THESIS

PLANT TANNIN INTERACTIONS DURING *Phytophthora ramorum* INFECTION

Submitted by

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ABSTRACT

PLANT TANNIN INTERACTIONS DURING *Phytophthora ramorum* INFECTION

Phytophthora ramorum, the responsible agent of "Sudden Oak Death" and the infection of over 100 different plants has the potential to disrupt oak forests leading to devastating consequences. Resistance to *P. ramorum* varies by pathogen race or plant cultivar, plant species or genus, plant non-host and partial resistance. During infection, *P. ramorum* produces a 10 kDa elicitor protein, i.e., elicitin, that can induce plant defenses. *P. ramorum* uses elicitins to acquire sterols from plants since the pathogen does not synthesize sterols. Factors influencing host resistance are largely unknown, although phytochemicals, such as phenolics, are found to influence resistance to *P. ramorum*. Tannins, a group of polyphenolic compounds found in plant tissues are able to precipitate proteins, such as elicitins. The studies presented here investigate the possibility of an elicitin-sterol-tannin interaction in plant resistance to *P. ramorum*. This research includes a series of in *vitro and in vivo* studies of sterol and tannin interactions with *P. ramorum*.

To explore the impact tannins may have on *P. ramorum*, media was treated with ground foliage, extracted tannins, or extracted sterols from three different tree types (Oregon white oak, California black oak and California bay laurel), or commercially-available sterols . Growth and sporulation of *P. ramorum* were higher on California bay laurel treatments as compared to the oaks. High concentrations of foliage from the oaks resulted in more rapid inhibition of *P. ramorum* growth and sporulation. Inhibition of *P. ramorum* growth and sporulation was also observed in response to plant sterols or tannins. This inhibition appears to be caused by two different mechanisms. Treatment with high concentrations of sterols reduced elicitin gene expression indicating a regulatory role. Tannins caused a decline in the amount of ELISA-detectable elicitin while there was no change seen in elicitin gene expression. All treatments showed a strong correlation between elicitin contents and *P. ramorum* growth and sporulation, suggesting a role for elicitin-sterol-tannin complexes in *P. ramorum* growth and sporulation in foliage.

In a second study, several evergreen varieties of Rhododendron, Kalmia and Azalea were assessed for constitutive tannin content, sterol content and leaf susceptibility to *P. ramorum*. Significant differences were seen between the different species and between the two trials for tannin content. Variation of sterol content was only seen in Kalmia plants. Azalea plants showed no susceptibility to *P. ramorum*, while susceptibility varied between trial 1 and trial 2 for Rhododendron and Kalmia varieties. Variation of tannin and leaf susceptibility was also seen between cultivars. A positive correlation between all tannin data and lesion size suggests a relationship between tannin and *P. ramorum*. We propose that the formation of elicitin-sterol-tannin complexes inhibits plant defense responses allowing for greater pathogen colonization and lesion development.

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DEDICATION

I dedicate this research to Susan and Gayvin Stong and my family.

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CHAPTER 1

INTRODUCTION

In the 1990's, an abnormal number of oaks were dying in California. Researchers identified the pathogen of this "Sudden Oak Death" as *Phytophthora ramorum* (Rizzo et al., 2002). Also around this time, a similar disease was observed on rhododendrons and viburnums in Europe, which was also caused by the pathogen *P. ramorum* (Werres et al., 2001). Since this time, over 100 plant species have been confirmed hosts for *P. ramorum*, including many nursery species that are shipped world-wide. This has led to a series of regulations (Rizzo et al., 2005) limiting the transport and sale of infected plants in an effort to contain and eradicate *P. ramorum* where possible (Kanaskie et al., 2011). Since 2001 this goal has not been reached. Furthermore, while efforts of containment, quarantine and eradication may have reduced the spread, new outbreaks of *P. ramorum* continue to be discovered (Kanaskie et al., 2011). It had been estimated that from 1994 to 2004 infected forests in California and Oregon will have lost 59-70% of their coast live oaks (Brown and Allen-Diaz, 2009).From 2005, approximately 60 new sites with *P. ramorum*-infected trees are confirmed every year (Kanaskie et al., 2011). To date, *P. ramorum* has not been identified in the vast oak forests of the Eastern United States; although many of the native oak species in these forests, such as red oaks, are potential hosts (Spaulding and Rieske, 2010).

Although the disease caused by *P. ramorum* is commonly called Sudden Oak Death it results in a wide range of symptoms depending upon the host species. These symptoms include, bole cankers commonly found in oak species, leaf necrosis as found on Rhododendrons and California bay laurel species; and shoot die back found in some *Ericaceae* and conifers (Davidson et al., 2011). In compatible species symptoms of bole cankers are often fatal, while leaf and shoot die back is typically non-fatal. Although non-fatal, *P. ramorum* produces a prolific amount of spores on these hosts, which makes it harder to contain the disease without extensive eradication efforts.

Phytophthora meaning "Plant Destroyer" is classified as an Oomycete. Oomycetes appear like fungi but have been molecularly identified as more closely related to the water molds or brown algae. One interesting difference between fungi and Oomycetes is the inability of Oomycetes to produce sterols, which are required for sporulation (Erwin and Ribeiro, 1996). Within the Oomycetes, *Phytophthora*, with at least 75 different species, include some of the most noted plant pathogens that play a role in many crop losses (Kamoun, 2003). For instance, *P. infestans*, one of the first identified *Phytophthora* and best known for the Irish potato famine, is still a leading pathogen in *solanaceous* crops such as potatoes and tomatoes. *P. sojae* causes problems in soybean production. Even strawberries have a *Phytophthora* pathogen, *P. fragariae* Understanding how one species (e.g., *P. ramorum*) interacts with its host plants may be critical for understanding how the wide array of *Phytophthora* spp. can be so destructive and cause disease in nearly every major crop species.

P. ramorum spreads through asexual and sexual spores. While sexual reproduction is possible and has been successfully achieved in the laboratory, this has not been observed in nature. Asexual reproduction occurs via three different spore structures: chlamydospores , sporangia and zoospores. Chlamydospores are formed directly from hyphae in the presence of sterols; they are the thick walled structures and have been shown to be the most resistant to environmental extremes (Erwin et al., 1983). Sporangia, organelles that hold zoozpores, are created from mycelium and are known to directly infect foliage. Zoospores are circular spores with two flagella that allow for motility in water, and once they come in contact with a host they encyst, germinate, and infect new susceptible plants (Erwin and Rubeiro, 1996). *P. ramorum*, like most *Phytophthora* spp., produces a family of elicitor proteins referred to as elicitins. While there are still questions as to the full function of these proteins, they are known to load, carry and transfer sterols between membranes (Mikes et al., 1998b; Osman et al., 2001a). Sterols, a sub group of steroids that play a role in cellular function, are precursors to fat-soluble vitamins and steroid hormones. In fungi they play a role in reproduction, growth stimulation or inhibition, hormones and plant disease resistance (Bloch, 1983; Hendrix, 1970) (s/t-3a). *Phytophthora* cannot produce their own sterols

so it is necessary for them to acquire those needed for reproduction and growth from plants (Hendrix, 1970; Kamoun, 1993). It has been suggested that the formation of a sterol-elicitin complex is a required step before elicitins can fasten to specific binding sites on cells of tobacco (Osman et al., 2001a). These complexes are the first event needed to trigger the hypersensitive response that occurs in tobacco preventing in plant spread of *P. ramorum* (Boissy et al., 1999). This connection between sterols and elicitins is further illustrated by findings that there is less elicitin gene expression when there is less sterol present (Yousef, 2010; Yousef et al., 2009). Elicitin gene expression is down regulated when *Phytophthora* infects a compatible host as opposed to an incompatible host as seen in potato (Kamoun et al., 1997) and tobacco plants (Colas et al., 2001) infected either with *P. infestans* or *P. parisitica*.

Susceptibility to *P. ramorum* varies from compatible to incompatible (Kamoun et al., 1999). Tobacco, an incompatible host, exhibits an hypersensitive response to *P. ramorum* (Boissy et al., 1999). In oaks, a group of compatible plants, some trees are able to resist infection while others are quite susceptible. A wide range of responses are seen in compatible plants including highly susceptible to partial resistance to *P. ramorum* (Grunwald et al., 2008; Tooley et al., 2007). This variability of response can also be seen within species such as is the case with *viburnum* , with varying susceptibilities between different cultivars (Grünwald et al., 2008a). The mechanism responsible for these differences is unknown however phytochemicals may be involved.

Plant phenolics have been correlated with plant resistance to fungi (Hakulinen and Julkunen-Tiitto, 2000; Witzell and Martín, 2008). Coast live oaks display a change of phenolics, gallic acid, tyrosol, and ellagic acid levels in *P. ramorum*-infected trees that survive as compared with those that die (Nagle et al., 2011; Ockels et al., 2007). *In vitro* bioassays showed that gallic acid and tyrosol can inhibit *P. ramorum* growth (Ockels et al., 2007). (+)-Catechin has been linked to inhibition of fungal growth in pine (Bonello and Blodgett, 2003; Evensen et al., 2000) . Gallic acid, ellagic acid, and (+)-catechin are all components of different phenolic molecules called tannins. Tannins are water soluble phenolics, capable of binding and or precipitating water soluble proteins (Bate-Smith and Swain, 1962). There are

three major classes of tannin: hydrolysable tannins, condensed tannins and phlorotannins. The last class, phlorotannin, is not very common and is mostly found in brown algae in the ocean. Condensed tannins, also known as proanthocyanindins, are flavonoid polymers with carbon-carbon bonds joining the individual flavonoid monomers. They are oxidatively degraded in strong acid to yield anthocyanidins (Porter et al., 1986). Hydrolysable tannins are made up of gallic or ellagic acid esters of glucose or other sugars (Haslam, 1979). Hydrolysable tannins get their name from the fact that they are easily hydrolyzed to esters. Tannins are found within leaves, wood, flowers and seeds of plants. Within a cell, tannins are predominantly found in the cell membrane bound to fibers or proteins or in vacuoles. One major function they perform is to provide protection against microbial pathogens, insects and larger herbivores (Dixon et al., 2005; Lattanzio et al., 2006). Tannins interact with attackers through toxic effects, binding of metals, oxidation or protein binding (Dixon et al., 2005). These interactions differ depending on the mode of infection. The full capability of the protein binding interaction is unknown, however it has been shown that tannins are induced by elicitor proteins produced by insects, bacteria and fungi (Haering et al., 2008). This suggests a relationship between tannin and elicitior proteins. Through binding of elicitor proteins these tannins in turn have an effect on pathogens attacking indicating a role for tannin in plant resistance.

In short, research shows that elicitin from *P. ramorum* retrieves sterols and induces plant defense responses, but other compounds may be involved in these plant-pathogen interactions. Tannin, with its protein binding capabilities, may affect plant susceptibility to *P. ramorum* through binding of elicitin proteins. With these concepts in mind, this project focused on the following two objects in order to better understand how sterol-tannin-elicitin complexes may be influencing host susceptibility to *P. ramorum*.

Objective 1- The first step in uncovering this interaction between tannin and *P. ramorum* included a series of *in vitro* studies to examine the effect tannins have on *P. ramorum* in the presence of sterols. This was studied by amending synthetic growth media with various amounts and types of sterols, tannins, or leaf extracts and monitoring *P. ramorum* growth and sporulation.

Objective 2- To examine potential *in vivo* tannin-sterol-elicitin interactions, a second study characterized constitutive tannin and sterol concentrations from a range of plant species: Azalea, Kalmia and Rhododendron, to determine if *P. ramorum* susceptibility is correlated with constitutive expression of these compounds.

