Metabolomics of *Quercus* spp. to understand and predict resistance to *Phytophthora ramorum*

DISSERTATION

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ABSTRACT

Sudden oak death (SOD) is a devastating disease of oak and tanoak in the western United States, caused by the non-native, generalist pathogen *Phytophthora ramorum* Werres et al. *Quercus agrifolia* Née (CLO—coast live oak) is a highly susceptible host of *P. ramorum* in California forests and *Quercus rubra* L. (NRO—northern red oak) is an important Eastern forest species that is also susceptible to *P. ramorum*. CLO naturally resistant to *P. ramorum* have been observed and include trees that fail to develop symptoms of infection or appear to recover after symptom development. Variability in CLO susceptibility has been associated with variation in the concentration of certain phenolic compounds produced in CLO phloem tissue, and putative phenolic biomarkers of resistance were identified from trees already infected with *P. ramorum*. However, the association between constitutive (i.e. pre-infection) levels of phenolics in naïve CLO and variation in host susceptibility has not been examined, and little is known about the relationship between NRO susceptibility and variation in phenolic levels.

This research aimed to elucidate chemical mechanisms of resistance and identify factors that may affect the production of phenolic defenses in susceptible *Quercus* spp., including CLO and NRO, before and after infection with *P. ramorum*. Time was a significant explanatory factor for the variability of certain phenolics, both within and between years, though overall variation was low and did not appear to be related to
variability in CLO susceptibility to \textit{P. ramorum}. NRO treated with a known, phosphonate-based elicitor of host defenses, were more resistant to \textit{P. ramorum}, and both constitutive and induced levels of certain phenolics were also significantly impacted.

Additionally, new approaches for identifying naturally resistant CLO based solely on constitutive concentrations of phenolics were tested. Four phenolic biomarkers of resistance were partially identified in CLO phloem collected before \textit{P. ramorum} infection and were used to estimate the probability of CLO resistance and survival following infection with \textit{P. ramorum}. Finally, this research demonstrated that Fourier-transform infrared (FT-IR) spectroscopy, combined with multivariate statistical analysis, can be used to distinguish between resistant and susceptible CLO prior to infection. The ability to identify resistant trees within forest stands could be a valuable tool for conserving and breeding resistant germplasm and identifying areas at risk of SOD. Moreover, the approaches utilized in these studies to identify resistant trees may be useful in other systems where forest pests and pathogens are of concern.
DEDICATION

In memory of my mother, Marcia Rees Conrad, whose unconditional love and endless support made everything possible.
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CHAPTER 1
LITERATURE REVIEW

**Generalized defense mechanisms in trees**

Trees are sessile organisms that have the capacity to live for hundreds and in some cases thousands of years. Because of their unique life histories, trees must be capable of responding to a variety of biotic (e.g. plant pests and pathogens) and abiotic disturbances (e.g. abnormal temperatures and drought). A tree’s longevity is a testament to its ability to successfully resist or tolerate a variety of threats over time. However, as human societies become increasingly globalized, forest ecosystems are increasingly becoming threatened by introduced organisms with the potential to adversely affect the health of the forests where they are introduced.

Trees are able to resist many of these challenges because they have evolved a suite of defense mechanisms. A tree’s longevity is dependent on the defense mechanisms that it possesses (Yamada, 2001) and the ability of those defense mechanisms to operate in a dynamic system, where the diversity of enemies is great and the environment is variable (Stamp, 2003). While some defense mechanisms are more specific in nature (e.g. they are a product of the co-evolution of a tree species with a specific biotic threat), many are less specific and therefore are capable of providing at least some degree of protection against
many different threats. These types of plant defenses can be referred to as generalized plant defenses, since they have activity against many different pest and pathogen species (Biere et al., 2004; Krischik et al., 1991). Compared to more specialized defenses, generalized defenses may be less costly to produce because they are effective against many different organisms (Andrew et al., 2007). Defense mechanisms that are less specific may be useful in situations where generalist plant pathogens or pests are of concern (e.g. organisms with broad host ranges), or where trees are facing a pest or pathogen for the first time (e.g. non-native, invasive organism) as a result of an introduction event.

Regardless of the specificity of a defense against a given pathogen or pest, trees are able to successfully defend themselves against many different threats in part due to the great diversity of defense mechanisms that each individual tree possesses. While different tree species possess different mechanisms of defense, all species possess a unique combination of physical and chemical defenses which can be used individually, additively, or synergistically to confer tolerance or resistance in most situations.

*Physical defenses*

Physical defenses exist primarily at cellular and tissue levels, and can be found throughout a tree. At the cellular level, the cell wall serves as the primary barrier to physically prevent the ingress of plant pathogens. This is possible because cell walls are fortified with compounds such as lignin and suberin, which increase the resistance of cell
walls to penetration and enzymatic degradation by pathogens and pests (Franceschi et al., 2005; Vance et al., 1980). Suberin is also found in tyloses, structures found occluding xylem vessels (Pearce and Holloway, 1984). Tyloses can prevent the spread of vascular pathogens by blocking the xylem from further ingress (Bostock and Stermer, 1989). Certain cell types can also play a role in blocking tree tissues from invading organisms. Parenchyma cells, which often store chemical defenses, may provide a barrier within certain tree tissues, like the xylem (Yamada, 2001). Once cells have been disrupted, callose or pectin-like substances (Bonello et al., 1991) are often deposited between cell walls and membranes in what are generically called cell wall appositions (CWA), papillae, or lignitubers. CWAs can contain silicon or be infused with phenolics and lignin, thus fortifying cells and preventing further spread of invading organisms (Bonello et al., 1991; Bostock and Stermer, 1989; Eyles et al., 2010; Hückelhoven, 2007).

While cellular level physical defenses cannot be observed with an unaided eye, some tissue level physical defenses can be. Spines, for example, are often visible on tree exteriors along branches and stems, and typically deter larger herbivores (e.g. ungulates) from feeding on tissues (Franceschi et al., 2005). Hair-like structures called trichomes are also located on the exterior of tissues, but are microscopic in scale and found primarily on leaves, buds, and roots where they function in water relations, and heat and light absorption and reflection (Coder, 2010). Trichomes also discourage or prevent small herbivores (e.g. insects) from feeding upon tree parts, make it more difficult for pathogen propagules to reach target surfaces, and can even prevent some pathogens from entering
through the stomata (Werres and Riedel, 2013). The density of trichomes on tissue surfaces may also increase following attack (Björkman et al., 2008), and some trichomes (i.e. glandular trichomes) exude chemicals with defensive properties. However, spines and trichomes are not the only physical defense in a tree’s arsenal. Tree bark, which can vary in thickness, texture, and toughness, is essential for preventing the ingress of many pests and pathogens (Franceschi et al., 2005; Pearce, 1996). In many cases, bark, which is composed of the periderm (outer bark), cortex, phloem, and cambial tissues, cannot be penetrated unless it has been weakened by enzymatic degradation or by wounding (Pearce, 1996). Still, the bark possesses natural cracks and openings, like lenticels on the surface of the periderm (Franceschi et al., 2005), which can be used by some organisms to enter the host. When the bark is wounded either by mechanical injury or by pest or pathogen attack, trees can produce cells in new spatial positions (Shigo, 1984). Tissue formed in this way is referred to as callus tissue; callus tissue closes wounds from the outside, while a layer of cells on the inside (i.e. barrier zone) protects trees through a process of compartmentalization (Shigo, 1984). Finally, trees can also physically defend themselves by growing quickly. Trees that shed infected or herbivorized leaves and grow new ones, or quickly form callus tissue to cover wound or infection sites, may be able to outlive those that cannot (Loehle, 1988).

Chemical defenses

Many of the cellular and tissue level physical defenses described in the preceding section function by preventing penetration or inhibiting the spread of pathogens and pests. In
many cases, physical defenses alone do not confer tolerance or resistance, which instead are conferred by a combination of physical and chemical defenses. Trees can afford to produce both types of defenses because the benefits of having multiple defenses often outweigh their costs for long lived organisms (Koricheva et al., 2004). For the purpose of this overview, chemical defenses will be divided into two categories: proteins and enzymes, and plant secondary metabolites, hereafter referred to as plant specialized metabolites (PSM) since secondary may imply that these compounds are less vital for plant livelihood.

**Defense related proteins and enzymes**

Like physical defenses, defense related proteins and enzymes are distributed throughout tree tissues (e.g. tissue layers of the bark) (Franceschi et al., 2005). Even though defense related proteins and enzymes are widely distributed, the exact function(s) of many of them is unknown. This is because functional elucidation of defense related protein and enzymes in trees is typically determined based on their structural or sequence homology to proteins and enzymes found in model herbaceous species (e.g. arabidopsis and tobacco).

Despite this limitation, a variety of defense protein and enzyme families have been identified in trees, primarily in model species of *Pinus* and *Populus*, and include various pathogenesis-related (PR) proteins, such as PR-1, PR-2 (β-1,3-glucanases), PR-3 (chitinases), PR-5 (thaumatin-like proteins), and antimicrobial peptides (reviewed in
Kovalchuk et al., 2013). For example, the induction of genes encoding the defense related proteins, β-1,3-glucanase and chitinase, were associated with mutant *Quercus petraea* (Matt.) Liebl. leaves that were more resistant to the powdery mildew fungus, *Erysiphe cichoracearum* DC. (Repka, 2002). Constitutive concentrations of PR-2 (glucanase) and PR-3 (chitinase) and the proteins encoding these enzymes were also found to be higher in resistant trees (Repka, 2002). Heightened resistance in this instance is not surprising, considering that glucanases and chitinases are capable of degrading fungal cell walls. Expression or up-regulation of PR-related proteins following infection, including PR-1, glucanases, and chitinases, has also been noted in other tree systems, including hybrid *Populus* infected with the rust *Melampsora medusae* Theum. (Miranda et al., 2007).

While many of the proteins mentioned in the previous paragraph have direct activity against the attacking microorganisms (e.g. bacterial and fungal) (van Loon et al., 2006), other defense related proteins function by detoxifying compounds produced by attacking organisms. For example, QsCAD1, a gene in *Quercus suber* L., which codes for a cinnamyl alcohol dehydrogenase, was up-regulated following root infection by *Phytophthora cinnamoni* Rands (Coelho et al., 2006). QsCAD1 is structurally homologous to an enzyme produced in grapevine that detoxifies eutypine, a toxin produced by the grapevine pathogen *Eutypa lata* (Pers: Fr.) Tul. & C. Tul., and therefore it is feasible that QsCAD1 has a similar role in oak (Coelho et al., 2006).
Finally, while many different defense-related proteins and enzymes are present within tree tissues, not all are active all the time. For example, the activity of multiple peroxidase enzymes were examined in *Picea abies* (L.) Karst. following infection by the pathogen *Ceratocystis polonica* (Siem.) C. Moreau (Nagy et al., 2004). Only highly basic peroxidase isoforms increased in activity following infection (Nagy et al., 2004). Therefore, when assessing the role of protein and enzymes in tree defense response, one must also consider that protein activity may be context specific.

**Plant specialized metabolites (PSMs)**

PSMs may be the most diverse group of defense compounds that a tree possesses. PSMs are diverse not only in structure but also in function; e.g. they can provide UV protection and contribute to alleviating drought, in addition to defense (Stamp, 2003). PSMs can be produced prior to infection (constitutive PSMs), or produced *de novo* or at elevated concentrations following infection (induced PSMs). They are found in cells and tissues throughout the tree, though they may differ in quality and quantity depending on the tissue type and age (Moore et al., 2014). There are five primary groups of PSMs: alkaloids, glucosinolates, cyanogenic glucosides, terpenes, and phenolics (Bennett and Wallsgrove, 1994). The first four groups will be reviewed in brief, while the fifth group, phenolics, the most prominent group of PSMs in the genus *Quercus*, will be reviewed more in depth in the succeeding section.
Alkaloids, which are low-molecular weight compounds containing nitrogen, are found in many different tree families, including, but not limited to, the Aceraceae, Fagaceae, and Ulmaceae, among temperate trees (Barbosa and Krischik, 1987; Facchini, 2001). They are structurally diverse (Facchini, 2001), and many are non-selective with respect to a given pathogen or pest (Franceschi et al., 2005). They are classified as qualitative defenses, due to their potency even at low concentrations (reviewed in Stamp, 2003). For example, the presence of alkaloids in a diverse group of tree families was associated with reduced favorability to the generalist pest, Lymantria dispar L.; L. dispar feeding preference was negatively correlated with alkaloid presence (Barbosa and Krischik, 1987). The alkaloid nicotine was also found to inhibit a generalist herbivore and five pathogenic Pseudomonas spp. (Krischik et al., 1991). Even though alkaloids are potent defenses, they are not always upregulated following pathogen or pest attack. Constitutive concentrations of alkaloids were relatively low in P. abies, and the concentration of alkaloids were induced in only some trees which were resistant to the European spruce bark beetle, Ips typographus L. (Schiebe et al., 2012).

Glucosinolates are sulfur and nitrogen containing compounds, which are found in a relatively limited number of plant species, but may be important for generalized defense in the species of trees in which they are found (Bennett and Wallsgrove, 1994). In trees, glucosinolates are found in Moringa spp., which are important tree crops in many tropical ecosystems (Bennett et al., 2003) However, most of the evidence to suggest their role as a generalized defense comes from non-tree host-pathosystems, where they have been
associated with reduced feeding and damage by generalist herbivores (reviewed in Bennett and Wallsgrove, 1994). Since glucosinolates are considered a qualitative defense, i.e. the compounds are potent at low concentrations, it is possible that constitutive concentrations of these compounds are enough to protect plants from pathogens and pests (Stamp, 2003; van Dam et al., 2008).

Cyanogenic glycosides, qualitative plant defense compounds containing hydrogen cyanide which is released following cell damage, are capable of providing broad protection against many pests (Moles et al., 2013; Stamp, 2003; Webber and Woodrow, 2008). Ryparosa kurrangii B.L. Webber, an understory tree species, contains high concentrations of cyanogenic glycosides, particularly in young leaves that have not yet developed physical defenses (Webber and Woodrow, 2008). Webber and Woodrow surmised that high cyanogenic glycoside concentrations protect tree leaves from herbivory by generalist pests, particularly when the foliage is young. A similar accumulation of cyanogenic glycosides was observed in leaves of mature Eucalyptus cladocalyx F. Muell. Young E. cladocalyx leaves contained more cyanide than older leaves (Gleadow and Woodrow, 2000). While the authors did not study the relationship between cyanide concentrations and herbivory directly, they concluded that vulnerable and/or high value tissues, like young foliage or reproductive organs (e.g. young flower buds), accumulate cyanogenic glycosides for defense against pests (Gleadow and Woodrow, 2000).
Finally, terpenes, which are derived from isoprene units, and include monoterpenes, diterpenes, and sesquiterpenes, are known to have generalized toxicity against many different pathogens and pests (Kovalchuk et al., 2013; Moore et al., 2014). Terpenes are often volatile and may directly or indirectly, through volatile attraction of hyperparasites, inhibit pests (reviewed in Tholl et al., 2006). Higher concentrations of terpenes were detected at the margin of Cytonaema sp. lesions on Eucalyptus globulus Labill., suggesting that terpenes are important for E. globulus defense against Cytonaema sp. (Eyles et al., 2003). Terpenes may also be important in general, i.e. less specific, plant defense responses, and may be important for resistance in some tree species. For example, P. abies that survived mass attack by L. typographus and that were treated with methyl jasmonate, a non-specific inducer of plant defenses, produced significantly higher amounts of many mono-, sesqui-, and diterpenes compared to trees that were killed (Schiebe et al., 2012). Finally, volatile emission of certain terpenes may be important for intra- and inter-plant communication, and may be associated with priming of host defenses (Baldwin et al., 2006).

Phenolics in tree defense

Phenolics are arguably the most ubiquitous and structurally diverse group of PSMs (Appel, 1993; Levin, 1971); they are derived from the shikimate and acetate/malonate pathways (Bennett and Wallsgrove, 1994; Salminen and Karonen, 2011). Compounds are classified within this group based on the presence of an aromatic ring and one or more hydroxyl groups, and based on biosynthesis and structure can be classified into different
classes, such as tannins (i.e. polyphenolic compounds containing multiple phenolic groups), flavonoids, and simple phenolic acids (Appel, 1993; Salminen and Karonen, 2011). In some instances (e.g. in nutrient limiting environments), carbon-based defenses, like some phenolics, may be less expensive to produce than nitrogen-based defenses (Coley et al., 1985). Phenolics have three primary modes of action in defense, i.e. as toxins (often after oxidation), cell wall modifiers (e.g. lignin is derived from phenolic precursors), and within plant signals following infection (Appel, 1993). In most plants, phenolics are stored in non-active glycosylated forms, usually within parenchyma cell vacuoles (Kovalchuk et al., 2013). Glycosylation makes phenolics more stable, soluble, and safe for storage, since aglycone forms can be toxic not only to the organisms that attack trees but to the trees themselves (Jones and Vogt, 2014; Kovalchuk et al., 2013).

Trees possess both constitutive and induced phenolics. Differences may exist between the two pools in composition and/or quantity of individual compounds (Bennett and Wallsgrove, 1994). The environment (e.g. nutrient, CO₂ availability, UV radiation, and temperature), tissue type, and tree or tissue age are all factors that can affect the production and activity of phenolics (Appel, 1993; Witzell and Martin, 2008). Ultimately, the efficacy of phenolic compounds in tree defense depends on their composition and concentration, as well as the organism, whether pest or pathogen, that is attacking the tree (Witzell and Martin, 2008).
Phenolics in *Quercus* spp. defense

For *Quercus* spp., phenolics are undoubtedly the most important chemical defense. *Quercus* (oak) is a widely distributed genus with approximately 300 species worldwide, the majority of which are found in North America, Europe, and Asia (Cochran, 2004). Fifty-five species, including both deciduous and evergreen trees (the latter often called live oaks), are native to the United States (Cochran, 2004). Many of these species possess both aesthetic and functional qualities. For example, oak wood is commonly used for furniture, flooring, and casks for wine and spirits, many wildlife feed on acorns, and oak trees are widely planted in the urban landscape because of their majestic nature and shady canopies (Cochran, 2004). Even though oaks do well in many different environments, they are not immune to attack by pests and pathogens. Like many of the other trees described in the preceding sections, they possess a suite of physical and chemical defenses that protect them from most organisms. Phenolics, including polyphenolic compounds such as tannins, have been the primary focus of research aimed at understanding the relationship between oak PSMs and defense against pests and pathogens.

The ability to identify and quantify individual phenolic compounds in plant tissues has improved greatly over the last several decades. This is primarily a result of advances in technology, such as the advent of high performance liquid chromatography (HPLC) and more sensitive mass spectrometers, which make it possible to identify individual oak phenolic compounds from complex mixtures. Even though quantifying phenolics does
not per se reveal which compounds are actually active in tree defense, these assays can provide hints as to which phenolics are important in a specific pest- or pathosystem. For example, in *Quercus agrifolia* Née, the concentration of foliar condensed tannins (CT) was found to increase as foliage matured (Mauffette and Oechel, 1989). During the time that foliar concentrations of CT were measured, trees were attacked by the herbivore *Phryganidia californica* Packard, the California oak moth. While the authors were unable to detect a clear relationship between foliar CT concentration and *P. californica* herbivory, they concluded that phenolics may explain, in part, variation in herbivory, at least early in the season (Mauffette and Oechel, 1989). In another example, Schultz and Baldwin (1982) found that red oak leaves formed following defoliation by the generalist pest, *Lymantria dispar* (gypsy moth), contained relatively high concentrations of hydrolysable tannins (HT) and undetectable levels of CT. This is of particular interest, considering that newer evidence suggests that HT, which are more oxidatively active at high pH (like the conditions of many insect guts) compared to CT, may be more important for active (induced) defense against insect herbivores than CT, whose main function is thought to be as protein precipitators (Appel et al., 2001; Barbehenn et al., 2006a, 2006b). Historically, CT were believed to be the most important compounds in oak defense.

Furthermore, oxidative activity of some phenolic groups has been measured in situ. For example, *Quercus rubra* L. and *Quercus alba* L. leaves contain predominantly CT and ellagitannins (ET), a subgroup of HT, respectively (Barbehenn et al., 2008). Barbehenn et
al. could detect semiquinone radicals (produced from ET) in the mid-gut of the generalist caterpillar *Orgyia leucostigma* JE Smith after feeding on *Q. alba* leaves, while CT radicals were detected in the gut of larvae that fed on *Q. rubra* leaves. Typically, radicals formed from CT are less oxidatively active than those formed from ET (Barbehenn et al., 2006a). However, measuring the oxidative activity of oak foliage and determining the defensive role of oxidation can be complicated by the fact that foliage may also contain low molecular weight compounds, like the antioxidant ascorbic acid, which can negate some of the pro-oxidant activity of ET in insect guts (Barbehenn et al., 2008).

Whether the phenolics in oak tissues are acting as protein precipitators (e.g. CT) or oxidizing agents (e.g. ET) *in situ*, phenolics can have measurable effects on pests (and pathogen) performance. For example, gypsy moth pupae were adversely impacted by maximum levels of total phenolics and HT, with pupal mass and fecundity negatively correlated with total phenolics concentration (Rossiter et al., 1988). In contrast, the concentration of HT was not significantly correlated with performance of *Operophtera brumata* L. larvae, while the survival and growth of larvae were negatively correlated with the concentration of CT in *Quercus robur* L. (Tikkanen and Julkunen-Tiitto, 2003). Therefore, the ability of an oak tree to successfully defend itself depends not only on the specific phenolics it possesses, but also on the specific organisms attacking it. Although it is possible to identify relationships between different classes of phenolics and oak defense against various pests, it is impossible to determine which phenolics are actually actively involved in defense unless more comprehensive analyses are performed.
to identify and quantify individual phenolic compounds. For example, vescalagin, a HT found in *Q. robur*, had a greater impact on two generalist herbivore moth species (*Acronicta psi* L. and *Amphipyra pyramidea* L.) compared to CT (Roslin and Salminen, 2008). Vescalagin, when added to an artificial diet fed to the two generalist moths, decreased larval growth, ingestion rate, and metabolic efficiency compared to that of larvae fed non-amended diets or diets amended with CT only (Roslin and Salminen, 2008). While vescalagin was effective in this scenario, other phenolic compounds may be effective in other pest- or pathosystems. For example, Moctezuma et al. (2014) examined the relationship between the concentration of individual phenolic compounds in *Quercus oleoides* Schltdl. & Cham. and herbivore damage caused by four different herbivore guilds. Mongolinin A and acutissimin B, both HT, were most associated with herbivory, regardless of guild, while there was no association between herbivory and the concentration of total phenolics, total HT, or total flavonols (Moctezuma et al., 2014). Additionally, higher concentrations of certain phenolics (e.g. acutissimin B, catechin, catechin dimer, and mongolinin A) were correlated with greater damage by herbivores (Moctezuma et al., 2014).

While the concentration of individual phenolics can impact the performance of the attacking organism, the timing of their accumulation or release may be more important in the defensive strategy of a given tree. For example, *Q. robur* trees that were more resistant to *Torix viridana* L., the oak leaf roller, relied more heavily on constitutive phenolic defenses (e.g. galloyl flavonol glycosides), while susceptible trees relied more
on induced phenolic defenses (e.g. flavonol glycosides) (Kersten et al., 2013). This conclusion was supported by the fact that resistant oaks contained higher constitutive concentrations of galloylated flavonol glycosides than susceptible oaks and higher concentrations of corilagin (ET) and ellagic acid (an ET derivative), while susceptible oaks contained higher concentrations of basic flavonol glycosides and free unconjugated flavonols (Kersten et al., 2013).

The common theme of the studies cited above is that oak foliar phenolics are important in defense against insect herbivores. Very few studies have examined the effect of oak phenolics on pathogen defense. Q. agrifolia, a geographically limited oak species, found primarily along the coast of California (Gaman and Firman, 2006), has become the “model” species for studying the role of oak phloem phenolics in pathogen defense, following the identification of a new disease on oak—sudden oak death. In regards to induced defenses, gallic acid and ellagic acid were found at higher concentrations within phloem tissue infected with the pathogen Phytophthora ramorum Werres, deCock & In’t Veld (the causal agent of sudden oak death) compared to trees that appeared to be healthy (i.e. showed no symptoms of P. ramorum infection) (Ockels et al., 2007). Gallic acid and tyrosol, two phenolic compounds detected at higher concentrations at the margin of P. ramorum cankers compared to regions away from cankers, both significantly reduced the in vitro growth of four Phytophthora spp., including P. ramorum (Ockels et al., 2007). Q. agrifolia that varied in susceptibility to P. ramorum also differed in respect to the concentration of three individual phenolic compounds and total peak area of
chromatograms at 370 nm (Nagle et al., 2011). Nagle et al. found that phloem collected from resistant trees (including those trees that never showed symptoms of infection or appeared to recover following infection) had higher concentrations of a tyrosol derivative compared to susceptible trees (trees that showed active symptoms of infection), and that the concentration of ellagic acid was greater in trees that recovered following infection compared to susceptible trees. Finally, a reanalysis of Nagle et al.’s phenolic data by McPherson et al. (2014) identified four putative phenolic biomarkers of resistance, including ellagic acid and an ellagic derivative; concentrations of these four compounds were then used to predict whether or not a tree was resistant to P. ramorum.

**Sudden oak death**

For the last 20 years, sudden oak death (SOD) has been recognized as the cause of extensive mortality of native red oak and tanoak species along the western coast of the United States. The disease is caused by Phytophthora ramorum, a relatively new, generalist (i.e. pathogen with a broad host range) oomycete species within a genus that is known to house many highly destructive and economically important plant pathogens (e.g. Phytophthora infestans (Mont.) de Bary the causal agent of potato late blight) (reviewed in Kamoun et al., 2014). SOD was first observed in Marin Co., CA in 1995 and was associated with dieback of Notholithocarpus densiflorus (Hook. & Arn.) Manos, C.H. Cannon & S. Oh (tanoak), and later Q. agrifolia (coast live oak), Quercus kelloggii Newb. (California black oak), and Quercus parvula var. shrevei (C.H. Muller) Nixon (Shreve oak) (McPherson et al., 2000; Rizzo et al., 2002). However, in the mid-1990s the
cause of tanoak and oak mortality was unknown. It was not until the early 2000s that \textit{P. ramorum} was identified as the causal agent (Rizzo et al., 2002), following its identification as a new pathogenic species causing twig blight on rhododendron and viburnum in Europe (Werres et al., 2001). \textit{P. ramorum} is believed to be a non-native species in North America, since molecular evidence suggests that epidemics in North American and Europe are products of separate and multiple introduction events, most likely facilitated by the global trade of live plant materials (Brasier et al., 2004; Croucher et al., 2013; Ivors et al., 2004). As a result, SOD in North America is an excellent example of what can happen when a generalist, alien invasive pathogen is introduced to naïve forests.

