure **4.3:** Pooled means of all monoterpenes (normalized by quartile ranks) in xylem 50 cm from inoculation sites or equivalent positions. Bars represent standard error, and letters indicate significant differences by LSD analysis ($P < 0.05$) for each variable.

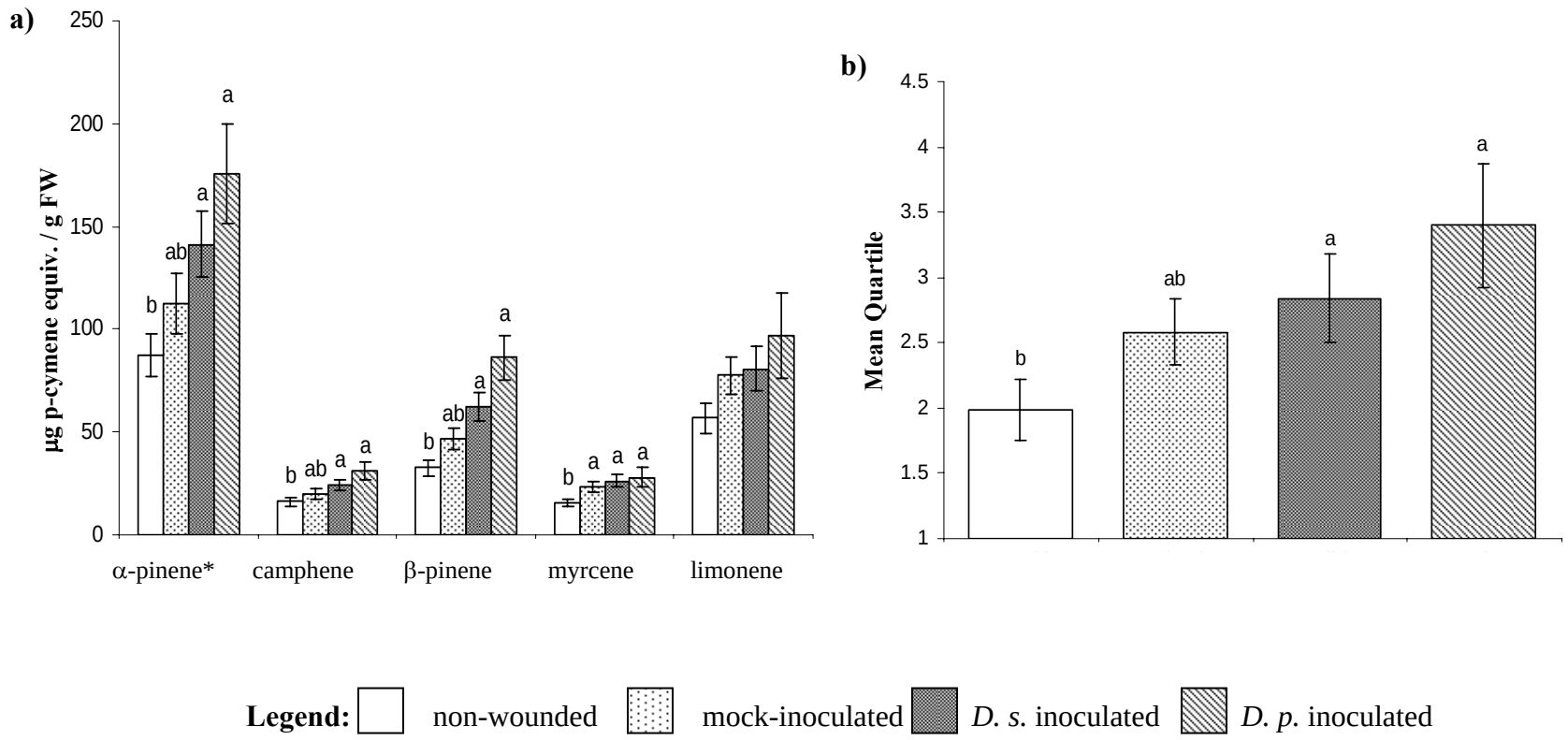


Figure **4.4:** a) Mean monoterpene concentrations in exuded resin taken within 5 cm from inoculation sites or equivalent positions. b) Pooled means of all monoterpenes (normalized by quartile ranks). Bars represent standard error, and letters indicate significant differences by LSD analysis ($P < 0.05$) for each variable. * The actual concentrations of α-pinene are 10 fold greater than the values used here.

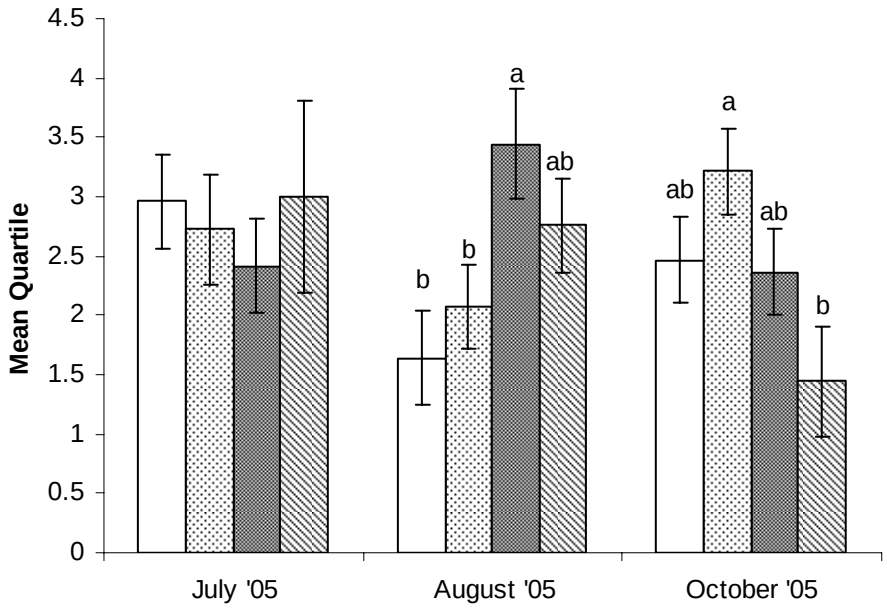


Figure **4.5:** Pooled means of all monoterpenes (normalized by quartile ranks in exuded resin taken 30 cm from inoculation sites or equivalent positions per each experimental trial. Bars represent standard error, and letters indicate significant differences by LSD analysis ($P < 0.05$) for each trial.

Figure **4.6:** Mean *Diplodia pinea* colony growth measurements for colonies grown on exuded resin from 5 cm of the inoculation site (a, b, c) or 25-30 cm from the inoculation site (d, e, f). Bars represent standard error, and letters indicate significant differences due to LSD. DCM = dichloromethane control

Figure 4.6

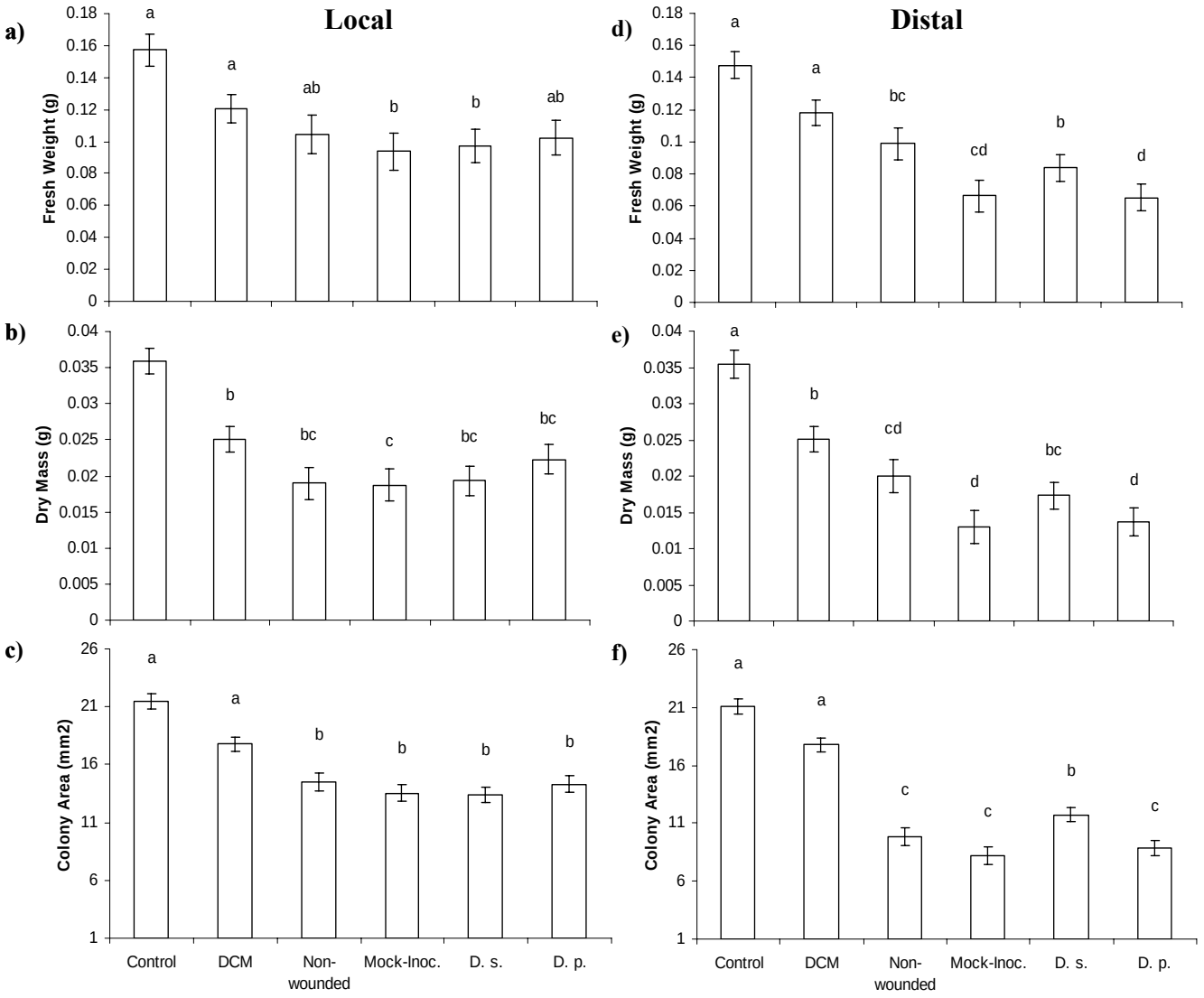
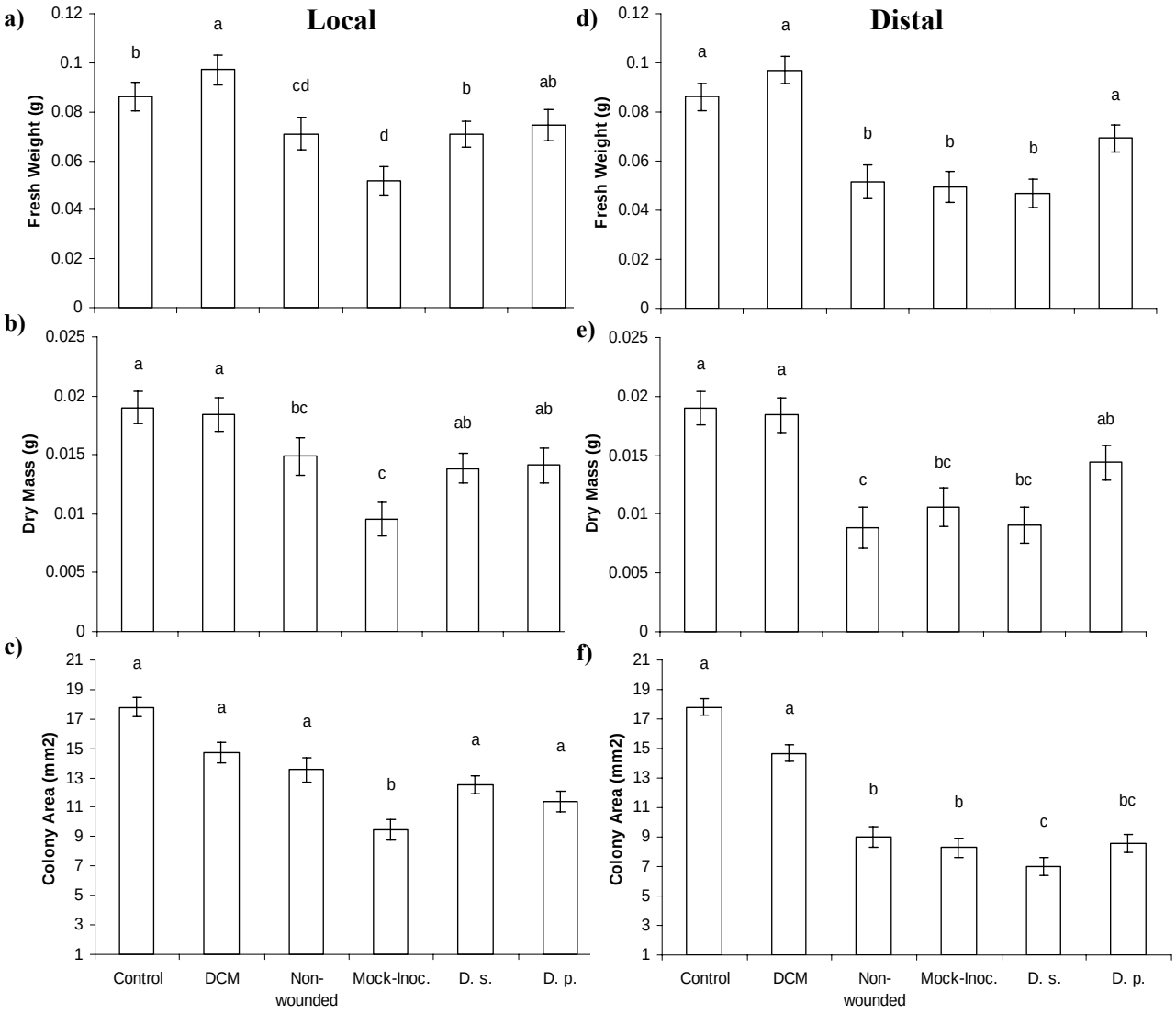

a)
Local
Fresh Weight (g)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
a
a
ab
b
b
ab
b)
Dry Mass (g)
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
a
b
bc
c
bc
bc
c)
Colony Area (mm2)
26
21
16
11
6
1
a
a
b
b
b
b
Control
DCM
Non-wounded
Mock-Inoc.
D. s.
D. p.
d)
Distal
Fresh Weight (g)
a
a
bc
cd
b
d
e)
Dry Mass (g)
a
b
cd
d
bc
d
f)
Colony Area (mm2)
a
a
c
c
b
c
Control
DCM
Non-wounded
Mock-Inoc.
D. s.
D. p.