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CHAPTER 2

EFFECT OF PLANT STEROLS AND TANNINS ON *Phytophthora ramorum* GROWTH AND SPORULATION[1]

Summary

Elicitin-mediated acquisition of plant sterols is required for growth and sporulation of *Phytophthora* spp. This study examines the interactions between elicitins, sterols, and tannins. Ground leaf tissue, sterols, and tannin-enriched extracts were obtained from three different plant species (California bay laurel, California black oak, and Oregon white oak) to evaluate the effect of differing sterol/tannin contents on *P. ramorum* growth. For all three species, high concentrations of foliage inhibited *P. ramorum* growth and sporulation, with a more rapid response to the two oak samples. *P. ramorum* growth and sporulation were inhibited by either phytosterols or tannin-enriched extracts. High levels of sterols diminished elicitin gene expression in *P. ramorum*; whereas the tannin-enriched extract decreased in the amount of 'functional' or ELISA-detectable elicitin, but not gene expression. Across all treatment combinations, *P. ramorum* growth and sporulation correlated strongly with elicitin content, suggesting a possible role for elicitin-sterol-tannin complexes in *P. ramorum* growth and sporulation in foliage.

[1] Submitted to Chemical Ecology

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Introduction

Elicitins, a unique class of elicitors, are a family of proteins secreted by members of the phytopathogenic Chromista, *Phytophthora* spp. and *Pythium* spp. (Kamoun, 1993; Panabieres et al., 1995; Vauthrin et al., 1999a). Elicitins play an important role in *Phytophthora* spp. infection and growth. In non-host species, elicitins can trigger a successful hypersensitive response (Pernollet et al., 1993; Vleeshouwers et al., 2000). In host species, the potential role of elicitins in disease development is incompletely understood. However, *Phytophthora ramorum* elicitins are linked to increased pathogen virulence (Manter et al., 2010), photosynthetic declines in tanoak, rhododendron, and bay laurel (Manter et al., 2007a), but not beech (Fleischmann et al., 2005), and ultra-structural changes in oak (Brummer et al., 2002a) and pepper (Ivanova and Singh, 2003).

One of the main biological functions of elicitins for *Phytophthora* spp. is sterol binding and transfer through phospholipid membranes (Mikes et al., 1998a; Vauthrin et al., 1999b). *Phytophthora* spp. lack the ability to synthesize sterols, which are required for sexual and asexual reproduction (Elliott et al., 1966; Haskins et al., 1964; Hendrix, 1970; Leal et al., 1964; Nes and Stafford, 1983). As a result, *Phytophthora* spp. take up and metabolize a variety of sterols from host plants (Gonzalez and Parks, 1981; Grant et al., 1988; Nes and Stafford, 1983, 1984). Sporulation rates differ depending upon the amount and type of sterol acquired (Nes and Stafford, 1983, 1984; Nes et al., 1980).

Due to the discrimination of *Phytophthora* spp. towards plant sterols and the associated changes in growth and spore formation, one might expect plants with quantitative and/or qualitative differences in plant sterol content to exhibit variable field resistance and/or influence sporulation rates of *Phytophthora* spp. In a study with various potato cultivars, Hazel et al. (Hazel, 1988) examined *P. infestans* sporulation on both detached potato leaves and artificial media amended with a mixture of sterols designed to mimic the potato leaf sterol profiles. Although sporangia production increased compared to non-sterol

treatments and differed significantly between the potato cultivars, the *in planta* and *in vitro* treatments were not well correlated, suggesting that additional plant compound(s) influenced sterol acquisition.

P ramorum is a highly virulent pathogen that infects diverse hosts including deciduous, evergreen, woody and herbaceous plants (Goheen et al., 2006; Grunwald et al., 2008). The result of infection varies depending on the plant host species, tissue type, and isolate of *P. ramorum* (Goheen et al., 2006; Grunwald et al., 2008; Manter et al., 2010; Rizzo et al., 2005). Factors influencing host resistance are largely unknown, although various phytochemicals have antimicrobial activity against *P. ramorum*. For example, three compounds from conifer heartwood had strong antimicrobial activity against *P. ramorum* (Manter et al. 2007a). Another study of phloem tissue in coast live oaks found significant differences between infected and non-infected tissue in several phenolic compounds, including gallic acid, catechin and ellagic acid (Nagle et al., 2011; Ockels et al., 2007).

One class of compounds that may influence sterol acquisition via elicitins are the polyphenolic compounds known as tannins. Tannins play a role in plant resistance by exhibiting direct toxicity against a wide variety of microbes (Latte and Kolodziej, 2000; Nelson et al., 1997; Sivakumaran et al., 2004). Tannins characteristically exhibit a strong ability to bind and precipitate proteins (Hagerman and Butler 1981; Shahidi and Naczk 1995).

The goal of this *in vitro* study was to explore the interaction between sterols and tannins on *P. ramorum* growth, sporulation and elicitin production. Leaf tissue, sterol, and tannin extracts were obtained from three different plant species, California bay laurel, California black oak, and Oregon white oak (*Umbellularia californica*, *Quercus kelloggii*, and *Q. garryana*) to obtain extracts with differing quantities and/or compositions of sterols and tannins.

Methods and Materials

Pathogen strain and plant material. A single North American isolate (PR-07-031, original name 15-WA-M isolated from soil in Washington 2006) of *P. ramorum* was grown on corn meal agar (Becton Dickinson, Rutherford, NJ) without light at room temperature to obtain starter cultures for sporulation experiments. A single 0.7 cm agar plug was taken from the outermost margins of 2-3 week-old starter cultures and used to inoculate agar (15 g l^{-1}, Bacto agar, Becton Dickinson) plates containing modified *Phytophthora* synthetic medium (PSM) formulated by Hoitink and Schmitthenner (1974). The medium was amended with various amounts of ground leaf tissue, sterols, and/or tannin-enriched extracts in lieu of the recommended cholesterol (10 mg l^{-1}). All treatments were replicated three times in each of three independent trials.

Leaf tissue preparations. Foliage for sterol extraction was collected from trees in Oregon between 29 September and 4 October, 2007. Leaves of Oregon white oak were gathered from randomly sampled branches of several native trees in Philomath, Benton County (44° 32' 21.42"N, 123° 20' 20.30"W, elevation 108 m). California black oak foliage was sampled from several trees growing near Jacksonville, Jackson County (42° 15' 48"N, 122° 59'15", elevation 824 m) and leaves of California bay laurel were gathered from a single ornamental tree in Albany, Linn County (44° 37' 22.25"N, 123° 5' 47.58"W, elevation 70 m). The leaves were removed from the stems, air dried in the laboratory and stored at room temperature until needed. Prior to extraction a subsample was ground in a Wiley mill to pass a 20 mesh screen.

Foliage used to amend culture media or for ground tissue and tannin extraction was gathered on 9 September, 2008, from the same Oregon white oak and bay laurel trees. Black oak was collected in Eugene (Lane County, 44° 0' 40.59"N, 123° 4' 59.08", elevation 144 m). After collection, foliage was allowed to air-dry at room temperature for 7 days before grinding with a Wiley-mill, sieved with a 5 mm

mesh screen, and stored at 4 °C until needed. The ground leaf tissue was added directly to PSM prior to autoclaving at a final concentration of 0.1, 0.5, 1.0, or 5.0 mg ml^{-1}.

Commercially available sterols. Several commercially available sterols – β-sitosterol, cholesterol, ergosterol, stigmasterol, and stigmastanol (Sigma-Aldrich, St. Louis, MO) – were selected for their different properties. β-Sitosterol is the most common plant sterol, and is structurally similar to cholesterol except for the ethyl substitution at position 24. Although the quantity of cholesterol in plants is typically low in terms of total lipid content, it is a frequent component of plant membranes and may be the major sterol on leaf surfaces (Behrman and Gopalan, 2005). Stigmasterol is the second most common plant sterol, and is nearly identical to stigmastanol except for the absence of a double bond. Ergosterol is similar to cholesterol but is almost exclusively found in fungi. All sterols were dissolved in ethanol and added directly to PSM prior to autoclaving at a final concentration of 0.1, 1, 10, 25, or 50 µM.

Plant sterol extracts. Leaf sterols were extracted according to the methods of Jeong and Lachance (Jeong and Lachance 2001). Air-dried, ground leaf tissue (10 g) was sealed in a glass bottle with 25 ml of 50% KOH and 100 ml 95% ethanol, and heated in an 80 ^{o}C water bath for 1 h. This solution was transferred to a separatory funnel with of 30 ml 95% ethanol, 50 ml warm distilled water, followed by 50 ml cold water. This mixture was rinsed six times with 100 ml petroleum ether. The combined petroleum ether fraction was divided into two aliquots (300 ml) and each was washed four times with 100 ml distilled water. The petroleum ether fractions were combined and concentrated to less than 50 ml in a rotary evaporator at 40 ^{o}C. Anhydrous sodium sulfate (1.0 g) was added and the solution was transferred to a weighed 50 ml round bottom flask with the aid of 5 ml methylene chloride, dried on the evaporator, and reweighed. Residual water in the air dried tissue was determined by mass using triplicate subsamples of ground tissue (250 mg) dried 16 h at 102 ^{o}C. The sterol extract yield was calculated on a dry weight basis.

The concentrated bay laurel extract retained a strong odor from monoterpenes, and the presence of these contaminants was confirmed by gas chromatography. These volatiles were removed before testing the extract on *P. ramorum*, as they can be inhibitory. The extract was redissolved in methylene chloride and a portion transferred to a small round-bottom flask. About 70% of the solution was removed on a rotary evaporator at 40 ^{o}C. Twenty ml of distilled water was then added and the temperature was increased to 70 ^{o}C. This completely removed the monoterpenes as determined by odor and gas chromatography. This terpene-free, sterol extract was used in the bioassays.

Sterol concentrations in the extracts were quantified on a Hewlett Packard (HP) 5890 Series II gas chromatograph with an Agilent (J&W Scientific, Inc.) DB-5 column (30 m x 0.25 mm, 0.25 μm film thickness) connected to a flame ionization detector. The helium carrier gas flow rate was 1.0 ml min^{-1} at 150 ^{o}C and a split of 1:20. The column temperature started at 150 ^{o}C and was increased 5 ^{o}C min^{-1} to 300 ^{o}C where it was held for 20 min. The injector and detector temperatures were 250 ^{o}C. Extracts were dissolved in hexanes, or 2:1 hexanes:methylene chloride (3 to 12 mg ml^{-1}) containing isophytol as an internal standard, and 2 μl was injected. Compounds were quantified with three point standard curves using solutions of the commercial samples dissolved in methylene chloride with isophytol added as an internal standard. Compounds in leaf extracts were identified using the same conditions as above in an Agilent (J&W Scientific, Inc.) DB-5MS column connected to an HP 5970 mass selective detector. The split was set at 1:10 and extract concentrations increased to about 20 mg ml^{-1}.

Plant sterol extracts were dissolved in ethanol and added directly to PSM media prior to autoclaving at a final concentration of 1, 5, 10, 50, or 100 μg ml^{-1}. Due to the presence of non-sterol components in the extract (Table 2.1), all weights were adjusted based on the amount of α- and β-sitosterol in the extract (9.51, 5.08, and 6.92% of the total sterol extract for bay laurel, black oak, and white oak samples, respectively).

Plant tannin-enriched extracts. Sub-samples (5 g) of the ground bay laurel, black oak, and white oak foliage were extracted three times with 100 ml of 70 % acetone for at least 4 h at room temperature. Tannin fractions were purified after evaporating the extracts under nitrogen and re-dissolving each extract in 50 ml of ethanol. The ethanol samples were applied to 5 ml Sephadex LH-20 columns which were washed with at least 50 ml ethanol or until absorbance at 280 nm in the effluent was no longer detected, then eluted with 50 ml of 70 % acetone (Strumeyer and Malin, 1975).