\textit{Hypothesized origin of \textit{P. ramorum}}

\textit{P. ramorum} is most closely related to \textit{Phytophthora lateralis} Tucker & Milbrath, another introduced \textit{Phytophthora} sp. that is the cause of root rot of Port-Orford-cedar (\textit{Chamaecyparis lawsoniana} (A. Murry) Parl.) in the Pacific Northwest (Hansen et al., 2000). While it is unknown exactly from where these species originate, recent evidence suggests that \textit{P. lateralis} may come from southeastern Asia, where \textit{Phytophthora} isolates, morphologically and molecularly similar to \textit{P. lateralis}, were isolated from soils in an old-growth \textit{Chamaecyparis} forest in Taiwan (Brasier et al., 2010). Because \textit{P. ramorum} is similar to \textit{P. lateralis}, not only in molecular phylogeny but also in growth habit, it is possible that the native range of \textit{P. ramorum} would be within this region, which houses
many native species of Fagaceae and Ericaceae, which include many host genera of *P. ramorum* in North America and Europe (Brasier et al., 2010, 2004).

*Phytophthora ramorum*

*P. ramorum* is a member of the genus that literally means “plant destroyer.” *P. ramorum* can be distinguished from other *Phytophthora* species based on morphology (e.g. sporangia, chlamydospores, and vegetative growth) and molecular analysis (e.g. ITS region, isozyme profiles, and AFLP analysis) (Rizzo et al., 2002; Werres et al., 2001). There are four known clades of *P. ramorum*: NA1, NA2, EU1, and the recently discovered EU2 (Ivors et al., 2006; reviewed in Manter et al., 2010; Van Poucke et al., 2012; Werres and Kaminski, 2005). NA1 and NA2 lineages are mating type A2, while EU1 and EU2 lineages are predominantly mating type A1 (Manter et al., 2010; Van Poucke et al., 2012; Werres and Kaminski, 2005; Werres et al., 2001). Landscape infestations of *P. ramorum* in California and Oregon can be attributed to genotypes within the NA1 lineage, while genotypes within the NA2 lineage have been found primarily within Washington and California nurseries (reviewed by Manter et al., 2010). The EU1 lineage is associated primarily with European epidemics, though it has also been isolated from nurseries in Oregon and Washington (reviewed in Manter et al., 2010). Interestingly, *P. ramorum* isolates found within North American forests (of the NA1 lineage), are less virulent (yield smaller lesions on inoculated rhododendron leaves), produce less sporangia, and have lower *in vitro* expression of ram-α2 elicitin, compared to isolates belonging to the NA2 or EU1 lineages (Manter et al., 2010). In Europe, a
second lineage, EU2, was first detected in 2007; it lacks genotypic diversity and thus is presumed to be a result of a recent introduction event (Van Poucke et al., 2012).

*P. ramorum* is mostly clonal in nature, reproducing asexually through the formation of sporangia, zoospores, and chlamydospores (Werres et al., 2001). Because *P. ramorum* is a heterothallic organism, i.e. incapable of self-fertilization, and requires two different mating types, A1 and A2, for sexual reproduction (Werres et al., 2001). In laboratory assays, *P. ramorum* can produce gametangia when crossed with different *Phytophthora* spp. of different mating types (Werres et al., 2001) or when crossed with isolates from different clonal lineages of different mating types (e.g. isolates from EU1 lineage crossed with isolates from NA1 lineage) (Van Poucke et al., 2012). Even though both mating types have been found in North American nurseries, there is no evidence to suggest that sexual reproduction is occurring regularly, and gametangia are rare even in fertile mixes in laboratory settings (Van Poucke et al., 2012).

*P. ramorum* sporangia are aerially dispersed primarily during rainy seasons (e.g. winter and spring months in coastal California), with sporulation and migration of different *P. ramorum* genotypes impacted by annual variation in rainfall patterns (Eyre et al., 2013; reviewed in Rizzo et al., 2005). *P. ramorum* can be isolated from rain water, stream water, soil, and litter (Davidson et al., 2005) and can persist both in soil and foliar environments during suboptimal climatic conditions, though the persistence of genotypes is most likely greater in foliage than in soil (Eyre et al., 2013). Epidemics are likely
driven by variation in climate (e.g. rainfall) and the presence of sporulating hosts of *P. ramorum* (Eyre et al., 2013; reviewed in Rizzo and Garbelotto, 2003).

*Host and associated species of P. ramorum*

*P. ramorum* has a broad host range, including species from over 40 plant genera (reviewed in Rizzo et al., 2005). In California forests, the primary species affected by SOD include tanoak (*N. densiflorus*) and red (section Lobatae) and intermediate (section Protobalanus) oaks; *P. ramorum* infects mainly the stems of these hosts (and also branches and leaves of tanoak) (reviewed in Rizzo and Garbelotto, 2003; Rizzo et al., 2002). There is no evidence to suggest that white oaks (section Quercus) become naturally infected (reviewed in Rizzo and Garbelotto, 2003), though chestnut and white oak seedlings (both species within the section Quercus) are susceptible when stem-inoculated in controlled assays (Tooley and Kyde, 2007). Other species associated with *P. ramorum* in California forests include California bay laurel (*Umbellularia californica* (Hook. & Arn.) Nutt.), Pacific madrone (*Arbutus menziesii* Pursh), redwood (*Sequoia sempervirens* (Lamb. ex D. Don) Endl.), and a variety of understory shrubs, including rhododendron and evergreen huckleberry; all of which primarily support brown or black sporulating lesions on foliage (reviewed in Rizzo and Garbelotto, 2003). While *P. ramorum* has not yet been found in landscapes of the Eastern United States, many plant species found within the region are known to be susceptible to *P. ramorum* infection. This includes *Q. rubra* (Northern red oak), which was found to be susceptible to natural
infections in Europe (Brasier et al., 2004) and as a result of artificial inoculation (Tooley and Kyde, 2007).

Interestingly, true oaks (Quercus spp.) are not known to support sporulating lesions, either on the surface of infected bark or from canker exudates, while both sporangia and chlamydoospores are produced on infected leaves of U. californica and other foliar hosts (Davidson et al., 2005). For this reason, oaks are considered terminal or dead-end hosts of P. ramorum (reviewed in Rizzo et al., 2005). P. ramorum isolates from non-oak hosts are more virulent (produce longer lesions) than those from oak hosts, on both U. californica leaves and Q. agrifolia stems (Huberli and Garbelotto, 2012). Whether or not variation in virulence is a direct result of P. ramorum’s ability to sporulate on some hosts and not on others is unknown. However, isolates with less virulent phenotypes (like those from non-sporeulating oak hosts) have a transposon-depressed phenotype with down-regulated expression of crinkler effector homologs compared to isolates with more virulent phenotypes (like those from sporulating U. californica) (Kasuga et al., 2012). Furthermore, seven simple sequence repeat markers in P. ramorum were analyzed and there was no indication of differentiation among the host species tested (tanoak, oak, or bay laurel) (Kasuga et al., 2012). These findings suggest that variability in isolate virulence may be a result of epigenetic silencing of transposable elements (i.e. epi-transposon hypothesis) (Kasuga et al., 2012).
SOD in Q. agrifolia and other true oaks

Foliar desiccation is the most obvious, quickly developing symptom of *P. ramorum* infection in oaks in some cases, hence the name “sudden oak death.” However, dieback is preceded by the formation of lesions (i.e. cankers) on the main stem of oak trees. Canker symptoms include brown or black discolored outer bark and a dark red, viscous exudate, commonly referred to as “blood” (Rizzo et al., 2002). When the outer bark is removed, subcortical cankers are revealed; cankers are restricted primarily to the phloem tissue, though they can reach into outer layers of the xylem (sapwood) (Rizzo et al., 2002). Individual subcortical lesions on *Q. agrifolia* are typically circular or elliptical in shape and are delineated by thin black lines (Rizzo et al., 2002). Following *P. ramorum* infection, some trees are attacked by various ambrosia (e.g. *Monarthrum scutellare* LeConte and *Xyleborinus saxeseni* Ratzeburg) and bark (e.g. *Pseudopityophthorus pubipennis* LeConte) beetles, even before foliar symptoms are evident (McPherson et al., 2008; Rizzo et al., 2002). Beetles are preferentially attracted to cankered regions on inoculated trees, and not to wounded or non-cankered (asymptomatic) infected trees (McPherson et al., 2008). While the exact mechanism of attraction is unknown, canker size may impact tree attractiveness, since basal cankers produce greater amounts of ethanol (a known attractant of many bark and ambrosia beetles) than spot cankers (Kelsey et al., 2013). Although *P. ramorum* alone can kill *Q. agrifolia* trees by girdling stems and cutting off the flow of water and nutrients to the canopy, the presence of beetle attacks on bleeding trees further reduces tree survival by an estimated 65 – 80% and can also make trees more susceptible to structural failure, i.e. stem snapping at the site of
beetle tunneling (McPherson et al., 2010). Finally, P. ramorum cankers on oak are also commonly associated with the presence of opportunistic, secondary organisms, like the fungus Annulohypoxylon thouarsianum (Lév.) Y.-M. Ju, J.D. Rogers & H.-M. Hsieh (Rizzo et al., 2002). A. thouarsianum and other fungi may thrive because of changing physiological conditions within the host tree or as a result of introduction by beetle colonizers (McPherson et al., 2013).

\textit{Q. agrifolia} susceptibility

Because of the interacting factors mentioned above, SOD can be devastating in some areas. 59 – 70% of \textit{Q. agrifolia} basal area was estimated to be lost over 20 years in sites heavily impacted by the disease in California (Brown and Allen-Diaz, 2009). On an annual basis, local infection rates can be as high as 5\% \textit{y}^{-1}, with a mortality rate of 3.1\% \textit{y}^{-1} (McPherson et al., 2010). In some areas, it is not unrealistic to expect 25\% of trees to be showing active symptoms of infection (e.g. bleeding) (McPherson et al., 2005). However, not all trees become infected, and of those that do become infected, some show limited canker development, the formation of callus tissue around cankered areas, and/or appear to recover (Dodd et al., 2005; McPherson et al., 2014; Nagle et al., 2011; reviewed in Rizzo et al., 2005; Swiecki and Bernhardt, 2005).

While the presence of uninfected trees in populations could be attributed to escape (e.g. hosts occupy ecological niches that the pathogen cannot occupy) (Dodd and Kashani 2003), variability in susceptibility to \textit{P. ramorum} in most areas, especially those where
rates of infection and mortality are high, likely has a genetic basis (Dodd et al., 2005). Dodd et al. (2005) found that variability in *Q. agrifolia* lesion sizes was greater within than between populations, and concluded that susceptibility to *P. ramorum* was most likely controlled by multiple genes. Genes associated with susceptibility have not yet been identified, though there is evidence to suggest that variability in susceptibility may be correlated with the timing of bud burst and cambial phenology (Dodd et al., 2008). Likewise, Dodd et al. (2008) showed that individuals from the same location varied in susceptibility to *P. ramorum* (varied lesion size), particularly in the spring, when mean lesion size was greatest. This study also suggested that susceptibility was heritable, since clones inoculated at different times during the year had lesion sizes that were highly correlated (Dodd et al., 2008).

While susceptibility to *P. ramorum* is very likely controlled by genetic components, the effect of the environment on variation in host susceptibility cannot be excluded. For example, variation in climate (e.g. temperature) was related to mean lesion size in the study by Dodd et al. (2008). Additionally, Swiecki and Bernhardt (2002) found that high *Q. agrifolia* stem water potential was positively associated with disease, more moist sites were at higher risk for disease, and that girdling severity and water stress were inversely related.
**SOD management**

Plant disease management can be costly, time consuming, and logistically challenging. This is particularly true in forest environments where diseases can be patchily distributed across the landscape and the benefit of saving a forest cannot be easily quantified. In addition, the level at which management occurs impacts what practices can or should be implemented. Regardless of level, for disease management to be successful, there must be comprehensive monitoring, accurate diagnosis, and effective eradication methods in place (Rizzo et al., 2005). This may be a challenge in situations where recently introduced organisms are of concern.

In the case of SOD, disease management occurs at three primary levels: individual tree, landscape or forest stand, and regional or international scale (Rizzo et al., 2005). Examples of management at the individual tree level include: preventative treatment with phosphonate based fungicides, either by direct injection into the stem or topically applied to the outer bark (Garbelotto and Schmidt, 2009; Garbelotto et al., 2009), lowering inoculum pressure by removing sporulating hosts (reviewed in Rizzo et al., 2005 and Kliejunas, 2010), and maintaining overall tree health (reviewed in Rizzo et al., 2005). At the landscape or forest stand level, Rizzo et al. (2005) suggests that management should include prevention, treatment, restoration, and conservation. Methods for implementing management at this level include cutting and burning of forests to create buffer zones to prevent further spread, and lowering inoculum pressure by removing sporulating hosts (reviewed in Rizzo et al., 2005). However, both methods may negatively impact
biodiversity, which can increase the risk of SOD in certain sites. Sites with greater biodiversity of (alternative) host species have been found to have lower disease incidence (Haas et al., 2011). Finally, at the regional and international scale, disease management is driven by prevention of pathogen introduction through regulation and management (Rizzo et al., 2005). In the case of SOD, quarantines within the United States and across foreign borders have been implemented to prevent the introduction of *P. ramorum* by preventing, regulating, and/or monitoring the trade of live plant materials that could be infested with the pathogen (Grunwald et al., 2008; reviewed in Kliejunas, 2010).

In nursery and ornamental settings, management practices, including treatment of water and soil to kill *P. ramorum*, and removal and destruction of infected plants, have been effective treatment options (reviewed in Kliejunas, 2010). Even with successful quarantines and intensive management in some areas (e.g. cut and burn and creation of buffer zones in Oregon), *P. ramorum* continues to spread in forest ecosystems. In areas where *P. ramorum* continues to spread, management plans may need to be reevaluated. In areas at the edge of the disease front, management could shift from prevention and treatment to conservation and utilization of naturally resistant germplasm. While utilizing naturally resistant trees for management may not be possible with all vulnerable host species (e.g. *N. densiflorus*) (Hayden et al., 2011), there is sufficient evidence to suggest that some host species (e.g. *Q. agrifolia*) have enough variability in susceptibility to implement this type of approach (Dodd et al., 2005; McPherson et al., 2014; Nagle et al., 2011; reviewed in Rizzo et al., 2005; Swiecki and Bernhardt, 2005).
New approaches to identify resistant trees for use in disease management

The concept of utilizing natural variability in host resistance to manage diseases of forest trees is not novel (see review by Telford et al., 2015). However, traditional approaches for identifying resistant trees are time intensive and may be destructive to threatened tree species. For example, identification of *Q. agrifolia* resistant to *P. ramorum* historically relied on monitoring trees in natural populations over several years, and/or artificially inoculating trees and waiting for symptom expression and disease development (e.g. McPherson et al., 2014; Nagle et al., 2011). These approaches cannot easily be applied over large spatial scales (e.g. at the landscape or forest stand level), due to economic, ecological, and regulatory restrictions. Additionally, genetic tools, like marker assisted selection are not widely used in forest tree research (Muranty et al., 2014), and in the case of *Q. agrifolia* no specific genes have even been linked to *P. ramorum* resistance.

Other approaches, like those that rely on the identification and utilization of chemical biomarkers or fingerprints, may provide alternative, less destructive and more rapid assays to identify resistant trees. Chemical biomarkers can be defined as compounds that can be used to distinguish between trees of varied susceptibility to a pest or pathogen, or to distinguish between healthy or infected trees. Differences can be quantitative (i.e. variation in concentration) or qualitative (i.e. presence or absence). While chemical biomarkers may include individual compounds, chemical fingerprinting does not necessarily focus on individual metabolites, but rather on the entire suite of metabolites in a given sample (Fiehn, 2002, 2001). Metabolites are not separated from one another using
this approach and compounds are not quantified individually. This allows for more rapid and high throughput analysis (Fiehn, 2002, 2001).

Chemical biomarkers or fingerprints that distinguish between plants of varied susceptibility to pests or pathogens have been identified in many systems (Ali et al., 2009; Augustyn et al., 2014; Batovska et al., 2008; Bollina et al., 2010; Hall et al., 2011; Martin et al., 2008; McPherson et al., 2014), though none of these studies actually used biomarkers or fingerprints to identify resistant (or susceptible) individuals explicitly for management purposes. This is surprising considering that Foley et al. (1998) discussed the ecological applications of one specific method of chemical fingerprinting (near infrared reflectance spectroscopy) over 15 years ago. In that paper, Foley et al. described the importance of accurate and precise predictive models generated from this approach and reviewed existing uses of near infrared reflectance spectroscopy (e.g. distinguishing between eucalyptus trees with variability in resistance to an insect pest). Even though the approach has not been used widely, it is being used on larger spatial scales (e.g. forest stand level). For example, Moore et al. (2010) used fingerprint data from near infrared reflectance spectroscopy and statistical modeling to predict variability in tree palatability, to identify potential mechanisms of variability, and to map variability across larger spatial scales. This study was an excellent example of how chemically based approaches can be used to detect variability within a forest stand, and provides evidence to suggest that approaches of this kind can be used to identify resistant trees at the landscape level.
Knowledge of resistant trees at landscape levels can guide what management practices, if any, are implemented.

**Research goals and objectives**

New approaches for managing forest diseases caused by all kinds of pathogens, but especially destructive, non-native and/or invasive organisms, are needed. Where variation in tree susceptibility has been observed, utilizing naturally resistant trees as a form of disease management may be the best option, particularly in situations where other management efforts have failed or proved to be broadly ineffective. Management efforts aimed at identifying, conserving, and breeding naturally resistant germplasm will help preserve genetic diversity in affected host species and may also help maintain the biodiversity of affected forests. However, in order to take advantage of natural resistance within forest tree populations, a better understanding of the mechanisms driving variability in host susceptibility is required. Once putative mechanisms are identified, the impact of outside factors (e.g. environment) on those mechanisms must be determined. Only then can approaches like chemical fingerprinting and biomarker discovery be used to their fullest potential, to identify resistant trees and subsequently facilitate the management of forest tree diseases.

Sudden oak death and the *Quercus – Phytophthora ramorum* pathosystem are the perfect system to test these approaches. *Q. agrifolia* is a highly susceptible host. Over the last 20+ years, variability in *Q. agrifolia* susceptibility within natural populations has been
observed. Susceptibility is believed to be controlled, at least in part, by genetic factors, and previous studies have shown that variation in disease phenotype (e.g. resistant/susceptible or healthy/symptomatic) is associated with variation in *Q. agrifolia* phenolics. Therefore, the goals of this research were to (1) elucidate chemical mechanisms of oak resistance to *P. ramorum* and to identify factors that might affect them, and (2) test new approaches for identifying naturally resistant trees before infection.

These goals are based on the central hypothesis that trees of differing susceptibility to *P. ramorum* have different phloem phenolic profiles, before (constitutive) and after (induced) infection. To test this central hypothesis, I performed a series of experiments to: (1) characterize phloem phenolics in *Q. agrifolia* before and after infection with *P. ramorum*; (2) test if chemical fingerprints can be used to distinguish between resistant and susceptible *Q. agrifolia* before infection; (3) identify phenolic biomarkers of resistance in *Q. agrifolia* and use them to predict resistance in a naïve population, and (4) identify differences in phenolic profiles of chemically induced resistant and naturally susceptible *Q. rubra*.

In the first experiment, *Q. agrifolia* phloem phenolic profiles were examined at various times before and after infection with *P. ramorum*, in resistant, susceptible, and control (non-inoculated) trees (Chapter 2). I hypothesized that tree phenology does not affect the composition and concentration of constitutive phloem phenolic compounds. I further
hypothesized that resistant trees contain higher concentrations of certain phenolic compounds compared to susceptible trees. Finally, I hypothesized that constitutive concentrations (not composition) of phenolic compounds vary over time. The objectives of this study were to (1) identify phloem phenolics in *Q. agrifolia* using high performance liquid chromatography (HPLC), photo diode array (PDA) detection, and mass spectrometry; (2) examine seasonal variation in phloem phenolic production, and (3) examine inter-annual variation in phloem phenolics in resistant, susceptible, and control *Q. agrifolia* over a three year period.

In the second and third experiments, extracts from *Q. agrifolia* phloem tissue collected before infection with *P. ramorum* were analyzed; chemical fingerprinting and metabolite profiling were used to develop models to predict whether or not a tree would be resistant to *P. ramorum* (Chapter 3 and Chapter 4, respectively). I hypothesized that chemical fingerprints and chemical biomarkers can distinguish between resistant and susceptible trees before infection, that FT-IR spectroscopy can be used to identify chemical features associated with resistant trees, and that constitutive concentrations of phenolic biomarkers can be used to estimate *Q. agrifolia* survival following *P. ramorum* infection. The primary objectives of these experiments were to: (1) determine if chemical fingerprints from Fourier-transform infrared (FT-IR) spectroscopy can distinguish between resistant and susceptible field trees before infection; (2) identify constitutive

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phloem phenolic biomarkers of resistance and develop a model to predict resistance based on the concentration of those compounds; (3) use FT-IR spectroscopy to predict the concentration of phloem phenolic biomarkers, and (4) determine if *Q. agrifolia* survival following infection can be estimated based on the constitutive concentration of biomarkers.

In the fourth experiment, I examined the effect of preventative application of phosphonate-based fungicide (a known inducer of host resistance) on *Q. rubra* susceptibility and production of chemical defenses, before and after infection with *P. ramorum* (Chapter 5). I hypothesized that *Q. rubra* preventatively treated with phosphonate-based fungicide are less susceptible to *P. ramorum* infection than trees not treated with fungicide. I also hypothesized that there are no effects of phosphonate-based fungicide application on the constitutive concentration of bark phenolics, but rather a significant effect on the induced concentration of certain phenolics. The objectives of this study were to: (1) assess the effect of preventative phosphonate based fungicide application on *Q. rubra* susceptibility to *P. ramorum*, and (2) determine the effect of phosphonate-based fungicide on constitutive and induced phenolic compounds.
CHAPTER 2

VARIATION IN QUERCUS AGRIFOLIA PHLOEM PHENOLICS OVER TIME AND IN RESPONSE TO PHYTOPHTHORA RAMORUM INFECTION

Abstract

Phenolics are important plant defense compounds in many tree-pathosystems. This is particularly true for Quercus agrifolia (CLO—coast live oak) defense against the alien, invasive pathogen Phytophthora ramorum. Pronounced differences in CLO susceptibility to P. ramorum have been observed, and include the presence of apparently resistant trees even in stands with high rates of infection and mortality. Resistant CLO are known to contain higher concentrations of certain phenolic compounds compared to susceptible trees. While variation in CLO susceptibility has been linked to variation in tree phenology, no study has yet examined whether phenology impacts the production of phenolic compounds in CLO phloem. Therefore, the objectives of this study were to characterize CLO phloem phenolics, and to examine variability in CLO phenolics over time and in relation to variability in CLO susceptibility to P. ramorum. Several diverse phenolic compounds were identified using mass spectrometry and included gallic acid derivatives, condensed tannins, and flavonoids. There was no change in the composition of phenolic compounds over time, though the levels of certain individual phenolics changed seasonally from December 2010 to November 2011 and year to year from 2010
to 2012. Inter-annual variation differed only slightly between resistant and susceptible CLO. A lack of pronounced seasonal variation and minimal inter-annual variation in CLO phloem phenolics suggests that phenological impacts on host susceptibility may not be related to variation in phloem phenolics. Lack of variability in phloem phenolics over time may be an adaptive trait, whose function is to buffer trees against environmental events, like drought, which can stress trees and alter their susceptibility to various pests and pathogens.

**Introduction**

Phenolics are ubiquitous in the genus *Quercus*, which includes over 300 species of trees (Cochran, 2004). Phenolics are structurally diverse compounds, and include relatively simple hydroxybenzoic acids, like gallic acid, and more complex ellagitannins, like oenothein A (a trimeric ellagitannin) (Karonen et al., 2010; Levin, 1971; Salminen and Karonen, 2011). Phenolic structure and concentration, as well as the identity and host specificity of an attacking organism are all factors that can impact phenolic function in defense against pathogens (Appel, 1993; Levin, 1971). Even though trees within the genus *Quercus* contain a diversity of phenolic compounds, they remain susceptible to many different pests and pathogens. Furthermore, susceptibility tends to vary over time, probably due, at least in part, to short-term (seasonal) and longer-term (inter-annual) phenological variability, including in phenolic compounds.
There is sufficient evidence to suggest that the concentration of phenolics in *Quercus* vary seasonally, i.e. within one year or growing season (Mauffette and Oechel, 1989; Parker, 1977; Salminen et al., 2004; Yarnes et al., 2008). The majority of studies in *Quercus* on seasonal variation has focused on foliar phenolics, and so changes in phenolics typically correspond with foliar maturation (Mauffette and Oechel, 1989; Salminen et al., 2004; Yarnes et al., 2008). Pronounced seasonal variation in bark phenolics was not detected in older *Quercus* spp., including trees that were 50 years or older and trees that were 10 m in height (Hathway, 1959; Parker, 1977), but have been found in younger trees (less than 10 years old) and corresponded with photosynthetic activity (Hathway, 1959).

Both seasonal variation in *Quercus* phenolics and variation in host susceptibility have been noted in some studies; however, the relationship between variation in phenolics and host susceptibility is not always clear (Mauffette and Oechel, 1989; Yarnes et al., 2008). Different phenolics have been positively and negatively associated with the abundance of Lepidopteran foliar pests within a *Quercus* hybrid zone (Yarnes et al., 2008), though seasonal variation in host phenolics does not always translate into differences in host susceptibility or pest preference (Mauffette and Oechel, 1989). Furthermore, neither of two studies that examined seasonal variation in *Quercus* spp. bark phenolics (Hathway, 1959; Parker, 1977) investigated the relationship between phenolic composition and host susceptibility.
However, within species variability in susceptibility is common and the relationship between susceptibility and phenolic composition is well established. For example, there were distinct differences in the concentration of foliar phenolics between *Quercus robur* that were resistant or susceptible to the oak leafroller, *Tortrix viridana* (Kersten et al., 2013). Resistant trees contained higher levels of galloylated flavonol glycosides, tannin and galloyl derivatives, and biosynthetic precursors of condensed tannins, while susceptible trees contained higher levels of basic flavonol glycosides and unconjugated flavonols (Kersten et al., 2013). Similarly, the content of inner bark (phloem) phenolics from asymptomatic tissue was examined in CLO following infection by *P. ramorum* (Nagle et al., 2011). Levels of total phenolics at 370 nm (i.e. total chromatographic peak area at 370 nm) were greater in CLO that were resistant to *P. ramorum* versus those that were susceptible (Nagle et al., 2011). Resistant trees also contained higher concentrations of a tyrosol derivative and ellagic acid, and both tyrosol and ellagic acid can inhibit the growth of *P. ramorum in vitro* (McPherson et al., 2014; Nagle et al., 2011; Ockels et al., 2007).