Figure **4.7:** Mean Diplodia scrobiculata colony growth measurements for colonies grown on exuded resin from 5 cm of the inoculation site (a, b, c) or 30 cm from the inoculation site (d, e, f). Bars represent standard error, and letters indicate significant differences due to LSD. DCM = dichloromethane control

Figure 4.7


a)
Local
d)
Distal
Fresh Weight (g)
0.12
0.1
0.08
0.06
0.04
0.02
0
b
a
cd
d
b
ab
a
a
b
b
b
a
b)
e)
Dry Mass (g)
0.025
0.02
0.015
0.01
0.005
0
a
a
bc
c
ab
ab
a
a
c
bc
bc
ab
c)
f)
Colony Area (mm2)
21
19
17
15
13
11
9
7
5
3
1
a
a
a
b
a
a
a
a
b
b
c
bc
Control
DCM
Non-wounded
Mock-Inoc.
D. s.
D. p.

CHAPTER 5

DIPLODIA SPP. INDUCED PHENOLIC ACCUMULATION IN THE XYLEM OF *PINUS NIGRA*

Introduction

Research aimed at discovering and confirming mechanisms that lead to SIR has only just begun (Bonello et al. 2006). The bulk of research on SIR and its mechanisms (e.g. the systemic induction of phenolics and terpenes) in conifers has focused on stem phloem responses to pathogen infection (e.g. Bonello and Blodgett 2003; Brignolas et al. 1995; Klepzig et al. 1995; Krekling et al. 2004). There is some evidence that SIR is organ dependent (Blodgett et al. 2007), and therefore other tissues and organs besides stem phloem should be examined for potential systemic responses to pathogen infection.

One such tissue that warrants further examination is the xylem. There is evidence that the xylem becomes more resistant to pathogen attack in trees infected by pathogens. The production of traumatic resin ducts (TRDs) within the xylem is known to occur locally and systemically in response to fungal infection (Krekling et al. 2004; Luchi et al. 2005). Within these TRDs, potentially antibiotic-enhanced oleoresin, whose makeup is roughly half monoterpenes and half diterpenes (Keeling and Bohlmann 2006), is stored *en mass*, presumably to quickly be released to protect wounds from secondary pathogen invasion (e.g. Christiansen et al. 1999; Krekling et al. 2004; Krokene et al. 2003; Luchi et

al. 2005). In addition to oleoresin, stilbenes and other antifungal phenolic compounds are present in conifer xylem (Hart 1981; Hart and Shrimpton 1979; Pearce 1996; Shrimpton and Whitney 1968).

Another gap in knowledge is in how conifers propagate defensive responses systemically (Bonello et al. 2006). Mediators of SIR are hypothesized to be chemical in nature, and to perhaps move in both the phloem and the xylem, both acropetally (xylem and phloem) and basipetally (phloem) from the infection (Blodgett et al. 2007; Krokene et al. 2003). Systemic induction of defense responses can be seen one week following a fungal inoculation with some fungi (Bonello and Blodgett 2003).

In herbaceous model systems such as *Arabidopsis*, salicylic acid has long been implicated in mediating an SIR response called systemic acquired resistance (SAR) (Durrant and Dong 2004). However, recent evidence has shown that a lipid-derived molecule, rather than salicylic acid (a phenolic derivative), might be the actual systemic signal (Durrant and Dong 2004). Likewise, another commonly described SIR phenomenon, known as induced systemic resistance (ISR) (van Loon et al. 1998), is thought to be signaled through the action of jasmonates and ethylene (Farmer et al. 2003). To the best of our knowledge, neither salicylates nor jasmonates have been described as being produced endogenously in *Pinus* spp. or other conifers.

However, it is possible that another endogenous hydroxybenzoic acid derivative similar to salicylic acid and/or another linolenic acid derivative similar to jasmonic acid might mediate SIR in conifers. Regarding the former, it was observed that 5-chlorosalicylic acid possibly induced defenses within pine (Reglinski et al. 1998). Exogenously applied methyl jasmonate also has been consistently shown to initiate

induced defenses, including SIR, with quantifiable increases in phenolics, terpenes, and traumatic resin ducts (Erbilgin et al. 2006; Franceschi et al. 2002; Hudgins et al. 2003; Hudgins et al. 2004; Martin et al. 2002; Martin et al. 2003; Zeneli et al. 2006). It was later determined that methyl jasmonate application is linked to ethylene metabolism in its elicitation of systemic defense responses (Hudgins and Franceschi 2004; Hudgins et al. 2006). Knowledge of what mediates the SIR response will greatly enhance our understanding of the regulation of defenses in conifers.

This study tests two hypotheses: 1) chemical changes occur within the xylem as part of the SIR response of *P. nigra* against *D. pinea* and *D. scrobiculata*; and 2) those changes comprise compounds that may be SIR mediators. This study concentrates on the xylem because of its basic function in mostly acropetal, rapid water and nutrient transport, thus providing a natural conduit for systemic movement of any signaling compound.

Materials and Methods

General Experimental Conditions

This experiment was conducted on potted *Pinus nigra* trees grown in the greenhouse. The trees were watered to field capacity twice daily and received a maximum of 200 Wm^{-2} of natural light. Each study was conducted as a completely randomized designed experiment, with experimental replicates acting as blocks in time. Different cohorts of trees were used in each separate experiment.

Analysis of Xylem Phenolics

This experiment was repeated three separate times in May, July, and August 2005. For each trial, four different induction treatments (non-wounded, mock-inoculation, *D. scrobiculata,* and *D. pinea* were applied to 5 randomized five-year-old trees according to the procedure described in Chapter 4, page 88.

Following a three week incubation period, a 10 cm segment of stem was obtained 55 to 65 cm from the soil line and debarked. These segments were quartered longitudinally using an axe, weighed, and each segment was placed in 50 mL centrifuge tube. The segments were then vacuum-infiltrated in HPLC-grade methanol (Fisher) for 30 minutes, removed and allowed to dry for 10 minutes, and then spun down in a new 50 mL tube at no less than 2,500 X g for 10 minutes in a tabletop centrifuge. The eluate volume was recorded and the xylem segments were reweighed. The methanol extracts were transferred to a 2 mL centrifuge tube and stored at -20^{o} C until HPLC analysis, which was performed according to the procedures described in Chapter 2, pages 42-43.

Branch Bioassay to Test for Local Activity of Methanol Extracts

Three experiments were performed, one for each of the three xylem chemistry trials described above. Methanol extracts from each induction treatment were pooled together. A total of 100 μL of each pooled methanol extract were pipetted onto the end of six 8 mm diameter sterile cotton swabs, which were cut to a height of 2 cm. As negative controls, methanol was applied to another 6 swabs and another 6 swabs were left untreated. For positive controls and to test the elicitation ability of SAR and ISR

mediating compounds, 10 mM solution of methyl jasmonate (Sigma, St. Louis, MO), salicylic acid (Sigma), and methyl salicylate were applied to 6 swabs each. Each of these swabs was allowed to dry for at least 1 hour.

Each tree in two sets of six three-year-old trees was randomly assigned to receive four of the eight treatments. On four randomized branches, 10 mm wounds were created 3 cm from the stem. The dried swabs were soaked in 1 mL of distilled water, and immediately placed treatment side down into the wounds and secured with duct tape. After 1 week, ~1 g of tissue was removed from around each swab. Additionally, a non-wounded branch was sampled from each tree.

The tissues were kept at -20^{o} C until they were ground in liquid nitrogen. A total of 100 mg of ground tissue was processed for extraction and quantification of phenolics as described in Chapter 2, pages 42-43.

Testing of Individual Compounds as Systemic Defense Elicitors

A total of three compounds were selected from the xylem extracts to see if they could elicit defense responses: ferulic acid glucoside (Bonello and Blodgett 2003), taxifolin (Sigma), and pinosylvin (Apin Chemicals, Abingdon, UK). These were examined in three separate, distinct experiments.

In the first experiment, solutions representing 1,000 times the concentrations calculated as occurring naturally within the xylem of infected pines (as detected in the xylem in this study, Table 5.1) were prepared in 5% methanol (to aid in their dissolution): approximately 1.06 mg/mL ferulic acid glucoside in 5% methanol, 0.21 mg/mL taxifolin

in 5% methanol, and 0.17 mg/mL pinosylvin in 5% methanol. The controls utilized in this study were: 10 mM solutions of methyl jasmonate, salicylic acid, and methyl salicylate prepared as positive controls; and 5% methanol and sterile water as negative controls. A independent treatment using a concentrated Fast Green FCF (Sigma) dye solution (10% dye w:v in water) was utilized to visualize translocation throughout the tree.

In the second experiment, the ferulic acid glucoside, taxifolin, pinosylvin, and dye solutions were further diluted 1:10 (v/v) in 5% methanol. In the third experiment, taxifolin, pinosylvin, salicylic acid, and methyl salicylate were excluded, but further dilutions of the ferulic acid glucoside solution were made to represent 100X, 10X, and 1X the amounts found in pathogen-induced xylem. The dye in the third experiment was used at the same concentration used in the first experiment.

Each treatment was applied to six randomized three-year-old trees, as follows. A drill with a 3/8 inch bit was used to make three holes per tree, with each hole at a ~30^{o} angle towards the soil. All holes were drilled between 1 and 10 cm from the soil line. An attempt was also made to ensure that each hole passed through the center of the tree.

Approx. 0.6-0.7 mL of each treatment solution was applied per tree, per time-point, for a total of 2 mL. The first experiment had just an initial application, while the second and third experiments had applications repeated at 4, 7, and 14 days (total of 4 applications / tree).

In addition to treatments above, six trees per experiment were inoculated with *Diplodia pinea* as described in the xylem induction experiments. An additional six trees were left unwounded as controls.

One week after the initial treatment application, trees were "challenge" inoculated on the stem with *D. pinea,* 30 cm from the soil line. This was done to quantify SIR (Blodgett et al. 2007). Also at this time, 0.5 g of phloem tissue was removed both around the drill sites and 30 cm from the soil (away from the challenge inoculation site). These samples were ground in liquid nitrogen and 100 mg of tissue was twice-extracted in methanol as described above and the extract analyzed by HPLC. At 28 days, a razor blade was used to remove the outer bark around the challenge inoculations and a ruler was used to measure challenge lesion lengths.

Statistical Analysis

All statistical analyses were performed using SPSS version 14.0 for Windows (SPSS Institute, Chicago). ANOVA with LSD multiple comparison tests ($\alpha = 0.05$) was performed on compound concentrations and lesion length data when normality assumptions were met. Data were transformed by quartile ranks by experiment as necessary to meet normality. If transformation failed to make data normal, equivalent non-parametric tests (Kruskal-Wallis and Mann-Whitney U) were performed. Data for individual phenolic peaks were quartile rank transformed and the mean across each of transformed individual peaks was taken for each sample. This was conducted to obtain a pooled representation of the phenolics as a whole in lieu of adding peak areas together.

Results

Identification of Xylem Phenolics

A total of 14 phenolic compounds were consistently detected in the xylem extracts and were quantified. Six of these compounds (coumaric acid hexoside, ferulic acid glucoside, taxifolin hexoside, taxifolin, pinosylvin, and pinosylvin monomethyl ether) were previously described as occurring in the stem phloem of *Pinus nigra* (Chapters 2 and 3), and these identities were confirmed by matching retention times and UV spectra. The remaining eight compounds were not identified and are labeled with their retention times and with the following UV maxima: pk7.0, 267 and 301 maximum absorbance; pk7.9, 257 and 295; pk9.4, 277 and 310 (this compound co-eluted with hydroxypropiovanillone hexoside); pk10.0, 305 and 333; pk11.4, 265; pk14.4, 342; pk15.0, 267 and 300; pk15.9, 300 and 315. Pk15.9 had been previously found in the stem phloem (Chapters 2 and 3).