This eluent was tested for total phenols and "tannin" contents. Total tannin content was established gravimetrically. A Folin-Denis assay (Folin and Denis, 1912) was used to assess total phenolics, using tannic acid (MP Biomedicals, Solon, OH) as a standard. Condensed tannin concentration was measured using the acid butanol assay using procyanidin C1 (PHY89537, Cerriliant, Round Rock, TX) as a standard (Porter et al., 1986). The concentration of galloyl-containing compounds(e.g., gallotannins, ellagitannins, and other galloyl esters) was measured using the rhodanine assay using gallic acid as a standard (Inoue and Hagerman, 1988). The remaining tannin extract fraction was evaporated under nitrogen, weighed, and re-dissolved in ethanol at a final concentration of 10 mg ml^{-1}. Tannins were added directly to PSM prior to autoclaving at a final concentration of 0.1, 1, 10, 25, and 50 µg ml^{-1}.

P. ramorum growth and sporulation . Inoculated plates were incubated for 11 d at 18 °C in darkness before measuring average colony diameter (two perpendicular measurements per plate). Plates were then flooded with 8 ml of sterile distilled H_2O to induce sporangium formation, and incubated overnight at 18 °C in darkness. After 18 h, a 200 µl aliquot of distilled H_2O was removed from each plate and stored at 4 °C for elicitin determination using the ELISA assay described below. Plates were then incubated for 2-4 h at 4 °C before warming to room temperature to stimulate zoospore release. The zoospore solution was poured from each plate into a 17 x 100 mm, 14.0 ml culture tube, and vortexed two times for 20 sec to

induce encysting of swimming zoospores. The zoospores were quantified using a hemocytometer at 40X magnification.

ELISA assay Elicitin concentrations were determined with a custom, indirect ELISA assay using rabbit anti-elicitin polyclonal antibodies (Covance Research, Denver, PA) as described previously (Manter et al., 2010). Absorbance at 650 nm was recorded every 30 sec for 15 min with shaking using a Biotek ELx808 microplate reader (Winooski, VT). Elicitin concentrations were determined using an external standard curve (100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0.2 μM) using a purified recombinant ram-α2 protein (Manter et al. 2010).

Elicitin RT-qPCR *P. ramorum* (PR-07-031) was inoculated to liquid PSM medium, amended with either foliar sterols or tannins at the same concentrations used for the bioactivity assays, and incubated for two weeks. Total RNA was extracted from the mycelium using the RNeasy Total RNA Extraction kit (Qiagen, Germantown, MD, USA) and cDNA was synthesized using the RETROscript kit (Applied Biosystems/Ambion, Austin, TX, USA) following the manufacturer's recommendations. TaqMan chemistry was used to determine gene expression for the elicitin ram-α2 gene (Manter et al. 2010). Gene espression for ram-α2 gene was determined using a for point external curve (.1-100 ng μl−1), and reported elicitin gene exspression values were relativized to β-tubulin

Statistical analysis The effect of the various foliage, sterol, or tannin amendments on vegetative growth (i.e., colony diameter), zoospore production, ELISA detected elicitin secretion, and elicitin gene expression was established using nonlinear regression PROC NLIN (SAS, Vers 9.3). For each

trial/treatment combination, a response curve was fit to calculate three different parameters (X @ Y_{max}, Y_{max}, and Y_i) (see Tables1, 3, 5). X @ Y_{max} is the optimum amendment concentration that resulted in the maximum response (Y_{max}). For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X) = 0$. Y_i is the calculated response at the highest amendment concentration tested. Significant differences in the calculated parameters (X @ Y_{max}, Y_{max}, and Y_i) between trial/treatment combinations were tested by ANOVA with Holm-Sidak post-hoc testing using PROC MIXED (SAS, Vers 9.3).

Results

Leaf tissue Colony diameter exhibited a log-normal relationship in response to the leaf tissue amendment of the culture medium (Fig. 2.1A). The amount of leaf tissue required to reach maximum colony growth (X @ Y_{max}) was significantly lower for the two oak extracts (Table 2.2). However, the maximum colony size did not differ significantly for the three plant-amended media (Table 2). Increasing the amount of leaf tissue significantly reduced colony diameter, with the largest change observed with the oak species(Table 2.2).

Similar to colony diameters, zoospore production also showed a log-normal response to leaf tissue amendment of the culture medium (Fig. 2A). However, unlike colony diameters, maximum zoospore production differed between the bay laurel and oak amendments, with nearly five-fold more zoospore production with bay laurel-containing medium (Table 2.3). An inhibitory effect (i.e., percent difference between Y_i and Y_{max}) was noted at the highest concentration tested for all three species, with about 40% inhibition for the bay laurel treatment and nearly 100% for the oaks (Table 2.3).

The amount of secreted elicitin in the samples amended with leaf tissue (Fig. 2.3A) was most similar to the pattern observed for zoospore production. For example, maximum elicitin secretion was

nearly 2.5-fold higher with bay laurel than with the oaks; and the inhibitory effect was only about 10 % with bay laurel as compared to approximately 50 % with the oaks (Table 2.4).

Plant and Commercial Sterol Amendments The foliage sterols extracts included a variety of sterol and non-sterol compounds: phytol, heptacosane, nonacosane, hentricontane, α-sitosterol and β-sitosterol (Table 2.1). Although a statistical comparison between the three species is not possible due to a lack of replicate trees, the three extracts exhibited a range of sterol (α- and β-sitosterol) contents (1745, 612, and 712 μg g^{-1} DW) for the bay laurel, black oak, and white oak extracts, respectively (Table 2.1). Since sterol extracts were added to petri dishes on a total weight basis (i.e., sterol and non-sterols), the reported sterol amendments were adjusted to reflect only sterols (Table 2.1). Similar to the leaf tissue amendments a log-normal relationship between leaf sterol amendments and *P. ramorum* colony diameters (Fig. 2.1B), zoospore production (Fig. 2.2B), or elicitin secretion (Fig. 2.3B) was observed. For all three parameters, similar patterns were observed regardless of the origin of the plant sterol extract (Tables 2.1-3). Similar to the leaf tissue amendments, an inhibitory effect (i.e., percent difference between Y_i and Y_{max}) was observed at the highest concentrations tested. For example, colony diameters declined by an average of 15.3 % (Table 2.2), zoospore production by an average of 24.7 % (Table 2.3), and elicitin secretion by an average of 3.1 % (Table 2.4). The effect of plant sterols on elicitin gene expression, as measured by RT-qPCR, showed a log-normal response curve for all three plant species (Fig. 2.4A). The curves did not differ between species for any of the descriptors (X @ Y_{max}, Y_{max}, or Y_i, Table 2.5). Similar to colony diameter and zoospore production, elicitin gene expression was inhibited at the highest concentration of foliar sterols (Table 2.5).

Qualitative differences in the effect of sterols on *P. ramorum* growth and sporulation were tested using a variety of commercially available sterols (β-sitosterol, cholesterol, egrosterol, stigmastanol, and stigmasterol). For colony diameters, the response to sterol amendments was either log-normal (β-

sitosterol, cholesterol) or exponential rise (ergosterol, stigmastanol, and stigmasterol) (Fig. 1D,E, Table1). The only sterol with any appreciable inhibitory effect on colony size was β-sitosterol, which delined at the highest concentration tested (Table 2.2). Each of the commercial sterols showed a similar response to each other and to the plant extracted sterols, for colony growth and ELISA detectable elicitin. Zoospore was more responsive to the type of sterol (Fig. 2.2D,E, Table 2.3). For example, an inhibitory effect of sterols on zoospore production could only be determined at the concentrations tested for β-sitosterol (84.2 %) and cholesterol (37.8 %) (Table 2.3). Ergosterol, stigmastanol, and stigmasterol exhibited a linear response to zoospore production indicting the effect of sterols on elicitin secretion was similar to the trends observed for zoospore production. For example, β-sitosterol had a X @ Y_{max} of 10.2 μM, while all others were > 50 μM (Fig. 2.3 D,E, Table 2.4); and an inhibitory effect of 54.3 %, while it could not be determined for any of the four other sterols (Fig. 2.3 D,E, Table 2.4).

Tannins. Gravimetric estimates of leaf tannin content after Sephadex LH-20 purification were greatest for black oak, followed by white oak then bay laurel with a lower concentration (Table 2.1). Further characterization into total phenolics (Folin-Denis Assay), gallotannin (Rhodanine Assay), or condensed (HCl-Butanol Assay) tannin components showed similar patterns, such that black oak > white oak > bay laurel (Table 1). These differences in tannin content of foliage by plant may be taken into consideration when assessing differing reactions to ground foliage. Tannin amendments had an inhibitory effect on all parameters measured, except elicitin gene expression (Fig. 2.1C, 2.2C, 2.3C, 2.4B). With increasing tannin concentrations, a decline was seen for colony diameter (Table 2.2), zoospore production (Table 2.3), and ELISA-detected elicitin secretion (Table 2.4) across all three species. In contrast, *P. ramorum* elicitin gene expression remained constant at all levels of tannin amendments tested, suggesting that elicitin expression and production is not affected by tannin concentrations (Fig. 2.4, Table 2.5).

Sterol-Tannin-Elicitin Relationship. Colony diameter growth and zoospore production are tightly correlated with the amount of ELISA detected elicitin across all treatment and trials (Fig. 2.5). It is worth noting that these highly significant relationships hold true across the three plant species tested despite differing sterol and tannin concentrations and compositions. Furthermore, the amount of elicitin required to maximize colony diameter growth is much lower level than that required for zoospore production (Fig. 2.5). Zoospore production did not reach its maximal level in this study.

Discussion

We examined the potential influence of sterols and 'tannins' on *P. ramorum* growth and sporulation and elicitin expression and function. Specifically, we showed that a tannin-enriched foliage extract (Sephadex LH20 purification) can negatively influence *P. ramorum* growth, colonization, and the amount of ELISA-detectable elicitin. However, due to the limitations of the tannin assays used here, additional studies are required to determine (i) the specific compound(s) in the tannin-enriched extract that interfere with sterol-elicitin binding, *P. ramorum* growth and sporulation, and (ii) any additional plant compounds that may interfere with sterol-elicitin binding. Although, it is clear from this work that the Sephadex LH20 tannin-enriched extract inhibits *P. ramorum* growth and sporulation; the enrichment is likely to omit simple phenolics, lower molecular weight tannins, and those with low affinity for proteins. Of these omitted compounds, several classes of compounds, including terpenes (Manter et al., 2006, 2007b) and phenolics (Nagle et al., 2011; Ockels et al., 2007), have also been shown to inhibit *P. ramorum* growth and sporulation.

Although we did not identify the specific compound(s) in the tannin-enriched extract responsible for inhibiting *P. ramorum* growth and sporulation, tannin-elicitin binding in favor of sterol-elicitin binding is consistent with our ELISA assay and the known ability of tannins to bind and precipitate proteins. For example, previous studies have shown that tannins can have a negative effect on some

microorganisms, likely due to their antioxidant activity (Hagerman et al., 1998) and/or their ability to bind proteins. The ability of tannins to bind and precipitate proteins is dependent upon many factors, including tannin structure, pH, and the size and structure of the protein (Hagerman and Butler, 1989). For example, tannins bind more readily to conformationally loose proteins, as compared to more tightly coiled globular proteins. Although we did not directly measure protein-tannin complexes in this study, we observed a decline in ELISA-detectable elicitin, but not elicitin gene expression, suggesting that tannin(s) in the enriched foliar extract are able to successfully bind and precipitate elicitins, or at least bind and interfere with antibody detection of elicitins. Irrespective of the type of elicitin-tannin interaction, our data are consistent with a decreased ability of elicitin, in the presence of tannins, to acquire the necessary sterols for *Phytophthora* growth and colonization. Recently, Ockels *et al.* (2007) observed differences in phenolic profiles of the phloem tissue of coastal live oaks infected with *P. ramorum* and uninfected tissue. Although some of these simple phenolics (e.g., tyrosol, gallic acid) can have direct antimicrobial activity against *P. ramorum* (Ockels et al., 2007), they are also precursors for the polyphenolic tannins (Bianco and Savolaninen, 1997). Therefore, they may be indicative of increased tannin production in infected tissue.