Moreover, Dodd et al. (2008) observed that variability in CLO susceptibility to *P. ramorum* was associated with differences in cambial phenology and the timing of bud burst. Both Ockels et al. (2007) and Nagle et al. (2011) alluded that variability in the concentration of CLO phloem phenolics and response to *P. ramorum* infection could be due, in part, to seasonal variation, i.e. phenological status of the trees at the time of infection. However, neither of those studies specifically examined whether variation in
cambial phenology or bud burst timing was associated with variation in the production of plant defenses like phenolic compounds. Therefore, the goal of this study was to examine variation in the composition and concentration of phenolic compounds in CLO over time. The objectives of this study were to (1) characterize phenolics in CLO phloem; (2) determine whether seasonal variation over one year affects levels of phloem phenolics, and (3) to examine variation in phloem phenolics over a three year period, before and after artificial inoculation with *P. ramorum*, in trees of varying susceptibility to *P. ramorum*.

**Materials and methods**

*Phloem collection*

Phloem was collected from 14 healthy (i.e. apparently asymptomatic) CLO within the Bear Creek staging area at Briones Regional Park (Contra Costa Co, CA, USA) to examine seasonal (intra-annual) variation in CLO phloem phenolics. Bear Creek trees were sampled four times from 2010 to 2011, in late fall (“winter”) (December 7, 2010), spring (April 14, 2011), summer (July 18, 2011), and fall (November 3, 2011). Using a cordless drill equipped with a 1.9 cm diameter drill bit, two phloem samples were collected and pooled from each tree at each time point. Following sampling at each time point, pooled phloem samples were stored on dry ice in the field. Samples were then temporary stored at −18°C until they were shipped on dry ice to The Ohio State University, Columbus, OH, where they were stored permanently at −80°C.
Phloem was also collected from thirty total CLO at two additional sites, Seaborg (37° 55’ 12.23” N, 122° 8’ 8.73” W) and Tabletop (37° 56’ 8.57” N, 122° 7’ 40.46” W), at Briones Regional Park to examine inter-annual variation in phloem phenolics. The thirty trees selected for this analysis were part of a larger study on CLO resistance. Phloem tissue from trees in this larger study had been collected and banked in July 2010, a subset of the trees had been artificially inoculated with *P. ramorum* in September 2010, and trees had been classified into groups based on external canker length measured in July 2011 (McPherson et al., 2014). Therefore, to analyze inter-annual variation in CLO phloem phenolics, five trees each from Seaborg and Tabletop (10 trees total) were randomly selected from each of three groups classified in July 2011: inoculated—resistant (canker length ≤ 25 cm) (n = 10), inoculated—susceptible (canker length ≥ 75 cm) (n = 10), and control—non-inoculated (asymptomatic) (n = 10). For each of the thirty trees selected for inter-annual phenolics analysis, phloem tissue previously banked and stored in July 2010 was used, and additional phloem samples were collected from away from canker (inoculated) or asymptomatic (control) tissue in July 2011 and July 2012 (Figure 2.1). For inoculated trees, tissue was collected from away from canker regions in order to avoid measuring locally induced levels of phenolics, which could confound the analysis (Nagle et al., 2011; Ockels et al., 2007). At all time points, tissue was collected and stored as described for Bear Creek CLO.
**Soluble phenolics extraction and analysis**

Pooled phloem samples were finely ground in liquid nitrogen and 100 mg ± 2 mg fresh weight (FW) were extracted twice with 500 µL HPLC grade methanol at 4°C for 24 hours (Nagle et al., 2011). Following centrifugation to remove particulates, extracts were pooled and stored at −20°C until analysis, and spun down one more time just prior to analysis to remove any residual particulate matter.

**General characterization of CLO phloem phenolics**

Phenolic compounds were putatively identified from pooled methanol extracts from Briones CLO phloem tissue collected in July 2010 (Figure 2.1) using a Varian 500 LC-MS (Varian Inc., Palo Alto, CA, USA) with electrospray ionization (ESI) in negative ion mode linked in-line with a Varian ProStar 335 photodiode array (PDA) detector scanning between 220 and 400 nm. Representative samples were analyzed with full scan and TurboDDS (i.e. MSn) modes, by injecting 10 µL into a Thermo Scientific 250 x 4.6 mm, 5-µm, C-18 reverse phase column at ambient temperature. Samples were separated using a water (A) and methanol (B) solvent gradient; each acidified with 0.1% (v/v) glacial acetic acid. The solvent gradient was modified from Nagle et al. (2011) (% represent proportion of solvent A): 100% (0-2 min); 100-90% (2-10 min); 90-83% (10-15 min); 83-77% (15-20 min); 77-52% (20-35 min); 52-0% (35-38 min); 0% (38-45 min); 0-100% (45-47 min); 100% (47-50 min) for the TurboDDS method. The full scan method was the same except 100% solvent B was held for an additional 1 minute (38-46 min) and the transition from 100% solvent B to 100% solvent A was reduced to 1 minute (46-47 min);
all other parameters were kept constant. The flow rate was 0.9 ml min$^{-1}$ throughout the entire run for both modes. For analysis using full scan mode, enhanced scan mode was used, with scanning from 50 – 1000 m/z. The capillary voltage was 80.0 V, and the needle voltage was $-5000$ V. For analysis using TurboDDS, enhanced scan mode was used, scanning between 50 – 1000 m/z. The capillary voltage was 80.0 V, the needle voltage was $-5000$ V, the nebulizer gas (compressed air) pressure was set to 25.0 psi (172.4 kPa), and the drying gas (nitrogen) pressure was set to 15.0 psi (103.4 kPa) with a drying gas temperature of 350$^\circ$C. Threshold ion counts were set at 5000 for MS$_2$, 500 for MS$_3$, and 50 for MS$_4$. Instrument parameters were selected based on Whitehill (2011). When possible, putative identities were confirmed with commercially available standards.

Characterization of seasonal and inter-annual effects on CLO phloem phenolics

Soluble phenolic extracts from CLO at Bear Creek (seasonal analysis) and Seaborg/Tabletop (inter-annual analysis) sites were analyzed using an Acquity H class Waters ultra-performance liquid chromatography (UPLC) system. 0.5 µL of each extract, spiked with the internal standard butylated hydroxyanisole (BHA), were injected into a Waters Acquity 2.1 x 50 mm, 1.7-µm, BEH C-18 reverse phase column (Waters, Milford, MA, USA) held at 40$^\circ$C. Samples were separated using a water and methanol solvent gradient acidified with 2% (v/v) glacial acetic acid. The solvent gradient was (% represents proportion of solvent A): 100% (0-0.01 min); 100-90% (0.01-1.6 min); 90-83% (1.6-2.6 min); 83-77% (2.6-3.6 min); 77-52% (3.6-6.6 min); 52-0% (6.6-8.0 min); 41
0% (8.0-8.6 min); 0-100% (8.6-9.4 min); 100% (9.4-10.2 min), with a constant flow rate of 0.21 mL min\(^{-1}\), and an injection delay of 5 minutes at 100% solvent A between each sample. A Waters Photo Diode Array (PDA) detector (Waters, Milford, MA, USA), scanning between 210 and 400 nm, was used for data acquisition. Data acquisition and processing were performed using Waters Empower Pro software (Waters, Milford, MA, USA) and visualizing data at 280 and 370 nm. The minimum peak area detection threshold was 8000 AU for 280 nm and 7500 AU for 370 nm. Any peak that was not present in 50% of biological samples was excluded from further analysis. Finally, the total amount of phenolics at 280 nm and 370 nm, respectively, was determined for each sample by summing together the area of all chromatographic peaks that met the minimum peak area detection threshold (Nagle et al., 2011). Hereafter, this value is referred to as total phenolics at 280 nm or 370 nm.

To identify phenolic peaks of interest from seasonal and inter-annual analyses, pools of phenolic extracts from Briones CLO tissue collected before and after infection with \(P. \text{ramorum}\) were analyzed using a hybrid UPLC-MS, as described in Sherwood (2014). However, for this analysis, all post-UPLC flow was directed into the Varian MS, and no in-line PDA detector was used. Samples were separated as stated above, with one exception: the percentage of glacial acetic acid in solvents A and B was reduced to 0.1% (v/v). Pooled phloem extracts from Briones CLO were analyzed using standard, full scan mode, scanning from 50 – 1000 m/z. The capillary voltage was 80.0 V, and the needle
voltage was −5000 V. Compound identification was confirmed by comparing unknown peaks with commercially available standards, when available.

**Statistical analysis**

Seasonal variation in the amount of each phenolic compound identified in Bear Creek CLO and total phenolics at 280 nm and 370 nm (normalized to the internal standard butylated hydroxyanisole, BHA) was assessed using a repeated measures general linear model (ANOVA). Season was the within-subjects factor. The Shapiro-Wilk test was used to assess normality of the data, and Mauchly’s test of sphericity (the assumption that variances are equal across all related treatment groups) was used to test the assumption of sphericity. Data violating the assumption of normality were analyzed using the non-parametric repeated measures ANOVA alternative, Friedman test, while the significance of data that failed Mauchly’s test of sphericity was assessed using the Greenhouse-Geisser degrees of freedom correction. All analyses were performed using IBM Statistics SPSS 21 and 22.

The level (peak area normalized to the internal standard BHA) of each phenolic compound identified in Seaborg and Tabletop CLO and total phenolics at 280 nm and 370 nm were then used to examine if inter-annual variation impacts the levels of phenolic compounds in CLO of varying susceptibility to *P. ramorum*. Assumptions of normality, homogeneity of variance, and sphericity were tested using the Shapiro-Wilk test, Levene’s test, and Mauchly’s test of sphericity, respectively. Data that satisfied the
assumptions were analyzed using repeated measures ANOVA. Time was the within-subjects factor and disease phenotype class (inoculated-resistant, inoculated-susceptible, control) was the between-subjects factor. The LSD test was used to make pairwise comparisons between disease phenotype classes. Data that violated the normality assumption were log transformed and then subjected to repeated measures ANOVA as detailed above. The Greenhouse-Geisser correction was used to assess the significance of any test where sphericity was not assumed. For repeated measures ANOVA with significant time by disease phenotype class interactions, a one-way ANOVA was run for each time point to determine in which year disease phenotype classes differed. The Friedman test was used to analyze data that violated the normality assumption, when transformation did not normalize that data, though only for within-subjects comparisons. All analyses were performed using IBM Statistics SPSS 21 and 22.

Results

General characterization of CLO phloem phenolics

LC-MS analysis in negative ion mode detected 29 distinct phenolic compounds in methanol extracts from CLO phloem. 21 of the 29 phenolic compounds were putatively identified by comparing unknown fragmentation patterns to the fragmentation patterns associated with known phenolic compounds as reported in peer-reviewed literature (Table 2.1). The majority of compounds that could be identified using this approach were classified into three groups: gallic acid derivatives (including gallic acid glycosides,
ellagitannins and ellagitannin derivatives), condensed tannins (including catechin monomers and dimers, and galloylated derivatives of catechin), and flavonoids (including myricitrin and a putative kaempferol derivative). Of the eight compounds that could not be identified based on fragmentation patterns, one compound (RT = 30.8) was classified based on part of its fragmentation pattern, as an unknown gallic acid derivative, because it had a neutral loss of 152, corresponding to a galloyl moiety. Four of the remaining unknown compounds contained the fragment 313 m/z (RT = 21.1, 30.0, 32.5, 40.2). However, these unknowns could not be assigned to a specific class because no matches for this fragment were found in the literature.

**Seasonal variation in CLO phloem phenolics**

A total of 28 phenolic peaks were detected in CLO from the Bear Creek staging area using UPLC-PDA analysis. The levels of each phenolic peak and total phenolics at 280 nm and 370 nm (normalized to the internal standard BHA) were compared within one year, at four different time points corresponding with late fall, i.e. “winter” (December 7, 2010), spring (April 14, 2011), summer (July 18, 2011), and fall (November 3, 2011) (Figure 2.2, Table 2.2) for 11 of the 14 trees—three trees had to be excluded due to missing data at one of the time points. In instances where normality was not assumed, the non-parametric repeated measures GLM alternative, Friedman test was used. Five phenolic peaks, P8, P19, P22, P24, and P28, were significantly affected by the season in which they were collected (Table 2.3). These peaks could not be identified using hybrid UPLC-MS analysis (Table 2.4).
Inter-annual variation in CLO phloem phenolics

A total of 30 phenolic peaks, plus total phenolics at 280 nm and 370 nm (Table 2.2), were compared between control, resistant, and susceptible CLO at Briones Regional Park from 2010 to 2012 (Figure 2.3). Peaks were putatively identified when possible based on UV spectrum and full scan fragments collected from analyzing pooled Briones CLO phenolic extracts on the UPLC-MS hybrid system (Table 2.4). Fourteen of the phenolic peaks and total phenolics at 280 nm were significantly impacted by time, i.e. year of collection (Table 2.5). The level of total phenolics at 280 nm was higher in 2010 for resistant and control trees, and lowest for susceptible trees in 2011 (Table 2.6). Other phenolic peaks significantly impacted by time included: P2, gallic acid, P4, P5, P6, P7, P8, P9, catechin, tyrosol, P12, P13, P15, and P17. Across all disease phenotype classes, levels of all peaks, except P2, gallic acid, P4, and P12 were slightly lower in 2011 compared to 2010 and 2012. In contrast, the normalized level of gallic acid and P12 decreased over time, while the normalized level of P2, a tentative galloyl glucoside, and P4 was slightly higher in 2012 versus 2010. For six phenolic peaks, P2, P7, P8, catechin, P28, and P29, the effect of time on levels of phenolics, depended on disease phenotype class (Table 2.5).

Resistant trees contained significantly higher levels of catechin in 2011 compared to susceptible trees (1-way ANOVA, $F_{2,27} = 3.356, p = 0.050$; LSD test, $p = 0.021$). The effect of time on the levels of P28 (Friedman test, $df = 2, \chi^2 = 7.400, p = 0.025$) and P29 (Friedman test, $df = 2, \chi^2 = 8.600, p = 0.014$) was observed only for susceptible trees, and only between 2010 and 2012 ($p_{P28} = 0.042$ and $p_{P29} = 0.011$). Finally, one phenolic peak, ellagic acid, was significantly affected by disease phenotype class (repeated measures evaluation).
GLM, $F_{2,27} = 3.614, p = 0.041$) (Table 2.5). The level of ellagic acid was consistently lower in resistant trees compared to control (LSD test, $p = 0.020$) and susceptible trees (LSD test, $p = 0.040$). There was no significant difference in the levels of ellagic acid between control and susceptible trees at any of the measured time points.

**Discussion**

Diverse, but predictable, classes of phenolic compounds were found in CLO phloem tissue, and included hydrolysable tannins (e.g. gallic acid derivatives), condensed tannins (e.g. catechin derivatives), and flavonoids. Previous work had identified gallic acid, tyrosol, catechin, and ellagic acid in CLO phloem based on comparison of unknown compounds with authentic standards; tyrosol and ellagic acid derivatives were also identified based on retention time and spectrum similarity to standards (Nagle et al., 2011; Ockels et al., 2007). LC-MS analysis corroborated the results of Nagle et al. and Ockels et al.; fragmentation patterns associated with gallic acid, tyrosol hexoside, catechin, and ellagic acid were all detected in CLO phloem extracts.

Additional hydrolysable tannins, condensed tannins, and flavonoids were putatively identified based on comparison of unknown fragmentation patterns to fragmentation patterns associated with known compounds. Hydrolysable tannins (and precursors) included gallic acid, galloyl glucose, digalloyl glucose, and a derivative of the ellagitannin, galloyl-bis-HHDP-glucopyranose (Barry et al., 2001; Cantos et al., 2003; Fernandes et al., 2009; Mammela et al., 2000; Meyers et al., 2007; Salminen et al., 1999;
Condensed tannins and precursors identified in *Q. agrifolia* phloem extracts included catechin, dimer and trimer procyanidins, (epi)gallocatechin, dimer (epi)gallocatechin-(epi)catechin, and monogalloyl-(epi)catechin-(epi)gallocatechin (Fraser et al., 2012; Rigaud et al., 1993; Souquet et al., 2000, 1996; Sun et al., 2007). Flavonoids included myricitrin (Lee et al., 2005; Romani et al., 2012) and kaempferol-3-glucuronide (Kosonen et al., 2012). The presence of myricitrin in CLO phloem tissue was confirmed by spiking an extract with authentic myricitrin and comparing UV spectrum and retention time (data not reported). Several additional phenolic compounds could not be identified based on fragmentation patterns; however, four of the unknowns all contained a 313 m/z fragment. The exact identity of this fragment is unknown; however, valoneic acid and a trigalloyl glucose, both hydrolysable tannins, were reported to contain this fragment (Fernandes et al., 2011, 2009). Valoneic acid can link two glucosyl moieties, and thus is an important constituent of many ellagitannins (Niemetz and Gross, 2005). Even though it was not possible to confirm the identities of many of these compounds because authentic standards were not available, it was not surprising that these compounds were found in CLO, given that essentially the same or similar compounds have been found in various oak species and tissues (Cantos et al., 2003; Fernandes et al., 2011, 2009; Meyers et al., 2007; Salminen et al., 2004). Finally, even though this study focused on characterizing constitutive phloem phenolics, many of the same phenolics identified in healthy or asymptomatic tissue prior to *P. ramorum* infection, can also be found in tissue along the margin of *P. ramorum* cankers. In fact, only one phenolic peak was detected *de novo* based on LC-MS analysis, and was identified as a pentagalloyl glucose based on a
full scan mass of 939 m/z in negative ion mode and a $\lambda_{\text{max}} = 279$ (data not shown) (Fernandes et al., 2009).

In addition to characterizing the phenolic composition of CLO phloem tissue, the effect of seasonal and inter-annual variation on the composition and levels of phenolics in CLO phloem tissue was examined. Fewer phenolics in healthy CLO phloem were affected by seasonal variation compared to inter-annual variation in trees of varying susceptibility to *P. ramorum*. Variation in the timing of bud burst and phenology of cambial activity are known to be associated with variability in CLO susceptibility to *P. ramorum* (Dodd et al., 2008). However, the impact of seasonal and inter-annual variation on the production of defense-related phenolic compounds in the phloem of CLO had not been previously addressed. Nagle et al. (2011) had suggested that examination of phloem phenolic profiles of CLO of differing susceptibilities to *P. ramorum* would be most relevant during the spring when the pathogen is most active. This may not be necessary. Based on the present analysis, seasonal variation over the course of one year (from December 2010 to November 2011) had a relatively minor effect (i.e. only levels 5 of 28 phenolics were impacted, and there was no effect on total phenolics at either 280 nm or 370 nm) on the level of phenolics in CLO phloem tissue and no effect on the composition, i.e. all phenolic peaks were detected at every time point. The five phenolic peaks significantly impacted by season of sampling included P8, P19, P22, P24, and P28. The relative amount of P8 was lowest in April 2011, while the level of P19 and P22 peaked at that time. All other peaks varied significantly only between December 2010 and November
2011, the furthest time points in this analysis. A lack of variability in levels of individual phenolics across seasons for most CLO phloem phenolics detected is not surprising considering that variation from May to February in the percentage of tannin in *Quercus pedunculata* Ehrh. (syn. *Quercus robur*) bark was not significant (Hathway, 1959), and only slight differences in the concentration of 39 phenolic compounds in the bark of *Quercus velutina* Lam. were detected monthly from May to November over two successive years (Parker, 1977). In contrast, seasonal variation has significant effects on the concentration of phenolics in oak foliage, though variation in the concentration of phenolics could be explained by ontogenetic process (Salminen et al., 2004; Yarnes et al., 2008). Compared to foliage, ontogenetic processes are not as pronounced in living phloem tissue, which could explain the proportionally small amount of variation that was observed in this study in the levels of CLO phloem phenolics. Whatever the origin of this minimal variation in CLO phloem phenolics, it is extremely important for the identification and practical utilization of chemical biomarkers of resistance, i.e. chemical compounds that can be used to identify disease-resistant trees (see Chapter 4). Concentrations of biomarkers ideally would not be affected by seasonal or inter-annual variation, which could potentially confound any analysis including them.

Variation in the composition and concentration of constitutive (or away from canker) phloem phenolics was assessed over a three year period in three disease phenotype classes: control (non-inoculated), resistant (inoculated), and susceptible (inoculated). As with seasonal variation, there were no qualitative changes in phenolics of CLO bark from
2010 to 2012, regardless of disease phenotype class. However, time (in this case, year of sampling) had a significant effect on the levels of many phenolic peaks, including seven unknown compounds, several tentatively identified compounds including two ellagitannins (P5 and P12), a condensed tannin (P9), a galloyl glucose (P2), and gallic acid, catechin, and tyrosol, which were confirmed with authentic standards (Cantos et al., 2003; Fernandes et al., 2009; Mammela et al., 2000; Meyers et al., 2006; Salminen et al., 1999; Souquet et al., 2000). The levels of most phenolics, except gallic acid, P2, P4, and P12, decreased slightly in 2011, suggesting that variation in the environment from year to year may have an effect on the amount of CLO phloem phenolics. A lack of difference in levels of phenolics between resistant, susceptible, and control trees, for most phenolics analyzed also suggests that phenology does not appear to affect the susceptibility of CLO to *P. ramorum*. A gradual increase in the concentration of P2 (a tentative galloyl glucoside) and P12 (a possible hydrolysable tannin), and a slight decrease in the amount of gallic acid (a biosynthetic precursor of galloyl glucoside) from 2010 to 2012, across all disease phenotype classes was also noted (Salminen and Karonen, 2011). This observation is consistent with Hathway’s hypothesis that the content of tannins in the bark of oak trees increases with age (Hathway, 1959).

While time was the predominant factor in influencing the levels of phenolics in CLO phloem, in some instances the impact of time depended on the disease phenotype class. Overall, the amount of catechin was affected by time, and in 2011 resistant trees contained higher levels of catechin compared to susceptible trees. Time did not impact
the levels of P28 or P29 in control or resistant trees, although it did have an effect on susceptible trees. For both phenolic peaks, 2010 and 2012 levels differed significantly with slightly higher levels of each phenolic in 2012 compared to 2010. This is interesting, considering that Ockels et al. (2007) found no significant difference between the concentration of *Q. agrifolia* phenolics in away from canker phloem and phloem from healthy trees. The same authors measured phenolics in trees only after they were infected with *P. ramorum*, which could explain this discrepancy. Since significant changes in the levels of each phenolic were only observed in susceptible trees, it is possible that these differences reflect systemic changes in the amount of phenolics following *P. ramorum* inoculation. Infections on resistant trees are relatively restricted, many resistant trees appear to recover over time, and in many cases callus tissue forms around infected tissues (McPherson et al., 2005; Nagle et al., 2011; Swiecki and Bernhardt, 2005). By 2014, 90% of resistant trees in this study showed no active symptoms of the disease, while 80% of susceptible trees had died (data not shown). This may explain why there was no apparent systemic effect of *P. ramorum* infection on the levels of P28 and P29 in resistant trees. Furthermore, UV spectrum and relative retention time of P28 matches that of FLV3, a constitutive phenolic biomarker (Chapter 4). Taken together, this suggests that P28 is important for CLO defense against *P. ramorum*, since higher constitutive levels of FLV3 are associated with higher probability of resistance to *P. ramorum* (Chapter 4), and higher systemic levels of P28 were observed in susceptible trees two years after infection with *P. ramorum*. While the identity of P28 is not known, P29, was tentatively identified as a gallic acid derivative (Fernandes et al., 2009). Gallic acid is known to inhibit the
growth of *P. ramorum in vitro*, and was detected at higher concentrations in infected versus healthy tissues, suggesting that it may be active in CLO defense (Ockels et al., 2007).

Finally, ellagic acid was the only phenolic compound found to be significantly affected by disease phenotype class, regardless of time. Resistant trees contained significantly lower amounts of ellagic acid compared to susceptible and control trees in 2010, 2011, and 2012. This was surprising because ellagic acid has been consistently associated with CLO resistance to *P. ramorum*. For example, ellagic acid and an ellagic acid derivative were identified as biomarkers of resistance in already infected CLO and ellagic acid inhibited the growth of *P. ramorum in vitro* (McPherson et al., 2014). Concentrations of ellagic acid were also higher in putatively resistant and in remission CLO versus susceptible CLO (Nagle et al., 2011). A different separation method and analytical instrument were used in this study to separate phenolic peaks from methanol extracts of CLO phloem. Peaks with similar polarity and structure can co-elute in complex plant samples (Tsao and Deng, 2004), so it is possible that peaks similar in retention time and structure to ellagic acid co-eluted in this analysis. Additionally, trees in this study were sampled before and 1- and 2-years after inoculation with *P. ramorum*, whereas trees in the Nagle et al. study were sampled 6 years following inoculation. Both factors could help explain the observed differences in the levels of ellagic acid between resistant and susceptible CLO in this study.
In conclusion, CLO phloem contains phenolic compounds, which were classified based on mass spectra fragmentation patterns as hydrolysable tannins, condensed tannins, and flavonoids. While the bioactivity of most of these compounds has not been tested against *P. ramorum*, past studies indicate that precursors and derivatives of many of the compounds detected in CLO phloem can inhibit *P. ramorum*’s growth. Future studies focused on confirming the identity of CLO phenolics could be combined with functional analyses to elucidate potential mode of actions in CLO defense responses. Furthermore, seasonal and inter-annual variation had no impact on the composition of CLO phloem phenolics, i.e. all phenolics were detected at all times. Although levels of some phenolics varied seasonally, levels of most phenolics did not vary and there was no impact on levels of total phenolics. In contrast, inter-annual variation in the levels of CLO phenolics was more pronounced; however, for the most part, it was not associated with variation in CLO susceptibility to *P. ramorum*. Taken together these results suggest that phenological effects on the levels of phenolics in CLO phloem are unlikely related to variation in CLO susceptibility to *P. ramorum*. 
Table 2.1 General characterization of phloem phenolics in CLO from Briones Regional Park. Putative identities based on Varian HPLC-MS-PDA analysis of pooled extracts from CLO phloem. CLO phloem tissue used for this analysis was collected from trees within Briones Regional Park that did not show any symptoms of *P. ramorum* infection.