Fungal Infection Induces Phenolic Accumulation in the Xylem

A total of nine compounds found within the xylem increased in concentration in pathogen-inoculated trees (Table 5.1). Pinosylvin, pk7.0, and pk15.9 increased significantly in *D. pinea* inoculated trees over all other induction treatments. *D. scrobiculata* inoculation induced the accumulation of taxifolin hexoside whereas *D. pinea* inoculation did not. Both pathogens significantly induced accumulation of pinosylvin monomethyl ether.

Treatment and trial had interactive effects on coumaric acid hexoside ($P < 0.05$). Within the July 2005 experiment, *D. pinea* infection significantly ($F_{3, 18} = 5.885$, $P = 0.007$) increased coumaric acid hexoside concentrations compared to both controls and *D. scrobiculata* infected trees. This was not observed in either of the other experiments.

Xylem Methanol Extracts Induce Accumulation of Phenolic in Pine Phloem

Application of crude methanol extracts to wounds made on branches of naïve trees significantly increased a variety of phenolic compounds in the phloem that are associated with local induced defenses (Chapter 2) (Fig. 4.1), including coumaric acid hexoside ($F_{3, 73} = 2.364$, $P = 0.050$), ferulic acid glucoside ($F_{3, 75} = 3.309$, $P = 0.010$), pinosylvin ($F_{3, 73} = 11.740$, $P < 0.001$), and pinosylvin monomethyl ether ($F_{3, 64} = 5.241$, $P = 0.001$). From this data, it appeared that the elicitation of pinosylvin was the best indication that localized induction of chemical defenses was triggered by the xylem methanol extracts (Fig. 4.1). However, the extracts from pathogen-infected trees did not elicit a stronger response than extracts from non-wounded and mock-inoculated trees (Fig. 4.1).

Ability of Individual Phenolics to Mediate SIR

Ferulic acid glucoside, taxifolin, and pinosylvin were chosen for this analysis because each was detected at significantly higher levels in the xylem of pathogen-infected trees than non-wounded trees and because they represent three classes of

phenolic derivatives (phenolic glycosides, flavonoids, and stilbenes, respectively). Fast Green FCF dye applied to the drill holes revealed uptake of the solutions in the first and third experiments, but not within the second experiment.

In the first experiment, no significant differences were found in lesion lengths overall according to treatment ($F_{8, 44} = 0.826$, $P = 0.585$). However, LSD analysis showed a significant ($P = 0.039$) reduction of lesion length by methyl jasmonate compared to the non-wounded control (data not shown).

In the second experiment, pinosylvin and taxifolin did not elicit SIR, but ferulic acid induced a significant reduction in challenge lesion length ($F_{8, 53} = 2.730$, $P = 0.015$) compared to non-wounded and methanol controls (Fig. 5.2a). However, ferulic acid glucoside did not significantly differ from any of the other treatments. No other chemical treatments, as well as the inoculation with *D. pinea* control, resulted in significantly decreased lesion length compared to non-wounded controls (Fig. 5.2a).

Based on the second experiment's results, a third experiment was performed with dilution series of ferulic acid glucoside (100X, 10X, and 1X the xylem concentration in induced trees). No significant reductions in lesion length occurred in this experiment ($F_{6, 35} = 2.636$, $P = 0.037$), except that the 1X concentration of ferulic acid glucoside actually resulted in significantly longer lesion lengths than the controls (Fig. 5.2b). The other ferulic acid glucoside treated trees had similar challenge lesion lengths as the controls (Fig. 5.2b).

Phloem samples taken from both around the site of compound injection and 30 cm away from that site were analyzed to determine whether systemic induction of phenolics occurred. Five compounds were analyzed at both sites to explore for induction

of phenolics: coumaric acid hexoside, hydroxypropiovanillone hexoside, ferulic acid glucoside, taxifolin hexoside, and taxifolin. Due to significant trial effects ($P < 0.05$) and different treatments used within each experiment, the experiments were analyzed separately.

Within 5 cm of application sites, no significant effects of treatment were found in the first experiment (data not shown). In the second experiment, taxifolin induced significantly ($P < 0.05$) greater accumulation of coumaric acid hexoside and hydroxypropiovanillone hexoside than the non-wounded and methanol controls, although the overall F-value was not significant. No other significant differences were observed in the second experiment. In the third experiment, trees treated with ferulic acid glucoside at 100X and 10X accumulated greater taxifolin levels than the non-wounded controls based on LSD tests, although the overall F-value was not significant. No other effects of treatments were observed.

No discernable changes in the concentrations of any compound due to treatments in any of the experiments were detected 30 cm away from the application site.

Discussion

This study determined that pathogen infection increased the levels of at least ten different compounds in the xylem distal to infected tissues. Application of methanol extracts of xylem tissue to wounds made in branches successfully triggered the accumulation of defense-related phenolic compounds including phenolic glycosides and stilbenes. However, methanol extracts from infected trees did not trigger a greater

induction of phenolic compounds than extracts from uninfected trees. Finally, no mediating role was determined for three selected phenolics (ferulic acid glucoside, taxifolin, and pinosylvin) that were induced within the xylem following *D. pinea* infection.

The levels of coumaric acid hexoside (in one trial), ferulic acid glucoside, taxifolin hexoside, taxifolin, pinosylvin, pinosylvin monomethyl ether, and four unknowns increased in trees induced by *Diplodia* infection. This mirrors similar systemic increases in the phloem following pathogen infection (e.g. Bonello and Blodgett 2003; Bonello et al. 2003; Brignolas et al. 1995; Cvikrova et al. 2006). Phenolic hexoside levels in the phloem have been inversely associated with fungal pathogen success (Chapter 2). Phenolic hexosides and stilbenes have are also known to have antifungal activity *in vitro* (Blodgett and Stanosz 1997a; Bois et al. 1999; Klepzig et al. 1996; Shain 1967; Shrimpton and Whitney 1968).

D. scrobiculata induced accumulation of taxifolin and its hexoside to greater levels than *D. pinea*, and *D. pinea* induced accumulation of the stilbenes (including pk15.9 which has the UV spectrum of a stilbene) to greater levels than *D. scrobiculata*. This implies that certain compounds might be produced more specifically in response to one of the pathogens over the other, and classes within the phenolics might therefore be somewhat independently regulated. Ecologically, this suggests that, as a defense strategy, pines may have evolved to rely on specific compound classes to counter one or the other *Diplodia* species. Alternatively, the *Diplodia* pathogens might have independent abilities to limit the production of particular classes of phenolic compounds.

It is likely that trees produce phenolics throughout stem tissues (both the phloem and xylem) to ensure maximum protection against fungal pathogens, as fungal pathogens other than *Diplodia* spp. can penetrate and destroy both the phloem and xylem. These results further suggest that phenolics might be unloaded into the xylem in part as a response to prevent xylem pathogens from gaining a foothold within the tree (Hart 1981; Pearce 1996).

This study also examined whether phenolics might have an additional role in the xylem to mediate SIR. In an exploratory study, it was determined that xylem methanol extracts can elicit the accumulation of higher levels of phenolic hexosides and stilbenes in the phloem, but only at levels much higher (at least 100 fold) than occur in the xylem. Because there was no detection of salicylates or jasmonates within these extracts, it is presumed that at least one novel, methanol-soluble elicitation compound exists in the xylem. However, there were no differences in elicitation capacity between extracts from uninfected and pathogen-infected trees. This could imply that there was no elicitor, but the methanol controls had lower levels of defense induction than any of the xylem extracts. Alternatively, these findings could suggest that the signaling agent is constitutively produced within the xylem of within the naïve trees, and this signal works in a dose-dependent manner to elicit phenolic production. It was possible that allowing the methanol extracts to evaporate prior to their application to naïve wounds resulted in elicitor compounds becoming artificially more concentrated within xylem extracts from uninfected trees. The result was then all xylem extracts, regardless if from uninfected or pathogen-infected trees, had elicitor concentrations at high enough dosages to induce the production of phenolics when applied to the wounds.

An attempt was made to identify mediating roles of individual compounds. Ferulic acid glucoside, pinosylvin, and taxifolin solutions were applied into the stem to examine if they could trigger an SIR response. These were chosen in part for their structural diversity (a phenolic glycoside, a stilbene, and a flavonoid) and their differential accumulation by *D. scrobiculata* and *D. pinea*. Methyl jasmonate was also applied as a positive control. One trial revealed a significant reduction of lesion length when ferulic acid glucoside was applied, compared to non-wounded and methanol controls, but could not be replicated. Alternatively, ferulic acid glucoside could have a direct inhibitory effect (it was associated with resistance in Chapter 2), but increases in ferulic acid glucoside levels were not observed to occur systemically in trees treated with this compound. This suggests that this compound might not have been translocated at levels great enough to affect fungal growth. Applications of taxifolin and pinosylvin also reduced lesion length, but not significantly.

Taken together, these results suggest that these compounds might not be signals, but rather elicitors that induce the pine host to produce the actual signaling compounds. This would in part explain the results shown in Figure 5.1, whereby all methanol extracts elicited localized increased phenolic accumulation over the controls. Perhaps particular phenolic compounds can function synergistically to induce pine defenses if they can reach sufficient concentrations. Phenolic accumulation could therefore be part of a positive feedback loop in the induction of SIR.

The failure of *D. pinea* inoculation and application of methyl jasmonate to elicit SIR revealed that potential problems existed in these elicitation experiments. The lack of any significant phenolic modulations due to any of the treatments further implied that this

methodology was not adequate to fully examine the signaling ability of the tested phenolics. Between-tree variation and seasonal effects of the trials were perhaps too great to obtain clear results. Additional efforts to develop more accurate methods to examine SIR-mediating ability of particular compound are thus warranted.

In conclusion, this study determined that increased concentrations of phenolic compounds occur throughout the xylem in infected trees, possibly as part of a SIR response against xylem pathogens. The application of methanol extracts from the xylem significantly induced phenolic production in naïve branches over controls, suggesting signaling molecules might exist within the polar (methanol-extractable) fraction of the xylem. However, the identity of these putative signaling compound(s) remains unknown and further method development and experimentation is needed to identify potential mediators of the SIR response in conifers.

Compound Name (RT, in minutes)	UV maxima	Non-wounded			Mock Inoculated			*D. scrobiculata* inoculated			*D. pinea* inoculated			F*	df	*P*
ferulic acid glucoside (10.3)	285, 320	21225	(5998)	ab	6200	(1174)	b	15304	(5261)	a	31652	(11993)	ab	2.667	57	0.059
taxifolin hexoside (15.4)	287	5528	(2129)	b	3458	(1086)	b	12511	(3226)	a	7969	(2423)	b	4.102	55	0.012
taxifolin (16.3)^	285	3687	(998)	ab	1691	(467)	b	5078	(1474)	a	3960	(1112)	ab	3.086	48	0.039
pinosylvin (24.8)	300, 315	855	(153)	b	718	(55)	b	4854	(2312)	b	7527	(2845)	a	6.949	36	0.001
pinosylvin monomethyl ether (27.8)	300, 315	872	(156)	b	718	(55)	b	2101	(614)	a	4926	(1779)	a	7.515	35	0.001
Pk. 7.0	267, 301	2903	(1209)	bc	3326	(965)	ab	1067	(418)	c	3483	(1132)	a	3.656	32	0.026
Pk. 9.4%	277, 310	77816	(34152)	ab	80326	(37452)	b	224063	(105442)	a	129744	(58991)	a	2.666	55	0.059
Pk. 15.0	265, 300	2738	(582)	a	1560	(325)	b	7330	(3138)	a	2409	(658)	ab	2.538	50	0.071
Pk. 15.9	300, 315	1280	(187)	b	1971	(395)	b	1220	(262)	b	14530	(6262)	a	11.29	36	<0.001

* these F-values represent ANOVAs run on transformed data to meet normality, except for coumaric acid hexoside

^ quantified at 280nm wavelength, all others peaks quantified at 308nm; % represents a co-elution of an unknown and hydroxypropiovanillone hexoside

Table 5.1: UV maxima, mean (standard error) of phenolic concentrations (AU), and ANOVA statistics for compounds altered by induction treatment within the xylem 60 cm from the induction. Letters indicate significant differences by LSD ($P < 0.05$).