Sterols are known to influence *Phytophthora* spp. growth and sporulation (Elliott et al., 1966; Haskins et al., 1964; Hendrix, 1970; Leal et al., 1964; Nes and Stafford, 1983) and a number of studies have examined quantitative and qualitative differences in sterols to support sexual reproduction (Elliott and Knights, 1974; Elliott et al., 1966; Knights and Elliott, 1976; Mikes et al., 1998a; Nes and Stafford, 1983, 1984). The mechanistic basis for sterol discrimination is unknown, however, it may be dependent upon differences in elicitin-sterol binding capacities (Mikes et al., 1998b; Nes and Stafford, 1983, 1984) and/or changes in elicitin gene expression (Yousef et al., 2009). In this study, at moderate sterol concentrations (i.e., 10 μM or prior to any inhibitory effects on elicitin gene expression) we observed maximal zoospore production and ELISA- detected elicitin, while higher sterol concentrations resulted in a decline in zoospore production and elicitin gene expression. We are unaware of any studies comparing

the binding efficiency of various sterols with *Phytophthora* elicitins. However, the large size of the sterol ligand relative to the elicitin protein and potential access cavity of only 7 Å (Boissy et al., 1999), might explain the discrimination against "left-handed" sterols (Nes and Stafford, 1984).

Due to the ability of elicitin-sterol complexes to trigger host defense responses (Osman et al., 2001b), it is possible that the down-regulation of elicitins represents an attempt by *Phytophthora* spp. to avoid host recognition (Yousef et al., 2009). In addition, our data suggest that the decline in ELISA-detectable elicitin is more sensitive to β-sitosterol, as opposed to other sterols (e.g., cholesterol). The mechanistic basis is unknown; however, it is interesting to speculate that this capacity arose in *Phytophthora* spp. due to the distribution of various sterols in leaf tissues. For example, cholesterol is a major component of leaf surface lipids (Noda et al., 1988); whereas, β-sitosterol is often the major sterol component within plant tissues (Gunstone et al. 1994). Therefore, elicitin gene expression, and sterol acquisition, may be maximized on the leaf surface where sporulation occurs, and minimized within plant cells to avoid host defense responses.

The studies reported here were conducted *in vitro*; therefore, it is unknown to what degree elicitins, tannins, and sterols interact *in planta*. There is some evidence that this interaction may occur; for example, both tannins (Evert, 2006) and elicitins (Brummer et al., 2002a; Osman et al., 2001b) are known to associate with plant cell walls suggesting that their interaction is spatially possible. Whether this truly happens or not can only be found through further *in planta* research. Furthermore, additional work with other plant tissues, i.e., bole/stems, needs to be pursued as *P. ramorum* colonization is often limited to the bole in a variety of oak species. Our data suggest that the amount of 'functional' elicitin, not necessarily elicitin gene expression, is critical for understanding the amount of sterols acquired by *Phytophthora* spp. for growth and sporulation and that 'functional' elicitin is impacted by tannin and sterol concentrations *in vitro*. While it may be assumed that tannins play a role in *P. ramorum*-plant interactions, exploration of the possible interactions *in planta* may show a potential effect of tannin on plant resistance or *P. ramorum*

virulence. In a previous study, it was shown that the sterol content of potatoes did not correlate with *P. infestans* sporulation or resistance *in planta* (Hazel, 1988) suggesting that another factor, perhaps tannin content, is also important. Therefore, resistance to *P. ramorum* may be better predicted using both sterol and tannin content. Even if not useful as biomarkers for *Phytophthora* spp., our work suggests that tannins may alternatively be useful as a fungicide due to their ability to reduce *Phytophthora* growth and sporulation. Tannin/phenolic expression in plants might also be potentially up-regulated *in planta* to provide increased tannin contents and reduced *Phytophthora* virulence. For example, a wide variety of genes and/or transcription factors have been implicated in the regulation of condensed tannins, such as Sn (Robbins et al., 2003), VvMYB5b (Deluc et al., 2008), and DkMyb4 (Akagi et al., 2011).

Additional studies on structural chemistry and make up of 'active' tannins could further separate out those tannins with the ability to bind *Phytophthora* elicitins. However, the studies reported here clearly show that a tannin-enriched foliar extract, consistent with the ability of tannins to bind and precipitate proteins, can reduce *P. ramorum* growth and sporulation. Thus additional studies are warranted to identify the specific tannins that may interfere with the elcitin/sterol binding.

TABLE 2.1. Foliar sterol and tannin concentrations.

Extract	Compound/Assay	Units	Bay laurel	Black oak	White oak
Sterol	Phytol	$\mu g\ g^{-1}$ DW	848.7	848.7	850.7
	Heptacosane	$\mu g\ g^{-1}$ DW	251.0	362.7	449.0
	Nonacosane	$\mu g\ g^{-1}$ DW	480.6	796.5	61.5
	Hentricontane	$\mu g\ g^{-1}$ DW	226.9	9.7	11.5
	α-sitosterol	$\mu g\ g^{-1}$ DW	95.1	20.3	11.0
	β-sitosterol	$\mu g\ g^{-1}$ DW	1649.6	591.5	700.9
Tannin	Gravimetric	$\mu g\ mg^{-1}$ DW	5.1	42.0	20.3
	Folin-Denis	μg TAE mg^{-1} DW[a]	33.7	129.5	88.3
	Rhodanine	μg GAE mg^{-1} DW[b]	8.2	68.4	19.3
	Acid butanol	μg C1E mg^{-1} DW[c]	3.8	41.2	14.3

[a]TAE = tannic acid equivalents
[b]GAE = gallic acid equivalents
[c]C1E = procyanidin C1 (PHY89537, Cerriliant, Round Rock, TX) equivalents

TABLE 2.2. *Phytophthora ramorum* colony diameter in response to various amendments. For each of three trials, response curves were fitted to each species/compound using PROC NLIN (SAS Vers 9.3). Values are the mean and SD of three independent trials. Values with different letters are significantly different ($p < 0.05$). X @ Y_{max} units are the same as those defined in the source column, Y_{max} and Y_i units are mm.

Source	Species / Compound	*Form of Fitted Curve*	X @ Y_{max} [1]	Y_{max} [2]	Y_i [3]
Foliage (mg mL^{-1})	bay laurel	Log-normal	0.54 (0.09) a	43.6 (1.1) a	38.5 (2.9) a
	black oak	Log-normal	0.28 (0.03) b	44.3 (0.4) a	15.4 (2.6) b
	white oak	Log-normal	0.24 (0.03) b	41.7 (1.7) a	17.3 (2.2) b
Foliar sterols (μg mL^{-1})	bay laurel	Log-normal	1.53 (0.29) a	41.3 (1.2) a	36.0 (1.1) a
	black oak	Log-normal	1.64 (0.18) a	42.3 (1.9) a	35.5 (2.7) a
	white oak	Log-normal	1.44 (0.29) a	42.4 (2.4) a	35.2 (1.5) a
Sterols (μM)	β-sitosterol	Log-normal	7.52 (1.91) b	39.2 (1.0) a	33.5 (2.8) b
	cholesterol	Log-normal	21.71 (2.51) a	40.3 (1.9) a	39.3 (1.9) a
	ergosterol	Exponential rise	nd	nd	42.5 (1.0) a
	stigmastanol	Exponential rise	nd	nd	40.5 (1.4) a
	stigmasterol	Exponential rise	nd	nd	40.9 (0.8) a
Foliar tannins (μg mL^{-1})	bay laurel	Exponential decay	0	41.2 (2.2) a	23.2 (1.8) a
	black oak	Exponential decay	0	40.4 (1.7) a	21.8 (1.7) a
	white oak	Exponential decay	0	39.9 (1.5) a	22.5 (2.1) a

[1] X @ Y_{max} is the optimum amendment concentration yielding the maximum response. For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X) = 0$. nd = not determined.

[2] Y_{max} is the maximum response value.

[3] Y_i is the calculated response at the highest amendment concentration tested (foliage: X = 5 mg ml^{-1}; foliar sterols: X = 10 μg mL^{-1}; foliar tannins: X = 500 μg mL^{-1}; sterols: X = 50 μM).

TABLE 2.3. *Phytophthora ramorum* zoospore production in response to various amendments. For each of three trials, response curves were fitted to each species/compound using PROC NLIN (SAS Vers 9.3). Values are the mean and SD of three independent trials. Values with different letters are significantly different ($p < 0.05$). X @ Y_{max} units are the same as those defined in the source column, Y_{max} and Y_i units are spores ml^{-1}.

Source	Species / Compound	*Form of Fitted Curve*	X @ Y_{max} [1]	Y_{max} [2]	Y_i [3]
Foliage	bay laurel	Log-normal	1.54 (0.18) a	695 (83) a	411 (52) a
(mg g^{-1})	black oak	Log-normal	0.41 (0.08) b	134 (48) b	0 (0) b
	white oak	Log-normal	0.35 (0.08) b	126 (49) b	1 (1) b
Foliar sterols	bay laurel	Log-normal	3.51 (0.15) a	298 (13) a	212 (18) a
(μg ml^{-1})	black oak	Log-normal	3.65 (0.70) a	292 (27) a	248 (39) a
	white oak	Log-normal	3.55 (0.28) a	293 (22) a	205 (22) a
Sterols	β-sitosterol	Log-normal	13.94 (3.17) b	1254 (206) a	197 (82) c
(μM)	cholesterol	Log-normal	35.88 (5.37) a	283 (282) b	176 (134) c
	ergosterol	Linear	≥ 50	≥ 866	866 (108) b
	stigmastanol	Linear	≥ 50	≥ 2754	2754 (333) a
	stigmasterol	Linear	≥ 50	≥ 2594	2594 (199) a
Foliar tannins	bay laurel	Exponential decay	0	138 (13) a	30 (9) a
(μg ml^{-1})	black oak	Exponential decay	0	134 (8) a	25 (6) a
	white oak	Exponential decay	0	130 (7) a	27 (8) a

[1] X @ Y_{max} is the optimum amendment concentration yielding the maximum response. For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X) = 0$.

[2] Y_{max} is the maximum response value.

[3] Y_i is the calculated response at the highest amendment concentration tested (foliage: X = 5 mg ml^{-1}; foliar sterols: X = 10 μg mL^{-1}; foliar tannins: X = 500 μg mL^{-1}; sterols: X = 50 μM).

TABLE 2.4. ELISA-detectable *Phytophthora ramorum* elicitin production in response to various amendments. For each of three trials, response curves were fitted to each species/compound using PROC NLIN (SAS Vers 9.3). Values are the mean and SD of three independent trials. Values with different letters are significantly different ($p < 0.05$). X @ Y_{max} units are the same as those defined in the source column, Y_{max} and Y_i units are nM.

Source	Species / Compound	*Form of Fitted Curve*	X @ Y_{max} [1]	Y_{max} [2]	Y_i [3]
Foliage (mg mL^{-1})	bay laurel	Log-normal	1.92 (1.82) a	26.2 (1.7) a	23.2 (1.8) a
	black oak	Log-normal	0.31 (0.55) b	10.8 (2.1) b	5.3 (1.2) b
	white oak	Log-normal	0.25 (0.46) b	11.1 (1.9) b	6.2 (1.6) b
Foliar sterols (μg mL^{-1})	bay laurel	Log-normal	15.34 (3.83) a	18.6 (1.0) a	18.5 (2.0) a
	black oak	Log-normal	17.62 (6.81) a	20.6 (1.3) a	19.2 (1.7) a
	white oak	Log-normal	15.89 (4.30) a	19.2 (1.6) a	18.8 (2.4) a
Sterols (μM)	β-sitosterol	Log-normal	10.21 (2.77)	34.4 (1.8)	15.7 (2.8) a
	cholesterol	Exponential rise	nd	nd	25.3 (2.5) b
	ergosterol	Exponential rise	nd	nd	26.7 (2.5) b
	stigmastanol	Exponential rise	nd	nd	43.2 (2.6) c
	stigmasterol	Exponential rise	nd	nd	42.7 (3.1) c
Foliar tannins (μg mL^{-1})	bay laurel	Exponential decay	0	15.5 (2.2) a	4.2 (1.8) a
	black oak	Exponential decay	0	17.6 (1.9) a	4.0 (0.8) a
	white oak	Exponential decay	0	16.1 (2.4) a	3.1 (1.6) a

[1] X @ Y_{max} is the optimum amendment concentration yielding the maximum response. For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X) = 0$.