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>MS1</th>
<th>MS2</th>
<th>MS3</th>
<th>MS4</th>
<th>Channel</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>Putative ID**</th>
</tr>
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<td><strong>241</strong>, 169</td>
<td>211, 167</td>
<td>187, 167, 192, 136</td>
<td>280</td>
<td>275</td>
<td>galloyl glucose&lt;sup&gt;2,11,14&lt;/sup&gt;</td>
</tr>
<tr>
<td>13.1</td>
<td>169</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>280</td>
<td>271</td>
<td>gallic acid&lt;sup&gt;2,9,11,17&lt;/sup&gt;</td>
</tr>
<tr>
<td>15.6</td>
<td>865</td>
<td><strong>695</strong>, 739</td>
<td>543, 451, 306</td>
<td>--</td>
<td>280</td>
<td>--</td>
<td>trimer procyanidin&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>17.6</td>
<td>477</td>
<td><strong>271</strong>, 211</td>
<td>211</td>
<td>168, 123</td>
<td>--</td>
<td>--</td>
<td>unknown (possible acetic acid adduct)</td>
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<td><strong>425</strong>, 289, 407, 467</td>
<td>407</td>
<td>285, 256, 243, 389</td>
<td>280</td>
<td>--</td>
<td>dimer (epi)catechin-(epi)gallocatechin&lt;sup&gt;5,16&lt;/sup&gt;</td>
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<td>179</td>
<td><strong>164</strong>, 151</td>
<td>120</td>
<td>280</td>
<td>--</td>
<td>(epi)gallocatechin&lt;sup&gt;5,17&lt;/sup&gt;</td>
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<tr>
<td>21.1</td>
<td>557</td>
<td><strong>313</strong>, 327</td>
<td>169</td>
<td>124</td>
<td>370</td>
<td>(274)</td>
<td>unknown (FLV1, Chapter 4)</td>
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<tr>
<td>21.8</td>
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<td>142, 118, 178</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>275</td>
<td>tyrosol hexoside&lt;sup&gt;3,7,18&lt;/sup&gt;</td>
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<td>22.3</td>
<td>289</td>
<td><strong>245</strong>, 205</td>
<td>203</td>
<td>175, 188, 161</td>
<td>280</td>
<td>279</td>
<td>catechin&lt;sup&gt;5,10,17&lt;/sup&gt;</td>
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<td>22.5</td>
<td>577</td>
<td>425</td>
<td>407</td>
<td>281, 285, 363</td>
<td>--</td>
<td>--</td>
<td>procyanidin B1 or B2&lt;sup&gt;17&lt;/sup&gt;</td>
</tr>
<tr>
<td>23.3</td>
<td>577</td>
<td>407</td>
<td><strong>285</strong>, 243, 281, 255</td>
<td>257</td>
<td>280</td>
<td>278</td>
<td>procyanidin B1 or B2&lt;sup&gt;17&lt;/sup&gt;</td>
</tr>
<tr>
<td>23.5</td>
<td>745</td>
<td><strong>593</strong>, 407, 575, 425</td>
<td><strong>289</strong>, 467, 407</td>
<td>245, 205, 120</td>
<td>370</td>
<td>--</td>
<td>monogallo-(epi)catechin-(epi)gallocatechin&lt;sup&gt;16&lt;/sup&gt;</td>
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<tr>
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<td>130</td>
<td>--</td>
<td>280</td>
<td>276</td>
<td>tyrosol glycoside&lt;sup&gt;3,18&lt;/sup&gt;</td>
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<td>25.7</td>
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<td><strong>331</strong>, 313</td>
<td>169</td>
<td>138</td>
<td>280</td>
<td>276</td>
<td>digalloyl glucose&lt;sup&gt;1,2,9,11,14&lt;/sup&gt;</td>
</tr>
<tr>
<td>26.3</td>
<td>579</td>
<td><strong>289</strong>, 469</td>
<td>245</td>
<td>203</td>
<td>280</td>
<td>277</td>
<td>benzylthioether of (epi)gallocatechin-3-O-gallate&lt;sup&gt;12,15&lt;/sup&gt;</td>
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Continued
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<tr>
<th>Peak Value</th>
<th>Wavelength</th>
<th>Retention Time</th>
<th>Mass Value</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>26.5</td>
<td>745</td>
<td>593</td>
<td>423</td>
<td>monogallo-(epi)catechin-(epi)gallocatechin&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
<tr>
<td>28.0</td>
<td>483</td>
<td>327</td>
<td>183</td>
<td>digalloyl glucose&lt;sup&gt;1,2,11&lt;/sup&gt;</td>
</tr>
<tr>
<td>28.3</td>
<td>729</td>
<td>577, 559, 407</td>
<td>407</td>
<td>monogalloylated derivative of (epi)catechin dimer&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
<tr>
<td>28.3</td>
<td>729</td>
<td>577, 559, 407</td>
<td>451</td>
<td>monogalloylated derivative of (epi)catechin dimer&lt;sup&gt;16&lt;/sup&gt;</td>
</tr>
<tr>
<td>28.9</td>
<td>467</td>
<td>313</td>
<td>169</td>
<td>galloyl-bis-HHDP-glucopyranos e derivative&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
<tr>
<td>30.0</td>
<td>451</td>
<td>313</td>
<td>169</td>
<td>unknown</td>
</tr>
<tr>
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<td>unknown gallic acid derivative</td>
</tr>
<tr>
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<td>451</td>
<td>271</td>
<td>211</td>
<td>unknown</td>
</tr>
<tr>
<td>32.5</td>
<td>497</td>
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<td>169</td>
<td>unknown</td>
</tr>
<tr>
<td>36.8</td>
<td>463</td>
<td>316</td>
<td>271</td>
<td>myricitrin (Chapter 4)&lt;sup&gt;8,13&lt;/sup&gt;</td>
</tr>
<tr>
<td>39.2</td>
<td>433</td>
<td>301, 300</td>
<td>229, 244, 257, 185</td>
<td>ellagic acid pentoside&lt;sup&gt;2,4,11&lt;/sup&gt;</td>
</tr>
<tr>
<td>39.5</td>
<td>447</td>
<td>--</td>
<td>--</td>
<td>unknown flavonoid&lt;sup&gt;4,9,10,17&lt;/sup&gt;</td>
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<td>461</td>
<td>328, 313</td>
<td>313</td>
<td>kaempferol-3-glucoronide&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
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<td>40.2</td>
<td>475</td>
<td>328, 313</td>
<td>313</td>
<td>unknown (FLV3, Chapter 4)</td>
</tr>
</tbody>
</table>

*Based on Varian PDA detector (unit = nm). **References: <sup>1</sup> Barry et al. 2001; <sup>2</sup> Cantos et al. 2003; <sup>3</sup> Eyles et al. 2007; <sup>4</sup> Fernandes et al. 2009; <sup>5</sup> Fraser et al. 2012; <sup>6</sup> Kosonen et al. 2012; <sup>7</sup> Krammerer et al. 2005; <sup>8</sup> Lee et al. 2005; <sup>9</sup> Mammela et al. 2000; <sup>10</sup> Mammela 2001; <sup>11</sup> Meyers et al. 2006; <sup>12</sup> Rigaud et al. 1993; <sup>13</sup> Romani et al. 2012; <sup>14</sup> Salminen et al. 1999; <sup>15</sup> Souquet et al. 1996; <sup>16</sup> Souquet et al. 2000; <sup>17</sup> Sun et al. 2007; <sup>18</sup> Whitehill 2011.
Table 2.2 Effect of season on CLO total phenolics at 280 nm and 370 nm. Peak area (± standard error) of total phenolics (normalized to internal standard BHA) at 280 nm and 370 nm in Bear Creek CLO phloem tissues (N = 11), collected in winter (December 2010), spring (April 2011), summer (July 2011), and fall (November 2011). There was no impact of seasonal variation on the levels of total phenolics at 280 nm and 370 nm.

<table>
<thead>
<tr>
<th>Time</th>
<th>Total 280</th>
<th>Total 370</th>
</tr>
</thead>
<tbody>
<tr>
<td>December</td>
<td>85.71 (7.67)</td>
<td>3.59 (0.43)</td>
</tr>
<tr>
<td>April</td>
<td>91.11 (10.39)</td>
<td>3.96 (0.53)</td>
</tr>
<tr>
<td>July</td>
<td>86.18 (8.00)</td>
<td>4.12 (0.64)</td>
</tr>
<tr>
<td>November</td>
<td>85.02 (7.92)</td>
<td>3.85 (0.65)</td>
</tr>
</tbody>
</table>
Table 2.3 Effect of season on CLO phenolics. Peak area (± standard error) of phenolics (normalized to internal standard BHA) significantly impacted by seasonal variation (repeated measures ANOVA, $p < 0.05$) are listed. Lower case letters indicate significant pairwise comparisons between seasons ($p < 0.05$). See Figure 2.2 for levels of phenolics that were not significantly impacted by seasonal variation.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Sampling date$^1$</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dec-10</td>
<td>Apr-11</td>
</tr>
<tr>
<td>P8*</td>
<td>0.847 (0.102)$^{ab}$</td>
<td>0.766 (0.071)$^a$</td>
</tr>
<tr>
<td>P19*</td>
<td>3.289 (0.283)$^{ab}$</td>
<td>4.439 (0.758)$^a$</td>
</tr>
<tr>
<td>P22**</td>
<td>3.171 (0.466)$^{ab}$</td>
<td>4.504 (0.870)$^a$</td>
</tr>
<tr>
<td>P24**</td>
<td>0.290 (0.105)$^a$</td>
<td>0.144 (0.048)$^{ab}$</td>
</tr>
<tr>
<td>P28**</td>
<td>0.349 (0.104)$^a$</td>
<td>0.342 (0.100)$^{ab}$</td>
</tr>
</tbody>
</table>

*Repeated measures general linear model (df = 3,30). **Results of non-parametric Friedman test (df = 3). $^1$Letter indicates significant pairwise comparison between seasons ($p < 0.05$).
Table 2.4 Phenolic peaks identified by UPLC-PDA analysis in CLO phloem extracts from trees sampled in Briones Regional Park at Bear Creek and Seaborg/Tabletop sites, for seasonal and inter-annual phenolics analysis, respectively. Putative identities assigned for some phenolic peaks based on hybrid UPLC-MS analysis. See Figure B.1 for chromatograms labeled with peaks.

<table>
<thead>
<tr>
<th>Peak</th>
<th>RT (min)*</th>
<th>[M-H]⁻ (m/z)</th>
<th>Channel (nm)</th>
<th>( \lambda_{max} ) (nm)**</th>
<th>Putative ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>0.84</td>
<td>-</td>
<td>280</td>
<td>275</td>
<td>--</td>
</tr>
<tr>
<td>P2</td>
<td>0.93</td>
<td>331</td>
<td>280</td>
<td>271</td>
<td>galloyl glucoside²,¹¹,¹²</td>
</tr>
<tr>
<td>gallic acid</td>
<td>1.08</td>
<td>-</td>
<td>280</td>
<td>271</td>
<td>--</td>
</tr>
<tr>
<td>P4</td>
<td>1.17</td>
<td>-</td>
<td>280</td>
<td>ND</td>
<td>--</td>
</tr>
<tr>
<td>P5</td>
<td>2.91</td>
<td>933</td>
<td>280</td>
<td>233</td>
<td>vescalagin/castalagin⁴,⁹</td>
</tr>
<tr>
<td>P6</td>
<td>3.25</td>
<td>-</td>
<td>280</td>
<td>277</td>
<td>--</td>
</tr>
<tr>
<td>P7</td>
<td>3.42</td>
<td>-</td>
<td>280</td>
<td>278</td>
<td>--</td>
</tr>
<tr>
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<td>3.52</td>
<td>-</td>
<td>280</td>
<td>277</td>
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<tr>
<td>P9</td>
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<td>catechin</td>
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<td>catechin⁵,¹⁰,¹⁴</td>
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<td>tyrosol</td>
<td>4.03</td>
<td><strong>299, 745</strong></td>
<td>280</td>
<td>276</td>
<td>tyrosol hexoside³,⁶,¹⁵</td>
</tr>
<tr>
<td>P12</td>
<td>4.19</td>
<td><strong>785, 786</strong></td>
<td>280</td>
<td>276</td>
<td>digalloyl-HHDP-glucose⁴,¹²</td>
</tr>
<tr>
<td>P13</td>
<td>4.31</td>
<td>-</td>
<td>280</td>
<td>276</td>
<td>--</td>
</tr>
<tr>
<td>P14</td>
<td>4.72</td>
<td>431</td>
<td>280</td>
<td>276</td>
<td>tyrosol hexoside pentoside³,¹⁵</td>
</tr>
<tr>
<td>P15</td>
<td>4.81</td>
<td>-</td>
<td>280</td>
<td>277</td>
<td>--</td>
</tr>
<tr>
<td>P16</td>
<td>5.27</td>
<td>-</td>
<td>280</td>
<td>278</td>
<td>--</td>
</tr>
<tr>
<td>P17</td>
<td>5.49</td>
<td>-</td>
<td>280</td>
<td>276</td>
<td>--</td>
</tr>
<tr>
<td>P18</td>
<td>5.64</td>
<td>-</td>
<td>280</td>
<td>276</td>
<td>--</td>
</tr>
<tr>
<td>P19</td>
<td>5.86</td>
<td>-</td>
<td>280</td>
<td>274</td>
<td>--</td>
</tr>
<tr>
<td>P20</td>
<td>5.95</td>
<td><strong>787, 788</strong></td>
<td>280</td>
<td>276</td>
<td>tetragalloyl glucose⁴</td>
</tr>
<tr>
<td>P21</td>
<td>6.09</td>
<td>451</td>
<td>280</td>
<td>276</td>
<td>(epi)catechin hexoside¹</td>
</tr>
<tr>
<td>P22</td>
<td>6.44</td>
<td>-</td>
<td>280</td>
<td>277</td>
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</tr>
<tr>
<td>P23</td>
<td>6.79</td>
<td>433</td>
<td>370</td>
<td>361, 253</td>
<td>ellagic acid pentoside²,⁴,¹⁰</td>
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<tr>
<td>P24</td>
<td>6.8</td>
<td>-</td>
<td>370</td>
<td>257, 243</td>
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</tr>
<tr>
<td>ellagic acid</td>
<td>7.08</td>
<td>447, 948</td>
<td>370</td>
<td>362, 253</td>
<td>ellagic acid rhamnoside⁸</td>
</tr>
<tr>
<td>P26</td>
<td>8.00</td>
<td><strong>461, 703</strong></td>
<td>370</td>
<td>368, 247</td>
<td>kaempferol-3-glucoronide⁷</td>
</tr>
<tr>
<td>P28</td>
<td>8.43</td>
<td>475</td>
<td>370</td>
<td>370, 247</td>
<td>FLV3 (Chapter 4)</td>
</tr>
<tr>
<td>P29</td>
<td>8.81</td>
<td><strong>613, 329</strong></td>
<td>370</td>
<td>370, 247</td>
<td>dehydrated tergallagic C-glucose⁴</td>
</tr>
</tbody>
</table>

Continued
Table 2.4 continued

*Average retention time (RT) of all biological samples from inter-annual analysis. RT with only 1 digit from seasonal analysis only. **$\lambda_{\text{max}}$ values from UPLC-PDA analysis; ND = $\lambda_{\text{max}}$ not detected. References cited: 1Brossa et al., 2009; 2Cantos et al., 2003; 3Eyles et al., 2003; 4Fernandes et al., 2009; 5Fraser et al., 2012; 6Kammerer et al., 2005; 7Kosonen et al., 2012; 8Lee et al., 2005; 9Mammela et al., 2000; 10Mammela, 2001; 11Meyers et al., 2006; 12Salminen et al., 1999; 13Souquet et al., 2000; 14Jianping Sun et al., 2007; 15Whitehill, 2011.
Table 2.5 Results of significant repeated measures ANOVA analyzing the effect of inter-annual variation (time) and disease phenotype class (control, resistant, and susceptible) on the production of phloem phenolics in CLO from Seaborg and Tabletop sites within Briones Regional Park. Phloem was sampled from all trees in 2010, before inoculation of resistant and susceptible trees with *P. ramorum*. Phloem tissue away from *P. ramorum* cankers was sampled in 2011 and 2012 in resistant and susceptible trees, while healthy (asymptomatic) phloem was sampled from control trees in 2011 and 2012.

<table>
<thead>
<tr>
<th>Phenolic peak</th>
<th>Time</th>
<th>Time*Class</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F^1$</td>
<td>$P$</td>
<td>$F^2$</td>
</tr>
<tr>
<td>P2</td>
<td>4.841</td>
<td>0.012</td>
<td>3.852</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>4.683</td>
<td>0.019</td>
<td>--</td>
</tr>
<tr>
<td>P4</td>
<td>6.274</td>
<td>0.004</td>
<td>--</td>
</tr>
<tr>
<td>P5</td>
<td>3.574</td>
<td>0.035</td>
<td>--</td>
</tr>
<tr>
<td>P6</td>
<td>3.723</td>
<td>0.031</td>
<td>--</td>
</tr>
<tr>
<td>P7</td>
<td>4.615</td>
<td>0.014</td>
<td>2.707</td>
</tr>
<tr>
<td>P8</td>
<td>4.738</td>
<td>0.013</td>
<td>2.789</td>
</tr>
<tr>
<td>P9</td>
<td>4.211</td>
<td>0.020</td>
<td>--</td>
</tr>
<tr>
<td>Catechin</td>
<td>3.866</td>
<td>0.027</td>
<td>4.055</td>
</tr>
<tr>
<td>Tyrosol</td>
<td>8.356</td>
<td>0.001</td>
<td>--</td>
</tr>
<tr>
<td>P12</td>
<td>3.624</td>
<td>0.033</td>
<td>--</td>
</tr>
<tr>
<td>P13</td>
<td>9.270</td>
<td>&lt; 0.001</td>
<td>--</td>
</tr>
<tr>
<td>P15</td>
<td>5.436</td>
<td>0.007</td>
<td>--</td>
</tr>
<tr>
<td>P17</td>
<td>5.830</td>
<td>0.005</td>
<td>--</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>P28*</td>
<td>7.400</td>
<td>0.025</td>
<td>--</td>
</tr>
<tr>
<td>P29*</td>
<td>8.600</td>
<td>0.014</td>
<td>--</td>
</tr>
<tr>
<td>Total 280</td>
<td>4.608</td>
<td>0.021</td>
<td>--</td>
</tr>
</tbody>
</table>

Degrees of Freedom (DF): $^1$DF = 2,54; $^2$DF = 4,54; $^3$DF = 2,27. Where data violated the assumption of sphericity, Greenhouse-Geisser correction was used to adjust the DF: $^4$DF = 1.7,44.7; $^5$DF = 1.6,44.4. *Results of Friedman test ($\chi^2$) for data that violated the assumption of normality: DF = 2; time was only significant for susceptible trees (test values noted).
Table 2.6 Effect of inter-annual variation on CLO total phenolics at 280 nm and 370 nm. Peak area (± standard error) of total phenolics (normalized to internal standard BHA) at 280 nm and 370 nm in Seaborg and Tabletop CLO phloem tissues collected in 2010, 2011, and 2012 from three disease phenotype classes: control, resistant, and susceptible. The level of total phenolics at 280 nm was significantly affected by year of sampling (repeated measures ANOVA, \( F_{1.6,44.4} = 4.608, p = 0.021 \)), though year-to-year variation was relatively small and did not significantly differ between disease phenotype classes. There was no effect of inter-annual variation or disease phenotype class on the level of total phenolics at 370 nm.

<table>
<thead>
<tr>
<th></th>
<th>Year</th>
<th>Control</th>
<th>Resistant</th>
<th>Susceptible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total 280</td>
<td>2010</td>
<td>17.14 (1.19)</td>
<td>16.56 (1.45)</td>
<td>15.53 (0.96)</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>15.11 (1.29)</td>
<td>15.13 (0.95)</td>
<td>15.13 (1.16)</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>15.08 (1.04)</td>
<td>15.90 (0.99)</td>
<td>16.58 (1.49)</td>
</tr>
<tr>
<td>Total 370</td>
<td>2010</td>
<td>0.73 (0.08)</td>
<td>0.64 (0.10)</td>
<td>0.72 (0.14)</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>0.72 (0.09)</td>
<td>0.60 (0.10)</td>
<td>0.76 (0.13)</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>0.65 (0.09)</td>
<td>0.60 (0.07)</td>
<td>0.73 (0.10)</td>
</tr>
</tbody>
</table>
Figure 2.1 Process for screening and selecting CLO for inter-annual phenolics analysis. Thirty CLO were selected from Seaborg and Tabletop sites at Briones Regional Park for soluble phenolics analysis, and included trees that were classified as resistant or susceptible to *P. ramorum* based on artificial inoculation and non-inoculated (control) trees. Asterisks indicate dates at which phloem, from the 30 trees at Seaborg and Tabletop sites, was collected to analyze inter-annual variation in phloem phenolics.
**Figure 2.2** Effect of season on CLO phenolics. Peak area (± standard error) of phenolics (normalized to internal standard BHA) at 280 nm and 370 nm in Bear Creek CLO phloem tissues collected in winter (December 2010), spring (April 2011), summer (July 2011), and fall (November 2011). Asterisks indicate phenolics whose levels significantly varied among seasons (repeated measures ANOVA, $p < 0.05$). Significant ANOVA test results are reported in Table 2.3.
Figure 2.3 Effect of inter-annual variation on CLO phenolics. Peak area (± standard error) of phenolics (normalized to internal standard BHA) at 280 nm and 370 nm detected in CLO from Seaborg and Tabletop sites in 2010, 2011, and 2012, in three disease phenotype classes: control (A), resistant (B), and susceptible (C). Significant ANOVA test results are reported in Table 2.5.
CHAPTER 3
IDENTIFICATION OF QUERCUS AGRIFOLIA (COAST LIVE OAK) RESISTANT TO THE INVASIVE PATHOGEN PHYTOPHTHORA RAMORUM IN NATIVE STANDS USING FOURIER-TRANSFORM INFRARED (FT-IR) SPECTROSCOPY

Abstract
Over the last two decades coast live oak (CLO) dominance in many California coastal ecosystems has been threatened by the alien invasive pathogen Phytophthora ramorum, the causal agent of sudden oak death. In spite of high infection and mortality rates in some areas, the presence of apparently resistant trees has been observed, including trees that become infected but recover over time. However, identifying resistant trees based on recovery alone can take many years. The objective of this study was to determine if Fourier-transform infrared (FT-IR) spectroscopy, a chemical fingerprinting technique, can be used to identify CLO resistant to P. ramorum prior to infection. Soft independent modeling of class analogy identified spectral regions that differed between resistant and susceptible trees. Regions most useful for discrimination were associated with carbonyl group vibrations. Additionally, concentrations of two putative phenolic biomarkers of

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resistance were predicted using partial least squares regression; > 99% of the variation was explained by this analysis. This study demonstrates that chemical fingerprinting can be used to identify resistance in a natural population of forest trees prior to infection with a pathogen. FT-IR spectroscopy may be a useful approach for managing forests impacted by sudden oak death, as well as in other situations where emerging or existing forest pests and diseases are of concern.

Introduction

Sudden oak death is a highly destructive disease that has caused extensive mortality of oaks and tanoaks in coastal central and northern California and southwest Oregon over the last two decades (Brown and Allen-Diaz, 2009; Cobb et al., 2012; Davis et al., 2010; McPherson et al., 2010, 2005; Meentemeyer et al., 2008; reviewed in Rizzo and Garbelotto, 2003; Rizzo et al., 2002). Few management options exist for controlling the disease and they are all centered on preventative management and silvicultural practices (reviewed in Rizzo and Garbelotto, 2003) since little, if anything, can be done once trees become infected (reviewed in Grunwald et al., 2008). Individual high value trees can be protected by treating them preventatively with phosphonate-based fungicides (Garbelotto and Schmidt, 2009). However, arguably the best management practice for sudden oak death in oak woodlands would focus on the identification and utilization of resistant germplasm, since genetic resistance is the cornerstone of plant protection against insect pests and diseases in conducive environments.
Coast live oak (CLO—*Quercus agrifolia* Née) is a highly susceptible host of *Phytophthora ramorum* Werres et al., the causal agent of sudden oak death. During the early to mid-2000s, the CLO infection rate in some populations was found to be as high as 5.0% y\(^{-1}\), with a mortality rate of 3.1% y\(^{-1}\) (McPherson et al., 2010). In sites heavily impacted by the disease, the loss of CLO basal area over a 20 year period was predicted to be 59-70% (Brown and Allen-Diaz, 2009). Even with high infection and mortality rates, variation in CLO susceptibility to the pathogen has been observed in laboratory assays (Dodd et al., 2005) and within natural populations in field studies (McPherson et al., 2005; Nagle et al., 2011; Ockels et al., 2007). Trees considered naturally resistant to *P. ramorum* never show symptoms of infection (e.g. bleeding exudate and discoloration of phloem tissue), they do not host bark and ambrosia beetles—often associated with infection (McPherson et al., 2008, 2005; Rizzo and Garbelotto, 2003), or appear to recover following infection (McPherson et al., 2014; Nagle et al., 2011). Additionally, when trees are artificially inoculated with a pathogen, resistance can be defined based on canker length, where resistant trees are those with canker lengths that do not differ significantly from mock inoculations or with canker lengths below some critical threshold—a criterion that has been used for pine (Gordon et al., 1998) and more specifically for CLO infected with *P. ramorum* (McPherson et al., 2014).

While the mechanism(s) of CLO resistance to *P. ramorum* is unknown, some studies support the hypothesis that plant specialized metabolites, in particular phenolic compounds, are important for CLO defense against *P. ramorum* (Nagle et al., 2011;
Moreover, Stong et al. (2013) found that tannin-enriched extracts and ground foliage from Oregon white oak (*Quercus garryana* Dougl. Ex Hook) and California black oak (*Q. kelloggii* Newberry), a susceptible host of *P. ramorum*, adversely affect the growth of *P. ramorum*. Tannin-enriched extracts also inhibited the production of *P. ramorum* zoospores and elicitin, which is positively correlated with *P. ramorum* growth and zoospore production (Stong et al., 2013). A reanalysis of Nagle et al. (2011) phenolic data (McPherson et al., 2014) revealed that concentrations of certain phenolic compounds (hereafter referred to as putative phenolic biomarkers of resistance), quantified from asymptomatic phloem of trees already infected with *P. ramorum*, could be used to identify resistant CLO. One of these biomarkers is ellagic acid, a byproduct of ellagitannin hydrolysis (Ascacio-Valdes et al., 2011), which has previously been associated with CLO defense and resistance (Nagle et al., 2011; Ockels et al., 2007).

Ellagic acid has also been shown to inhibit the growth of certain invasive *Phytophthora* species *in vitro*, including *P. cinnamomi* Rands (Cahill and McComb, 1992), a generalist pathogen associated with oak decline throughout North America and Europe (Brasier, 1996; Nagle et al., 2010; Tainter et al., 2000) and *P. ramorum* (McPherson et al., 2014), in the latter case at *in planta*-relevant concentrations. Still, CLO resistance cannot be predicted by measuring the concentration of a single phenolic compound, but instead can be predicted only when several phenolic compounds are used concurrently in a predictive model (McPherson et al., 2014). Thus, techniques that examine a broader spectrum of plant-derived chemicals may be more useful for the identification of resistant CLO.
One technique that is capable of producing comprehensive chemical fingerprints is Fourier-transform infrared (FT-IR) spectroscopy. FT-IR spectroscopy has many advantages over more traditional methods of chemical fingerprinting (e.g. high performance liquid chromatography-mass spectrometry), such as its rapidity and reproducibility in the analysis of solid, liquid, or gaseous samples (Fiehn, 2001). Infrared (IR) spectroscopy can be used to produce chemical fingerprints, which then can be used to identify or discriminate between samples, because spectra, which are produced by measuring changes in the molecular absorption of IR radiation, are determined based on qualitative and quantitative attributes of the chemicals (i.e. functional groups) present in a given sample (Diem, 1993; Guillén and Cabo, 1997; reviewed in Rodriguez-Saona and Allendorf, 2011). This is because the molecular structure of compounds influences how IR radiation is absorbed and consequently the mechanical motion of the molecules (either vibrational or rotational) (Diem, 1993; Guillén and Cabo, 1997; reviewed in Rodriguez-Saona and Allendorf, 2011).