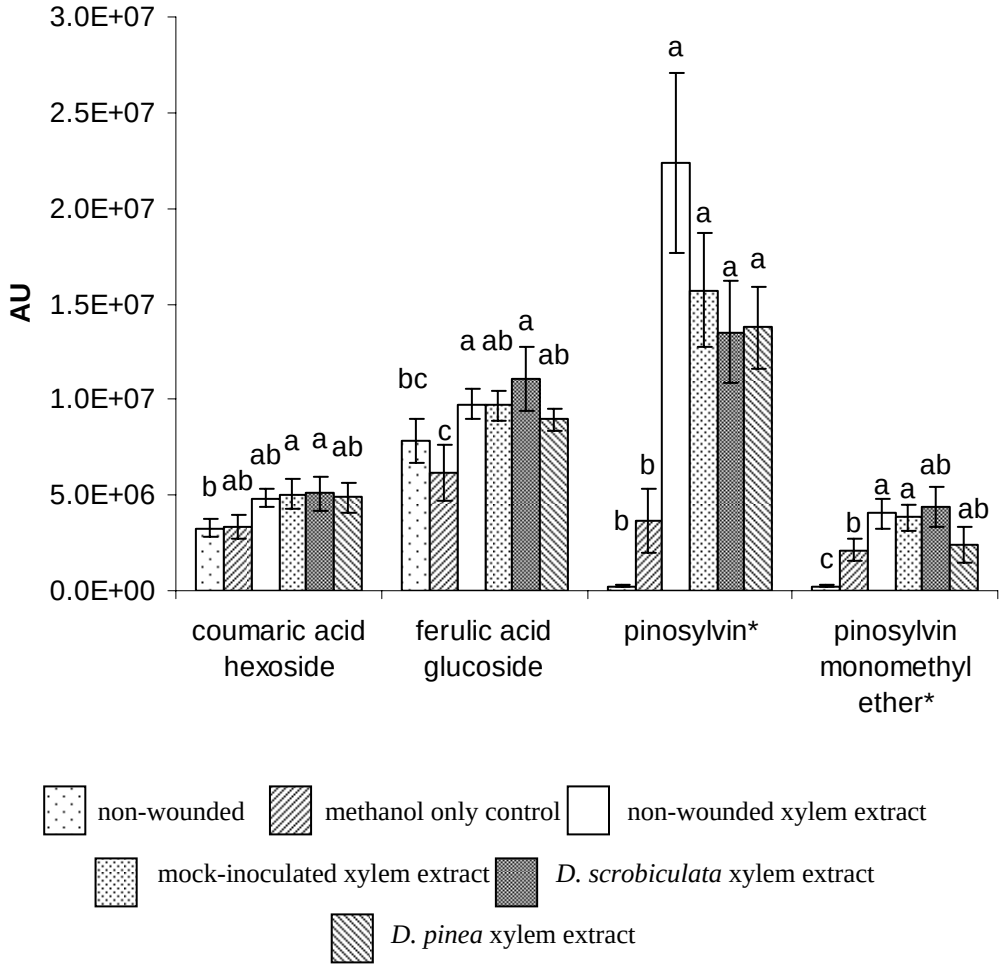


Figure **5.1:** Phenolic concentrations (AU) within the phloem taken around the application sites of the methanol extracts or controls. Letters indicate significant differences by LSD analysis ($P < 0.05$) for each compound. Bars represent the standard error. * Both pinosylvin and pinosylvin monomethyl ether are represented at 10 X their actual amounts here to make differences easier to observe.

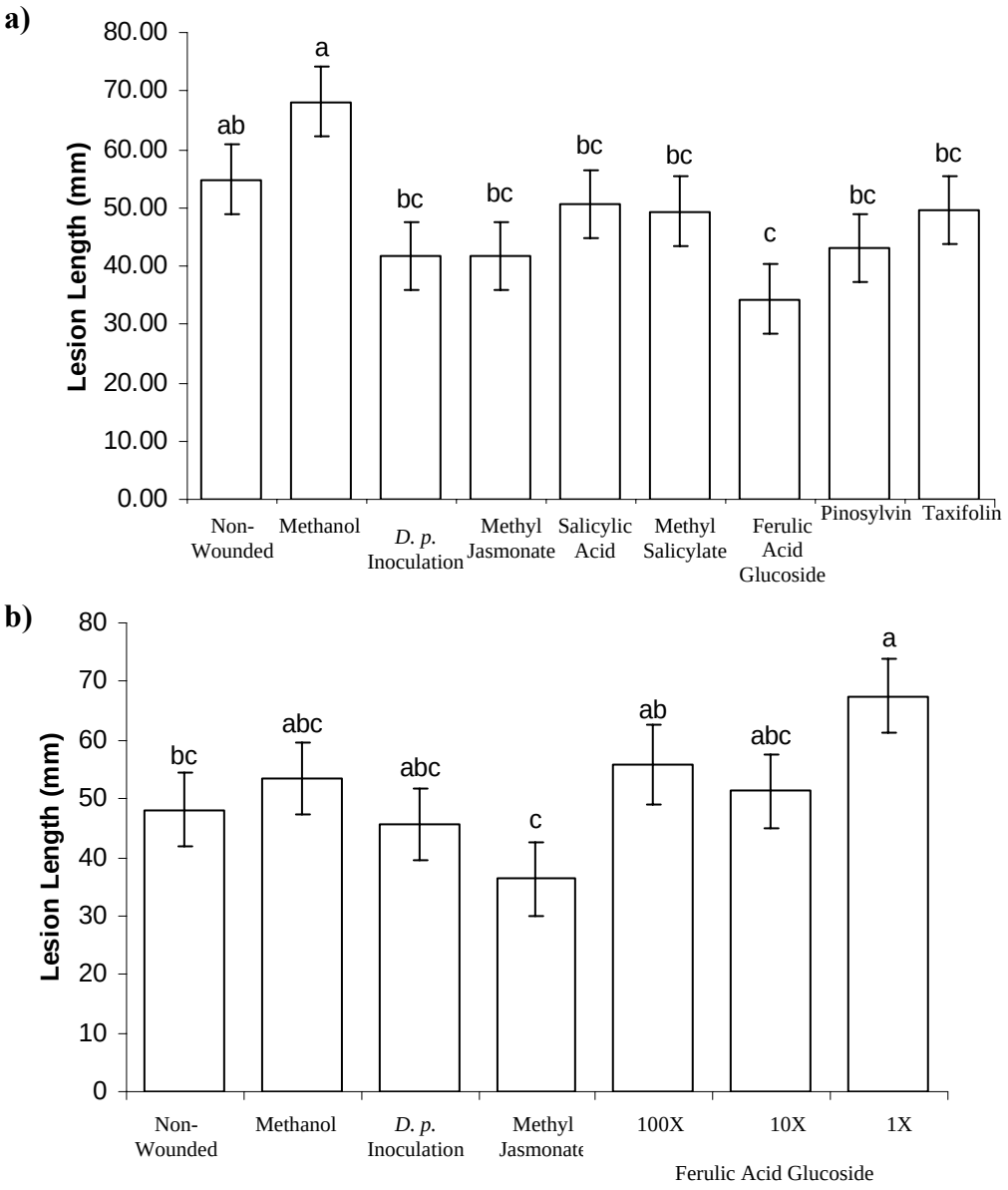


Figure **5.2:** Challenge lesion lengths taken 21 days following *D. pinea* challenge inoculations for each elicitation treatment in a) elicitation experiment 2, and b) elicitation experiment 3. Letters indicate significant differences by LSD analysis ($P < 0.05$). Bars represent the standard error.

CHAPTER 6

TOWARDS A BETTER UNDERSTANDING OF PINE DEFENSES AGAINST PATHOGENS

The central aim of this work was to better understand how pines defend themselves against pathogens. The knowledge gained could eventually be used to help preserve the ecological, environmental, economical, and aesthetical values of pines. A wide variety of experiments was conducted to begin filling some important gaps in our understanding of how pines naturally combat pathogens.

The *Pinus nigra* and *Diplodia* spp. pathosystem was utilized in all experiments to accomplish the aims and objectives of this work. Many of the findings of this research should be applicable to other conifers such as spruce, larch, and fir, as this pine pathosystem has already proven to be quite useful to characterize many basic phenomena involved in pine defense (Bonello et al. 2006).

This work had three major findings about pine defenses against fungal pathogens. First, phenolics were associated with constitutive and induced (both local and systemic) resistance, whereas terpenes seem only associated with local induced resistance. Second, nutrient availability affected constitutive terpene levels and pathogen-inducible phenolic levels. Lastly, differential regulation was observed between phenolics and terpenes,

revealing that two defense regulatory pathways might be at work in pines. These observations might have far-reaching impacts on the understanding of how pines respond to pests in the environment. They could also lead to novel management options to protect pines from their pests.

The Role of Phenolics and Terpenes in the Fight against Pathogens

Previous studies suggest that both phenolics and terpenes are likely involved in combating fungal pathogens in conifers. Various phenolics, e.g. phenolic glycosides and stilbenes, have antifungal activity against pathogens *in vitro* (e.g. Blodgett and Stanosz 1997a; Bois et al. 1999; Klepzig et al. 1995; Klepzig et al. 1996; Lindberg et al. 1992; Shain 1967; Shrimpton and Whitney 1968; Woodward and Pearce 1988; Zou and Cates 1997). This, coupled with evidence of induction both locally and systemically (e.g. Bonello and Blodgett 2003; Bonello et al. 2003; Brignolas et al. 1995; Cvikrova et al. 2006; Evensen et al. 2000; Krekling et al. 2004; Lieutier et al. 1991; Shain 1971; Viiri et al. 2001), strongly supports the hypothesis that phenolics are crucial components in defense against pathogens. However, attempts to correlate phenolic production and accumulation with the expression of resistance have been mostly unsuccessful to date (e.g. Bonello and Blodgett 2003; Viiri et al. 2001), although Blodgett et al. (2007) did find a positive correlation between phenolics (including lignin) and resistance.

In this work, a significant, positive correlation was found between *D. pinea* resistance and phenolics (both phenolic glycosides and stilbenes) *in vivo*. Multivariate analyses also showed that Austrian pine produces phenolic glycosides and stilbenes as

co-regulated compound classes. The roles of individual compounds in pine resistance to *D. pinea* remains less clear, as the ability of particular compounds to affect pathogen growth has not been fully studied. Bioassays examining the relative antimicrobial properties of individual compounds and compounds in combination in pure culture could generate focused hypotheses regarding the role of individual compounds *in planta*. These bioassays could support the hypothesis that Austrian pine uses all of these compounds in a coordinated manner in resisting *D. pinea* attack.

Terpenes have been shown to accumulate following induction of defense and it has been suggested that they are associated with pathogen containment (Bohlmann et al. 1998; Christiansen et al. 1999; Hudgins et al. 2005; Krekling et al. 2004; Luchi et al. 2005; Zeneli et al. 2006). In this work increased levels of monoterpenes were found in tissues taken around an infection (Figure 4.1) and they were negatively correlated with lesion length (Table 4.1), supporting the conclusion that terpenes may play roles in local induced resistance to *D. pinea*. However, I generally found no evidence to show that monoterpenes may be involved in constitutive or systemic induced resistance. The only exception was β-pinene, which was associated with systemic induced resistance in one study (Table 2.3). These findings suggest that assumptions found in the literature that monoterpene levels prior to infection are important in resistance need to be reassessed.

Effects of Soil Fertility on Pine Defenses against Pathogens

Austrian pines grown at low, medium, and high fertility levels were assessed for their ability to produce and accumulate phenolics and terpenes constitutively and under

induction by *D. pinea*. In the study described in Chapter 3, fertility did not affect constitutive accumulation of phenolics, while constitutive levels of monoterpenes increased with increasing fertility (Figures 3.1 and 3.2). However, when all induction treatments were considered, phenolics were found to decrease with increasing fertility and terpenes were unaffected (Table 2.2). These results suggest that differential resource regulation occurs between phenolic and terpene metabolic networks, as fertility had different effects on each compound class in different forms of resistance.

Differential Regulation of Phenolics and Terpenes

Results from this work suggest that phenolics and terpenes follow different and contrasting regulatory pathways in pine. It was known that both insects and fungal pathogens can induce both phenolics and terpenoids (Franceschi et al. 2005). Less clear was whether phenolics and terpenes are coordinately regulated in the overall defense response.

This work suggests that the induction processes for the phenolic and terpene networks are controlled independently, at least in the SIR-associated response. The first line of evidence to support this interpretation was the multivariate analysis performed to identify co-regulated compound classes in systemically induced phloem tissues. This analysis showed that phenolics and monoterpenes were consistently clustered in separate groups, with very little overlap. The second line of evidence was that the phenolic and terpene classes were strongly negatively correlated with one another, which suggests a competition for resources resulting in a negative trade-off in defense. A final line of

evidence that these compounds are regulated differently is the effects that methyl jasmonate application had on induced defense. When applied to the xylem, methyl jasmonate increased oleoresin flow whereas *D. pinea* infection had the opposite effect, as infected trees produced less oleoresin. Where both methyl jasmonate and *D. pinea* inoculations were used (i.e. experiment described in Chapter 5 on page 125) an increase, albeit non-significant ($P < 0.10$), of phenolic glycosides was observed in trees inoculated with *D. pinea* compared to methyl jasmonate treated trees (data not shown).

Interestingly, both methyl jasmonate and *D. pinea* inoculation elicited SIR, despite having different effects on the chemistry of systemic defense induction. It is possible that methyl jasmonate-induced trees produced elevated quantities of oleoresin that physically prevented establishment of the challenge inoculations. Furthermore, visual observations suggest that wound healing is quicker in methyl jasmonate treated trees than in the other treatments. *D. pinea* inoculation, on the other hand, induced accumulation of larger amounts of phenolics, which was correlated with resistance *in vivo*, suggesting a triggering of antifungal compounds systemically to combat a second infection by the same pathogen. These observations lead to a model in which terpenes and phenolics have different induction patterns and roles in resistance to fungal pathogens, yet are still integral components of an effective resistance response. Perhaps the terpenes are more important in quickly sealing a wound while phenolics act directly as antibiotics within the infected tissues to limit fungal development.