[2] Y_{max} is the maximum response value.

[3] Y_i is the calculated response at the highest amendment concentration tested (foliage: X = 5 mg ml^{-1}; foliar sterols: X = 10 μg mL^{-1}; foliar tannins: X = 500 μg mL^{-1}; sterols: X = 50 μM).

TABLE 2.5. *Phytophthora ramorum* ram-*a*2 elicitin gene expression in response to various amendments. For each of three trials, response curves were fitted to each species/compound using PROC NLIN (SAS Vers 9.3). Values are the mean and SD of three independent trials. Values with different letters are significantly different ($p < 0.05$). X @ Y_{max} units are the same as those defined in the source column, Y_{max} and Y_i units are dimensionless.

Source	Species / Compound	*Form of Fitted Curve*	X @ Y_{max} [1]	Y_{max} [2]	Y_i [3]
Foliar sterols ($\mu g\ mL^{-1}$)	bay laurel	Log-normal	4.03 (1.82) a	0.82 (0.12) a	0.61 (0.11) a
	black oak	Log-normal	3.54 (1.44) a	0.77 (0.17) a	0.57 (0.08) a
	white oak	Log-normal	3.98 (2.06) a	0.80 (0.09) a	0.64 (0.05) a
Foliar tannins ($\mu g\ mL^{-1}$)	bay laurel	Linear	nd	nd	0.75 (0.09) a
	black oak	Linear	nd	nd	0.64 (0.15) a
	white oak	Linear	nd	nd	0.79 (0.10) a

[1] X @ Y_{max} is the optimum amendment concentration yielding the maximum response. For lognormal curves, values were determined from the 1st derivative of the response curve, such that $f'(X) = 0$.

[2] Y_{max} is the maximum response value.

[3] Y_i is the calculated response at the highest amendment concentration tested (foliage: $X = 5\ mg\ ml^{-1}$; foliar sterols: $X = 10\ \mu g\ mL^{-1}$; foliar tannins: $X = 500\ \mu g\ mL^{-1}$; sterols: $X = 50\ \mu M$).

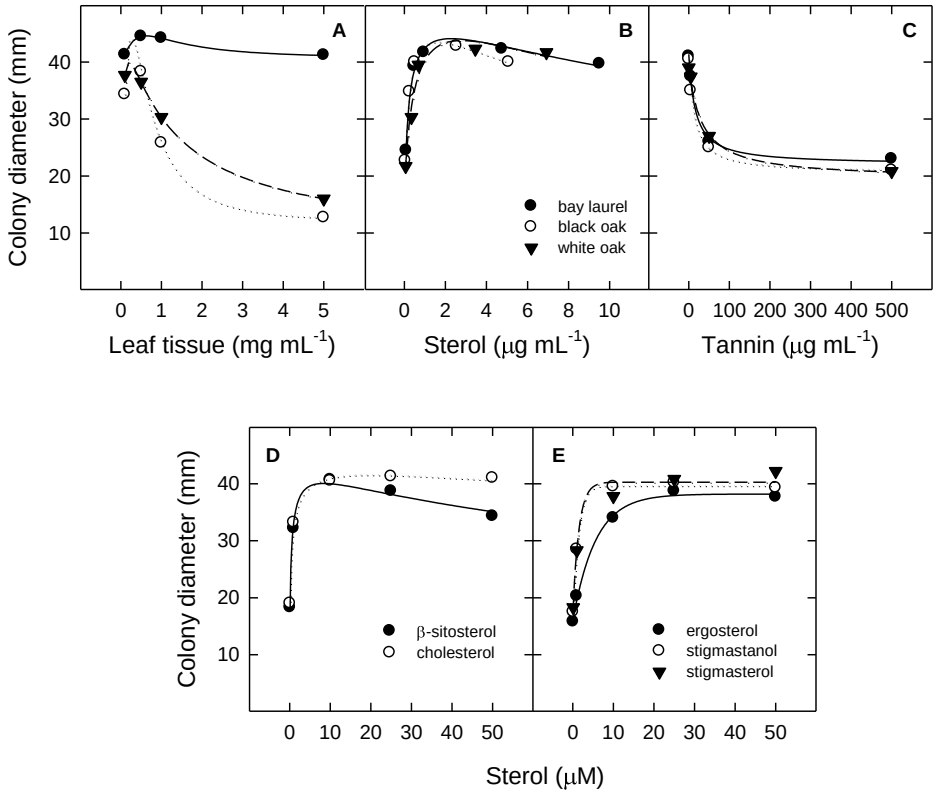

Figure 2.1. *Phytophthora ramorum* colony diameter in response to A) ground foliage, B) foliar sterol extracts, C) foliar tannin extracts, D) β-sitosterol and cholesterol, and E) ergosterol, stigmastanol and stigmasterol. Each point is the average (n = 9) value for all three trials.

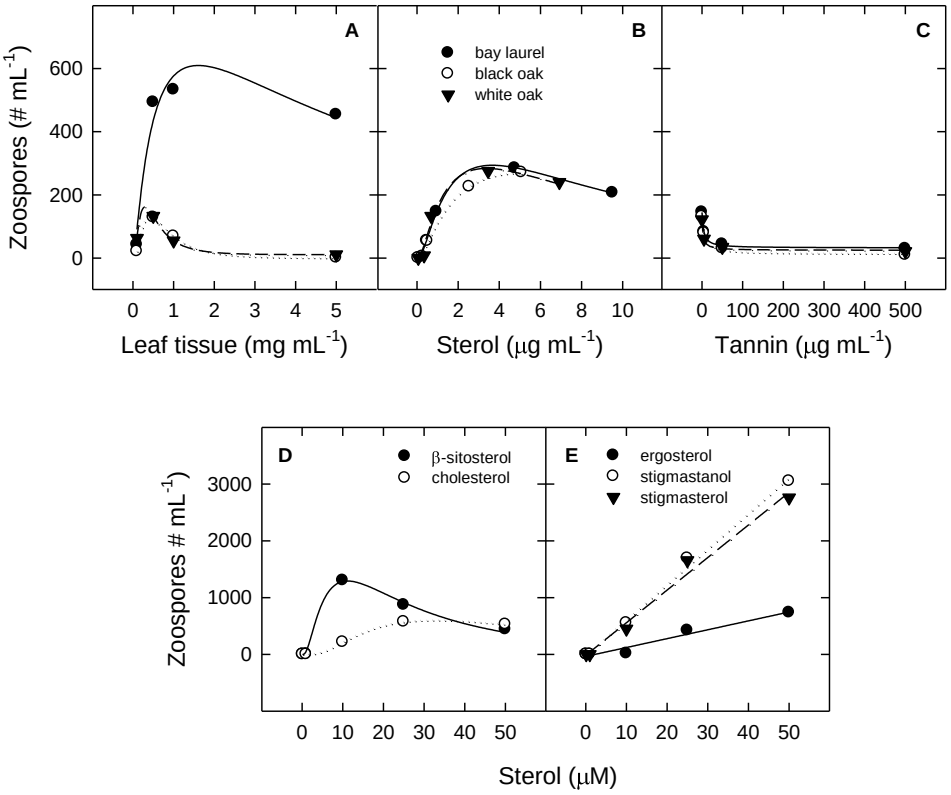

Figure 2.2. *Phytophthora ramorum* zoospore production in response to A) ground foliage, B) foliar sterol extracts, C) foliar tannin extracts, D) β-sitosterol and cholesterol, and E) ergosterol, stigmastanol and stigmasterol. Each point is the average (n = 9) value for all three trials.

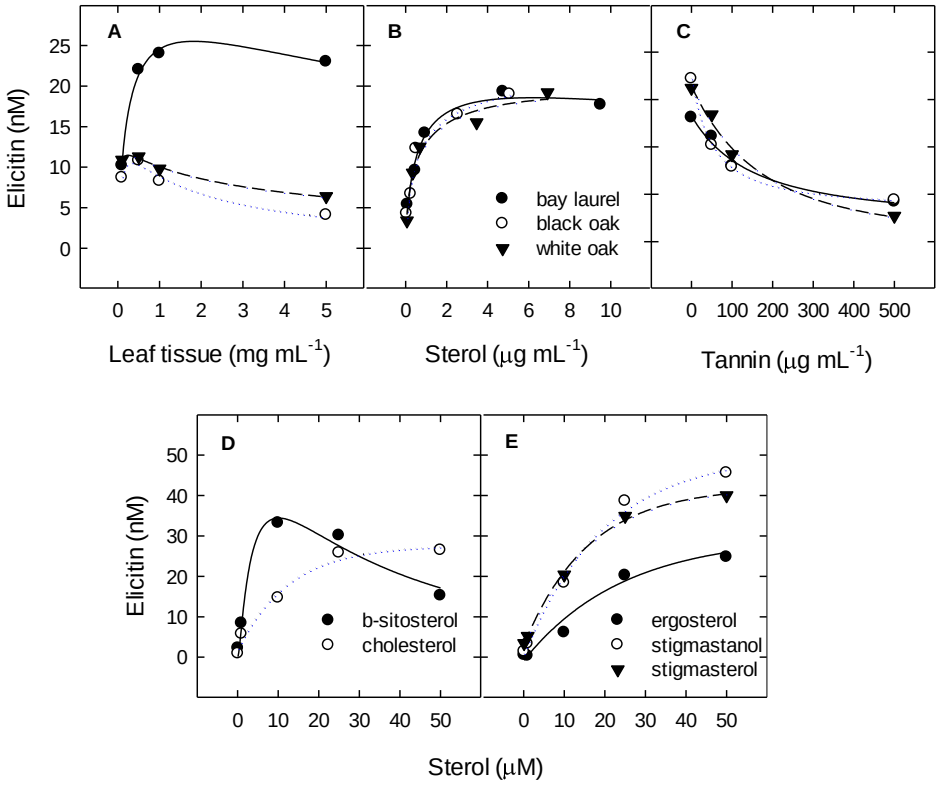

Figure 2.3. ELISA-detectable *Phytophthora ramorum* elicitin secretion in response to A) ground foliage, B) foliar sterol extracts, C) foliar tannin extracts, D β-sitosterol and cholesterol, and E) ergosterol, stigmastanol and stigmasterol. Each point is the average (n = 9) value for all three trials.

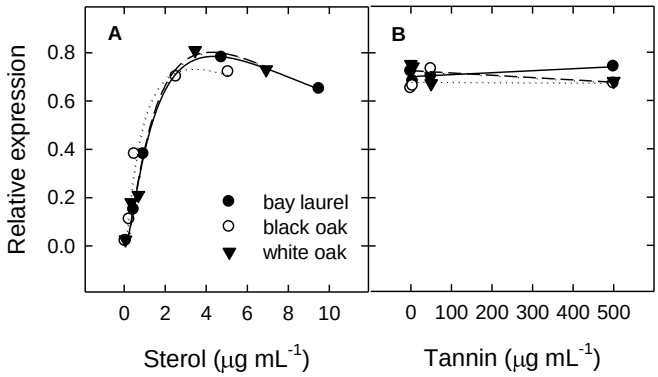

Figure 2.4. RT-qPCR Expression analysis of *ramorum* ram-α2 elicitin gene expression in response to A) foliar sterol extracts and B) foliar tannin extracts. Each point is the average (n = 9) value for all three trials.