While spectroscopy has been used to determine the water status of CLO foliage (Cheng et al., 2011; Hunt and Rock, 1989; Pu et al., 2003) there are no reports of its use for chemically fingerprinting CLO phloem tissue. However, FT-IR spectroscopy has been used to monitor specialized metabolite production in grapevine (Schmidtke et al., 2012) and for qualitative and semi-quantitative analysis of birch bark extracts (Cîntă-Pințaru et al., 2012). The technique was also used successfully to identify markers of potato resistance to late blight disease caused by *Phytophthora infestans* (Mont.) de Bary
(Taoutaou et al., 2012), to monitor chemical changes in elm wood following infection with the pathogen *Ophiostoma novo-ulmi* Brasier (a causal agent of Dutch elm disease) (Martín et al., 2005), to distinguish between resistant and susceptible elms post-inoculation (Martín et al., 2005), and was able to discriminate between elm clones of differing levels of susceptibility to *O. novo-ulmi* based on the analysis of healthy tissue (Martin et al., 2008).

Based on the evidence that quantitative differences in the constitutive chemical composition of CLO phloem tissue are associated with resistance to *P. ramorum*, the objectives of this study were to determine if FT-IR spectroscopy could be used to (1) discriminate between resistant and susceptible trees, and (2) predict the concentration of putative phenolic biomarkers of resistance, by analyzing phloem tissue collected prior to infection.

**Materials and methods**

**Inoculation and resistance screening**

In July 2010, two phloem samples were collected with a cordless drill equipped with a 1.9 cm diameter drill bit from the main stem of 154 haphazardly selected, apparently disease-free (asymptomatic) CLO from two sites (37° 55’ 12.23” N, 122° 8’ 8.73” W and 37° 56’ 8.57” N, 122° 7’ 40.46” W), covering ~ 8 ha in total, within Briones Regional Park (Contra Costa Co., CA, USA), an area just outside the then-known area of
infestation, for which no records of natural disease incidence were known prior to the start of the study (Brown and Allen-Diaz, 2009) and in which only a small number of symptomatic CLO were observed in the vicinity of the plots at the initiation of the study. Phloem samples from each tree were pooled, placed on dry ice in the field, and then frozen at −18°C until October 2010, when they were shipped on dry ice to The Ohio State University. Following shipping, all samples were stored at −80°C.

In September 2010, each tree stem was inoculated at breast height at the two ends of a 1/3 circumference arc with a plug of P. ramorum isolated from an Umbellularia californica (Hook. & Arn.) Nutt. (California bay laurel) in Contra Costa Co. and grown on 1/3 V8 medium (inoculum kindly provided by Dr. David Rizzo, UC Davis). Inoculations were performed according to the methods of McPherson et al. (2008). Inoculations were conducted under a California Department of Food and Agriculture permit released to Dr. David Rizzo and were necessary because, at the initiation of this study, no other methods were available to screen trees for resistance.

Resistance was determined by observing trees 10, 14, 22, and 34 months following inoculation and separating them into three groups based on the disease phenotypes of Nagle et al. (2011): in remission—trees were initially symptomatic (only bleeding exudate observed) but appeared to recover quickly (no more bleeding)—trees considered most resistant; symptomatic—trees continued to bleed and/or beetle activity and/or Annulohypoxylon fruiting bodies were observed; and susceptible—trees had brown or
leafless crowns, or inoculated stems had snapped (some with green foliage)—trees considered dead and most susceptible.

Support for the phenotypic groupings was sought by calculating the mean length of the two external cankers on each tree approximately 10 months following inoculation (McPherson et al., 2014). Only phloem from trees classified as resistant (i.e. in remission) (n = 22) or susceptible (n = 24) 22 months post-inoculation (see Chapter 4) was used for the present analysis, since these trees were considered most resistant and susceptible, respectively, and thus were ideal for testing whether or not FT-IR spectroscopy could be used to distinguish between resistant and susceptible CLO.

**FT-IR spectroscopy**

Phloem tissue was finely ground in liquid nitrogen and stored at −80°C. 100 ± 1 mg fresh weight (FW) of finely ground phloem tissue was extracted two times with 0.5 mL of HPLC grade methanol (Fisher Scientific, Pittsburgh, PA, USA) for 24 hours at 4°C, as described in Nagle et al. (2011). Extracts were pooled and stored at −80°C until analysis.

The ability of FT-IR spectroscopy and chemometric analysis to discriminate between extracts from resistant and susceptible CLO was then tested on two separate instruments. The first was an Excalibur 3500GX FT-IR spectrometer (benchtop) (Digilab, Randolph, MA, USA), equipped with a triple-bounce zinc selenide, attenuated total reflectance (ATR) accessory and a potassium bromide beamsplitter. The second instrument was a
Cary 630 FT-IR spectrometer (portable) (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a five-bounce zinc selenide ATR accessory. Bounce number indicates the number of times the sample comes in contact with the IR beam. Spectra were collected over a range of 4000–700 cm\(^{-1}\) at 4 cm\(^{-1}\) resolution and an interferogram of 64 scans was co-added for each sample. Spectral data were displayed in terms of absorbance and viewed using Win-IR Pro Software (Agilent Technologies Inc., Santa Clara, CA, USA).

Methanol extracts were concentrated prior to analysis on the benchtop unit. Methanol was completely evaporated from aliquots of extract using a Savant SpeedVac DNA 120 (Thermo Scientific, Asheville, NC, USA) at room temperature and with a low drying rate. Resulting pellets were re-suspended in methanol to a final concentration of 10 times (10x) that of the original extract. Five \(\mu\)l of 10x extract were loaded onto the ATR accessory crystal and allowed to sit for \(~ 60\) seconds; this allowed methanol, which interferes with the spectra of plant extracts, to evaporate before analysis. For analysis with the portable unit, 50 \(\mu\)l of crude methanol extract were loaded onto the ATR accessory crystal. Samples were brought to dryness using a water aspirator and then analyzed. Two technical replicates were analyzed on each unit for each extract (biological replicate).
**Statistical analysis**

**Confirming resistance phenotypes**

Canker length was compared between resistant and susceptible trees using an independent samples t-test (IBM SPSS Statistics 21). Assumptions were tested prior to analysis using Shapiro-Wilk’s and Levene’s tests for normality and homoscedasticity, respectively. Canker length data were log transformed in order to satisfy the assumption of normality and the Welch-Satterthwaite method was used to account for a lack of homoscedasticity between groups (reviewed in Ruxton, 2006; Satterthwaite, 1946; Welch, 1947, 1938).

**FT-IR spectroscopy**

Data collected from the portable unit and benchtop unit were analyzed using the chemometric software Pirouette version 4.0 (Infometrix Inc., Woodville, WA, USA). Soft independent modeling of class analogy (SIMCA) was used to discriminate between resistant and susceptible trees, while partial least squares regression (PLSR) was used to predict the concentration of two putative phenolic biomarkers of resistance, ellagic acid and an uncharacterized flavonoid (FLV1). Ellagic acid and FLV1 were quantified (mg g\(^{-1}\) FW) based on HPLC analysis using a modification of the method described in Nagle et al. (2011) and using an ellagic acid standard curve for compound quantification, as described in Ockels et al. (2007) (Chapter 4). SIMCA is a classification technique that develops principal components models for each training group (i.e. resistant and susceptible CLO) and identifies variables that are important for discriminating between
groups (Subramanian et al., 2007). PLSR uses multivariate analysis to reduce high dimensional, potentially collinear data (e.g. spectral frequencies), and regression analysis to estimate the concentration of variables of interest (e.g. concentrations of phenolic compounds), while maximizing covariance (Wilkerson et al., 2013).

Technical replicates from each biological replicate were analyzed separately. For SIMCA analysis, data were transformed using the standard normal variate (SNV) approach and by taking the second derivative (with a 21 points Savitzky and Golay polynomial filter) (Savitzky and Golay, 1964). For PLSR, data were transformed using the divide by sample 2-norm function. SIMCA 3D class projection plots were used to visualize clustering patterns of resistant and susceptible trees. SIMCA Coomans plots and discriminating power plots were used to identify spectral region(s) that had the highest discriminating power between resistant and susceptible trees (Coomans and Broeckaert, 1986; Subramanian et al., 2007). Coomans plots and 3D class projection plots were also used to identify outliers, which were then removed from the model. For PLSR, loadings and scores plots were used to visualize data and identify the infrared region that best explained the observed variation, respectively. PLSR model performance was evaluated in terms of outlier diagnostics, leave-one-out cross validation, and number of factors included in the model (Wilkerson et al., 2013). Outliers were trimmed based on the methods of Wilkerson et al. (2013), and sample sizes reported reflect the number of technical replicates used for each analysis.
Results

Resistant phenotypes

CLO classified as resistant (n = 22) in 2012 based on symptom expression had significantly smaller external canker lengths 10 months post-inoculation, compared to trees classified as susceptible (n = 24) (t = 8.475, df = 27, P < 0.001) (Figure 3.1).

FT-IR spectra and SIMCA analysis

Spectral data were collected from the mid-IR region (4000 to 700 cm\(^{-1}\)), and overlapping peaks were resolved by using SNV and second derivative functions (Figure 3.2, Table 3.1). Only quantitative spectral differences were observed between resistant and susceptible trees (Figure 3.3). Differences between extracts from resistant and susceptible trees were observed only after spectral transformation, and were most visible around 1305, 1735, and 1772 cm\(^{-1}\).

A 4-factor SIMCA identified two spectral regions that were most important for reliably discriminating between resistant and susceptible trees, regardless of instrument used. This included spectra from approximately 1250 to 1350 cm\(^{-1}\) and 1700 to 1800 cm\(^{-1}\), which corresponded primarily to carbonyl (C=O) group stretching vibrations. Using data collected from the benchtop system (with outliers removed), 100% of extracts from resistant trees (n = 24) and 100% of extracts from susceptible trees (n = 36) were correctly classified, with an interclass distance of 2.4 (the larger the interclass distance,
the less likely samples will be classified as both resistant and susceptible by the SIMCA model (Figure 3.4, Figure 3.5). For data collected from the portable unit (with outliers removed), 100% of extracts from resistant trees (n = 25) and 97% of extracts from susceptible trees (n = 31) were correctly classified, with an interclass distance of 2.3 (Figures A.1 and A.2).

**PLSR analysis**

Normalized (divide by sample 2-norm) spectra between 1202 – 1802 cm⁻¹ (benchtop unit) and 1200 – 1801 cm⁻¹ (portable unit) could be used to predict the concentration of two putative phenolic biomarkers of resistance, ellagic acid and FLV1, independently (Table 3.2). For ellagic acid, a 4-factor PLSR analysis explained > 99.9% of the variation in the concentration of ellagic acid, regardless of instrument used, with a strong positive correlation \( r_{\text{benchtop}} = 0.84; r_{\text{portable}} = 0.75 \) between the predicted and measured concentrations (Figure 3.6, Figure A.3). The standard error of cross-validation (SECV), an approximation of the anticipated error when independent samples are predicted using the model, for ellagic acid was 0.08 – 0.09%. A 3-factor PLSR analysis explained > 99.9% of the variation in the concentration of FLV1, regardless of instrument used, with a strong positive correlation \( r_{\text{benchtop}} = 0.78; r_{\text{portable}} = 0.84 \) between measured and predicted concentrations and a SECV of 0.03% (Figure 3.7, Figure A.4). Loadings plots for factor 4 (ellagic acid) and factor 3 (FLV1) overlaid with preprocessed spectral data indicate areas of the spectrum which correspond with high loading values (either positive or negative) for ellagic acid (Figure 3.8, Figure A.5) and FLV1 (Figure 3.9, Figure A.6).
Areas of the spectrum overlapped with high loading values are likely important for predicting the concentration of each biomarker.

**Discussion**

For the first time, we demonstrate that chemical fingerprinting, based on FT-IR spectroscopy of phloem extracts combined with chemometric analysis, can be used to predict resistance in a natural population of CLOs prior to infection by an important invasive pathogen, *P. ramorum*. Chemical fingerprints were also used to predict the concentration of two putative phenolic biomarkers of CLO resistance, ellagic acid (McPherson et al., 2014) and FLV1 (Chapter 4). Variability in spectral intensities was observed within resistant and susceptible trees; however, resolution of overlapping peaks by normalization and transformation, along with outlier trimming, made it possible to develop strong predictive models. Spectral bands corresponding to carbonyl group vibrations were consistently important for distinguishing between resistant and susceptible CLO, regardless of instrument sensitivity. Instrument sensitivity was dependent on the specific configuration of the ATR accessory, with the five-bounce ATR accessory (portable unit) more likely to detect subtle differences between groups than the triple-bounce ATR accessory (benchtop unit) (Agilent Technologies, 2013).

Two spectral ranges, corresponding primarily to carbonyl group vibrations, had the greatest discriminating power between resistant and susceptible trees in 3-dimensional space using a 4-factor SIMCA. While spectral bands with the greatest discriminating
power differed slightly depending on the instrument used, in general, bands previously associated with plant specialized metabolites were most important. Band 1305 cm\(^{-1}\) was previously found in free quercetin (Torreggiani et al., 2005). Quercetin may be important for plant defense because under certain conditions it scavenges free radicals and chelates metal ions (Torreggiani et al., 2005). Furthermore, quercetin inhibits the growth of *Phytophthora megasperma* Drechsler *in vitro*, a pathogen of olive roots and many other woody species (Báidez et al., 2006). Two bands at 1735 cm\(^{-1}\) (Bovi Mitre et al., 2004) and 1772 cm\(^{-1}\) (Genta et al., 2010) were previously associated with lactones. While we do not know exactly which compounds in our extracts are responsible for these two bands, we do know that ellagic acid is a dilactone (Ascacio-Valdes et al., 2011) with absorbance in the same range that we used for the SIMCA analysis (Figure A.7). Moreover, this corroborates previous studies, which examined the relationship between CLO phenolics and defense and/or resistance to *P. ramorum*. Ockels et al. (2007) found that ellagic acid was associated with CLO defense, and Nagle et al. (2011) found that trees more resistant to *P. ramorum* had higher levels of ellagic acid in their phloem tissue compared to more susceptible CLO. McPherson et al. (2014) identified ellagic acid as one of four putative phenolic biomarkers of resistance in asymptomatic tissue of already infected trees, and also found that ellagic acid inhibited the growth of *P. ramorum* at *in planta*-relevant concentrations *in vitro*.

In addition to using FT-IR spectra to discriminate between resistant and susceptible CLO, PLSR was used to predict the concentration of two putative phenolic biomarkers of
resistance, ellagic acid (McPherson et al., 2014) and FLV1 (Chapter 4). Predicted concentrations of each phenolic biomarker were strongly positively correlated with measured concentrations of each compound, regardless of instrument used, confirming that FT-IR spectroscopy can be used to identify and/or quantify phytochemical features associated with resistant trees. Bands associated with aromatic ring (C=C) and carbonyl (C=O) group vibrations had the highest loading values. Some of the bands identified as being important, based on correlation spectrum plots (data not shown), were previously found to be associated with oak tannin (Gust, 1991), C=O stretching associated with elm defense (Martín et al., 2005), and phenols and C-C bending in gallic acid (Mohammed-Ziegler and Billes, 2002). The potential association of one of these bands with gallic acid is of particular interest, since gallic acid was found at higher concentrations in *P. ramorum* infected phloem tissue and has been shown to inhibit the growth of multiple *Phytophthora* species *in vitro*, including *P. ramorum* (Ockels et al., 2007).

Taken together these results suggest that FT-IR spectroscopy is a viable approach for chemically fingerprinting methanol extracts from CLO phloem tissue. By performing chemometric analysis on data collected from FT-IR spectroscopy, we were able to (1) discriminate between CLO resistant and susceptible to *P. ramorum* prior to infection with the pathogen and (2) estimate the concentration of two putative constitutive phenolic biomarkers of resistance. In the future, these models can be used to predict whether or not an uninfected CLO will be resistant to *P. ramorum*, though they may need to be refined.
(by incorporating data from additional CLO), depending on the accuracy required in future predictions.

Knowledge of resistant (or susceptible) CLO in the landscape may be useful for homeowners, extension agents, or forest managers interested in protecting high-value trees with chemical treatments, protecting stands with high levels of resistance from development and fire, or for the development of sudden oak death management and risk assessment plans. In some areas where many resistant trees are present, the best form of management may be no intervention (allowing naturally resistant trees to replenish the seed bank), or may be limited only to the removal of hazardous trees. Furthermore, the approach detailed in this study may be appropriate for use in other forest pathogen and pest systems where the main objective is to identify resistant germplasm.
Table 3.1 Wavenumber ranges and associated functional groups. Spectral ranges based on peak presence in raw infrared spectrum.

<table>
<thead>
<tr>
<th>Wavenumber range (cm(^{-1}))</th>
<th>Assignment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2840 – 3040</td>
<td>(-\text{C-H (CH}_2\text{)}) stretching</td>
<td>Diem, 1993; Koca et al., 2010</td>
</tr>
<tr>
<td>2860 – 2760</td>
<td>(-\text{C-H (CH}_2\text{)}) stretching</td>
<td>Diem, 1993; Koca et al., 2010</td>
</tr>
<tr>
<td>1650 – 1740</td>
<td>(\text{C=O stretching})</td>
<td>Diem, 1993</td>
</tr>
<tr>
<td>1520 – 1650</td>
<td>(\text{C=C (benzene ring)})</td>
<td>Martín et al., 2005</td>
</tr>
<tr>
<td>1400 – 1480</td>
<td>(\text{C-O stretching, CH}_2\text{, CH}_3)</td>
<td>Diem, 1993; Guillén and Cabo, 1997</td>
</tr>
<tr>
<td>1400 – 1180</td>
<td>(\text{C-O stretching, CH}_2\text{ stretching, C=O})</td>
<td>Koca et al., 2010; Carballo-Meilan et al., 2014</td>
</tr>
<tr>
<td>1200 – 800</td>
<td>(\text{C-O stretching (carbohydrate region)})</td>
<td>Martín et al., 2005</td>
</tr>
</tbody>
</table>
Table 3.2 Results of PLSR analysis for ellagic acid and FLV1.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Range (mg g⁻¹ FW)</th>
<th>Instrument</th>
<th># Factors</th>
<th>% Variance Explained</th>
<th>( r_{val} )</th>
<th>SECV (^{**} ) (mg g⁻¹ FW)</th>
<th>% N Removed (^{***} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellagic acid</td>
<td>0.04 - 0.62</td>
<td>Benchtop</td>
<td>4</td>
<td>99.94</td>
<td>0.84</td>
<td>0.08</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Portable</td>
<td>4</td>
<td>99.94</td>
<td>0.75</td>
<td>0.09</td>
<td>13</td>
</tr>
<tr>
<td>FLV1****</td>
<td>0.03 - 0.47</td>
<td>Benchtop</td>
<td>3</td>
<td>99.91</td>
<td>0.78</td>
<td>0.03</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Portable</td>
<td>3</td>
<td>99.92</td>
<td>0.84</td>
<td>0.03</td>
<td>22</td>
</tr>
</tbody>
</table>

*Correlation coefficient of cross-validation—correlation coefficient describing the direction and strength of the relationship between cross-validated predicted and actual concentrations.
**Standard error of cross-validation—standard error of cross-validated predicted concentrations.
***N = 93.
****In ellagic acid equivalents.
Figure 3.1 External canker length (± standard error) measured 10 months following inoculation with *P. ramorum*. Resistant trees (n = 22) have significantly smaller canker lengths than susceptible trees (n = 24) (independent t-test, *p* < 0.001). Statistical analysis performed using log transformed data.
Figure 3.2 Solid line—raw infrared spectrum from coast live oak phloem extracts; dashed line—second derivative infrared spectrum. The second derivative spectrum was used to resolve overlapping bands (peaks) and to identify differences in the spectra of resistant and susceptible trees.
Figure 3.3 Second derivative and SNV-transformed spectra. Spectral bands with high discriminating power are indicated with arrows. Black—resistant trees; Red—susceptible trees.
Figure 3.4 SIMCA 3D class projection plot for spectral data, transformed using SNV and second derivative. Data collected from the Excalibur 3500GX (benchtop) unit equipped with triple-bounce ATR accessory. Closed diamonds—resistant trees; open diamonds—susceptible trees. Dashed lines indicate the 95% confidence interval for each group.
Figure 3.5 SIMCA Coomans plot with 4 factors (dashed lines indicate critical sample residual thresholds) based on transformed (SNV and second derivative) data. Data from the Excalibur 3500GX (benchtop) unit equipped with triple-bounce ATR accessory. Closed diamonds—resistant trees; open diamonds—susceptible trees. This plot shows the relative, dimension-free distance of a sample from a given class, resistant (x-axis) or susceptible (y-axis), based on the 4 factor SIMCA analysis.
Figure 3.6 PLSR correlation plot showing the relationship between the concentration of the phenolic biomarker of resistance, ellagic acid, determined by HPLC analysis, and the predicted concentration of ellagic acid based on FT-IR spectra. Spectra collected from the Excalibur 3500GX (benchtop) unit equipped with triple-bounce ATR accessory. Spectral data were normalized with divide by sample 2-norm transformation. Closed diamonds—resistant trees; open diamonds—susceptible trees. Statistical analysis reported in Table 3.2.
Figure 3.7 PLSR correlation plot showing the relationship between the concentration of the phenolic biomarker of resistance, FLV1, in ellagic acid equivalents (mg g\(^{-1}\) FW), determined by HPLC analysis, and the predicted concentration of FLV1 based on FT-IR spectra. Spectra collected from the Excalibur 3500GX (benchtop) unit equipped with triple-bounce ATR accessory. Spectral data were normalized with divide by sample 2-norm transformation. Closed diamonds—resistant trees; open diamonds—susceptible trees. Statistical analysis reported in Table 3.2.
**Figure 3.8** Ellagic acid PLSR loadings plot with divide by sample 2-norm transformed data for the 4\textsuperscript{th} factor (solid line), the main factor of discrimination between resistant and susceptible trees, with raw spectra (absorbance) overlaid (dashed line). Data collected from the Excalibur 3500GX (benchtop) unit equipped with triple-bounce ATR accessory. High loading values, either positive or negative, indicate informative spectra.
Figure 3.9 FLV1 PLSR loadings plot with divide by sample 2-norm data for the 3rd factor (solid line), the main factor of discrimination between resistant and susceptible trees with raw spectra (absorbance) overlaid (dashed line). Data collected from the Excalibur 3500GX (benchtop) unit equipped with triple-bounce ATR accessory. High loading values, either positive or negative, indicate informative spectra.
CHAPTER 4

CONSTITUTIVE CONCENTRATIONS OF PHENOLIC BIOMARKERS IDENTIFY QUERCUS AGRIFOLIA RESISTANT TO PHYTOPHTHORA RAMORUM AND CAN BE USED TO ESTIMATE SURVIVAL FOLLOWING INFECTION

Abstract

Sudden oak death, caused by the introduced oomycete Phytophthora ramorum, can be deadly for Quercus agrifolia Née (CLO—coast live oak). Still, resistant trees have been observed in natural populations. Putative phenolic biomarkers of resistance have been identified in asymptomatic tissue of infected CLO, though no previous work has examined whether constitutive concentrations of phenolic compounds can be used as biomarkers to identify resistant trees before infection. To test this, naïve trees were selected from a natural population of CLO. Constitutive concentrations of phenolic compounds in the phloem of trees classified as resistant or susceptible, following P. ramorum inoculation, were quantified using high-performance liquid chromatography and photo diode array detection. Sparse linear discriminant analysis and stepwise logistic regression analysis using individual phenolic compounds as predictor variables were used to identify constitutive phenolic biomarkers of resistance. Four phenolic biomarkers of resistance, including myricitrin and 3 incompletely characterized phenolics, were
identified and together correctly classified 80% of the trees in the training set. Logistic regression analysis was then used to estimate the proportion of resistant trees within a subset of non-inoculated (control) trees from the same population. CLO survival, another measure of tree resistance, was estimated following inoculation using a Weibull survival model and Cox Proportional Hazards analysis, as a function of the constitutive concentrations of phenolic biomarkers. Biomarkers did provide estimates of survival following infection, though to varying degrees depending on the analysis used. These results suggest that pre-infection concentrations of certain phenolic compounds (i.e. biomarkers) can be used to identify trees naturally resistant to an invasive forest pathogen. Knowledge of resistant trees within natural populations may be useful for disease management and efforts aimed at conserving and breeding resistant germplasm.

Introduction

The generalist pathogen *P. ramorum* is an aggressive, alien-invasive oomycete that causes sudden oak death (SOD) and ramorum blight on trees and woody shrubs (Garbelotto and Hayden, 2012; Grunwald et al., 2012; Huberli and Garbelotto, 2012; Rizzo and Garbelotto, 2003; Rizzo et al., 2002; Werres et al., 2001). Several species of California oak are susceptible to *P. ramorum*, including CLO, *Quercus kelloggii* (California black oak), *Quercus parvula var. shrevei* (Shreve oak), and *Notholithocarpus densiflorus* (tanoak) (McPherson et al., 2000; Rizzo et al., 2002). A dark red, viscous, sap-like substance, referred to as “blood,” exuding from the stem of these trees is characteristic of *P. ramorum* infection in areas where the disease is known to occur.
Often, bleeding is accompanied by cracking and discoloration of the outer bark that overlays the cankers, which are primarily restricted to the inner bark (phloem) (Dodd et al., 2008; Grunwald et al., 2008; Rizzo et al., 2002). Several species of ambrosia (e.g. *Monarthrum scutellare* and *Xyleborinus saxeseni*) and bark (e.g. *Pseudopityophthorus pubipennis*) beetles have been observed on *P. ramorum* infected trees, preferentially attacking cankered areas, even when tree canopies appear heathy (McPherson et al., 2008, 2000; Rizzo et al., 2002). Beetles are believed to be attracted to kairomones (e.g. ethanol) emitted from cankered regions (Kelsey et al., 2013; McPherson et al., 2008). Beetle tunneling and the introduction of their fungal associates can weaken the structural integrity of infected trees, and may hasten tree death (McPherson et al., 2008). Beetle presence is typically also associated with opportunistic fungi, e.g. *Annulohypoxylon thouarsianum*; these fungi may further reduce tree survival (McPherson et al., 2013, 2008; Rizzo et al., 2002).