Finally, these results suggest that at least two separate chemical mediators may be necessary in SIR: one that mediates terpenes and one that mediates phenolics. Functionally, this would be analogous to what is known to occur in the best understood

model systems, in which salicylic acid regulates SAR pathways and jasmonic acid regulates ISR pathways. The molecular basis of any potential cross-talk occurring between these pathways in conifers is unknown, but the outcome may be both positive and negative depending on the specific pathosystems and environmental conditions (Rostas et al. 2003). This is similar to what is known to occur in herbaceous model studies, in which cross-talk between SAR and ISR has been studied much more extensively, and has been shown to result in both synergistic and antagonistic interactions [as reviewed by Beckers and Spoel (2006)].

Ecological and Evolutionary Considerations of this Research

As a whole, this work paints a clearer picture of pine defenses against fungal pathogens. Phenolics appear to play an antibiotic role in defense and are likely to directly combat fungal pathogens in the stem. Terpenes, on the other hand, appear to be more associated with wound repair and physical exclusion or expulsion of secondary invaders. Both phenolics and terpenes work together to allow pines to survive potentially devastating attacks. Without either phenolics or terpenes pines would likely succumb to pathogen attack. These two distinct pathways likely allow for fine-tuning of defenses to achieve the optimal and most cost-efficient defense response to whatever threat the tree faces (Beckers and Spoel 2006). Pines are constantly under pathogen and insect pest threat, and must have mechanisms to prevent draining of resources whenever an

infection/infestation occurs. Otherwise, resources within the pines might quickly become overtaxed, and the pines might enter a state of systemic induced susceptibility, as predicted by Bonello et al. (2006).

These two distinct, resource-competing pathways could help explain, at least in part, the evolution of bark beetle/fungal complexes. In the these associations, fungal partners are thought to benefit from the interaction by being dispersed into new hosts by their bark beetle partners, while the insects are thought to benefit because the fungi kill host tissue making it more amenable to colonization by the beetles. However, a further non-mutually exclusive mechanism may explain the overall benefit of the association to the insect vectors. By preferentially triggering the phenolic defense pathway, fungi may impair the tree's ability to mount an effective terpenoid response, which is associated with resistance to insects (Franceschi et al. 2005), in a biochemical tradeoff that was partly demonstrated in this work. Alternatively, with both pathways triggered simultaneously, the bark beetle/fungal complex could cause the simultaneous depletion of resources for both of these defenses. Such an event might become overwhelming for the host, and could lead to both defensive pathways losing their effectiveness against the pests.

Practical Considerations of this Research

Beyond ecological impacts of this research, these findings suggest that trees that inherently accumulate more phenolics and/or terpenes should be selected for in breeding programs, as such trees would be better able to defend against pathogen attack.

Furthermore, this work shows some promise in finding signaling and mediating elements of the SIR response. Discovery of these mediators could lead to novel immunization treatments that render trees better able to fend off insect pests and pathogens. As a whole, this work has taken us closer to the discovery of novel management options that would allow for increased preservation of the intrinsic ecological, environmental, economic, and aesthetical value of pines.

BIBLIOGRAPHY

Anon. 2006. Mountain Pine Beetle Action Plan 2006-2011. British Columbia. http://www.gov.bc.ca/pinebeetle

Ashton, P.M.S., S. Gamage, I. Gunatilleke and C.V.S. Gunatilleke 1997. Restoration of a Sri Lankan rainforest: using Caribbean pine *Pinus caribaea* as a nurse for establishing late-successional tree species. Journal of Applied Ecology. 34:915-925.

Ayres, P. 1984. The interaction between environmental stress injury and biotic disease physiology. Annual Review of Phytopathology. 22:53-75.

Baker, C.J., B.D. Whitaker, D.P. Roberts, N.M. Mock, C.P. Rice, K.L. Deahl and A.A. Aver'yanov 2005. Induction of redox sensitive extracellular phenolics during plant-bacterial interactions. Physiological and Molecular Plant Pathology. 66:90-98.

Bakker, P., C.M.J. Pieterse and L.C. van Loon 2007. Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology. 97:239-243.

Baldwin, I.T., R. Halitschke, A. Paschold, C.C. von Dahl and C.A. Preston 2006. Volatile signaling in plant-plant interactions: "Talking trees" in the genomics era. Science. 311:812-815.

Baldwin, I.T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proceedings of the National Academy of Sciences of the United States of America. 95:8113-8118.

Beckers, G.J.M. and S.H. Spoel 2006. Fine-tuning plant defence signalling: Salicylate versus jasmonate. Plant Biology. 8:1-10.

Beckman, C.H. 2000. Phenolic-storing cells: keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? Physiological and Molecular Plant Pathology. 57:101-110.

Benitez, M., F. Baysal Tustasa, D. Rotenberg, M. Kleinhenz, J. Cardina, D. Stinner, S. Miller and B. McSpadden Gardener 2007. Multiple statistical approaches of community fingerprint data reveal bacterial populations associated with general disease suppression arising from the application of different organic field management strategies. Soil Biology & Biochemistry. 39:2289-2301.

Bergelson, J., C.B. Purrington, C.J. Palm and J.C. LopezGutierrez 1996. Costs of resistance: A test using transgenic *Arabidopsis thaliana*. Proceedings of the Royal Society of London Series B-Biological Sciences. 263:1659-1663.

Berrymann, A. 1969. Responses of *Abies grandis* to attack by *Scolytus ventralis* (Coleoptera-Scolytidae). Canadian Entomologist. 101:1033-42.

Berrymann, A. 1972. Resistance of conifers to invasion by bark beetle-fungus associations. Bioscience. 22:599-601.

Biere, A., H.B. Marak and J.M.M. van Damme 2004. Plant chemical defense against herbivores and pathogens: generalized defense or trade-offs? Oecologia. 140:430-441.

Blanchette, R. and A. Biggs 1992. Defense mechanisms of woody plants against fungi. Springer, Berlin, New York.

Blodgett, J.T. and P. Bonello 2003. The aggressiveness of *Sphaeropsis sapinea* on Austrian pine varies with isolate group and site of infection. Forest Pathology. 33:15-19.

Blodgett, J.T., A. Eyles and P. Bonello 2007. Organ-dependent induction of systemic resistance and systemic susceptibility in *Pinus nigra* inoculated with *Sphaeropsis sapinea* and *Diplodia scrobiculata*. Tree Physiology. 27:511-517.

Blodgett, J.T., D.A. Herms and P. Bonello 2005. Effects of fertilization on red pine defense chemistry and resistance to *Sphaeropsis sapinea*. Forest Ecology and Management. 208:373-382.

Blodgett, J.T., E.L. Kruger and G.R. Stanosz 1997. Effects of moderate water stress on disease development by *Sphaeropsis sapinea* on red pine. Phytopathology. 87:422-428.

Blodgett, J.T. and G.R. Stanosz 1997a. Differential inhibition of *Sphaeropsis sapinea* morphotypes by a phenolic compound and several monoterpenes of red pine. Phytopathology. 87:606-609.

Blodgett, J.T. and G.R. Stanosz 1997b. *Sphaeropsis sapinea* morphotypes differ in aggressiveness, but both infect nonwounded red or jack pines. Plant Disease. 81:143-147.

Bohlmann, J., G. Meyer-Gauen and R. Croteau 1998. Plant terpenoid synthases: Molecular biology and phylogenetic analysis. Proceedings of the National Academy of Sciences of the United States of America. 95:4126-4133.

Bois, E. and F. Lieutier 1997. Phenolic response of Scots pine clones to inoculation with *Leptographium wingfieldii*, a fungus associated with *Tomicus piniperda*. Plant Physiology and Biochemistry. 35:819-825.

Bois, E., F. Lieutier and A. Yart 1999. Bioassays on *Leptographium wingfieldii*, a bark beetle associated fungus, with phenolic compounds of Scots pine phloem. European Journal of Plant Pathology. 105:51-60.

Bojovic, S., M. Jurc, D. Drazic, P. Pavlovic, M. Mitrovic, L. Djurdjevic, R.S. Dodd, Z. Afzal-Rafii and M. Barbero 2005. Origin identification of *Pinus nigra* populations in southwestern Europe using terpene composition variations. Trees-Structure and Function. 19:531-538.

Bonello, P. and J.T. Blodgett 2003. *Pinus nigra-Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. Physiological and Molecular Plant Pathology. 63:249-261.

Bonello, P., J. Blodgett and D. Herms 2001a. Progress in research on systemic induced resistance in Austrian pine against shoot blight (formally known as Diplodia tip blight). *In* Ornamental plants annual reports and research reviews 2001 Eds. J.A. Chatfield, J.F. Boggs, E.A. Draper, H. Mathers and A.K. Stone.

Bonello, P., T.R. Gordon, D.A. Herms, D.L. Wood and N. Erbilgin 2006. Nature and ecological implications of pathogen-induced systemic resistance in conifers: A novel hypothesis. Physiological and Molecular Plant Pathology. 68:95-104.

Bonello, P., T. Gordon and A. Storer 2001b. Systemic induced resistance in Monterey pine. Forest Pathology. 31:99-106.

Bonello, P., W. Heller and H. Sandermann Jr 1993. Ozone effects on root-disease susceptibility and defence responses in mycorrhizal and non-mycorrhizal seedlings of Scots pine (*Pinus sylvestris* L.). New Phytologist. 124:653-63.

Bonello, P. and R. Pearce 1993. Biochemical defence responses in primary roots of Scots pine challenged *in vitro* with *Cylindrocarpon destructans*. Plant Pathology. 42:203-11.

Bonello, P., R.B. Pearce, F. Watt and G.W. Grime 1991. An induced papilla response in primary roots of Scots pine challenged *in vitro* with *Cylindrocarpon destructans*. Physiological and Molecular Plant Pathology. 39:213-228.

Bonello, P., A.J. Storer, T.R. Gordon and D.L. Wood 2003. Systemic effects of *Heterobasidion annosum* on ferulic acid glucoside and lignin of presymptomatic ponderosa pine phloem, and potential effects on bark-beetle-associated fungi. Journal of Chemical Ecology. 29:1167-1182.

Bostock, R.M. 2005. Signal crosstalk and induced resistance: Straddling the line between cost and benefit. Annual Review of Phytopathology. 43:545-580.

Bostock, R.M., R. Karban, J.S. Thaler, P.D. Weyman and D. Gilchrist 2001. Signal interactions in induced resistance to pathogens and insect herbivores. European Journal of Plant Pathology. 107:103-111.

Bridges, J.R. 1987. Effects of terpenoid compounds on growth of symbiotic fungi associated with the southern pine-beetle. Phytopathology. 77:83-85.

Brignolas, F., B. Lacroix, F. Lieutier, D. Sauvard, A. Drouet and A. Claudot 1995. Induced responses in phenolic metabolism in two Norway spruce clones after wounding and inoculations with *Ophiostoma polonicum*, a bark-beetle-associated fungus. Plant Physiology. 109:821-7.

Bryant, J.P., F.S. Chapin and D.R. Klein 1983. Carbon nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos. 40:357-368.

Bull, J. and J. Farrand Jr. 2003. National Audubon Society Field Guide to Birds. Alfred A. Knopf, Inc., New York.

Burdon, J.J. and P.H. Thrall 2003. The fitness costs to plants of resistance to pathogens. Genome Biology. 4:227.

Byun-McKay, A., K.A. Godard, M. Toudefallah, D.M. Martin, R. Alfaro, J. King, J. Bohlmann and A.L. Plant 2006. Wound-induced terpene synthase gene expression in sitka spruce that exhibit resistance or susceptibility to attack by the white pine weevil. Plant Physiology. 140:1009-1021.

Castello, J.D., D.J. Leopold and P.J. Smallidge 1995. Pathogens, Patterns, and Processes in Forest Ecosystems. Bioscience. 45:16-24.

Cates, R.G., C.B. Henderson and R.A. Redak 1987. Responses of the western spruce budworm to varying levels of nitrogen and terpenes. Oecologia. 73:312-316.

Celimene, C., D. Smith, R. Young and G. Stanosz 2001. *In vitro* inhibition of *Sphaeropsis sapinea* by natural stilbenes. Phytochemistry. 56:161-165.

Cheniclet, C. 1987. Effects of wounding and fungus inoculation on terpene producing systems of maritime pine. Journal of Experimental Botany. 38:1557-1572.

Cherubini, P., G. Fontana, D. Rigling, M. Dobbertin, P. Brang and J.L. Innes 2002. Tree-life history prior to death: two fungal root pathogens affect tree-ring growth differently. Journal of Ecology. 90:839-850.

Chiron, H., A. Drouet, F. Lieutier, H. Payer, D. Ernst and H. Sandermann 2000. Gene induction of stilbene biosynthesis in Scots pine in response to ozone treatment, wounding, and fungal infection. Plant Physiology. 124:865-872.

Christiansen, E., P. Krokene, A.A. Berryman, V.R. Franceschi, T. Krekling, F. Lieutier, A. Lonneborg and H. Solheim 1999. Mechanical injury and fungal infection induce acquired resistance in Norway spruce. Tree Physiology. 19:399-403.