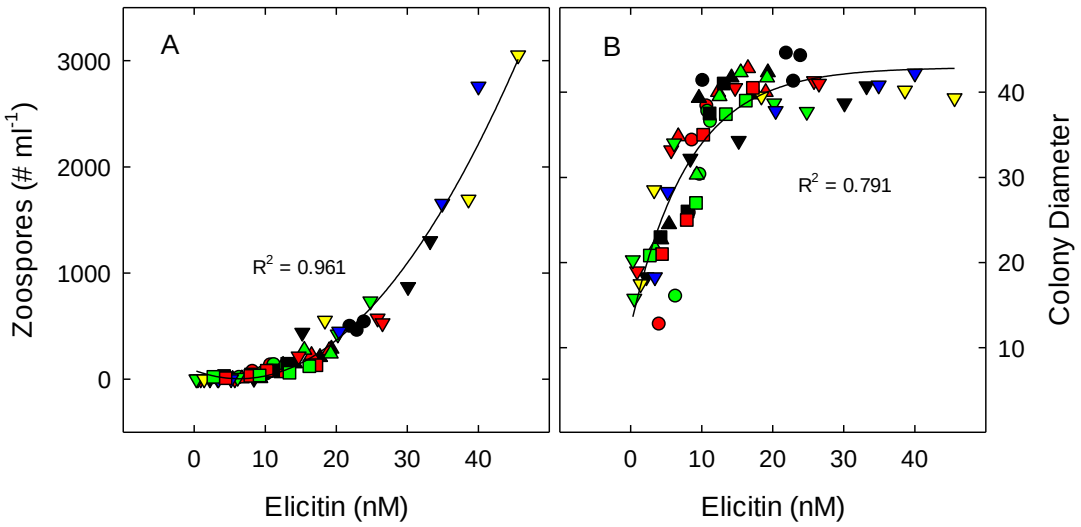

Figure 2.5. Relationship between ELISA-detectable *Phytophthora ramorum* elicitin secretion and A) zoospore production or B) colony diameter growth. Each point is the average (n = 9) value for all three trials. Symbol shapes denote amendment type -- leaf tissue (circles), foliar sterol extracts (upward triangles), commercial sterols (downward triangles), foliar tannin extracts (squares); symbol colors denote plant species -- bay (black), black oak (red), white oak (green) or commercial sterols -- β-sitosterol (black), cholesterol (red), ergosterol (green), stigmastanol (yellow), stigmasterol (blue).

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CHAPTER 3

TANNINS: KEY PLAYERS IN COMPATIBLE HOST INTERACTIONS WITH *Phytophthora ramorum*

Summary

Several cultivars of evergreens, Rhododendron, Kalmia and Azalea, were assessed for constitutive tannin content, sterol content, and leaf susceptibility to *Phytophthora ramorum*. Significant differences were seen between the trials and species for tannin contents. Azalea plants showed no susceptibility to *P. ramorum*, while susceptibility varied between Rhododendron and Kalmia varieties. Tannin content and susceptibility was higher for trial 2 than trial 1, and across all treatment combinations a significant positive correlation was observed between tannin content and *P. ramorum* susceptibility. We hypothesize that the increase in susceptibility of compatible hosts to *P. ramorum* is due to tannin binding of elicitin-sterol complexes reducing the plants ability to mount an effective plant defense response.

Introduction

Phytophthora ramorum is an aggressive pathogen that infects over 100 species of plants (APHIS, 2012) causing bole cankers or leaf and shoot dieback (Grünwald et al., 2008b). *Phytophthora* secrete a family of elicitor proteins, i.e. elicitins, that trigger a hypersensitive response (HR) in incompatible hosts (Kamoun, 1993) and induce systemic acquired resistance (Kamoun, 1993; Ricci et al., 1989). Sterols are necessary for *Phytophthora* reproduction (Boissy et al., 1999); however, *Phytophthora* lack the ability to synthesize sterols themselves, therefore they must acquire them from plant hosts. Labeling has shown that elicitins carry and transfer sterols between membranes providing *Phytophthora* spp. with the necessary sterol supply (Brummer et al., 2002b; Lherminier et al., 2003). This interaction is further emphasized by indications that sterols regulate the expression of class I elicitin genes in

Phytophthora spp. (Yousef, 2010). A defense mechanism of plants has been proposed based on the recognition of elicitins via specific sites on plant plasma membranes (Wendehenne et al., 1995). Further research has shown that these sites have a higher affinity for elicitin-sterol complexes as compared to empty elicitins (Osman et al., 2001a). Thus any compounds, or other factors, that limit the formation of elicitin-sterol complexes may reduce plant defense responses and increase plant susceptibility to *P. ramorum* colonization.

Phenolics have been indicated as having a role in resistance to *P. ramorum* (Ockels et al., 2007). Several simple phenolics, ellagic acid, tyrosol and some unidentified phenolics, were found to occur at higher levels in resistant coast live oaks as compared to symptomatic oaks (Nagle et al., 2011). One group of phenolics that may prove interesting in *P. ramorum* host interactions is tannins. Tannins are a group of protein-binding poly-phenolic compounds, found in plant cell walls and vacuoles, which are used by plants as structural components and for defense against herbivores, insects, and microbial attack (Dixon et al., 2005; Lattanzio et al., 2006). Large and significant differences have been found in the tannin concentrations between leaves, stems and roots; and variation of these concentrations can also occur throughout the season (Häring et al., 2007). The protein binding property of tannins is most interesting in terms of defense. Haering (2008) showed that different elicitor proteins released by insects, bacteria and fungi induced tannins. In addition extractable leaf tannins also can bind *P. ramorum* elicitins (unpublished data), and as described in Chapter 2 of this thesis, high tannin concentrations reduce the amount of ELISA-detectable elicitin without a corresponding change in elicitin gene expression. One group of tannins, the condensed (proanthoyanidin) tannins, may be involved in such interactions as it has been argued that a major function of the condensed tannins is to

provide protection against microbial pathogens, insect pests and larger herbivores (Dixon et al., 2005).

Various types of resistance occur in plant-*Phytophthora* interactions such as pathogen race or plant cultivar specific resistance, plant species or genus resistance, non-host and partial resistance (Kamoun et al., 1999). Within the species of Viburnum, a host plant, susceptibility to *P. ramorum* varied across varieties from highly susceptible to not susceptible (Grünwald et al., 2008a). Clonal repeatability confirmed that lesion size (a measure of susceptibility) is an heritable trait (Dodd et al., 2008; Nagle et al., 2011).

In this study, we further characterized the potential role of tannin-sterol-elicitin interactions in plant susceptibility to *P. ramorum* by examining the potential relationship between constitutive levels of leaf tannin contents and *P. ramorum* colonization in 4-6 cultivars of three evergreen plant species (i.e., Azalea, Rhododendron and Kalmia). Previously, Rhododendrons and Kalmia have shown susceptibility to *P. ramorum* (Linderman et al., 2006; Tooley et al., 2007); whereas, only deciduous Azaleas have shown to be susceptible to artificial inoculation (Tooley et al., 2007) .

Material and Methods

Plant Material- All plant material consisted of plants grown in 4" pots with standard potting soil. For trial 1, six cultivars of Rhododendron and Azalea and five cultivars of Kalmia were selected (Table 1). From each plant (six per cultivar), 3-6 leaves (ca. 0.5 g) were removed for tannin extractions, 3-6 leaves (ca. 0.5g) were used to determine leaf fresh weight / dry weight ratios and later for sterol extraction, and 3-6 leaves (Table 1) were also selected for *P. ramorum*

inoculations. For trial 2, five cultivars of Azalea were selected and four cultivars of Rhododendron and Kalmia were selected. For each cultivar, nine replicate plants were used (Table 1) with sampling for tannin extraction and *P. ramorum* inoculation conducted the same as in trial 1.

Extraction of tannins- Using the protocols laid out in the Tannin handbook (Hagerman, 2002) as a guide, fresh leaves were ground in liquid nitrogen using a hand grinder (IKA,Wilmington NC). Samples were then placed in a 15 ml plastic culture tube, 70% acetone was added to each sample to achieve a ratio of 20 ml 70% acetone per 8 g fresh weight. Samples were then sonicated for 30 min and then centrifuged for 10 min at 2500 g. This extraction procedure was repeated three more times and all supernatants were combined and dried under an air stream at room temperature. The dried samples were dissolved in 1 ml of ethanol and loaded onto Sephedex LH 20 columns (6 mm x 75 mm) and washed with ethanol until the eluent was colorless. Bound tannins were eluted with 70% acetone, dried under an air stream at room temperature and dissolved in methanol using the same volume (8 g leaf fresh weight / 20 mL methanol) as used for the initial extraction.

Total phenolic quantification by Folin-Denis - Total phenolic contents of the Sephedex LH20 eluents were determined based on the Folin-Denis assay of Hagerman (Hagerman, 2002). Briefly 95 µl of methanol, 5 µl sample or standard, and 20 µl Folin-Denis reagent (Sigma) were combined in a single well of a 96 well plate. After 3 min, 30 µl H_2O and 20 µl Na_2CO_3 (22 g / 500 ml) were added to each well. Following 60 min incubation at room temperature, absorbance at 650 nm was determined spectrophotometrically (PowerWave XS2 Bio-Tek). Total phenolic content (mg tannin acid equivalents [TAE] / g leaf DW) for each sample was determined using a

external calibration curve using a tannic acid (MP Biomedicals, Solon OH) as a standard (25, 10, 5, 4, 3, 2, 1, 0.5, 0.1, 0 mg / ml).

Condensed tannin quantification by vanillin- Condensed tannins were measured using the vanillin assay based on the methods outlined by Butler et al (1982). To each well of a 96-well plate 50 µl 10% HCL (in methanol), 50 µl methanol, and 5 µl sample or standard. After determining the background absorbance at 550 nm, 50 µl 4% vanillin (in methanol) was added to each well. Following a 30 min incubation at room temperature the absorbance at 550 nm was measured again. Concentration was calculated by subtracting background read from final read. Condensed tannin content (mg (+)-catechin equivalents [CE] / g leaf DW) for each sample was determined using a 5-point external calibration curve using a (+) catchin (TCI, Kita-ku, Tokyo, Japan) as a standard (50, 20, 15, 10, 5, 2, 1, 0.5, 0.1, 0 mg / ml).

Condensed tannin quantification by acid butanol- Further analysis of condensed tannins was done by running a modified acid butanol assay (Hagerman, 2002). 60 µl of acid butanol (95% n-butanol in concentrated HCL), 10 µl of sample and 2 µl of iron reagent (2% $FeNH_4(SO_4)_2 \cdot 12H_2O$ in 2 M HCl) were added to a 1 ml tube then vortexed and placed in boiling water for 50 min. Cooled samples were added at 20 µl to 100 µl of methanol in 96-well plate. Condensed tannin content (mg procyanidin C1 equivalents [PCE] / g leaf DW) for each sample was determined using a 5-point external calibration curve using procyanidin C1 (PHY89537, Cerriliant, Round Rock, TX) as a standard. (0.01, 0.005, 0.025, 0.001, 0 mg / µl)

Sterol Extraction and assay- Samples were ground in a hand grinder and 15 ml per 0.5 g KOH (150 g / L) were added to each. These saponified at 95 C for 1 h. Petroleum ether was then added at same volume as KOH, vortexed, centrifuge and top layer taken off. This was repeated three

times. Samples were dried down and then brought up in 1ml of methanol. Sterol content was measured using Wacko Cholestorol E enzymatic colormetric kit (Wako Pure Chemical Industries, Richmond VA Based on method set by W. Richard 1972). Five µl of sample was added to 96 well plates with 200 µl of color reagent. Plate was read at 600 nm after shaking for 5min at 37C. Sterol content (mg Cholesterol equivalents [CSE] / g leaf DW) for each sample was determined using a 5-point external calibration curve using cholesterol standard provided in kit.)

Cultures, Inoculations and Lesion measurement- Isolates of *P. ramorum* were cultured from soil in Washington in 2006 (Table 1). Cultures were maintained on dilute V8 agar plates stored at room temperature in the dark (Ribeiro, 1978). Prior to inoculation leaves were first wounded with three needle pricks approximately 1 mm from the center vein in the middle upper side of the leaf. Mycelium plugs (6 mm diameter) were taken from the margins of two week old colonies, inverted and placed over the wounded area. Inoculated leaves were placed on a moist paper towel in plastic boxes and hand-misted with distilled water before closing. These plastic box chambers were placed in a dark room at room temperature for 7 days. Mycelium plugs were removed after 4 days and the chambers were misted. After 7 days, leaves were removed and scanned on a flat-bed scanner. Total leaf length and lesion length were measured and the percent lesion (% mm lesion / mm leaf) covering the leaf was calculated.