CLO is a highly susceptible, terminal host of *P. ramorum* (Davidson et al., 2002), with high infection and mortality rates in some populations (Brown and Allen-Diaz, 2009; McPherson et al., 2010). Even though CLO as a species is susceptible, there is significant evidence to indicate that a subset of trees are resistant to *P. ramorum* (Dodd et al., 2005; McPherson et al., 2014; Nagle et al., 2011; Rizzo et al., 2005; Swiecki and Bernhardt, 2005). Still, very few studies have examined the mechanisms by which trees successfully defend themselves against *P. ramorum* (Dodd et al., 2008; McPherson et al., 2014; Nagle et al., 2011; Ockels et al., 2007; Stong et al., 2013). From studies focused on the chemical
composition of CLO phloem, i.e. the tissue showing the most conspicuous symptoms of
*P. ramorum* infection, it is known that phenolic profiles of healthy and cankered tissues
(Ockels et al., 2007) and asymptomatic phloem phenolic profiles from putatively
resistant, in remission and susceptible trees (Nagle et al., 2011), differ quantitatively.
Therefore, metabolite profiling, a focused analysis on a subset of metabolites (Fiehn,
2001), may be the most useful approach for identifying resistant CLO while also
contributing to the elucidation of CLO resistance mechanisms. A metabolite profiling
approach focused on the identification of chemical biomarkers of CLO resistance is
supported by the fact that chemical fingerprinting, a less analytically sensitive approach
(Fiehn, 2001), was able to distinguish between CLO that were resistant and susceptible to
*P. ramorum* before infection (see Chapter 3). Furthermore, the identification of
biomarkers is not a novel concept in the study of plant pathosystems. Metabolites have
been identified as potential biomarkers of resistance in other plant pathosystems,
including barley for resistance to *Fusarium graminearum* Schwabe, the causal agent of
Fusarium Head Blight (Bollina et al., 2010; Kumarsawamy et al., 2011), and grapevine
for resistance to downy mildew, powdery mildew, and *Botrytis* (Ali et al., 2009;
Batovska et al., 2008). Biomarkers have also been identified in tree pest- and
pathosystems. For example, constitutive concentrations of select resin monoterpenes
were higher in white pine weevil-resistant than susceptible Sitka spruce (Hall et al., 2011)
and phenolic biomarkers were identified from mango leaf extracts that were associated
with cultivars that were tolerant or susceptible to *Fusarium* (Augustyn et al., 2014).
Putative phenolic biomarkers of CLO resistance to *P. ramorum* have been identified from the work of Nagle et al. (2011) and Ockels et al. (2007), and also from the reanalysis of Nagle et al. (2011) data by McPherson et al. (2014). The compounds identified as putative biomarkers include ellagic acid and one of its derivatives, total phenolics, as well as several other incompletely characterized phenolic compounds (McPherson et al., 2014; Nagle et al., 2011; Ockels et al., 2007). There is even evidence to suggest that some of these phenolics may play a role in CLO defense against *P. ramorum*. *In vitro* bioassays confirmed that gallic acid, tyrosol, ellagic acid, and crude soluble phenolics extract (i.e. total phenolics) independently inhibit the growth of *P. ramorum* in a dose-dependent manner and that total phenolics can be fungicidal at in planta-relevant concentrations (McPherson et al., 2014; Ockels et al., 2007). While the mechanism(s) by which these compounds inhibit the growth of *P. ramorum* is unknown, phytosterols and tannin-enriched extracts decrease *P. ramorum* elicitin gene expression and ELISA-detectable elicitin, respectively, and also adversely affect *P. ramorum* growth and sporulation (Stong et al., 2013).

McPherson et al. (2014) reported the identification of four phenolic biomarkers of resistance from the analysis of phloem tissue collected away from *P. ramorum* cankers on resistant and susceptible CLO. Discrimination between resistant and susceptible individuals was based on the levels of these compounds, rather than qualitative differences. Since it was not possible to determine whether or not the concentrations of phenolic compounds were actually at constitutive (pre-infection) levels, the goal of the
present study was to determine if constitutive concentrations of phenolic biomarkers can be used to identify resistant individuals from a naïve, previously non-infected population of CLO. Specifically, the objectives of this study were to: (1) measure constitutive phloem phenolics in a naïve population of CLO; (2) identify constitutive biomarkers of resistance and develop a model based on biomarker concentrations to predict resistance in naïve trees, and (3) estimate CLO survival following *P. ramorum* infection using the concentration of biomarkers as predictor variables.

**Materials and methods**

*Plant material and resistance screening*

Phloem tissue collected from apparently asymptomatic CLO within Briones Regional Park was used in the study; a description of sample collection and storage can be found in Chapter 3. Trees were subsequently inoculated (see Chapter 3 for description of inoculation method) and response phenotype was determined based on symptom expression 10, 22, 34, and 44 months post-inoculation. Trees were classified as either resistant (i.e. in remission: trees once showed evidence of infection but appeared to recover), symptomatic (trees continued to show symptoms of infection), or susceptible (trees succumbed to the disease). For identification of phenolic biomarkers of resistance, only tissue samples collected from trees that were eventually classified as resistant or susceptible 22 months post-inoculation were used, while for survival analysis, tissue
samples collected from trees that were eventually classified as resistant or susceptible 44 months following inoculation were used (see Table 4.1 for detailed study timeline).

Phloem tissue was also collected from approximately 450 non-inoculated control CLO, i.e. asymptomatic, in Briones Regional Park, that were monitored yearly for natural disease development. A subset of control trees (N = 103) was then selected for analysis of constitutive phenolics.

*High performance liquid chromatography (HPLC) analysis and data processing*

Pooled phloem samples were finely ground in liquid nitrogen and 100 mg ± 1 mg fresh weight (FW) were extracted twice with 500 µL HPLC grade methanol at 4°C for 24 hours (Nagle et al., 2011). Extracts were pooled and stored at -20°C until analysis. Pooled extracts were briefly centrifuged to remove residual particulate matter, and then analyzed with a Waters 2690 HPLC equipped with an auto sampler (Waters, Milford, MA, USA), with samples held at 4°C. 10 µL of each extract was then injected into a Thermo Scientific 250 x 4.6 mm, 5-µm, C-18 reverse phase column at 40°C. Samples were separated using a water (A) and methanol (B) solvent gradient adapted from Nagle et al., 2011; each solvent was acidified with 2% (v/v) glacial acetic acid. The solvent gradient was as follows (% represent proportion of solvent A): 100% (0-2 min); 100-90% (2-10 min); 90-83% (10-15 min); 83-77% (15-20 min); 77-52% (20-35 min); 52-0% (35-38 min); 0% (38-41 min); 0-100% (41-42 min); 100% (42-45 min), with a 5 min injection delay between samples. The flow rate was 1.0 ml min⁻¹ from 0-10 min, 1.0-1.75
ml min\(^{-1}\) from 10-15 min, 1.75 ml min\(^{-1}\) from 15-20 min, 1.75-1.0 ml min\(^{-1}\) from 20-35 min, and 1.0 ml min\(^{-1}\) from 35-45 min. A Waters 996 Photodiode array (PDA) detector, scanning between 210 and 400 nm was used for data acquisition. Data acquisition and processing (with smoothing by a 21 point Savitzky-Golay filter) were performed using Empower Pro software (Waters, Milford, MA, USA). Wavelengths of 280 and 370 nm were used to aid in peak selection. The minimum peak area detection threshold was 24464 AU for 280 nm and 1609 AU for 370 nm.

Phenolic biomarkers of resistance were putatively identified using a Varian 500 LC-MS (Varian Inc., Palo Alto, CA, USA) with electrospray ionization (ESI) in negative ion mode. Representative samples were analyzed with full scan and TurboDDS (i.e. MSn) modes, using the same separation method as described above with three exceptions: flow rate was reduced to 0.9 ml min\(^{-1}\) throughout the entire run; percentage of acetic acid was reduced to 0.1% (v/v), and the run time was extended to 50 min (see Chapter 2 for a description of LC-MS gradients). For analysis using full scan mode, enhanced scan mode was used, with scanning from 50 – 1000 m/z. The capillary voltage was 80.0 V, and the needle voltage was −5000 V. For analysis using TurboDDS, enhanced scan mode was used, scanning between 50 – 1000 m/z. The capillary voltage was 80.0 V, the needle voltage was −5000 V, the nebulizer gas (compressed air) pressure was set to 25.0 psi (172.4 kPa), and the drying gas (nitrogen) pressure was set to 15.0 psi (103.4 kPa) with a drying gas temperature of 350°C. The MSn was 4, with threshold counts at 5000 for MS\(_2\),
500 for MS$_3$, and 50 for MS$_4$. Instrument parameters were selected based on Whitehill (2011).

To confirm that fragmentation patterns were associated with biomarkers, fractions containing each biomarker were collected using a Waters Fraction Collector II connected to the Waters 2690 HPLC described above. For fraction collection, injection volume was increased to 20 µL; all other parameters remained unchanged. Fractions were pooled for each biomarker and concentrated using an Eppendorf Vacufuge (Eppendorf North American, Inc., Westbury, NY, USA). Concentrated fractions were then subjected to HPLC analysis to confirm biomarker presence based on peak retention time and UV absorbance. After confirming peak presence in the concentrated fraction, fractions were diluted (1:10) with methanol and directly infused into an AB SCIEX QTRAP 5500 mass spectrometer (AB SCIEX, Framingham, MA, USA) at a flow rate of 7 µL min$^{-1}$. Putative identities from mass spectrometry analysis were confirmed by spiking samples with commercially available standards, when available (Ockels et al., 2007), and peaks of interest (at 370 nm) were quantified in ellagic acid equivalents using a 3-point calibration curve with concentrations ranging from 0.01 to 1.0 mg ml$^{-1}$.

**Statistical analysis**

**Constitutive phenolic biomarkers**

A multi-faceted approach was utilized to identify constitutive phenolic biomarkers in CLO (Figure 4.1). Sparse linear discriminant analysis (SLDA) was used to identify the 15
most important soluble phenolic peaks (normalized peak area units at 280 nm and 370 nm; peak area normalized to have a zero mean for each phenolic) discriminating between resistant \((n = 24)\) and susceptible \((n = 31)\) trees classified in 2012, hereafter referred to as the training set \((R\) software, \(R\) development core team, version 2.15.3) \((\text{McPherson et al., 2014})\). \(\text{SLDA}\) is a supervised technique which classifies samples based on measured characteristics of each sample \((\text{e.g. phenolic levels})\), incorporating dimension reduction, and may be more appropriate for smaller sample sizes \((\text{McPherson et al., 2014; Shao et al., 2011})\). In certain biological samples, individual peaks could not be quantified because they were not present or below detection thresholds. In these instances, the peak area threshold was used in lieu of a zero.

Untransformed peak areas, from the 15 peaks selected by \(\text{SLDA}\), were then subjected to backwards stepwise logistic regression \((\text{LR})\) analysis using the likelihood ratio method \((\text{IBM SPSS Statistics 22})\). The final model was selected based on the following criteria: results of Hosmer and Lemeshow test \((\text{goodness of fit test}; \text{only non-significant models were considered})\), number of model predictors \((\text{maximize parsimony})\), and accurate prediction of cases used to generate the model \((\text{Hosmer and Lemeshow, 2000})\). The linearity assumption was then tested for each of the predictors selected for the final \(\text{LR}\) model. Model coefficients were calculated using the concentration of selected peaks in ellagic acid equivalents \((\text{mg g}^{-1} \text{ FW})\). The general \(\text{LR}\) model was as follows:

\[
(1) \quad P_{R}(Y=1) = \frac{\exp(a + b_1 \cdot \text{Phenolic}_1 + b_2 \cdot \text{Phenolic}_2 + b_i \cdot \text{Phenolic}_i)}{[1 + \exp(a + b_1 \cdot \text{Phenolic}_1 + b_2 \cdot \text{Phenolic}_2 + \ldots + b_i \cdot \text{Phenolic}_i)]}
\]
where the probability of being resistant (belonging to the R group) is 1 and i phenolic compounds can be used as predictor variables in the model (McPherson et al., 2014).

A nonparametric receiver operating characteristic (ROC) curve was used to evaluate the classification performance of the final LR model and to test whether or not predicted probabilities were better than those from chance alone (IBM SPSS Statistics 22). An ROC curve compares predicted and actual probabilities of resistance, and curve coordinates can be used to determine the probability cut-off that optimizes specificity and sensitivity, where sensitivity is the proportion of cases correctly classified as resistant and [1-specificity] is the proportion of susceptible cases that were incorrectly classified as resistant, i.e. false positive (Hosmer and Lemeshow, 2000; Hughes and Madden, 2003). A ROC curve was also used to optimize the probability cut-off of the final LR model. The final LR model with optimized probability cut-off was then used to estimate the probability of resistance for each control CLO selected for constitutive phenolic analysis, since a true testing set could not be used for model validation due to an insufficient number of resistant and susceptible trees.

Finally, the relationship between each phenolic biomarker and susceptibility to P. ramorum was tested using phenolic data collected from all trees in the training set. Spearman’s rank order correlation analysis was used to ascertain the level of association between external canker length measured 10 months post-inoculation and the constitutive
concentration of each biomarker (IBM SPSS Statistics 22). Correlations with \( p \leq 0.05 \) were considered significant.

**Constitutive phenolic biomarkers and survival**

Since those trees that survive the longest are subsequently considered the most resistant trees to *P. ramorum*, the constitutive concentration of phenolic biomarkers identified by SLDA and LR were used as covariates in two different survival analyses. Mortality was assessed 10, 14, 22, 34, and 44 months following inoculation for all trees classified as resistant \( (n = 37) \) and susceptible \( (n = 62) \) in 2014 (44 months post-inoculation).

A censored Weibull survival analysis (parametric model) was used to estimate future CLO survival as a function of the concentration of phenolic biomarkers (R software, R development core team, version 3.1.1). The Weibull model allowed for survival to be estimated into the future, beyond the 44 month study period (Lee and Wang, 2003; McPherson et al., 2005). However, a relatively short study length may limit the accurate modeling of survival using the Weibull function. For that reason, a Cox proportional hazards (PH) model (semi-parametric model) was also used to determine if phenolic biomarkers were significant predictors of survival within the context of the study period (IBM SPSS Statistics 22). For both models, overall model significance was assessed using a Chi Square test. Both Weibull and Cox PH analyses allow for the inclusion of censored cases (i.e. cases where trees are still alive) and can be used to estimate survival in new cases, given that variables used as predictors have been measured.
Results

Constitutive phenolic biomarkers

Using SLDA on the training set, the top 15 HPLC peaks discriminating between resistant and susceptible trees were selected from 33 total peaks detected at 280 and 370 nm and the sum of all phenolics (total phenolics) at 280 and 370 nm, respectively. Peaks selected by SLDA included: tyrosol, ellagic acid, tyrosol glycoside, myricitrin, 6 unknown phenolics detected at 370 nm, and 5 unknown catechin-like compounds detected at 280 nm (based on spectral similarity to authentic catechin standard). Then, four compounds were selected as predictor variables (i.e. biomarkers of resistance) using backwards stepwise LR analysis, and included myricitrin (M) and 3 incompletely characterized phenolics detected at 370 nm (FLV1, FLV2, and FLV3) (see Table 4.2 for retention time, UV absorbance and MS fragmentation patterns of selected biomarkers). Since all predictor variables, i.e. phenolic compounds, were detected at 370 nm, LR model coefficients were calculated in ellagic acid equivalents (mg g\(^{-1}\) FW) (a commercially available analytical standard) (Table 4.3). The LR equation is as follows:

\[
(2) \quad P_R(Y=1) = \frac{\exp(-0.81 + 15.26*FLV1 -16.15*FLV2 - 20.25*M + 1.86*FLV3)}{[1 + \exp(-0.81 + 15.26*FLV1 -16.15*FLV2 - 20.25*M + 1.86*FLV3)]}
\]

where the probability of resistance, \(P_R\), ranges from 1 (trees are resistant) to 0 (trees are susceptible). Greater concentrations of FLV1 and FLV3 were associated with a higher probability of resistance (coefficients for FLV1 and FLV3 were positive), while greater
concentrations of FLV2 and myricitrin were associated with a higher probability of susceptibility (negative coefficients for FLV2 and myricitrin).

Using a ROC curve (Figure 4.2), the probability cutoff that optimized specificity and sensitivity was determined. Trees with $P_R \geq 0.46$ (determined using equation 2) were classified as resistant, while trees with a probability less than the 0.46 cutoff were classified as susceptible. The ROC curve also confirmed that predicted probabilities from the model were better than those from chance alone (area under curve = 0.847, SE = 0.054, $p < 0.001$). Overall, the model was a good fit (Hosmer and Lemeshow test, $\chi^2 = 4.931$, df = 7, $p = 0.668$) with 80% of trees (N = 55) correctly classified as either resistant or susceptible in the training set. 79.2% of resistant trees (n = 24) were correctly classified by the model, while only 19.4% of susceptible trees (n = 31) were misclassified as resistant.

The proportion of resistant trees in a subset of control CLO (N = 103) at Briones Regional Park was estimated using equation 2, the optimized $P_R$ cutoff of 0.46, and constitutive concentrations of phenolic biomarkers quantified in ellagic acid equivalents. If control trees become infected with $P. ramorum$, 20% are predicted to be resistant. In May 2014 (46 months after tree monitoring began), 6 of the 103 control trees analyzed showed possible symptoms of natural $P. ramorum$ infection, i.e. bleeding exudate. All 6 trees were predicted to be susceptible ($P_R < 0.46$) based on equation 2, though none of these trees have shown additional symptoms of SOD, e.g. presence of beetles or
Annulohypoxylon fruiting bodies, or have died as a result of an apparent *P. ramorum* infection.

Spearman’s rank correlation analysis revealed that 3 of the 4 phenolic biomarkers were significantly correlated with external canker length, a measure of CLO susceptibility to *P. ramorum*. FLV1 and FLV3 were negatively correlated with canker length, such that increased concentrations of FLV1 and FLV3 were associated with more resistant CLO (i.e. smaller external canker lengths). FLV2 was positively correlated with canker length; greater concentrations of FLV2 were associated with more susceptible CLO (i.e. larger external canker lengths). While myricitrin was identified as a biomarker of resistance, there was no relationship between its constitutive concentration and CLO susceptibility following *P. ramorum* infection based on external canker length. (Table 4.4).

**Constitutive phenolic biomarkers and survival**

Constitutive concentrations of the four phenolic biomarkers could be used to estimate CLO survival following infection with *P. ramorum*, though to different degrees depending on which model was used ($\chi^2_{\text{Weibull}} = 10.5$, $df_{\text{Weibull}} = 4$, $p_{\text{Weibull}} = 0.033$; $\chi^2_{\text{CoxPH}} = 8.1$, $df_{\text{CoxPH}} = 4$, $p_{\text{CoxPH}} = 0.087$) (Table 4.5). Higher concentrations of FLV2 and myricitrin were associated with shorter survival times (Weibull model) and increased hazard, i.e. likelihood of mortality, following *P. ramorum* inoculation (Cox PH).
Using *in planta* concentrations of phenolic biomarkers from CLO of varying susceptibility to *P. ramorum* (susceptibility quantified by external canker length) the median survival time of the most resistant and the most susceptible trees in the study was estimated using the Weibull model (Table 4.6, Figure 4.3). The median survival time for the most resistant tree in the study was approximately 1 year longer than the most susceptible tree in the study. The effect of biomarkers on CLO survival was also examined using the Cox PH model. Trees were 23.7 times more likely to die following inoculation within the 44 month study period, for every 0.1 mg g\(^{-1}\) FW increase in the constitutive concentration of myricitrin (Cox PH model, Exp(B) = 237.2, 95% CI = 1.9 – 30292.4), assuming all other variables are kept constant.

**Discussion**

This study confirms that constitutive concentrations of phenolic biomarkers can be used to predict resistance to *P. ramorum*, corroborating the findings of McPherson et al. (2014). Four phenolic compounds, FLV1, FLV2, FLV3, and myricitrin, together correctly classified 80% of resistant and susceptible trees in the training set. Although the model could not be validated using a testing set, the high percentage of correctly classified trees in the training set in combination with a significant area under the ROC curve support the hypothesis that constitutive concentrations of phenolic biomarkers can be used to identify resistant CLO. In addition, the proportion of control (uninoculated) trees predicted to be resistant, 20% (N = 103), falls between the actual proportion of resistant trees based on artificial inoculation with *P. ramorum* observed in 2012 (16%)
and 2014 (25%) (N = 153) (data not shown). At this time it is not possible to assess the accuracy of the LR model, since validation relies on natural infection and disease progression in control trees. Still, phloem phenolic compounds appear to be ideal biomarkers for CLO because levels of most phenolics were not impacted by tree phenology (see Chapter 2). This is important, since a necessary condition for biomarkers to be useful is that they display little environmental and population variation (Steinfath et al., 2010).

Because time from infection to death varies within CLO populations (Rizzo and Garbelotto, 2003), classifying trees as resistant based on disease progression and ultimately survival is limited by the short duration of most scientific studies when compared to the lifetime of a tree. Models predicting tree survival based on pre-infection properties may be of use in situations where study duration is a limiting factor. To combat this limitation and also to confirm that biomarkers can be used to predict CLO resistance, the relationship between the concentration of the four phenolic biomarkers of resistance and tree survival within the context of the study period (Cox PH), and into the future (Weibull), was assessed. When considered in combination, concentrations of all four biomarkers could be used to model CLO survival at $p < 0.1$. For both FLV2 and myricitrin, tree survival decreased with increasing concentrations of each compound. This is consistent with the finding that both compounds were associated with a higher probability of susceptibility in the LR resistance model (equation 2). Based on median survival time, the most resistant tree in the population was estimated to survive ~1 year.
longer than the most susceptible tree. This difference seems small, considering that even symptomatic CLO may live over 7 years following artificial inoculation (McPherson et al., 2014; Nagle et al., 2011) and symptomatic trees can survive years after natural disease development (Rizzo and Garbelotto, 2003; Swiecki and Bernhardt, 2005). The Weibull model is limited by the relatively short duration of this study, the time intervals in which mortality was assessed (i.e. 10, 14, 22, 34, and 44 months post-inoculation), and by the lack of inclusion of other relevant data that can be recorded only after infection (e.g. beetle presence or absence, canker length) (McPherson et al., 2014, 2005). However, predicting survival using variables measured only before infection was the main objective, and the only other variable measured before infection in this study, diameter at breast height, did not significantly improve the fit of the model (data not shown).

These analyses show that phenolic biomarkers can be used to identify CLO resistant to *P. ramorum* before infection. However, identification of chemical biomarkers does not necessarily imply that these compounds are of biological relevance (Fiehn, 2001). Therefore, the relationship between biomarker concentration and CLO external canker length, a measure of tree resistance (Gordon et al., 1998; McPherson et al., 2014), was assessed using Spearman’s rho correlation analysis. Concentrations of 3 of the 4 biomarkers identified in this study, FLV1, FLV2, and FLV3, were significantly correlated with canker length. Based on the retention time, spectra, and relatedness of these compounds to commercially available standards, including, but not limited to, ellagic acid, quercetin, kaempferol, and myricitrin, one of the phenolic biomarkers was
identified as the flavonoid myricitrin and it is possible that the remaining biomarkers could also be flavonoids. Flavonoids play important roles in plant defense responses in many pathosystems. For example, flavonoids from geranium are known pro-oxidants, generating reactive oxygen species or inhibiting the antioxidant activity of other, potentially phytotoxic compounds (Tuominen, 2013). However, extracts from *Eucalyptus* known to contain flavonoids also possess antioxidant activity, which can protect plants from damaging free radicals (Okamura et al., 1993; Witzell and Martin, 2008). While oxidant (e.g. pro-oxidant or antioxidant) activity is context specific (e.g. environmental conditions like pH may alter the oxidative activity of certain compounds), it has been implicated as a defense in many different plant systems (Appel, 1993; Barbehenn et al., 2006b). The findings in *Geranium sylvaticum* L. are particularly relevant because *G. sylvaticum* contains a suite of phytochemical classes that overlap with those found in oak, including but not limited to ellagitannins, proanthocyanidins, gallotannins, and flavonoids (Salminen et al., 2004; Tuominen, 2013). In addition to possessing oxidative activity, flavonoids have been shown to inhibit the growth of some microbes *in vitro*, including the plant pathogen *Verticillium dahliae* Kleb. (Báidez et al., 2007). Flavonoids can also play important roles in constitutive and induced defense responses in trees. Genes related to flavonoid biosynthesis were induced in poplar following infection by the rust, *Melampsora medusae* (Miranda et al., 2007). In the present study, two biomarkers, FLV1 and FLV3, were positively associated with CLO resistance. However, the converse may also be true, depending on the specific compound. Significant differences in the enrichment of transcripts related to constitutive flavonoid biosynthesis in *Q. robur* were
found between trees resistant and susceptible to the oak leaf roller, *Tortrix viridana* (Kersten et al., 2013). Kersten et al. observed greater constitutive flavonoid gene expression in susceptible trees. Likewise, FLV2 and myricitrin were associated with CLO susceptibility (increased concentrations were associated with a lower probability of resistance and a greater risk of mortality), though the concentration of myricitrin was not significantly correlated with CLO canker length. This is not inconsistent with other reports of myricitrin in the literature. UV-B stressed poplar leaves contained higher concentrations of myricitrin-3-galactoside and were more susceptible to winter moth larval feeding compared to non-stressed leaves (Lavola et al., 1998). However, an artificial diet amended with myricitrin (and the flavonoid-glycoside quercitrin) did not stimulate larval feeding in vitro, but decreased feeding at the highest concentration tested (Lavola et al., 1998). In another study, myricetin glycosides (myricetin is the aglycone of myricitrin) from plant extracts were easily oxidized at a high pH (Vihakas et al., 2014). The oxidative (e.g. pro-oxidant and antioxidant) activity of phenolic compounds may contribute to their ecological function, since phenolic action in many cases requires oxidative activation resulting in the formation of toxins and oxygen radicals (Appel, 1993). A comprehensive functional analysis would be needed to know the exact mechanisms by which these compounds operate in CLO defense against *P. ramorum*. An analysis of this nature was outside the scope of this study, since it is not necessary for the identification of resistant trees.
The SOD epidemic shows no signs of fading anytime soon, as the disease continues to spread within the native range of CLO. The identification of phenolic biomarkers of resistance, in combination with the development of statistical models that identify resistant trees as a function of the constitutive concentration of biomarkers, may be a useful tool for the management of SOD in forests on the edge of the outbreak or where naïve hosts are found. Although biomarkers can be used to identify resistant trees, they may not directly interact with *P. ramorum in planta* to confer resistance. Still, the association of biomarkers with variability in CLO susceptibility does suggest putative resistance mechanisms, which could be a topic of future studies. Identifying resistant trees using biomarkers provides an alternative to traditional, inoculation-based methods, which are more time intensive and potentially carry a larger ecological cost, since not all trees will become naturally infected with the pathogen. Finally, biomarkers can and should be used to identify potentially resistant trees for conservation and breeding efforts aimed at producing and preserving resistant germplasm.
### Table 4.1 Timeline of research activities.