Christiansen, E., R.H. Waring and A.A. Berryman 1987. Resistance of conifers to bark beetle attack - searching for general relationships. Forest Ecology and Management. 22:89-106.

Ciesla, W. 1995. Climate change, forests and forest management, an overview. *In* FAO Forestry Paper 126.

Cipollini, D. 1998. Induced defenses and phenotypic plasticity. Trends in Ecology & Evolution. 13:200-200.

Cipollini, D. 2004. Stretching the limits of plasticity: Can a plant defend against both competitors and herbivores? Ecology. 85:28-37.

Cipollini, D., C.B. Purrington and J. Bergelson 2003. Costs of induced responses in plants. Basic and Applied Ecology. 4:79-89.

Coley, P.D., J.P. Bryant and F.S. Chapin 1985. Resource availability and plant antiherbivore defense. Science. 230:895-899.

Conrath, U., O. Thulke, V. Katz, S. Schwindling and A. Kohler 2001. Priming as a mechanism in induced systemic resistance of plants. European Journal of Plant Pathology. 107:113-119.

Cook, S. and F. Hain 1988. Toxicity of host monoterpenes in resistance of Douglas fir to western spruce budworm defoliation. Journal of Entomological Science. 23:287-292.

Croteau, R., S. Gurkewitz, M. Johnson and H. Fisk 1987. Biochemistry of oleoresinosis: monoterpene and diterpene biosynthesis in lodgepole pine saplings infected with *Ceratocystis calvigera* or treated with carbohydrate elicitors. Plant Physiology. 85:1171-7.

Cvikrova, M., J. Mala, M. Hrubcova and J. Eder 2006. Soluble and cell wall-bound phenolics and lignin in *Ascocalyx abietina* infected Norway spruces. Plant Science. 170:563-570.

de Groot, P. and J.J. Turgeon 1998. Insect-pine interactions. *In* Ecology and Biogeography of *Pinus* Ed. D.M. Richardson. Cambridge University Press, New York, pp. 354-380.

de Wet, J., T. Burgess, B. Slippers, O. Preisig, B.D. Wingfield and M.J. Wingfield 2003. Multiple gene genealogies and microsatellite markers reflect relationships between morphotypes of *Sphaeropsis sapinea* and distinguish a new species of *Diplodia*. Mycological Research. 107:557-566.

Dewar, R.C. and M.G.R. Cannell 1992. Carbon sequestration in the trees, products and soils of forest plantations - an analysis using UK examples. Tree Physiology. 11:49-71.

Donaldson, J.R., E.L. Kruger and R.L. Lindroth 2006. Competition- and resource-mediated tradeoffs between growth and defensive chemistry in trembling aspen (*Populus tremuloides*). New Phytologist. 169:561-570.

Dubey, V.S., R. Bhalla and R. Luthra 2003. An overview of the non-mevalonate pathway for terpenoid biosynthesis in plants. Journal of Biosciences. 28:637-646.

Durrant, W. and X. Dong 2004. Systemic acquired resistance. Annual Review of Phytopathology. 42:185-209.

Enebak, S.A. and W.A. Carey 2006. Evidence for induced systemic protection to fusiform rust in loblolly pine by plant-growth promoting rhizobacteria. Plant Disease 84: 306-308.

Ennos, R. and K. Swales 1991. Genetic-variation in a fungal pathogen - response to host defensive chemicals. Evolution. 45:190-204.

Entry, J., K. Cromack, E. Hansen and R. Waring 1991. Response of western coniferous seedlings to infection by *Armillaria ostoyae* under limited light and nitrogen. Phytopathology. 81:89-94.

Erbilgin, N., P. Krokene, E. Christiansen, G. Zeneli and J. Gershenzon 2006. Exogenous application of methyl jasmonate elicits defenses in Norway spruce (*Picea abies*) and reduces host colonization by the bark beetle *Ips typographus*. Oecologia. 148:426-436.

Erbilgin, N. and K.F. Raffa 2002. Association of declining red pine stands with reduced populations of bark beetle predators, seasonal increases in root colonizing insects, and incidence of root pathogens. Forest Ecology and Management. 164:221-236.

Evensen, P.C., H. Solheim, K. Hoiland and J. Stenersen 2000. Induced resistance of Norway spruce, variation of phenolic compounds and their effects on fungal pathogens. Forest Pathology. 30:97-108.

Eyles, A., R. Chorbadjian, C. Wallis, R. Hansen, D. Cipollini, D. Herms and P. Bonello 2007a. Cross-effects of systemic induced resistance between an insect and a fungal pathogen in Austrian pine over a fertility gradient. Oecologia. 153:365-374.

Eyles, A., K. Riedl, W. Jones, S. Schwartz, K. Chan, D. Herms, D. Cipollini and P. Bonello 2007b. Comparative phloem chemistry of Manchurian (*Fraxinus mandshurica*) and two North American ash species (*F. americana* and *F. pennsylvanica*). Journal of Chemical Ecology. 33:1430-1448.

Eyles, A., N. Davies and C. Mohammed 2003. Novel detection of formylated phloroglucinol compounds (FPCs) in the wound-associated wood of *Eucalyptus globulus* and *E. nitens*. Journal of Chemical Ecology. 29:863-880.

Faldt, J., D. Martin, B. Miller, S. Rawat and J. Bohlmann 2003. Traumatic resin defense in Norway spruce (*Picea abies*): Methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. Plant Molecular Biology. 51:119-133.

Faldt, J., H. Solheim, B. Langstrom and A. Borg-Karlson 2006. Influence of fungal infection and wounding on contents and enantiomeric compositions of monoterpenes in phloem of *Pinus sylvestris*. Journal of Chemical Ecology. 32:1779-1795.

Farmer, E.E., E. Almeras and V. Krishnamurthy 2003. Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Current Opinion in Plant Biology. 6:372-378.

Felton, G.W., K.L. Korth, J.L. Bi, S.V. Wesley, D.V. Huhman, M.C. Mathews, J.B. Murphy, C. Lamb and R.A. Dixon 1999. Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. Current Biology. 9:317-320.

Ferreira, R.B., S. Monteiro, R. Freitas, C.N. Santos, Z.J. Chen, L.M. Batista, J. Duarte, A. Borges and A.R. Teixeira 2006. Fungal pathogens: The battle for plant infection. Critical Reviews in Plant Sciences. 25:505-524.

Flor, H.H. 1971. Current status of gene-for-gene concept. Annual Review of Phytopathology. 9:275-&.

Franceschi, V. 2001. Calcium oxalate in plants. Trends in Plant Science. 6:331-331.

Franceschi, V., P. Krokene, E. Christiansen and T. Krekling 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. New Phytologist. 167:353-75.

Franceschi, V., P. Krokene, T. Krekling and E. Christiansen 2000. Phloem parenchyma cells are involved in local and distant defense responses to fungal inoculation or bark-beetle attack in Norway spruce (Pinaceae). American Journal of Botany. 87:314-26.

Franceschi, V.R., T. Krekling, A.A. Berryman and E. Christiansen 1998. Specialized phloem parenchyma cells in Norway spruce (Pinaceae) bark are an important site of defense reactions. American Journal of Botany. 85:601-615.

Franceschi, V.R., T. Krekling and E. Christiansen 2002. Application of methyl jasmonate on Picea abies (Pinaceae) stems induces defense-related responses in phloem and xylem. American Journal of Botany. 89:578-586.

Franceschi, V.R. and P.A. Nakata 2005. Calcium oxalate in plants: Formation and function. Annual Review of Plant Biology. 56:41-71.

Friedli, J. and S. Bacher 2001. Mutualistic interaction between a weevil and a rust fungus, two parasites of the weed *Cirsium arvense*. Oecologia. 129:571-576.

Funk, C., E. Lewinsohn, B.S. Vogel, C.L. Steele and R. Croteau 1994. Regulation of oleoresinosis in grand fir (*Abies grandis*) - coordinate induction of monoterpene and diterpene cyclases and 2 cytochrome-P450 dependent diterpenoid hydroxylases by stem wounding. Plant Physiology. 106:999-1005.

Gershenzon, J. 1994. Metabolic costs of terpenoid accumulation in higher plants. Journal of Chemical Ecology. 20:1281-1328.

Glynn, C., D.A. Herms, C.M. Orians, R.C. Hansen and S. Larsson 2007. Testing the growth-differentiation balance hypothesis: dynamic responses of willows to nutrient availability. New Phytologist. 176:623-634.

Goodale, C.L., M.J. Apps, R.A. Birdsey, C.B. Field, L.S. Heath, R.A. Houghton, J.C. Jenkins, G.H. Kohlmaier, W. Kurz, S.R. Liu, G.J. Nabuurs, S. Nilsson and A.Z. Shvidenko 2002. Forest carbon sinks in the Northern Hemisphere. Ecological Applications. 12:891-899.

Grossnickle, S.C. and C.P.P. Reid 1982. The use of ectomycorrhizal conifer seedlings in the revegetation of a high-elevation mine site. Canadian Journal of Forest Research. 12:354-361.

Hale, B.K., D.A. Herms, R.C. Hansen, T.P. Clausen and D. Arnold 2005. Effects of drought stress and nutrient availability on dry matter allocation, phenolic glycosides, and rapid induced resistance of poplar to two lymantriid defoliators. Journal of Chemical Ecology. 31:2601-2620.

Harrington, T.C. and M.J. Wingfield 1998. Disease and the ecology of indigenous and exotic pines. *In* Ecology and Biogeography of *Pinus* Ed. D.M. Richardson. Cambridge University Press, New York, pp. 381-404.

Harris, R. 1976. Arboriculture: Care of trees, shrubs and vines. Prentice Hall, Inc., Upper Saddle River, NJ.

Hart, J.H. 1981. Role of phytostillbenes in decay and disease resistance. Annual Review of Phytopathology. 19:437-458.

Hart, J.H. and D.M. Shrimpton 1979. Roles of stilbenes in resistance of wood to decay. Phytopathology. 69:1138-1143.

Hatcher, P. and N. Paul 2000. Beetle grazing reduces natural infection of *Rumex obtusifolius* by fungal pathogens. New Phytologist. 146:325-333.

Hatcher, P.E., J. Moore, J.E. Taylor, G.W. Tinney and N.D. Paul 2004. Phytohormones and plant-herbivore-pathogen interactions: Integrating the molecular with the ecological. Ecology. 85:59-69.

Hatcher, P.E., N.D. Paul, P.G. Ayres and J.B. Whittaker 1994. Interactions between *Rumex* Spp, herbivores and a rust fungus - *Gastrophysa viridula* grazing reduces subsequent infection by *Uromyces rumicis*. Functional Ecology. 8:265-272.

Heil, M. 2001. The ecological concept of costs of induced systemic resistance (ISR). European Journal of Plant Pathology. 107:137-146.

Herms, D.A. 2002. Effects of fertilization on insect resistance of woody ornamental plants: Reassessing an entrenched paradigm. Environmental Entomology. 31:923-933.

Herms, D.A. and W.J. Mattson 1992. The dilemma of plants - to grow or defend. Quarterly Review of Biology. 67:283-335.

Heyworth, C.J., G.R. Iason, V. Temperton, P.G. Jarvis and A.J. Duncan 1998. The effect of elevated CO2 concentration and nutrient supply on carbon-based plant secondary metabolites in Pinus sylvestris L. Oecologia. 115:344-350.

Himejima, M., K. Hobson, T. Otsuka, D. Wood and I. Kubo 1992. Antimicrobial terpenes from oleoresin of ponderosa pine tree *Pinus ponderosa* - a defense-mechanism against microbial invasion. Journal of Chemical Ecology. 18:1809-1818.

Huber, D.P.W., R.N. Philippe, L.L. Madilao, R.N. Sturrock and J. Bohlmann 2005. Changes in anatomy and terpene chemistry in roots of Douglas-fir seedlings following treatment with methyl jasmonate. Tree Physiology. 25:1075-1083.

Hudgins, J.W., E. Christiansen and V.R. Franceschi 2003. Methyl jasmonate induces changes mimicking anatomical defenses in diverse members of the Pinaceae. Tree Physiology. 23:361-371.

Hudgins, J.W., E. Christiansen and V.R. Franceschi 2004. Induction of anatomically based defense responses in stems of diverse conifers by methyl jasmonate: a phylogenetic perspective. Tree Physiology. 24:251-264.

Hudgins, J.W. and V.R. Franceschi 2004. Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation. Plant Physiology. 135:2134-2149.

Hudgins, J.W., G.I. McDonald, P.J. Zambino, N.B. Klopfenstein and V.R. Franceschi 2005. Anatomical and cellular responses of *Pinus monticola* stem tissues to invasion by *Cronartium ribicola*. Forest Pathology. 35:423-443.

Hudgins, J.W., S.G. Ralph, V.R. Franceschi and J. Bohlmann 2006. Ethylene in induced conifer defense: cDNA cloning, protein expression, and cellular and subcellular localization of 1-aminocyclopropane-1-carboxylate oxidase in resin duct and phenolic parenchyma cells. Planta. 224:865-877.

Johnson, T.G., J.S. Vissage and D.P. Stratton 2001. The United States. *In* Assessment of timber product output and use, 1996 Ed. T. Johnson. USDA Forestry Service Southern Research Station, Asheville, NC, pp. 1-16.