Statistical Analysis- Significant differences ($p < 0.05$) between trials and species were determined using a two way analysis of variance (ANOVA) and post-hoc comparisons with Sidak's corrections (PROC GLM, SAS Vers 9.3, SAS Institute, Cary, NC). Significant differences ($p < 0.05$) between cultivars for tannin contents, sterol content, and percent lesion lengths were analyzed using a one way analysis of variance (ANOVA) and post-hoc comparisons with Sidak's corrections (PROC GLM, SAS Vers 9.3). Pearson correlations

coefficients between each dependent variable were calculated using the arithmetic means for each cultivar/trial combination.

Results

Total Phenolics - The Folin-Denis assay showed no significant statistical differences between trials and species in total phenolic content of Kalmia or Rhododendrons, however differences between the trial means show at least 20 TAE (tannic acid equivalent) mg /g increase from trial 1 to trial 2 (Figure 3.1). Azaleas had significantly less phenolics in trial 1 versus trial 2. Significant differences were also seen between cultivars but these differences were not as great as those observed between species (Table 3.2). For instance in trial 1 TAE for cultivars from Rhododendron and Kalmia species were between 15-30 TAE mg / g; whereas, the range for Azalea cultivars was more than double or 1-30 TAE mg / g. The variation for all three species was much smaller, ranging between 45-60 TAE mg / g, except for one cultivar of Rhododendron, which was 26 TAE mg/g.

Condensed Tannin- Condensed tannin concentrations measured by the vanillin assay varied significantly between trials and species (Figure 3.2). The greatest difference between trials was seen for the Rhododendron cultivars, increasing approx. 3-fold or 100 CE ((+)-catechin equivalent) mg / g from trial 1 to trial 2. A similar, but much smaller increase from trial 1 to trial 2 was seen for the other two species. Significant differences were also seen between cultivars (Table 3.3). In trial 1, the Kalmia cultivars did not differ significant and ranged between 50-85 CE mg / g, Rhododendron varied from 10-30 CE mg / g, and Azalea at 3-5 CE mg / g. Similar

species differences were observed in Trial 2 despite condensed tannin contents significantly increasing for all species (10-200 CE mg / g).

The acid butanol assay showed condensed tannin concentration to be significantly different between trials and species (Figure 3.3). The most notable differences are seen in trial 2 which had higher concentration of condensed tannins than trial 1. The greatest difference was seen in Kalmia species where it increased by about 700 PCE (procyanidin C1 equivalent) mg / g, followed by Rhododendron differing by about 250 PCE mg / g, and Kalmia increasing by 25 PCE mg / g from trial 1 to 2. There was greater variability seen in trial 1 than trial 2 at the cultivar level (Table 3.4). For both trials, species were significantly different; for instance in trial 1 Kalmia cultivars ranged between 130-190 PCE mg / g, Azalea 55-100 PCE mg / g, Rhododendrons 30-80 PCE mg / g. In trial 2, much less variability was observed between cultivars, with no differences in Kalmia, all Rhododendrons falling into group at about 130 PCE mg / g except one at 100 PCE mg / g, and Azalea ranging from 250-650 PCE mg / g.

Sterols – A significant increase of about 50 CSE (Cholesterol Equivalent) mg / g was seen from trial 1 to trial 2 (Figure 3.4) in sterol concentration. No significant differences were found between trials for Azalea or Rhododendron. Between all trials of Azalea and Rhododendron and trial 1 of Kalmia there is not much variation. Significant variations with groupings from 15-150 CSE mg / g are seen between cultivars with no distinct separation of species for trial 1 (Table 3.5). For trial 2 there is little variation, Kalmia and Azaleas fall between 10-30 CSE mg / g, and Rhododendron cultivars range 1-3 CSE mg/g.

Percent Lesion Size - No lesions were observed on inoculated Azalea leaves. Percent lesion size by species differed significantly for trial 1, whereas for trial 2 there are no significant differences

(Figure 3.5). Rhododendrons exhibit a significant increase in lesion length of about 35 % points from trial 1 to trial 2, whereas there was no significant difference seen between trials for Kalmia. Significant differences were seen between cultivars (Table 3.6). In trial 1, no difference between Kalmia cultivars observed, ranging from 60-80%; whereas, for the Rhododendrons significant cultivar differences were observed with average lesion size ranging from 20-56%. For trial 2, lesion size did not differ significantly between the Rhododendron and Kalmia species (Figure 3.5).

Comparison of Lesion to Tannin Content- Correlation analysis between tannins and lesion across trials indicated a significant positive correlation with all p values less than 0.0001 and Pearson correlation coefficients values around 0.5, for all the tannin assays measured (Table 3.7). Sterol content showed a negative correlation. For all measures, the correlation between tannins and lesion size were highly significant ($p < 0.001$) in trial 1 but not trial 2 ($p > 0.03$) consistent with the higher tannin levels and less variation between cultivars for lesion size for trial 2, and the observed curvilinear relationship between condensed tannins and lesion size (Figure 3.6).

Discussion

My research found variation in tannin content between trials, species and cultivar for all tannin assays. In particular tannin content was lower for trial 1 compared with trial 2. Significant differences between trial 1and 2 were most prominent in the acid butanol and vanillin assay measurements of condensed tannins. Percent lesion length also increased from trial 1 to 2 in Rhododendrons. Increase in percent lesion length was not significant for Kalmia but this maybe

a product of trial 1, already displaying high susceptibility masking any additional susceptibility from this mode of measurement. The increase in percent lesion length and tannin from trial 1 and trial 2 indicated a possible correlation to lesion and tannin content. Little variation was seen in sterol content between trials indicating that change in percent lesion length of this study was not due to availability of sterol. Variation between trials is not due to genetic difference as both lesion size (Dodd et al., 2008; Nagle et al., 2011) and condensed tannin content (Vailcourt et al. 1986) are linked to heritable traits. Tannin content can vary based on environmental impacts such as soil or stress and leaf age (Haering et al., 2008; Häring et al., 2007; Haviola et al., 2007; McLeod, 1974). All plants in this study were relatively young plants (growth had only reached about 9 in). Trial 1 was conducted over the course of a calendar year as the plants were analyzed upon availability and receipt from nursery stock; whereas, all plants in trial 2 plants were received in November. Therefore, it is impossible to attribute the differences in season per se but appear to be influenced by nursery growing conditions and or handling.

In plant-pathogen interactions, cell death may be a result of toxins or a hypersensitive response (HR). Research on *Phytophthora* elicitin toxicity indicates a wide range of responsiveness to elicitins. Previous studies have found varying degrees of necrosis or cell apoptosis in Solanaceae plants (Qutob et al., 2002; Vleeshouwers et al., 2000), ultrastructural changes in oak (Brummer et al., 2002b) and pepper (Ivanova and Singh, 2003), and no change in net photosynthesis in beech (Fleischmann et al., 2005). The majority of work on elicitin has focused on their ability to induce HR and systemic acquired resistance (SAR) in incompatible hosts such as tobacco (Keller et al., 1996; Ricci, 1997). During HR a suppression of photosynthesis is initiated before cell death and photosynthetic declines have been observed following *P. ramorum* elicitin treatment in tobacco, an incompatible host and three compatible hosts: tanoak, myrtle and rhododendron. This decline in photosynthesis and an associated production of ethylene in all four species suggests an HR or HR-like response in all hosts (Manter et al., 2007a). This HR- like in compatible hosts may be due to slow triggering of HR or an incomplete HR pathway response and

differences in the degree of the HR-like response is one possible explanation for the varying lesion sizes observed in this study. Elicitin-sterol complexes are known triggers for the HR to *Phytophthora* (Osman et al., 2001a). The positive correlation between tannin and lesion size could indicate tannin binding of elicitin- sterol complexes inhibiting binding to trigger sites and reducing the amount of HR-like response.

Tannins have the potential to impact *P. ramorum* interaction at several points during infection. For example, through immunocytochemical labeling, Brummer (2002) uncovered that *Phytophthora* produce elicitins during the whole growth phase. The first step in *Phytophthora* infection occurs when zoospores germinate, encyst and either enter the stomata using mycelium or directly penetrate into the leaf using appressorium (Bircher and Hohl, 1997). Tannins may be able to interact with elicitin here but no point of contact is known. Once within the plant, *Phytophthora* moves through intercellular and intracellular spaces releasing elicitin. Intercellular elicitins may interact with tannins in transport or bound to cell walls. *Phytophthora* penetrates cell walls using haustorium (Enkerli et al., 1997) and the cell reacts by strengthening the cell wall (Fellbrich et al., 2002) and releasing phytoalexins, pathogenesis related proteins and toxins. Some toxins that may be released from vacuoles are tannins. Within the cell *Phytophthora* releases elicitin into the cytoplasm where they bind to sterols from cell membranes (Boissy et al., 1999). These sterol-elicitin complexes then attach to binding sites and trigger a HR-like response or are taken up into *Phytophthora* for growth and reproduction. In compatible plants tannins may bind sterol-elicitin complexes preventing binding to HR binding sites and slowing down HR-like responses. It is at this point that spread of pathogen is either stopped by HR in incompatible plants (Haitema 2003, Takemoto et al 2003) or *Phytophthora* is able to spread at different rates in compatible plants due to differing degrees of an HR-like response.

The tannins measured by the assays in this study are only representative of leaf contents. Through extraction and purification using Sephedex LH 20 the enrichment of extracted samples is likely to omit simple phenolics, lower molecular weight tannins, and those with low affinity for proteins. The assays themselves further limit quantification of tannin. The Folin-Denis assay measures oxidation reduction.

This measurement is not specific for tannins but will quantify many phenolics (Waterman and Mole, 1994). The vanillin assay depends on the reaction of vanilla with condensed tannins. This reaction is affected by temperature (Waterman and Mole, 1994), water content (Hagerman et al., 1998) and more importantly variability of the reactivity of the subunits of the tannin polymer (Butler et al., 1982). The acid butanol assay measures cleavage of condensed tannin yielding anthocyanidins. While considered by many to be the most accurate of colorimetric tannin assays, it is dependent upon the type and structure of the various anthocyandins present,(Schofield et al., 2001). Therefore, due to the limitations of the assays employed in this study, additional studies will be required to better understand the specific tannin(s) that are involved in binding of *Phytophthora* elicitins.

Although the current study shows a correlation between tannin content and lesion size, further studies are needed to determine the extent of tannin-elicitin interactions. A closer look to chemically identify what tannins are involved in reaction with elicitin through liquid chromatography or HPLC or LC-MS will lead to a better understanding of what is occurring in elicitin-sterol-tannin interaction. A study of membrane interaction with elicitin-sterol complex in presence of tannin may further prove the interaction of an elicitin sterol complex. It has been shown that plant tannins increased in response to infection (Walkinshaw, 1990). An examination of induced tannins as it relates to lesion size may better help us see what is occurring during *P. ramorum* infection. Research should also be done to see what impact inducing or inhibiting higher tannin concentrations has on *Phytophthora* infection.

In conclusion, we observed a positive, curvilinear relationship between leaf tannin content and lesion size in compatible hosts; although, the mechanism remains unknown, it is consistent with elicitin-sterol-tannin complexes that reduce triggering of HR-like reactions in compatible hosts.