<table>
<thead>
<tr>
<th>Date</th>
<th>Months post-inoculation</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 2010</td>
<td>--</td>
<td>Phloem is sampled from naïve trees, including trees that were then artificially inoculated and those that were left to become naturally infected.</td>
</tr>
<tr>
<td>September 2010</td>
<td>0</td>
<td>A subset of CLO (N ~ 150) is inoculated with <em>P. ramorum</em></td>
</tr>
<tr>
<td>July 2011</td>
<td>10</td>
<td>Inoculated trees are monitored for symptom development; external lesions measured; control trees monitored for symptoms of natural infection.</td>
</tr>
<tr>
<td>November 2011</td>
<td>14</td>
<td>Inoculated trees are monitored for symptom progression; subcortical lesions (cankers) measured.</td>
</tr>
<tr>
<td>July 2012</td>
<td>22</td>
<td>Inoculated trees are monitored for symptom progression; control trees monitored for symptoms of natural infection.</td>
</tr>
<tr>
<td>July 2013</td>
<td>34</td>
<td>Inoculated trees are monitored for symptom progression; control trees monitored for symptoms of natural infection.</td>
</tr>
<tr>
<td>May 2014</td>
<td>44</td>
<td>Inoculated trees are monitored for symptom progression; control trees monitored for symptoms of natural infection.</td>
</tr>
</tbody>
</table>
**Table 4.2** Chromatographic and mass spectral (MS) characteristics of phenolic biomarkers of resistance. Direct infusion of concentrated fractions containing each biomarker was used to confirm fragmentation patterns. All MS data were obtained in negative ion mode. Bolded numbers indicate ions that were subjected to further fragmentation, where one or more daughter fragment ions were detected. See Figure B.2 for HPLC-PDA chromatogram with biomarkers labeled.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT (min)</th>
<th>$\lambda_{max}$ at 370 nm</th>
<th>MS1</th>
<th>MS2</th>
<th>MS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLV1</td>
<td>12.9</td>
<td>372, 254</td>
<td>557</td>
<td>313, 327</td>
<td>169</td>
</tr>
<tr>
<td>FLV2</td>
<td>23.1</td>
<td>359, 233</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Myricitrin</td>
<td>27.6</td>
<td>351, 260</td>
<td>463</td>
<td>316</td>
<td>271</td>
</tr>
<tr>
<td>FVL3</td>
<td>37.2</td>
<td>367, 247</td>
<td>475</td>
<td>328, 313</td>
<td>313, 298, 285</td>
</tr>
</tbody>
</table>
Table 4.3 Logistic regression variables for selected constitutive phenolic biomarkers of resistance.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Estimate</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLV1</td>
<td>15.26</td>
<td>5.30</td>
<td>0.004</td>
</tr>
<tr>
<td>FLV2</td>
<td>−16.15</td>
<td>8.96</td>
<td>0.071</td>
</tr>
<tr>
<td>Myricitrin</td>
<td>−20.25</td>
<td>9.27</td>
<td>0.029</td>
</tr>
<tr>
<td>FVL3</td>
<td>1.86</td>
<td>0.98</td>
<td>0.057</td>
</tr>
<tr>
<td>(Intercept)</td>
<td>−0.81</td>
<td>1.06</td>
<td>0.447</td>
</tr>
</tbody>
</table>
Table 4.4 Association between the concentration of each phenolic biomarker of resistance and CLO susceptibility to *P. ramorum*, based on external canker length measured 10 months post-inoculation (Spearman’s rank-order correlation test) (N = 55).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\rho$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLV1</td>
<td>-0.35</td>
<td>0.01</td>
</tr>
<tr>
<td>FLV2</td>
<td>0.28</td>
<td>0.04</td>
</tr>
<tr>
<td>Myricitrin</td>
<td>0.07</td>
<td>0.63</td>
</tr>
<tr>
<td>FLV3</td>
<td>-0.29</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Table 4.5 Weibull and Cox PH model variables for constitutive phloem phenolic biomarkers of resistance.

<table>
<thead>
<tr>
<th>Model</th>
<th>Compound</th>
<th>Coefficient</th>
<th>SE</th>
<th>Test statistic</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weibull</td>
<td>FLV1</td>
<td>0.86</td>
<td>0.59</td>
<td>1.46</td>
<td>0.144</td>
</tr>
<tr>
<td></td>
<td>FLV2</td>
<td>−1.86</td>
<td>0.95</td>
<td>−1.97</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>Myricitrin</td>
<td>−2.50</td>
<td>0.96</td>
<td>−2.62</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>FVL3</td>
<td>0.17</td>
<td>0.13</td>
<td>1.25</td>
<td>0.210</td>
</tr>
<tr>
<td></td>
<td>(Intercept)</td>
<td>3.91</td>
<td>0.14</td>
<td>28.63</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>Log (scale)</td>
<td>−1.00</td>
<td>0.11</td>
<td>−9.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cox PH</td>
<td>FLV1</td>
<td>−2.08</td>
<td>1.59</td>
<td>1.72</td>
<td>0.189</td>
</tr>
<tr>
<td>model</td>
<td>FLV2</td>
<td>4.82</td>
<td>2.58</td>
<td>3.50</td>
<td>0.061</td>
</tr>
<tr>
<td></td>
<td>Myricitrin</td>
<td>5.47</td>
<td>2.47</td>
<td>4.89</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>FLV3</td>
<td>−0.36</td>
<td>0.36</td>
<td>0.97</td>
<td>0.324</td>
</tr>
</tbody>
</table>
Table 4.6 Concentrations of phenolic biomarkers (mg g\textsuperscript{-1}FW, in ellagic acid equivalents) used to estimate median survival in months (95% confidence interval) using the Weibull model for the most resistant (R) and the most susceptible (S) tree in the study based on external canker length.

<table>
<thead>
<tr>
<th>Tree</th>
<th>Canker length, cm</th>
<th>PR</th>
<th>FLV1</th>
<th>FLV2</th>
<th>M</th>
<th>FVL3</th>
<th>Median survival (95% CI), mo.</th>
<th>Actual survival\textsuperscript{1}, mo.</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>2</td>
<td>0.84</td>
<td>0.24</td>
<td>0.02</td>
<td>0.08</td>
<td>0.31</td>
<td>45.1 (36.6 – 53.6)</td>
<td>44</td>
</tr>
<tr>
<td>S</td>
<td>167</td>
<td>0.09</td>
<td>0.11</td>
<td>0.03</td>
<td>0.17</td>
<td>0.41</td>
<td>31.6 (25.9 – 37.3)</td>
<td>14</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Maximum actual survival post-inoculation is 44 mo., i.e. the end of the observation period.
Figure 4.1 Statistical pipeline for phenolic biomarker of resistance identification, i.e. discriminating between resistant (R) and susceptible (S) trees.
The receiver operating characteristic (ROC) curve, generated from predicted probabilities and actual tree classification, indicates that predicted probabilities from the logistic regression model (equation 2) are better than those from chance alone (area under curve = 0.847, SE = 0.054, \( p < 0.001 \)). Sensitivity is the proportion of resistant trees correctly classified, and 1-specificity is the proportion of susceptible trees incorrectly classified as resistant. Sensitivity (horizontal dashed line) and 1-specificity (vertical dashed line) are optimized when \( P_R = 0.46 \). When \( P_R \geq 0.46 \) (determined using equation 2), a tree is classified as resistant.
Weibull survival models based on *in planta* concentrations of phenolic biomarkers in Briones CLO with varying susceptibility to *P. ramorum* based on canker length measured 10 months following *P. ramorum* infection (see Table 4.2 for values): A (most susceptible) and B (most resistant).

**Figure 4.3** Weibull survival models based on *in planta* concentrations of phenolic biomarkers in Briones CLO with varying susceptibility to *P. ramorum* based on canker length measured 10 months following *P. ramorum* infection (see Table 4.2 for values): A (most susceptible) and B (most resistant).
CHAPTER 5
CHEMICAL MECHANISMS OF PHOSPHONATE INDUCED RESISTANCE IN
QUERCUS RUBRA AGAINST PHYTOPHTHORA RAMORUM

Abstract
Phosphonate-based fungicides are well-known inducers of plant host resistance, particularly against pathogens within with genus Phytophthora. Phytophthora ramorum is a generalist plant pathogen with the ability to infect a wide array of host species, including various species within the red oak subgroup of the genus Quercus. While preventative treatment with phosphonate-based fungicides is known to make susceptible oaks more resistant P. ramorum, the effect of phosphonate-based fungicides on oak defense in this instance is unknown. Therefore, the primary objective of this study was to determine the effect of the phosphonate-based fungicide, Agrifos on susceptibility and phenolic defense production in Quercus rubra, a keystone species in many eastern North American forests. Q. rubra preventatively treated with Agrifos were less susceptible to P. ramorum and contained higher levels of certain phenolic compounds, compared to untreated trees. The elicited amount of only one phenolic compound, a tentative hydrolysable tannin precursor, was significantly affected by Agrifos, although levels of several phenolics were affected after wounding or P. ramorum infection. These results
suggest that Agrifos primes *Q. rubra* phenolic defenses. However, follow-up studies are needed to confirm these results. Furthermore, the identification of mechanisms of chemically induced resistance may be useful for identifying and understanding natural mechanisms of resistance in *Q. rubra* and other related species.

**Introduction**

*Phytophthora ramorum*, the causal agent of sudden oak death (SOD), has the potential to impact a diverse array of natural and urban forests across the United States and around the world, due to its broad host range and its ability to cause lethal stem girdling lesions (i.e. cankers) on various species of red oak (*Quercus* section Lobatae) and tanoak (*Notholithocarpus densiflorus*) (reviewed in Rizzo et al., 2005; Rizzo et al., 2002). As a result, trade within and between the United States and numerous other countries (e.g. United Kingdom, Australia) has been impacted. For example, the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) has implemented regulations and quarantines on infested or potentially infested plant materials to prevent the spread of *P. ramorum*. Regulations imposed to prevent the spread of *P. ramorum* to other parts of the United States are necessary, since the ecological and economic threat to Eastern United States forests is significant. Current models predict the risk of SOD in these areas as medium to high (Kelly et al., 2007; reviewed in Kliejunas, 2010). Furthermore, *Q. rubra* (Northern red oak—NRO), an important eastern timber species, has been found naturally infected with *P. ramorum* in Europe (Brasier et al., 2004) and is susceptible to stem inoculations in greenhouse assays (Tooley and Kyde,
If *P. ramorum* were to be introduced to Ohio, for example, the effect could be devastating, since 25% of the standing timber in Ohio forests is represented by oak (including both red and white oak) (Letson et al., 2006). Across the United States, the economic threat of *P. ramorum* to oak forests is even larger and is estimated at over $30 billion (Kliejunas, 2003).

In spite of these threats, few options exist for managing SOD, with most efforts still focused on attempting to prevent or interrupt the spread of the disease. High value trees can be treated preventatively, and therefore protected from infection, with phosphonate-based fungicides (Garbelotto and Schmidt, 2009; Garbelotto et al., 2009, 2007), though the approach is expensive and unfeasible on landscape scales. One phosphonate-based fungicide registered for prevention of SOD is Agrifos (Agrichem, Queensland, Australia) (Garbelotto and Schmidt, 2009). Agrifos, a systemic fungicide, can be directly injected into the tree stem or applied topically to the bark when combined with the surfactant Pentrabark™ (Agrichem, Queensland, Australia), which facilitates the absorption of Agrifos through the bark (Garbelotto and Schmidt, 2009; Garbelotto et al., 2007; Schmidt et al., 2006). Preventative topical application and/or injection of phosphonate-based fungicides significantly reduces the size of *P. ramorum* lesions on *Quercus agrifolia*, *Quercus parvula* var. *shrevei*, and *N. densiflorus* (Garbelotto and Schmidt, 2009; Garbelotto et al., 2009, 2007; Schmidt et al., 2006). Still, the exact mode of action of phosphonate-based fungicides in conferring oak resistance to *P. ramorum* is unknown. Some phosphonate-based fungicides are capable of inhibiting pathogens directly, for
example by impacting pathogen metabolism or production of host defense suppressors, while others activate host defense responses (reviewed in Guest and Grant, 1991).

Phosphonic acid reduces *P. ramorum* hyphal growth, colony formation from germinating zoospores, and number of sporangia compared to controls *in vitro*. Nevertheless, Garbelotto et al. (2009) concluded that the direct fungistatic effect *in planta* may be only modest at best. Finally, phosphonate-based fungicides are not known to effectively cure oak trees with existing *P. ramorum* infections, suggesting that the primary action of fungicides is not direct pathogen inhibition (reviewed in Kliejunas, 2010).

Instead it is hypothesized that the mode of action of phosphonate-based fungicides is by enhancing the production of plant specialized metabolites (PSM) following infection with *P. ramorum* (Garbelotto et al., 2009; Guest and Grant, 1991). If this is accurate, then phosphonate-based fungicides act as primers of host resistance, i.e. most changes in oak PSMs associated with phosphonate application will not be apparent until after plants are challenged by *P. ramorum*. Changes associated with phosphonate treatment could include deposition of lignin and suberin at lesion margins, callus formation, upregulation of enzymes (e.g. phenylalanine ammonium lyase) associated with PSM production, or the accumulations of phenolics around infected cells (Daniel and Guest, 2006; reviewed in Guest and Grant, 1991; Pilbeam et al., 2011). For example, changes in phenolic accumulation were noted in *Arabidopsis thaliana* (L.) Heynh. seedlings infected with *Phytophthora palmivora* E.J. Butler (Daniel and Guest, 2006) and physical differences in
lesion margins were observed in *Eucalyptus marginata* Donn ex. Sm. infected with the root pathogen *P. cinnamomi* (Pilbeam et al., 2011).

The hypothesis that PSM induction is one mode of action of phosphonate-based fungicides in conferring oak resistance to *P. ramorum* was tested using NRO, an ecologically and economically important and susceptible species of oak. The objectives of this study were to examine the effect of preventative phosphonate-based fungicide treatment on (1) the susceptibility of NRO to *P. ramorum*, and (2) the induction of constitutive and induced phenolic defenses in NRO.

**Materials and methods**

*Experimental design*

In March 2014, 35 nursery-produced NRO trees, approximately 4.5 years old, from Boething Treeland Farms, Inc., Portola Valley, CA, USA, growing in 2.09 cubic feet (~59 l) containers containing 8 parts redwood shavings and 1 part topsoil were placed in quarantine at the National Ornamental Research Site at Dominican University of California (NORS-DUC). Quarantine was necessary to ensure that trees were not naturally infected with *P. ramorum* prior to the beginning of the study. After six weeks all trees were found to be asymptomatic and were moved to a research plot within the north research nursery site at NORS-DUC. Trees were approximately evenly spaced around a 8.5 x 6.9 m pond and organized into a randomized complete block design, in
order to minimize possible effects of variation in sunlight and shading within the plot. Trees were watered by drip irrigation with 2-gallon (7.6 l) drip emitters for approximately 20 min day$^{-1}$. Trees were randomly assigned into one of six blocks. Each block contained 6 unique treatment groups, combining an inoculation treatment—three levels: control, mock inoculated, $P$. ramorum inoculated—and chemical treatment with Agrifos fungicide—two levels: no Agrifos (water) or Agrifos. The unique treatment groups were as follows: (1) untreated control (no Agrifos); (2) Agrifos application only; (3) water, mock inoculated; (4) Agrifos, mock inoculated; (5) water, inoculated with $P$. ramorum; (6) Agrifos, inoculated with $P$. ramorum. An equal mixture of Agrifos (45.8% mono- and di-potassium salts of phosphorous acid) and water, with 2.5% Pentrabark$^\text{TM}$ was applied to the main stems in groups 2, 4, and 6 (Garbelotto et al., 2007). Due to an insufficient number of trees, there was no treatment group 4 in the sixth block. The mixture was applied using a sponge from the root crown (or soil surface if root crown was not visible) up to the first lateral branch, until just before run-off. A sponge was used for application to prevent fungicide drift, due to the relatively small diameter of tree stems. Using the same application method, water was applied to the bark of trees in groups 3 and 5 to account for any potential effect of application method. No Pentrabark only treatment was applied because Pentrabark alone is not known to affect disease resistance.
Tissue sampling and inoculation

One week following Agrifos application, bark samples were collected from the main stem of each tree using a 10 mm diameter arch punch at 15 cm, 45 cm, and 75 cm above the soil line (or root crown) for groups 3 – 6 and 15 cm and 75 cm for groups 1 and 2. Locations were staggered not only laterally but also longitudinally around the main stem of each tree. Bark (outer bark plus phloem) from each sampling location was then pooled together in a paper coin envelope; after pooling, samples were flash frozen and stored in liquid nitrogen until returning to the laboratory where they were stored at −80°C.

Following bark sampling, trees in groups 5 and 6 were inoculated at the three points from where tissue was sampled, with a 10 mm diameter plug of three-week old P. ramorum Pr52 grown on 1/3-strength clarified V8 medium, or mock inoculated (groups 3 and 4) three times with a 10 mm plug of 1/3-strength clarified V8 medium only (Tooley and Kyde, 2007). Pr52 (kindly provided by Prof. David Rizzo, UC Davis) was originally isolated from rhododendron, and has been used widely and successfully to assess the susceptibility of different tree hosts of P. ramorum (Hayden et al., 2014; Nagle et al., 2011). Parafilm, followed by aluminum tape, were wrapped around all inoculations and wounds to minimize desiccation and hold agar plugs in place.

Approximately six weeks following inoculation, the outer bark was carefully removed using a razor blade from the margins of wounds and lesions from the top and bottom sites, 15 and 75 cm above the soil surface/root crown (tissue from the center point, 45 cm above soil line, was removed five days post-inoculation for a separate study). Lesion
length and width were measured and photographed for each inoculation site on each tree. From each tree and inoculation site, phloem at the actual under bark lesion margin was sampled. Tissue from top and bottom inoculation sites was pooled together in a paper coin envelope, and then flash frozen in liquid nitrogen. Samples were stored as described in the preceding paragraph. At the completion of the study, frozen samples from all time points were shipped on dry ice to a BSL-3 laboratory at The Ohio State University, Columbus, OH for further processing and permanent storage at −80°C.

*Analysis of soluble phenolics*

Phloem samples were ground in liquid nitrogen and 0.10 g aliquots of finely ground phloem were extracted twice with ~500 μL HPLC grade methanol at 4°C (each extraction for 24 hours). The exact volume of methanol added to each phloem sample was determined by measuring the weight of methanol in each sample tube. Supernatants from the two successive extractions were pooled and stored at −80°C until analysis. Just before analysis, extracts were briefly centrifuged to remove any residual particulate matter.

Soluble phenolic extracts were analyzed using an Acquity H class Waters ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA). 0.5 μL of each extract was injected into a Waters Acquity 2.1 x 50 mm, 1.7-μm, BEH C-18 reverse phase column maintained at 40°C. Samples were separated using a water and methanol solvent gradient acidified with 2% (v/v) glacial acetic acid. The solvent gradient was (% represents proportion of solvent A): 98% (0-0.01 min); 98-94% (0.01-
1.5 min); 94-88% (1.5-3.0 min); 88-80% (3.0-4.0 min); 80-60% (4.0-7.5 min); 60-0% (7.5-8.0 min); 0% (8.0-8.2 min); 0-98% (8.2-8.7 min); 98% (8.7-9.0 min), with a constant flow rate of 0.40 mL min\(^{-1}\) and a 5 min injection delay between each sample. A Waters Photo Diode Array (PDA) detector (Waters, Milford, MA, USA), scanning between 210 and 400 nm, was used for data acquisition. Data acquisition and processing were performed using Waters Empower Pro software (Waters, Milford, MA, USA), visualizing data at 280 and 370 nm. The minimum peak area threshold for detection was 70000 AU for 280 nm and 1000 AU for 370 nm. Any peak that was not present in at least 50% of the samples was excluded from the analysis, and individual samples with missing peaks (e.g. peak present, but below detection threshold or no peak present) were given a value equal to the minimum peak area threshold at its respective wavelength.

Phenolic defense classification

Phenolic peaks measured in extracts from NRO bark collected before and after implementation of inoculation treatment were classified based on treatment group (Table 5.1). Phenolics detected in no Agrifos treated trees prior to wounding (1), mock-inoculation (3), or inoculation (5) were considered to be at constitutive levels, whereas levels of phenolics in Agrifos treated trees (2, 4, and 6) at the same time were considered to be elicited. Following wounding, mock-inoculation, or inoculation, phenolics were considered to be at induced (1, 3, and 5) or induced-elicited (2, 4, and 6) levels.
Classification of phenolics

Unknown compounds of interest were classified based on their retention time and UV profile compared to the retention time and UV profile of authentic standards (Nagle et al., 2011; Ockels et al., 2007). A standard methanol mix, with seven individual compounds pooled together, was used. The compounds included: (+)-catechin ([X] = 1.3 mg ml\(^{-1}\)), ellagic acid ([X] = 2.5 mg ml\(^{-1}\)), gallic acid ([X] = 0.9 mg ml\(^{-1}\)), tyrosol ([X] = 2.2 mg ml\(^{-1}\)), quercetin ([X] = 0.45 mg ml\(^{-1}\)), kaempferol ([X] = 0.55 mg ml\(^{-1}\)), and myricitrin ([X] = 0.65 mg ml\(^{-1}\)). The standard mix was diluted and a 5-point calibration curve (X, 0.5X, 0.1X, 0.02X, and 0.01X), with three technical replicates per level of standard mix, was generated (Ockels et al., 2007).

Statistical analysis

Lesion length

The effect of Agrifos application and inoculation on lesion length (where tree lesion length = mean of top and bottom lesion lengths at each inoculation/wounding site) was determined using a univariate general linear model (ANOVA) with two factors: inoculation (three levels: wound, mock-inoculated, or inoculated) and Agrifos treatment (two levels: no Agrifos or Agrifos). Block was excluded from the model after preliminary analysis determined that it was not a significant factor associated with lesion length. Boxplots were used to detect and remove outliers as needed, and the assumptions of
normality and homogeneity of variance were tested using the Shapiro-Wilk test and Levene’s test, respectively.

**Soluble phenolics analysis**

A repeated measures ANOVA with one within subjects factor (time) and two between subjects factors (Agrifos and inoculation) was used to examine whether the amount (peak area corrected for tissue weight and extraction volume) of individual phenolic compounds changed over time (i.e. before and after inoculation), in relation to Agrifos application and inoculation type. The effect of Agrifos application and inoculation was tested separately at each time point for any phenolic compound where a significant interaction in the repeated measures ANOVA was detected, in order to examine the simple main effects. Using a univariate ANOVA, the impact of between subjects treatments were tested to determine at which time point (before or after infection/wounding) an effect was present. For univariate ANOVA analyses, the model examined the effect of Agrifos and inoculation (both fixed factors), block (random factor), and the interaction between Agrifos application and inoculation. For each unique treatment combination, normality was tested using the Shapiro-Wilk test and visually assessed using Q-Q plots, and the homogeneity of variance assumption was tested using Bartlett’s test. Data that violated either assumption were log transformed, and/or significant outliers were removed when necessary based on preliminary box plots. Finally, the LSD test was used to assess the significance of pairwise comparisons between inoculation treatments in both repeated measures and univariate ANOVA.
All statistical analyses were conducted using the IBM Statistics SPSS 22 package, except for the Bartlett’s test, which was conducted using R software (R development core team, version 3.1.2).

**Results**

*Lesion length*

Differences in lesion length and symptom development were evident between trees that were inoculated with *P. ramorum* but either did or did not receive preventative Agrifos treatment. Characteristic symptoms of *P. ramorum* infection were observed on trees inoculated with the pathogen, but which did not receive preventative Agrifos treatment and included bleeding exudate, sunken outer bark tissue, and the presence of asymmetrical under-bark lesions with definitive zone lines (Figure 5.1). A dark zone of tissue surrounding wounds/inoculation points was observed on many Agrifos treated trees. Inoculation treatment had a significant impact on lesion length (univariate ANOVA, $F_{2,27} = 10.2, p = 0.001$), though the effect was dependent on Agrifos treatment (significant interaction between Agrifos and inoculation) (univariate ANOVA, $F_{2,27} = 8.87, p = 0.001$) (Figure 5.2). Agrifos treated *P. ramorum* inoculated trees (group 6) had lesions that were 38% shorter than inoculated trees that did not receive Agrifos treatment (group 5), though there was considerable variation in lesion length within group 5. In order to satisfy the assumption of normality, two outliers were removed, one each from groups 5 and 6; however, the assumption of homogeneity of variance could not be
satisfied due to the natural variation in *P. ramorum* lesion length that was observed in group 5 and the lack of variation in lesion length associated with the other treatment groups (Levene’s test, $F_{5,27} = 19.47$, $p < 0.001$). While the ANOVA test statistic and associated p-value may be affected by a lack of homogeneity of variance, there was a clear effect of Agrifos treatment on NRO susceptibility that cannot be disregarded.

**Soluble phenolics**

A total of 42 soluble phenolic peaks were detected at 280 nm and 370 nm, before and after inoculation with *P. ramorum* (Table 5.2). Total phenolics at 280 nm and 370 nm were measured by determining the total integrated chromatographic peak area for each sample at 280 nm and 370 nm, respectively. Levels of 59% of individual phenolic peaks ($N = 39$; 3 peaks excluded from repeated measures analysis because criteria for peak inclusion was not met at both time points) and total phenolics at 280 nm and 370 nm significantly differed before and after wounding (1 and 2), mock-inoculation (3 and 4), or inoculation (5 and 6) (Table 5.3). Of those peaks, only 3 peaks, P7, P21, and P24 were detected at higher levels in induced or induced-elicited extracts (Figures 5.3 and 5.4).

For each repeated measures ANOVA with a significant time by between-subjects factor interaction (e.g. with Agrifos and/or inoculation) or a significant between-subjects factor or interaction, a univariate ANOVA was performed to determine at which time point the amount of phenolic compound “X” was impacted by Agrifos treatment and/or inoculation.
Levels of 15 individual phenolic peaks and total phenolics at 370 nm were significantly impacted by Agrifos and/or inoculation.

**Constitutive and elicited phenolics**

Treatments (e.g. Agrifos, inoculation) had a significant impact on constitutive or elicited levels of 12 phenolic peaks and total phenolics at 370 nm. However, Agrifos treatment alone impacted the level of only one peak, P1, a putative galloyl glycoside—classified based on retention time and UV spectrum (Table 5.2) (univariate ANOVA, $F_{1,23} = 11.610$, $p = 0.002$). Constitutive or elicited levels of all other phenolic peaks were significantly impacted by inoculation or by the interaction between Agrifos and inoculation treatment. The inoculation treatment had not yet been implemented at the time these tissues were sampled, and there was no clear relationship between treatment group and variation in levels of individual phenolics (Figure 5.3) or total phenolics at 370 nm (Figure 5.5). Therefore, variation among inoculation treatment groups, or significant Agrifos x inoculation interactions in this instance, most likely reflects inherent differences in phenolic profiles of individual NRO used in this study, and not treatment effects, since at the time of sampling inoculation treatments had not yet been implemented.