Jurc, D., S. Bojovic and M. Jurc 1999. Influence of endogenous terpenes on growth of three endophytic fungi from the needles of *Pinus nigra* Arnold. Phyton-Annales Rei Botanicae. 39:225-229.

Kammerer, D., R. Carle and A. Schieber 2004. Characterization of phenolic acids in black carrots (*Daucus carota* ssp *sativus* var. *atrorubens* Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. Rapid Communications in Mass Spectrometry. 18:1331-1340.

Karonen, M., M. Hamalainen, R. Nieminen, K.D. Klika, J. Loponen, V.V. Ovcharenka, E. Moilanen and K. Pihlaja 2004. Phenolic extractions from the bark of *Pinus sylvestris* L. and their effects on inflammatory mediators nitric oxide and prostaglandin E_2. Journal of Agricultural and Food Chemistry. 52:7532-7540.

Keeley, J.E. and P.H. Zedler 1998. Evolution of life histories in *Pinus*. *In* Ecology and Biogeography of *Pinus* Ed. D.M. Richardson. Cambridge University Press, New York, pp. 219-250.

Keeling, C.I. and J. Bohlmann 2006. Diterpene resin acids in conifers. Phytochemistry. 67:2415-2423.

Kersten, P., B. Kopper, K. Raffa and B. Illman 2006. Rapid analysis of abietanes in conifers. Journal of Chemical Ecology. 32:2679-2685.

Klepzig, K.D., E.L. Kruger, E.B. Smalley and K.F. Raffa 1995. Effects of biotic and abiotic stress on induced accumulation of terpenes and phenolics in red pines inoculated with bark beetle-vectored fungus. Journal of Chemical Ecology. 21:601-626.

Klepzig, K.D., D.J. Robison, G. Fowler, P.R. Minchin, F.P. Hain and H.L. Allen 2005. Effects of mass inoculation on induced oleoresin response in intensively managed loblolly pine. Tree Physiology. 25:681-688.

Klepzig, K.D., E.B. Smalley and K.F. Raffa 1996. Combined chemical defenses against an insect-fungal complex. Journal of Chemical Ecology. 22:1367-1388.

Kloepper, J.W., C.M. Ryu and S.A. Zhang 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology. 94:1259-1266.

Kopper, B.J., B.L. Illman, P.J. Kersten, K.D. Klepzig and K.F. Raffa 2005. Effects of diterpene acids on components of a conifer bark beetle-fungal interaction: Tolerance by *Ips pini* and sensitivity by its associate *Ophiostoma ips*. Environmental Entomology. 34:486-493.

Koricheva, J., S. Larsson, E. Haukioja and M. Keinanen 1998. Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. Oikos. 83:212-226.

Koricheva, J., H. Nykanen and E. Gianoli 2004. Meta-analysis of trade-offs among plant antiherbivore defenses: Are plants jacks-of-all-trades, masters of all? American Naturalist. 163:E64-E75.

Kraus, T.E.C., R.A. Dahlgren and R.J. Zasoski 2003. Tannins in nutrient dynamics of forest ecosystems - a review. Plant and Soil. 256:41-66.

Krekling, T., V.R. Franceschi, P. Krokene and H. Solheim 2004. Differential anatomical response of Norway spruce stem tissues to sterile and fungus infected inoculations. Trees-Structure and Function. 18:1-9.

Krokene, P., E. Christensen, H. Solheim, V.R. Franceschi and A.A. Berryman 1999. Induced resistance to pathogenic fungi in Norway spruce. Plant Physiology. 121:565-569.

Krokene, P., H. Solheim and E. Christensen 2001. Induction of disease resistance in Norway Spruce (*Picea abies*) by necrotizing fungi. Plant Pathology. 50:230-233.

Krokene, P., H. Solheim, T. Krekling and E. Christiansen 2003. Inducible anatomical defense responses in Norway spruce stems and their possible role in induced resistance. Tree Physiology. 23:191-7.

Kytö, M., P. Niemela and E. Annila 1998. Effects of vitality fertilization on the resin flow and vigour of Scots pine in Finland. Forest Ecology and Management. 102:121-130.

Kytö, M., P. Niemela, E. Annila and M. Varama 1999. Effects of forest fertilization on the radial growth and resin exudation of insect-defoliated Scots pines. Journal of Applied Ecology. 36:763-769.

Lambert, M. 1986. Sulfur and nitrogen nutrition and their interactive effects on *Dothistroma* infection in *Pinus radiata*. Canadian Journal of Forestry Research. 16:1055-62.

Langenheim, J.H. 1994. Higher-plant terpenoids - a phytocentric overview of their ecological roles. Journal of Chemical Ecology. 20:1223-1280.

Latta, R.G., Y.B. Linhart, L. Lundquist and M.A. Snyder 2000. Patterns of monoterpene variation within individual trees in ponderosa pine. Journal of Chemical Ecology. 26:1341-1357.

Lee, I.B., P. Fynn and T. Short 2000. Development and evaluation of a computer-controlled fertigation system. Applied Engineering in Agriculture. 16:279-284.

Le Maitre, D.C. 1998. Pines in cultivation: a global view. *In* Ecology and Biogeography of *Pinus* Ed. D.M. Richardson. Cambridge University Press, New York, pp. 407-431.

Lewinsohn, E., M. Gijzen and R. Croteau 1991. Defense-mechanisms of conifers - Differences in constitutive and wound-induced monoterpene biosynthesis among species. Plant Physiology. 96:44-49.

Lieutier, F., D. Sauvard, F. Brignolas, V. Picron, A. Yart, C. Bastien and C. JayAllemand 1996. Changes in phenolic metabolites of Scots-pine phloem induced by *Ophiostoma brunneo-ciliatum*, a bark-beetle-associated fungus. European Journal of Forest Pathology. 26:145-158.

Lieutier, F., A. Yart, C. Jayallemand and L. Delorme 1991. Preliminary investigations on phenolics as a response of Scots pine phloem to attacks by bark beetles and associated fungi. European Journal of Forest Pathology. 21:354-364.

Lindberg, M., L. Lundgren, R. Gref and M. Johansson 1992. Stilbenes and resin acids in relation to the penetration of *Heterobasidion annosum* through the bark of *Picea abies*. European Journal of Forest Pathology. 22:95-106.

Lindgren, B., G. Nordlander and G. Birgersson 1996. Feeding deterrence of verbenone to the pine weevil, *Hylobius abietis* (L.) (Col., Curculionidae). Journal of Applied Entomology. 120:397-403.

Little, E.L. 2001. Austrian Pine. *In* National Audubon Society Field Guide to North American Trees Eastern Region. Alfred A. Knopf, New York, pp. 290-291.

Liu, R.J., A.K.M. Ekramoddoullah and A. Zamani 2005. A class IV chitinase is up-regulated by fungal infection and abiotic stresses and associated with slow-canker-growth resistance to *Cronartium ribicola* in Western white pine (*Pinus monticola*). Phytopathology. 95:284-291.

Lombardero, M.J., M.P. Ayres and B.D. Ayres 2006. Effects of fire and mechanical wounding on *Pinus resinosa* resin defenses, beetle attacks, and pathogens. Forest Ecology and Management. 225:349-358.

Luchi, N., R. Ma, P. Capretti and P. Bonello 2005. Systemic induction of traumatic resin ducts and resin flow in Austrian pine by wounding and inoculation with *Sphaeropsis sapinea* and *Diplodia scrobiculata*. Planta. 221:75-84.

Mackey, D., B.F. Holt, A. Wiig and J.L. Dangl 2002. RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell. 108:743-754.

Manion, P.D. 1996. Tree Disease Concepts., 2nd edition. Prentice Hall, Inc., Upper Saddle River, NJ.

Maresi, G., P. Ambrosi, A. Battisti, P. Capretti, R. Danti, E. Feci, S. Minerbi and S. Tegli 2001. Pine dieback by *Sphaeropsis sapinea* in Northern and Central Italy. *In* The Proceedings of the Shoot and Foliage Diseases Conference, IUFRO Working Party, Hyytiälä, Finland, pp. 60-67.

Martin, D.M., J. Gershenzon and J. Bohlmann 2003. Induction of volatile terpene biosynthesis and diurnal emission by methyl jasmonate in foliage of Norway spruce. Plant Physiology. 132:1586-1599.

Martin, D., D. Tholl, J. Gershenzon and J. Bohlmann 2002. Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems. Plant Physiology. 129:1003-1018.

Mattila, U., R. Jalkanen and A. Nilula 2001. The effects of forest structure and site characteristics on probability of pine twisting rust damage in young Scots pine stands. Forest Ecology and Management. 142:89-97.

Mauch-Mani, B. and J.P. Metraux 1998. Salicylic acid and systemic acquired resistance to pathogen attack. Annals of Botany. 82:535-540.

McSpadden Gardener, B. 2006. Statistical analyses of microbiological and environmental data. *In* Modern Soil Microbiology, 2nd Edition Eds. J. van Elsas and J. Jannson. CRC Press, Boca Raton, FL, pp. 555-585.

Metraux, J.P. 2001. Systemic acquired resistance and salicylic acid: current state of knowledge. European Journal of Plant Pathology. 107:13-18.

Metraux, J.P., C. Nawrath and T. Genoud 2002. Systemic acquired resistance. Euphytica. 124:237-243.

Miller, B., L.L. Madilao, S. Ralph and J. Bohlmann 2005. Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. Plant Physiology. 137:369-382.

Mirov, N. and J. Hasbrouck 1976. The story of pines. Indiana University Press. 148 p.

Moran, P.J. 1998. Plant-mediated interactions between insects and a fungal plant pathogen and the role of plant chemical responses to infection. Oecologia. 115:523-530.

Moran, P.J. and J.C. Schultz 1998. Ecological and chemical associations among late-season squash pests. Environmental Entomology. 27:39-44.

Moreira, A.C. and J.M.S. Martins 2005. Influence of site factors on the impact of *Phytophthora cinnamomi* in cork oak stands in Portugal. Forest Pathology. 35:145-162.

Mumm, R. and M. Hilker 2006. Direct and indirect chemical defence of pine against folivorous insects. Trends in Plant Science. 11:351-358.

Nagy, N.E., V.R. Franceschi, H. Solheim, T. Krekling and E. Christiansen 2000. Wound-induced traumatic resin duct development in stems of Norway spruce (Pinaceae): Anatomy and cytochemical traits. American Journal of Botany. 87:302-313.

Newmaster, S.G., F.W. Bell, C.R. Roosenboom, H.A. Cole and W.D. Towill 2006. Restoration of floral diversity through plantations on abandoned agricultural land. Canadian Journal of Forest Research. 36:1218-1235.

Nordlander, G. 1990. Limonene inhibits attraction to alpha-pinene in the pine weevils *Hylobius abietis* and *H. pinastri*. Journal of Chemical Ecology. 16:1307-1320.

Nosetto, M.D., E.G. Jobbagy and J.M. Paruelo 2006. Carbon sequestration in semi-arid rangelands: Comparison of *Pinus ponderosa* plantations and grazing exclusion in NW Patagonia. Journal of Arid Environments. 67:142-156.

Osier, T.L. and R.L. Lindroth 2001. Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. Journal of Chemical Ecology. 27:1289-1313.

Oven, P. and N. Torelli 1994. Wound response of the bark in healthy and declining silver firs (*Abies alba*). IAWA Journal. 15:407-415.

Padgett, G.B., J.S. Russin, J.P. Snow, D.J. Boethel and G.T. Berggren 1994. Interactions among the soybean looper (Lepidoptera, Noctuidae), threecornered alfalfa hopper (Homoptera-Membracidae), stem canker, and red crown rot in soybean. Journal of Entomological Science. 29:110-119.

Pan, H. and L.N. Lundgren 1996. Phenolics from the inner bark of *Pinus sylvestris*. Phytochemistry. 42:1185-1189.

Park, J.M. and K.H. Paek 2007. Recognition and response in plant-pathogen interactions. Journal of Plant Biology. 50:132-138.

Pearce, R.B. 1996. Antimicrobial defences in the wood of living trees. New Phytologist. 132:203-233.

Phillips, M.A. and R.B. Croteau 1999. Resin-based defenses in conifers. Trends in Plant Science. 4:184-190.

Pieterse, C.M.J., J.A. Van Pelt, S.C.M. Van Wees, J. Ton, K.M. Leon-Kloosterziel, J.J.B. Keurentjes, B.W.M. Verhagen, M. Knoester, I. Van der Sluis, P. Bakker and L.C. Van Loon 2001. Rhizobacteria-mediated induced systemic resistance: Triggering, signaling and expression. European Journal of Plant Pathology. 107:51-61.

Pieterse, C.M.J., J.A. Van Pelt, B.W.M. Verhagen, J. Ton, S.C.M. Van Wees, K.M. Leon-Kloosterziel and L.C. Van Loon 2003. Induced systemic resistance by plant growth-promoting rhizobacteria. Symbiosis. 35:39-54.

Purrington, C.B. 2000. Costs of resistance. Current Opinion in Plant Biology. 3:305-308.

Raffa, K.F., A.A. Berryman, J. Simasko, W. Teal and B.L. Wong 1985. Effects of grand fir monoterpenes on the fir engraver, *Scolytus ventralis* (Coleoptera, Scolytidae), and its symbiotic fungus. Environmental Entomology. 14:552-556.