**Induced and induced-elicited phenolics**

Induced levels of 11 phenolic peaks (Figure 5.4) and total phenolics at 370 nm (Figure 5.5) were significantly impacted by Agrifos and/or inoculation, though homogeneity of variance was not assumed in all instances (Table 5.4). Regardless of inoculation
treatment, levels of P12 were higher in Agrifos-treated trees (univariate ANOVA, $F_{1,22} = 7.392, p = 0.013$). Agrifos application also had an overall significant effect on the level of P25 ($F_{A,1,22} = 5.394, p = 0.030$); however, the effect of Agrifos depended on inoculation treatment ($F_{A\times I,2,22} = 5.352, p = 0.013$). Induced-elicited levels of P25 were only higher than induced levels for control (wound) and mock-inoculated trees. Levels of P41 were also significantly affected by Agrifos treatment ($F_{1,22} = 6.362, p = 0.019$), except that levels of P41 were lower in Agrifos treated trees, regardless of inoculation treatment.

Levels of phenolic peaks P20, P24, and P37 were significantly affected by inoculation treatment (Table 5.4; Figure 5.4); however Agrifos application had no obvious effect on the levels of these compounds. The impact of Agrifos treatment on the induced-elicited level of phenolic peaks P26, P27, P28, P29, P32, and total phenolics at 370 nm, depended on the specific inoculation treatment (Table 5.4). Variation in induced and induced-elicited levels of these phenolic peaks between treatment groups was consistent with variation observed between treatment groups for constitutive and elicited levels. Since this variation most likely represents inherent variation in phenolic profiles between NRO in this study, the results of these ANOVAs will not be discussed further.

Finally, there was a significant effect of inoculation on the level of P29 (univariate ANOVA, $F_{2,22} = 4.473, p = 0.023$). The amount of P29 differed in inoculated trees compared to control (wounded) trees (LSD test, $p = 0.012$) (Figure 5.7). UV spectra properties, retention time, and putative identity/classify phenolic peaks that were
significantly affected by Agrifos treatment and/or inoculation only following inoculation can be found in Table 5.2.

Discussion

Preventative application of phosphonate-based fungicide (Agrifos) had a significant effect on the susceptibility of NRO to *P. ramorum* and on the constitutive and induced production of certain phenolic compounds in NRO bark. Agrifos-treated trees were less susceptible to *P. ramorum* infection than untreated trees, with lesions more similar in length to those that were wounded. This finding was expected, considering that preventative application of Agrifos has been shown to reduce or prevent the formation of *P. ramorum* lesions on other oak species, including *Q. agrifolia* and *Q. parvula var. shrevei*, and on highly susceptible tanoak (*N. densiflorus*) (Garbelotto and Schmidt, 2009; Garbelotto et al., 2009, 2007; Schmidt et al., 2006). Still, the precise mode of action of phosphonate in conferring oak resistance to *P. ramorum* had not been tested, though it had been hypothesized to be associated with the induction of antimicrobial PSMs following *P. ramorum* infection (Garbelotto et al., 2009; Guest and Grant, 1991). Past studies in other tree-pathosystems, including *Persea americana* Mill.-*P. cinnamomi*, *Eucalyptus marginata*-*P. cinnamomi*, and *Mangifera indica* L.-*Ceratocystis fimbriata* Ellis & Halst., had supported this hypothesis, by showing that the concentration of certain phenolic compounds increase in phosphonate (or phosphite) treated trees following pathogen infection and that trees treated with phosphonate were less susceptible to
pathogen attack (Araujo et al., 2014; Bekker et al., 2014; Jackson et al., 2000). However, before this study, no such information was available for NRO.

The constitutive amount of only one phenolic peak, P1, tentatively classified as a galloyl glucose based on retention time and spectrum, was found to be significantly affected by Agrifos application alone. The lack of effect of Agrifos on the pre-infection (i.e. elicited) amount of the majority of phenolics detected in NRO was not surprising considering most changes in plant defense following phosphonate application are detected after pathogen attack (Araujo et al., 2014; Bekker et al., 2014; Daniel and Guest, 2006; reviewed in Guest and Grant, 1991; Jackson et al., 2000). This suggests that phosphonate-based fungicides act as priming agents (Garbelotto et al., 2009; Jackson et al., 2000), i.e. plants respond more quickly and effectively to pathogen attack, but defense responses are not detectable until after attack (Aranega-Bou et al., 2014).

Elevated pre-inoculation/wounding levels of P1 in Agrifos-treated trees suggest two potential biochemical modes of action in Agrifos-induced resistance. First, gallic acid is a key component of plant defense responses associated with the hydrolysable tannin pathway, and is the precursor of simple gallic acid derivatives, more structurally sophisticated gallo- and ellagi-tannins, and their derivatives (Fernandes et al., 2009; Salminen and Karonen, 2011). Second, gallic acid may be directly active in oak defense. Gallic acid can inhibit the growth of several different Phytophthora species in vitro, including P. ramorum (Cahill and McComb, 1992; Ockels et al., 2007), and has been
associated with *Q. agrifolia* defense against *P. ramorum in planta* — tissues infected with *P. ramorum* contained higher concentrations of gallic acid than asymptomatic or away from canker tissues (Ockels et al., 2007). However, based on the results of this study the role of P1 in conferring NRO resistance to *P. ramorum* is likely not direct. Higher elicited amounts of P1 in Agrifos-treated trees could indicate that Agrifos application promotes the biosynthesis of hydrolysable tannins and other gallic acid derivatives by accumulating precursor compounds prior to infection. This is supported by the finding that pathogen-induced amounts of P1, although slightly higher in Agrifos-treated trees, were not significantly affected by any other treatment or interaction. If P1 was to be playing a direct and active role in oak defense, then induced amounts of P1 should be significantly higher in both Agrifos-treated trees and non-treated trees inoculated with *P. ramorum*.

The effect of Agrifos application was more pronounced when pathogen-induced (induced or induced-elicited) levels of phenolics were analyzed. For example, the amount of P12, classified as an unknown tannin based on retention time and UV spectrum, was significantly impacted by Agrifos treatment. P12 increased regardless of inoculation treatment, i.e. induced-elicited levels of P12 were higher even in trees that were only wounded, suggesting the Agrifos-primed response does not require pathogen infection for activation, but instead is more of a general response to wounding and/or inoculation. This conclusion is further supported by the observation that tissue surrounding wound sites darkens, presumably as a result of PSM accumulation.
Still, elevated levels of potential derivatives of P1 — hydrolysable tannins and ellagitannins are both derived from gallic acid (Salminen and Karonen, 2011) — provide support, albeit minor, for phenolic priming as the mode of action of phosphonate in oak resistance. However, potentially more significant is the induction of P25, classified as an ellagic acid derivative. The level of P25 was significantly affected by Agrifos application depending on inoculation treatment. Trees that were not inoculated with \textit{P. ramorum}, but which were treated with Agrifos, contained greater wound-induced-elicited amounts of P25 compared to non-Agrifos treated control and mock-inoculated trees. Only when trees were inoculated with \textit{P. ramorum} did non-Agrifos treated trees contain higher levels of P25. A similar pattern was observed for P37, another tentative ellagic acid derivative, except the amount of induced P37 in control trees did not appear to be affected by Agrifos application. Since ellagic acid derivatives are derived from gallic acid precursors (Fernandes et al., 2009), the association between non-specific Agrifos induction of phenolics and \textit{P. ramorum} induction of the same compounds provide further support for the hypothesis that Agrifos primes phenolic defenses in oaks and induces resistance following attack.

The induction of tentative ellagic acid derivatives following Agrifos application and inoculation also reveal potential mechanisms by which Agrifos-induced PSMs could alter oak resistance to \textit{P. ramorum}. Ellagic acid has been repeatedly associated with \textit{Q. agrifolia} resistance to \textit{P. ramorum}. \textit{Q. agrifolia} phloem infected with \textit{P. ramorum} contained higher concentrations of ellagic acid compared to phloem from asymptomatic
trees or phloem collected away from cankers (Ockels et al., 2007), and was higher in resistant versus susceptible *Q. agrifolia* (Nagle et al., 2011). Ellagic acid and an ellagic acid derivative were also identified as putative phenolic biomarkers of resistance in *Q. agrifolia* (McPherson et al., 2014). Moreover, ellagic acid may confer resistance by acting directly as an antibiotic against *P. ramorum*, since it can inhibit *P. ramorum* mycelial growth *in vitro* at *in planta*-relevant concentrations (McPherson et al., 2014), act as an antioxidant, even at low concentrations, and can scavenge free radicals (Priyadarsini et al., 2002). However, the association between ellagic acid antioxidant activity and/or free radical scavenging and *P. ramorum* growth and development has yet to be tested.

Finally, the last phenolic peak found to be impacted by Agrifos application following inoculation treatment was P41, a tentative quercetin derivative. P41 was classified based on the comparison of its UV spectrum and retention time to that of authentic quercetin. The effect of Agrifos application was non-specific, i.e. the amount of P41 decreased regardless of inoculation treatment. A general decrease in the amount of P41 following wounding and/or infection suggests that Agrifos treatment may favor the biosynthesis and upregulation of certain phenolic compounds over others, which could result in a decrease in the amount of phenolic compounds that are produced from divergent PSM pathways. For example, quercetin, a flavonoid, is derived from both the acetate/malonate and shikimate pathways, whereas hydrolysable tannins are products of the shikimate pathway only (Salminen and Karonen, 2011). Since certain pathway precursors are
shared (e.g. 3-hydroshikimic acid), it is possible that the reduction in the induced amount of P41 in Agrifos-treated trees compared to non-treated trees could be a result of a biosynthetic trade-off in the production of phenolics (Salminen and Karonen, 2011). If certain upstream precursors are being funneled into the production of hydrolysable tannins, then a reduction in the production of phenylpropanoid precursors necessary for the biosynthesis of flavonoids, like quercetin, is possible.

In conclusion, NRO treated preventatively with the phosphonate-based fungicide Agrifos were less susceptible to *P. ramorum* inoculation and differed in elicited and induced levels of select phenolics compared to untreated trees. The results of the phenolics analysis suggest that phosphonate-based fungicide acts as a primer of oak resistance. Resistance may be conferred by enhancing the production of phenolics associated with the hydrolysable tannin pathway, which could result in biosynthetic trade-offs in the production of phenolic compounds found in other pathways. Knowledge of putative resistance mechanisms and potential trade-offs of chemically induced resistance could be useful for understanding natural mechanisms of resistance in oak to *P. ramorum*. 
Table 5.1 Description of treatment groups and classification of phenolic defenses in NRO bark collected before and after inoculation treatment was applied.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Description</th>
<th>Phenolic defense types</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No Agrifos (control)</td>
<td>constitutive</td>
</tr>
<tr>
<td>2</td>
<td>Agrifos only</td>
<td>elicited</td>
</tr>
<tr>
<td>3</td>
<td>No Agrifos/mock-inoculated</td>
<td>constitutive</td>
</tr>
<tr>
<td>4</td>
<td>Agrifos/mock-inoculated</td>
<td>elicited</td>
</tr>
<tr>
<td>5</td>
<td>No Agrifos/inoculated</td>
<td>constitutive</td>
</tr>
<tr>
<td>6</td>
<td>Agrifos/inoculated</td>
<td>elicited</td>
</tr>
</tbody>
</table>

Before inoculation\(^1\) | After inoculation\(^2\)  
---|--------------------------|
constitutive | wound-induced
elicited | wound-induced-elicited
constitutive | mock-inoc. induced
elicited | mock-inoc. induced-elicited
constitutive | *P. ramorum*-induced
elicited | *P. ramorum*-induced-elicited

\(^1\)One week post-Agrifos application (“constitutive”). \(^2\)Six weeks post-inoculation (“induced”).
Table 5.2 Phenolic peaks identified by UPLC-PDA analysis in NRO phloem extracts at 280 nm and 370 nm, and putative classification of phenolic peaks of interest based on unknown peak retention time and spectral similarity to authentic standards. See Figure B.3 for chromatograms labeled with peaks.

<table>
<thead>
<tr>
<th>Phenolic peak</th>
<th>Retention time (min)</th>
<th>Channel</th>
<th>$\lambda_{max}$</th>
<th>Putative ID</th>
<th>Reference</th>
</tr>
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1 Average of all runs. 2 Representative $\lambda_{\text{max}}$. Only $\lambda_{\text{max}}$ values above 255 nm were included, due to variability in values below that threshold.
Table 5.3 Results of repeated measures general linear model (ANOVA), with within subjects factor time (T) and between subjects factors Agrifos (A) and inoculation (I). Significant interactions between within and between factors are also noted. Only phenolic peaks with significant test results are reported.

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*Peak area data log transformed for analysis. 1Within subjects degrees of freedom: DF_T = 1, 26; DF_TxA = 2, 26; DF_Txl = 2, 26; DF_TxAxl = 2, 26. 2Between subjects degrees of freedom: DF_A = 1, 26; DF_I = 2, 26; DF_AxI = 2, 26. Exceptions to noted degrees of freedom: 3DF_T = 1, 23; 4DF_T = 1, 25, DF_Txl = 2, 25, DF_AxI = 2, 25; 5DF_T = 1, 24, DF_I = 2, 24. 6Data did not pass assumption of homogeneity of variance (p < 0.05). 7Data did not pass normality assumption (p < 0.05).
Table 5.4 Results of univariate general linear model (ANOVA) for phenolic compounds with significant within subject interaction terms (e.g. TxI) and/or significant between subjects terms from repeated measures ANOVA for factors Agrifos (A) and inoculation (I). Only phenolic compounds with significant test results are included in the table.

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</table>

*Peak area data log transformed for analysis. ¹Degrees of freedom for constitutive ANOVA: DF_A = 1,23; DF_I = 2, 23; DF_AxI = 2,23. ²Degrees of freedom for induced ANOVA: DF_A = 1,22; DF_I = 2,22; DF_AxI = 2,22. Exceptions to noted degrees of freedom: ³DF_AxI = 2,22; ⁴DF_I = 2,21. ⁵Data did not pass homogeneity of variance assumption (p < 0.05). ⁶Data did not pass normality assumption (p < 0.05).
Figure 5.1 Characteristic symptoms of *P. ramorum* infection on NRO. Symptoms apparent on the outside of the bark (left), include sunken tissue (A) and viscous, red exudate (B), often referred to as blood. Subcortical symptoms of *P. ramorum* infection include asymmetrical, oval-shaped lesions, with dark lines delineating lesion margins.
Figure 5.2 NRO lesion lengths (exclusive of trimmed data) measured six weeks following wounding, mock-inoculation, or inoculation with *P. ramorum*. There was a significant effect of inoculation on lesion length (univariate ANOVA, $F_{2,27} = 10.2, p = 0.001$) and a significant interaction between treatment with Agrifos and inoculation (univariate ANOVA, $F_{2,27} = 8.87, p = 0.001$) (data did not satisfy homogeneity of variance assumption). Trees that were inoculated with *P. ramorum* but which did not receive preventative Agrifos treatment had longer lesions compared to any of the other treatment groups.
Figure 5.3 Relative levels of constitutive phenolic peaks at 280 nm (top) and 370 nm (bottom). Peaks are labeled in order of retention time at each wavelength (see Table 5.2 for peak retention time and spectrum). Asterisks indicate significant univariate ANOVA ($p < 0.05$) (refer to Table 5.4 for ANOVA results).
Figure 5.4 Relative levels of constitutive phenolic peaks at 280 nm (top) and 370 nm (bottom). Peaks are labeled in order of retention time at each wavelength (see Table 5.2 for peak retention time and spectrum). Asterisks indicate significant univariate ANOVA ($p < 0.05$) (refer to Table 5.4 for ANOVA results).
Figure 5.5 Relative levels of total phenolics at 280 nm (top) and 370 nm (bottom), before (constitutive) and after (induced) NRO inoculation with *P. ramorum*. There was no treatment effect on total phenolics at 280 nm, while there was a significant Agrifos x inoculation interaction for total phenolics at 370 nm at both times (univariate ANOVA, $p < 0.05$) (refer to Table 5.4 for ANOVA results).
The goals of my dissertation research were to elucidate chemical mechanisms of *Quercus* spp. (oak) resistance to the non-native, generalist pathogen *Phytophthora ramorum*, and to test new approaches for identifying resistant trees from within natural populations before infection. These goals were based on the central hypothesis that trees that differ in susceptibility to *P. ramorum* possess different amounts of constitutive and induced phloem phenolics.

Metabolic profiling and chemical fingerprinting combined with univariate and/or multivariate statistical analyses were used throughout my research to achieve these goals. Using these approaches, I examined variation in *Quercus agrifolia* (CLO—coast live oak) phloem phenolic profiles over time and in trees that varied in susceptibility to *P. ramorum* (Chapter 2), tested whether Fourier-transform infrared (FT-IR) spectroscopy combined with chemometric analysis could be used to distinguish between resistant and susceptible CLO (Chapter 3), identified phenolic biomarkers of resistance from a naïve population of CLO and developed models to estimate the probability that a tree would be resistant to *P. ramorum* based on constitutive concentrations of those compounds.
(Chapter 4), and examined chemical mechanisms of phosphonate-induced resistance in *Quercus rubra* (NRO—northern red oak) against *P. ramorum* (Chapter 5). Ultimately, a better understanding of what factors (e.g. environmental and genetic) effect levels of chemicals associated with oak resistance is necessary, if approaches that utilize variation in phenolics to identify resistant trees are to be implemented most effectively and used to their fullest potential.

**Seasonal and inter-annual variation in CLO phloem phenolics**

In Chapter 2, seasonal and inter-annual variation in CLO phloem phenolics was examined to determine whether or not variation in CLO susceptibility to *P. ramorum* could be explained by changes in the levels of phenolics over time. Seasonal and inter-annual variation had no effect on the composition of phenolics in CLO phloem, i.e., all phenolic peaks were detected at all times and in all disease phenotype groups (control, resistant, and susceptible), though levels of certain phenolics varied over time. However, variation in phenolic levels over time and variation in CLO susceptibility to *P. ramorum* did not appear to be related. The environment and the phenological status of a tree should not impact markers used to determine the level of susceptibility of a tree. A lack of variation in phenolic levels, particularly for biomarkers, is essential if biomarkers are to be used to reliably predict tree resistance. Furthermore, increasing levels of certain phenolics in away from canker phloem of susceptible CLO could indicate a systemic induction of phenolic defenses, which was not observed in resistant trees in this study or in CLO in previous studies (Ockels et al., 2007). This suggests that induced phenolic
defenses may be less important for resistant trees, than for susceptible trees. Future studies should address the relationship between variation in CLO susceptibility and phenolic defense strategies (e.g. constitutive vs. induced). Phenolic data already collected from CLO at Briones Regional Park could be used for an analysis of this nature.

**Chemical fingerprinting and CLO resistance**

In Chapter 3, FT-IR spectroscopy, a chemical fingerprinting method, combined with chemometric (i.e. multivariate) analysis was used to distinguish between phloem extracts collected from resistant and susceptible CLO prior to infection with *P. ramorum*. While it was not possible to identify specific chemical compounds important for identifying resistant trees, the models developed from this study can be used to rapidly screen naïve trees for resistance to *P. ramorum*. Analysis of CLO samples from additional populations could also be used to validate, refine, and/or improve models. Finally, whether or not handheld or portable FT-IR devices can be used to screen trees for resistance needs to be tested. If identification of resistant CLO is to be used as a practical tool to manage sudden oak death (SOD) on landscape or forest-stand scales, then handheld or field-capable devices will most likely be the best option for doing so, since they could assess resistance most rapidly and may be less destructive (e.g. only a small window of phloem would need to be revealed versus collection and storage of several grams of phloem tissue).
Constitutive phenolic biomarkers of resistance

Chapter 4 described how constitutive concentrations of CLO phenolic biomarkers, including myricitrin and three unknown phenolics detected at 370 nm, were used to predict resistance to *P. ramorum* and estimate tree survival following inoculation. At this time it is impossible to determine the accuracy of the predictive model that was developed, since validation relies on natural infection of control trees. Even so, the use of phenolic biomarkers of resistance appears to be a viable option to identify resistant CLO, and one that does not rely on artificial or natural infection of trees with *P. ramorum*. Knowledge of resistant trees within natural populations can be used to better assess disease risk (if variability in host resistance is taken into account in predictive models), and guide conservation and restoration efforts focused on protecting and producing resistant germplasm. However, further work focused on confirming identities of unknown phenolic biomarkers may reveal mechanisms of CLO resistant to *P. ramorum*, and would allow for a functional analysis of these compounds to determine their mode of action, if any, in tree defense responses.

Chemical mechanisms of phosphonate-induced resistance

Finally, in Chapter 5, chemical mechanisms of phosphonate-induced resistance to *P. ramorum* were tested in NRO, another oak species which is susceptible to *P. ramorum*. Preventative phosphonate-based fungicide (Agrifos) application essentially inhibited *P. ramorum* growth and lesion development in NRO. Analysis of constitutive, elicited, and induced phenolics revealed that Agrifos most likely functions as a primer for the
production of phenolics in response to *P. ramorum* infection. Agrifos-induced resistance may be associated with enhanced production of phenolics in the hydrolysable tannin pathway. This could result in reduced levels of other phenolics, which share upstream biosynthetic precursors, but which are produced through divergent pathways. In order to test this hypothesis, compounds significantly impacted by Agrifos-application need to be identified using LC-MS analysis. The expression of genes associated with each biosynthetic pathway should also be measured, and the phenomenon needs to be tested on a larger sample size, since natural variation in susceptibility to *P. ramorum* and in levels of phenolic defenses was observed and may have obscured treatment effects in this study. Understanding mechanisms of chemically induced resistance in oak may be useful for elucidating natural resistance mechanisms, and may also be useful for identifying potential defense trade-offs. For example, determining whether trees naturally resistant to *P. ramorum* are more resistant (or susceptible) to other impending biotic and abiotic threats, such as the gold spotted oak borer or prolonged drought.

**New approaches to assess tree resistance in natural populations**

Increasingly forests are being threatened by the introduction of non-native, invasive pests and pathogens. The widespread mortality of trees due to the introduction and spread of *P. ramorum* in western coastal forests is a testament to this new reality. The presence of naturally resistant trees in areas of high infection and mortality suggests that one option for managing SOD might be to utilize naturally resistant CLO. However, identifying resistant trees, in general to any biotic threat, historically relied on observing disease
development in trees after artificial or natural infection. This process of screening trees for resistance based on pathogen (or pest) infection is time intensive and economically and ecologically unfeasible in some situations. Therefore, alternative methods for identifying resistant trees are needed, which is why a major portion of my dissertation research focused on testing chemical-based assays for identifying CLO resistant to *P. ramorum*. Still, utilization of chemical-based assays to rapidly screen trees for resistance should not replace basic biological research focused on identifying and understanding mechanisms of resistance and determining the genetic basis for resistance where appropriate. This type of research is especially important in instances where hosts are highly susceptible or where the pathogen is likely to overcome host defenses overtime.

I found that alternative methods, including the identification and utilization of phenolic biomarkers of resistance and FT-IR spectroscopy, combined with predictive models, could be used to identify resistant trees from within natural populations. While the ability of these approaches to identify resistant trees needs to be tested on larger scales, predictive models, particularly those from FT-IR analysis, can be relatively easily updated and revised as more data becomes available. These approaches can be utilized in other forest pest- and patho-systems, where identification of resistant trees is a priority, and more rapid and high-throughput identification of resistant trees is needed. Finally, approaches like these may be most valuable in systems where genetic markers of resistance are not readily available and/or where resistance is controlled by multiple genes that cannot be easily measured.
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APPENDIX A: SUPPLEMENTARY FIGURES FROM CHAPTER 31

Figure A.1 SIMCA 3D class projection plot for spectral data, transformed using SNV and second derivative. Data collected from the Cary 630 (portable) FT-IR unit equipped with 5-bounce ATR accessory. Closed diamonds—resistant trees; open diamonds—susceptible trees. Dashed lines indicate the 95% confidence interval for each group.
Figure A.2 SIMCA Coomans plot with 4 factors (dashed lines indicate critical sample residual thresholds) based on transformed (SNV and second derivative) data. Data collected from the Cary 630 (portable) unit equipped with 5-bounce ATR accessory. Closed diamonds—resistant trees; open diamonds—susceptible trees. This plot shows the relative dimension-free distance of a sample from a given class, resistant (x-axis) or susceptible (y-axis), based on the 4 factor SIMCA analysis.
Figure A.3 PLSR correlation plot showing the relationship between the concentration of the phenolic biomarker of resistance, ellagic acid, determined by HPLC analysis, and the predicted concentration of ellagic acid based on FT-IR spectra. Data collected from the Cary 630 (portable) unit equipped with 5-bounce ATR accessory. Spectral data were normalized with divide by sample 2-norm transformation. Closed diamonds—resistant trees; open diamonds—susceptible trees. Statistical analysis reported in Table 3.2.
Figure A.4 PLSR correlation plot showing the relationship between the concentration of the phenolic biomarker of resistance, FLV1, in ellagic acid equivalents (mg g\(^{-1}\) FW), determined by HPLC analysis, and the predicted concentration of FLV1 based on FT-IR spectra. Spectra collected from the Cary 630 (portable) unit equipped with 5-bounce ATR accessory. Spectral data were normalized with divide by sample 2-norm transformation. Closed diamonds—resistant trees; open diamonds—susceptible trees. Statistical analysis reported in Table 3.2.
Figure A.5 Ellagic acid PLSR loadings plot with divide by sample 2-norm transformed data for the 4th factor (solid line), the main factor of discrimination between resistant and susceptible trees, with raw spectra (absorbance) overlaid (dashed line) for data. Data collected from the Cary 630 (portable) unit equipped with 5-bounce ATR accessory. High loading values, either positive or negative, indicate informative spectra.
Figure A.6 FLV1 PLSR loadings plot with divide by sample 2-norm data for the 3rd factor (solid line), the main factor of discrimination between resistant and susceptible trees with raw spectra (absorbance) overlaid (dashed line) for data. Data collected from the Cary 630 (portable) unit equipped with 5-bounce ATR accessory. High loading values, either positive or negative, indicate informative spectra.
Figure A.7 Ellagic acid standard FT-IR spectra. Solid line—raw data; dashed line—second derivative data.
APPENDIX B: LABELED CHROMATOGRAMS
Figure B.1 Labeled overlaid representative chromatograms from UPLC-PDA analysis of CLO phloem in Chapter 2. (A) Chromatogram at 280 nm. (B) Chromatogram at 370 nm. See Table 2.4 for individual peak retention time and spectral properties.
Figure B.2 Labeled overlaid chromatograms from representative samples at 370 nm from HPLC-PDA analysis of CLO in Chapter 4. Arrows indicate phenolics that were identified as phloem phenolic biomarkers of resistance, as described in Table 4.2.
Figure B.3 Labeled representative chromatograms from UPLC-PDA analysis of NRO bark in Chapter 5. (A) Chromatogram at 280 nm. (B) Chromatogram at 370 nm. See Table 5.2 for retention time and spectral properties of each phenolic peak.