Raffa, K.F., S.C. Krause and P.B. Reich 1998. Long-term effects of defoliation on red pine suitability to insects feeding on diverse plant tissues. Ecology. 79:2352-2364.

Raffa, K.F. and E.B. Smalley 1995. Interaction of pre-attack and induced monoterpene host conifer defense against bark beetle-fungal complexes. Oecologia. 102:285-295.

Ralph, S.G., H. Yueh, M. Friedmann, D. Aeschliman, J.A. Zeznik, C.C. Nelson, Y.S.N. Butterfield, R. Kirkpatrick, J. Liu, S.J.M. Jones, M.A. Marra, C.J. Douglas, K. Ritland and J. Bohlmann 2006. Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. Plant Cell and Environment. 29:1545-1570.

Read, D.J. 1998. The mycorrhizal status of *Pinus*. *In* Ecology and Biogeography of *Pinus* Ed. D.M. Richardson. Cambridge University Press, New York, pp. 324-340.

Reglinski, T., F.J.L. Stavely and J.T. Taylor 1998. Induction of phenylalanine ammonia lyase activity and control of *Sphaeropsis sapinea* infection in *Pinus radiata* by 5-chlorosalicylic acid. European Journal of Forest Pathology. 28:153-158.

Rey, F. and F. Berger 2006. Management of Austrian black pine on marly lands for sustainable protection against erosion (Southern Alps, france). New Forests. 31:535-543.

Rhoades, D. 1979. Evolution of plant chemical defense against herbivores. *In* Herbivores: Their interaction with secondary plant metabolites Eds. G. Rosenthal and D. Janzen. Academic Press, New York, pp. 1-55.

Richardson, D.M. and P.W. Rundel 1998. Ecology and biogeography of *Pinus*: and introduction. *In* Ecology and Biogeography of *Pinus* Ed. D. Richardson. Cambridge University Press, New York, pp. 3-40.

Rosner, S. and B. Hannrup 2004. Resin canal traits relevant for constitutive resistance of Norway spruce against bark beetles: environmental and genetic variability. Forest Ecology and Management. 200:77-87.

Rostas, M. and M. Hilker 2002. Feeding damage by larvae of the mustard leaf beetle deters conspecific females from oviposition and feeding. Entomologia Experimentalis Et Applicata. 103:267-277.

Rostas, M., M. Simon and M. Hilker 2003. Ecological cross-effects of induced plant responses towards herbivores and phytopathogenic fungi. Basic and Applied Ecology. 4:43-62.

Ruel, J.J., M.P. Ayres and P.L. Lorio 1998. Loblolly pine responds to mechanical wounding with increased resin flow. Canadian Journal of Forest Research. 28:596-602.

Russo, V.M., B.M. Russo, M. Peters, P. PerkinsVeazie and B. Cartwright 1997. Interaction of *Colletotrichum orbiculare* with thrips and aphid feeding on watermelon seedlings. Crop Protection. 16:581-584.

Saikkonen, K., S. Neuvonen and P. Kainulainen 1995. Oviposition and larval performance of European pine sawfly in relation to irrigation, simulated acid rain and resin acid concentration in Scots pine. Oikos. 74:273-282.

Sampson, R., G. Moll and J. Keilbaso 1992. Opportunites to increase urban forests and the potential impacts on carbon storage and conservation. *In* Forests and Global Change, American Forests, Washington, DC, pp. 51-67.

Sarma, B.K. and U.P. Singh 2003. Ferulic acid may prevent infection of *Cicer arietinum* by *Sclerotium rolfsii*. World Journal of Microbiology & Biotechnology. 19:123-127.

Savage, T.J., M.W. Hatch and R. Croteau 1994. Monoterpene synthases of *Pinus contorta* and related conifers - a new class of terpenoid cyclase. Journal of Biological Chemistry. 269:4012-4020.

Schmidt, A., G. Zeneli, A. Hietala, C. Fossdal, P. Korkene and E. Christiansen 2005. Induced chemical defenses in conifers: biochemical and molecular approaches to studying their function. *In* Chemical ecology and phytochemistry of forest ecosystems Ed. J. Romeo. Elsevier, Amsterdam, pp. 1-28.

Schneider, H. 1980. Deposition of wound gum, callose, and suberin as responses to diseases and wounding of citrus. Bulletin De La Societe Botanique De France-Actualites Botaniques. 127:143-150.

Schoeneweiss, D. 1975. Predisposition, stress, and plant disease. Annual Review of Phytopathology. 13:193-211.

Schoenholtz, S.H., J.A. Burger and J.L. Torbert 1987. Natural mycorrhizal colonization of pines on reclaimed surface mines in Virginia. Journal of Environmental Quality. 16:143-146.

Seppanen, S., L. Syrjala, K. von Weissenberg, T. Teeri, L. Paajanen and A. Pappinen 2004. Antifungal activity of stilbenes in in vitro bioassays and in transgenic *Populus* expressing a gene encoding pinosylvin synthase. Plant Cell Reports. 22:584-593.

Shain, L. 1967. Resistance of sapwood in stems of loblolly pine to infection by *Fomes annosus*. Phytopathology. 57:1034-45.

Shain, L. 1971. The response of sapwood of Norway spruce to infection by *Fomes annosus*. Phytopathology. 61:301-307.

Sharma, P., D. Borja, P. Stougaard and A. Lonneborg 1993. PR-proteins accumulating in spruce roots infected with a pathogenic *Pythium* sp isolate include chitinases, chitosanases and beta-1,3-glucanases. Physiological and Molecular Plant Pathology. 43:57-67.

Shrimpton, D. and H. Whitney 1968. Inhibition of growth of blue stain fungi by wood extractive. Canadian Journal of Botany. 46:757-61.

Simon, M. and M. Hilker 2003. Herbivores and pathogens on willow: do they affect each other? Agricultural and Forest Entomology. 5:275-284.

Sinclair, W., H. Lyon and W. Johnson 1987. Diseases of Trees and Shrubs. Cornell University Press, Ithaca, NY.

Snoeijers, S.S., A. Perez-Garcia, M. Joosten and P. de Wit 2000. The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. European Journal of Plant Pathology. 106:493-506.

Stamp, N. 2003. Out of the quagmire of plant defense hypotheses. Quarterly Review of Biology. 78:23-55.

Stamp, N. 2004. Can the growth-differentiation balance hypothesis be tested rigorously? Oikos. 107:439-448.

Stanosz, G.R., J.T. Blodgett, D.R. Smith and E.L. Kruger 2001. Water stress and *Sphaeropsis sapinea* as a latent pathogen of red pine seedlings. New Phytologist. 149:531-538.

Stanosz, G.R. and J. Cummings Carlson 1996. Association of mortality of recently planted seedlings and established saplings in red pine plantations with Sphaeropsis collar rot. Plant Disease. 80:750-753.

Sticher, L., B. MauchMani and J.P. Metraux 1997. Systemic acquired resistance. Annual Review of Phytopathology. 35:235-270.

Storer, A.J., D.L. Wood and T.R. Gordon 2002. Effects of pitch canker pathogen on gallery excavation and oviposition by Ips paraconfusus (Coleoptera : Scolytidae). Canadian Entomologist. 134:519-528.

Stout, M., J. Thaler and B. Thomma 2006. Plant mediated interactions between pathogenic microorganisms and herbaceous arthropods. Annual Review of Entomology. 51:663-689.

Stout, M.J., A.L. Fidantsef, S.S. Duffey and R.M. Bostock 1999. Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. Physiological and Molecular Plant Pathology. 54:115-130.

Sullivan, K.B. 1969. From open pit mines to grass and pines. Soil Conservation. 35:29.

Swedjemark, G., B. Karlsson and J. Stenlid 2007. Exclusion of *Heterobasidion parviporum* from inoculated clones of *Picea abies* and evidence of systemic induced resistance. Scandinavian Journal of Forest Research. 22:110-117.

Tholl, D. 2006. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. Current Opinion in Plant Biology. 9:297-304.

Thomas, B. and M.A. Hall 1979. Control of wound callose formation in willow phloem. Journal of Experimental Botany. 30:449-458.

Tomova, L., S. Braun and W. Fluckiger 2005. The effect of nitrogen fertilization on fungistatic phenolic compounds in roots of beech (*Fagus sylvatica*) and Norway spruce (*Picea abies*). Forest Pathology. 35:262-276.

Ton, J., J.A. van Pelt, L.C. van Loon and C.M.J. Pieterse 2002. Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. Molecular Plant-Microbe Interactions. 15:27-34.

Trapp, S. and R. Croteau 2001. Defensive resin biosynthesis in conifers. Annual Review of Plant Physiology and Plant Molecular Biology. 52:689-724.

Turtola, S., A.M. Manninen, J.K. Holopainen, T. Levula, H. Raitio and P. Kainulainen 2002. Secondary metabolite concentrations and terpene emissions of Scots pine xylem after long-term forest fertilization. Journal of Environmental Quality. 31:1694-1701.

Tuzun, S. 2007. Terminology related to induced systemic resistance: Incorrect use of synonyms may lead to a scientific dilemma by misleading interpretation of results. *In* Multigenic and Induced Systemic Resistance in Plants Eds. S. Tuzun and E. Bent. Springer, New York, pp. 1-8.

USDA 1998. USDA Census of Agriculture:1998. Census of Horticultural Specialties, Vol 2

Vallauri, D.R., J. Aronson and M. Barbero 2002. An analysis of forest restoration 120 years after reforestation on badlands in the Southwestern Alps. Restoration Ecology. 10:16-26.

Valluri, J.V. and E.J. Soltes 1990. Callose formation during wound-inoculated reaction of *Pinus elliottii* to *Fusarium subglutinans*. Phytochemistry. 29:71-72.

van Akker, L., R. Alfaro and R. Brockley 2004. Effects of fertilization on resin canal defences and incidence of *Pissodes strobi* attack in interior spruce. Journal of Forest Research. 34:855-862.

van Haverbeke, D.F. 1990. European Black Pine. *In* Silvics of North America. USDA Forestry Service, Washington, DC, pp. 395-404.

van Loon, L. and E. van Strien 1999. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiological and Molecular Plant Pathology. 55:85-97.

van Loon, L.C., P. Bakker and C.M.J. Pieterse 1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology. 36:453-483.

van Loon, L.C., W. Pierpoint, T. Boller and V. Conejero 1994. Recommendations for naming plant pathogenesis-related proteins. Plant Molecular Biology Reporter. 12:245-264.

Viiri, H., E. Annila, V. Kitunen and P. Niemela 2001. Induced responses in stilbenes and terpenes in fertilized Norway spruce after inoculation with blue-stain fungus, *Ceratocystis polonica*. Trees-Structure and Function. 15:112-122.

Wainhouse, D., D.R. Rose and A.J. Peace 1997. The influence of preformed defences on the dynamic wound response in Spruce bark. Functional Ecology. 11:564-572.

Wallin, K.F. and K.F. Raffa 1999. Altered constitutive and inducible phloem monoterpenes following natural defoliation of jack pine: Implications to host mediated interguild interactions and plant defense theories. Journal of Chemical Ecology. 25:861-880.

Wang, D., A. Eyles, D. Mandich and P. Bonello 2006. Systemic aspects of host-pathogen interactions in Austrian pine (*Pinus nigra*): A proteomics approach. Physiological and Molecular Plant Pathology. 68:149-157.

Waters, E.R. 2003. Molecular adaptation and the origin of land plants. Molecular Phylogenetics and Evolution. 29:456-463.

Werner, R. 1995. Toxicity and repellency of 4-allylanisole and monoterpenes from white spruce and tamarack to the spruce beetle and eastern larch beetle (Coleoptera: Scolytidae). Environmental Entomology. 24:372-379.

Whitehill, J.G.A., J.S. Lehman and P. Bonello 2007. *Ips pini* (Curculionidae : Scolytinae) is a vector of the fungal pathogen, *Sphaeropsis sapinea* (Coelomycetes), to Austrian pines, *Pinus nigra* (Pinaceae). Environmental Entomology. 36:114-120.

Woodward, S. and R. Pearce 1988. The role of stilbenes in resistance of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) to entry of fungal pathogens. Physiological and Molecular Plant Pathology. 33:127-149.

Wu, H. and Z.H. Hu 1997. Comparative anatomy of resin ducts of the Pinaceae. Trees-Structure and Function. 11:135-143.

Zeneli, G., P. Krokene, E. Christiansen, T. Krekling and J. Gershenzon 2006. Methyl jasmonate treatment of mature Norway spruce (*Picea abies*) trees increases the accumulation of terpenoid resin components and protects against infection by *Ceratocystis polonica*, a bark beetle-associated fungus. Tree Physiology. 26:977-988.

Zou, J.P. and R.G. Cates 1995. Foliage constituents of Douglas-fir (*Pseudotsuga menziesii* (Mirb) Franco (Pinaceae)) - Their seasonal-variation and potential role in Douglas-fir resistance and silviculture management. Journal of Chemical Ecology. 21:387-402.

Zou, J.P. and R.G. Cates 1997. Effects of terpenes and phenolic and flavonoid glycosides from Douglas fir on western spruce budworm larval growth, pupal weight, and adult weight. Journal of Chemical Ecology. 23:2313-2326.