Table 3.1. Plants material and Phytophthora *ramorum* isolates used for inoculation by trial

Host scientific name	Host common name	Trial	*Phytophthora ramorum* isolate	Number of Plants	Leaves inoculated per isolate per plant
Rhododendron catawbiense album	Cat. Album Rhododendron	1	Pr-031,Pr-002	6	2
		2	Pr-031		
Rhododendron catawbiense 'Independence Day'	Independence Day Rhododendron	1	Pr-031,Pr-002	6	2
		2	Pr-031		
Rhododendron catawbiense 'Borsalt'	Borsalt Rhododendron	1	Pr-031,Pr-002		2
		2	Pr-031	9	3
Rhododendron catawbiense 'Nova Zembla'	Nova Zembla Rhododendron	1	Pr-031,Pr-002	6	2
		2	Pr-031	9	3
Rhododendron catawbiense 'Scintillation'	Sintillation Rhododendron	1	Pr-031,Pr-002	6	2
		2	Pr-031	9	3
Rhododendron catawbiense 'Lee's Dark Purple'	Lee's Dark Purple Rhododendron	1	Pr-031,Pr-002		2
		2	Pr-031	9	3
Rhododendron catawbiense 'Percy Wiseman'	Percy Wiseman Rhododendron	1	Pr-031,Pr-002	6	2
		2	Pr-031		
Rhododendron catawbiense ' Pink Butterfly'	Pink Butterfly Rhododendron	1	Pr-031,Pr-002	6	2
		2	Pr-031		
Kalmia Latifolia 'Ostbo Red'	Ostbo Red Mountain Laurel	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3
Kalmia Latifolia 'Peppermint'	Peppermint Mountain Laurel	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3
Kalmia Latifolia 'Pink Charming'	Pink Charming Mountain Laurel	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3
Kalmia Latifolia 'Pristine'	Pristine Mountain Laurel	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3
Kalmia Latifolia 'Sarah'	Sarah Mountain Laurel	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3
Kalmia Latifolia 'Starburst'	Starburst Mountain Laurel	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031		

Table 3.1 (cont'd). Plants Inoculated and isolates.

Host scientific name	Host common name	Trial	*Phytophthora ramorum* isolate	Number of Plants	Leaves inoculated per isolate per plant
Rhododendron tsutsuji 'Arneson Gem'	Arneson Gem Azalea	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031		
Rhododendron tsutsuj 'Candy Lights'	Candy Lights Azalea	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3
Rhododendron tsutsuj 'Fragrant Star'	Fragrant Star Azalea	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3
Rhododendron tsutsuj 'Golden Lights'	Golden Lights Azalea	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3
Rhododendron tsutsuj 'Mandarin Lights'	Mandarin Lights Azalea	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3
Rhododendron tsutsuj 'Rosy Lights'	Rosy Lights Azalea	1	Pr-057,Pr-031, Pr-002	6	3
		2	Pr-031	9	3

Table 3.2. Folin-Denis total phenolics (Tannic acid equivalent mg/g) ANOVA across cultivar SAS Back transformed

		Trial 1			Trial 2		
Species	**Cultivar**	**LSmean**	**High**	**Low**	**LSmean**	**High**	**Low**
Rhododendron	Borsalt				61.13 a	2.86	2.73
	Cat Album	21.17 ab	24.33	18.41			
	Independence Day	14.71 abc	16.91	12.79	57.64 ab	2.69	2.57
	Lee's Dark Purple				26.19 d	1.22	1.17
	Nova Zembla	26.01 ab	29.91	22.63	57.07 ab	2.67	2.55
	Percy Wiseman	14.74 abc	16.95	12.82			
	Pink butterfly	24.60 ab	28.28	21.40			
	Scintillation	10.24 abc	11.78	8.91			
Kalmia	Ostbo Red	32.74 a	36.00	29.77	56.35 ab	2.33	2.23
	Peppermint	21.11 ab	23.21	19.20	48.39 abc	2.00	1.92
	Pink Charming	30.06 a	33.06	27.34	47.33 abc	1.95	1.88
	Sarah	26.81a	29.49	24.39	44.37 bc	1.83	1.76
	Starburst	24.33 ab	26.76	22.13			
Azalea	Arneson Gem	32.74 cd	36.00	29.77	48.77 abc	4.28	3.94
	Candy Lights	3.1 6f	5.45	1.83	41.23 c	3.62	3.33
	Fragrant Star	0.07 ef	0.13	0.04	48.72 abc	4.02	3.72
	Golden Lights	0.36 de	0.63	0.21	48.72 abc	4.02	3.72
	Mandarin Lights	1.14 e	2.08	0.62	53.64 abc	4.43	4.09
	Rosy Lights	0.46 bcd	0.80	0.26			

Table 3.3. Vanillin condensed tannins ((+)-Catechin equivalent mg/g) ANOVA by cultivar SAS Back transformed

		Trial 1			Trial 2		
Species	**Cultivar**	**LSmean**	**High**	**Low**	**LSmean**	**High**	**Low**
Rhododendron	Borsalt				229.87 a	47.77	39.55
	Cat Album	32.19 bcd	38.51	26.91			
	Independence Day	29.45 bcd	35.24	24.62	214.85 a	44.65	36.96
	Lee's Dark Purple				46.05 bcd	9.57	7.92
	Nova Zembla	24.86 cd	29.75	20.78	146.93 abc	30.53	25.28
	Percy Wiseman	17.41 de	20.83	14.55			
	Pink butterfly	30.88b cd	36.94	25.81			
	Scintillation	10.25 ef	12.26	8.57			
Kalmia	Ostbo Red	85.37 a	99.56	73.21	71.51 abc	16.06	13.12
	Peppermint	42.52 abc	49.59	36.46	96.69 abc	21.72	17.74
	Pink Charming	77.03 a	89.84	66.06	188.39 a	42.32	34.55
	Sarah	58.38 ab	68.09	50.06	120.46 abc	27.06	22.10
	Starburst	51.88 abc	60.50	44.48			
Azalea	Arneson Gem	3.97 g	4.45	3.54			
	Candy Lights	3.72 g	4.17	3.32	14.53 de	6.63	4.55
	Fragrant Star	5.63 gf	6.31	5.02	9.72 e	4.43	3.05
	Golden Lights	4.23 g	4.79	3.73	67.50 abc	28.69	20.13
	Mandarin Lights	4.12 g	4.62	3.68	36.85 cde	15.66	10.99
	Rosy Lights	4.29 g	4.86	3.78	79.01 abc	33.58	23.57

Table 3.4. Acid Butanol condensed tannins (Procyanadin C1 mg/g) ANOVA by cultivar SAS Back transformed

		Trial 1			Trial 2		
Species	**Cultivar**	**LSmean**	**High**	**Low**	**LSmean**	**High**	**Low**
Rhododendron	Borsalt				257.07 b	25.67	23.34
	Cat Album	62.95 ef	70.02	56.59			
	Independence Day	38.39 hg	42.70	34.51	653.86 b	65.29	59.36
	Lee's Dark Purple				241.41 b	24.10	21.92
	Nova Zembla	80.64 cde	89.70	72.50	296.14 a	29.57	26.89
	Percy Wiseman	57.15 efg	63.58	51.38			
	Pink butterfly	65.55 def	72.92	58.93			
	Scintillation	30.32 h	33.72	27.25			
Kalmia	Ostbo Red	136.90 a	148.95	125.82	776.07 a	42.37	40.18
	Peppermint	184.23 ab	192.81	176.04	751.86 a	41.05	38.92
	Pink Charming	150.18 a	157.17	143.50	720.34 a	39.33	37.29
	Sarah	179.00 a	187.33	171.03	609.19 a	33.26	31.54
	Starburst	189.53 ab	198.35	181.10			
Azalea	Arneson Gem	81.12 cde	87.61	75.10			
	Candy Lights	50.42 fg	54.46	46.69	134.24 cd	11.99	11.01
	Fragrant Star	108.97 bc	117.69	100.89	99.02 d	8.85	8.12
	Golden Lights	100.23 bce	109.06	92.12	133.21 cd	11.19	10.33
	Mandarin Lights	80.00 cde	86.41	74.07	134.42 cd	11.29	10.42
	Rosy Lights	81.12 ab	87.61	75.10	154.43 c	12.98	11.97

Table 3.5. Sterols (cholesterol equivalent mg/g) ANOVA by cultivar SAS Back transformed

		Trial 1			Trial 2		
Genus	**Cultivar**	**LSmean**	**High**	**Low**	**LSmean**	**High**	**Low**
Rhododendron	Borsalt				1.52 c	0.60	0.43
	Cat Album	15.25 f	17.51	13.28			
	Independence Day	45.12b cd	51.18	39.78	2.05 c	0.80	0.58
	Lee's Dark Purple				1.64 c	0.64	0.46
	Nova Zembla				2.86 c	1.12	0.80
	Percy Wiseman	46.64 bcd	52.91	41.12			
	Pink butterfly	49.50 bcd	56.83	43.12			
	Scintillation	55.34 bcd	64.57	47.43			
Kalmia	Ostbo Red	27.58 def	31.00	24.54	15.93 ab	3.01	2.53
	Peppermint	28.17 cdef	33.23	23.88	16.41 ab	3.10	2.61
	Pink Charming	28.17b cde	33.23	23.88	9.51 ab	1.80	1.51
	Sarah	21.52 ef	24.46	18.93	18.08 ab	3.42	2.87
	Starburst	35.63 bcde	42.04	30.21			
Azalea	Arneson Gem	62.50 ab	70.73	55.24			
	Candy Lights	69.15 ab	79.18	60.40	21.36 ab	2.23	2.02
	Fragrant Star	45.39 bcd	51.36	40.11	13.13 ab	1.37	1.24
	Golden Lights	55.92 bcd	65.05	48.06	18.06 ab	1.78	1.62
	Mandarin Lights	154.16 a	190.96	124.46	18.16 ab	1.79	1.63
	Rosy Lights	57.20 bc	65.50	49.96	30.40 ab	2.99	2.72

Table 3.6. Lesion (mm) ANOVA by cultivar SAS Back transformed

		Trial 1			Trial 2		
Genus	**Cultivar**	**LSmean**	**High**	**Low**	**LSmean**	**High**	**Low**
Rhododendron	Boursalt				65.11 bcd	4.40	4.13
	Cat Album	41.94cd	44.82	39.25			
	Independence Day	56.37bc	60.24	52.74	74.52 abc	5.04	4.72
	Lee's Dark Purple				56.15 cd	3.80	3.56
	Nova Zembla	24.82ef	26.53	23.23	69.84 abcd	4.72	4.43
	Percy Wiseman	24.82de	26.53	23.23			
	Pink butterfly	19.42f	20.75	18.17			
	Scintillation	27.38ef	29.26	25.62			
Kalmia	Ostbo Red	69.23ab	73.99	64.78	93.49 a	81.26	43.48
	Peppermint	61.50ab	65.73	57.55	51.86 d	45.08	24.12
	Pink Charming	65.57ab	70.08	61.36	87.76 ab	76.28	40.81
	Sarah	80.92a	86.48	75.72	26.06 abcd	22.65	12.12
	Starburst	64.22ab	68.63	60.09			
Azalea	Arneson Gem	0.00	0.00	0.00	0.00	0.00	0.00
	Candy Lights	0.00	0.00	0.00	0.00	0.00	0.00
	Fragrant Star	0.00	0.00	0.00	0.00	0.00	0.00
	Golden Lights	0.00	0.00	0.00	0.00	0.00	0.00
	Mandarin Lights	0.00	0.00	0.00	0.00	0.00	0.00
	Rosy Lights	0.00	0.00	0.00	0.00	0.00	0.00

Table 3.7. Pearson Correlation Coefficients (P values)

	Lesion		
	Across Trials	**Trial 1**	**Trial 2**
Total phenolics (Folin-Denis)	0.5296 (<0.0001)	0.2311 (0.05)	0.094 (0.40)
Condensed tannin (Vanillin)	0.4565 (<0.0001)	0.3877 (0.001)	-0.236 (0.03)
Condensed tannin (Acid Butanol)	0.5412 (<0.0001)	0.5559 (<0.0001)	-0.020 (0.85)
Sterol	-0.4327 (<0.0001)	-.4754 (0.0005)	0.019 (0.86)

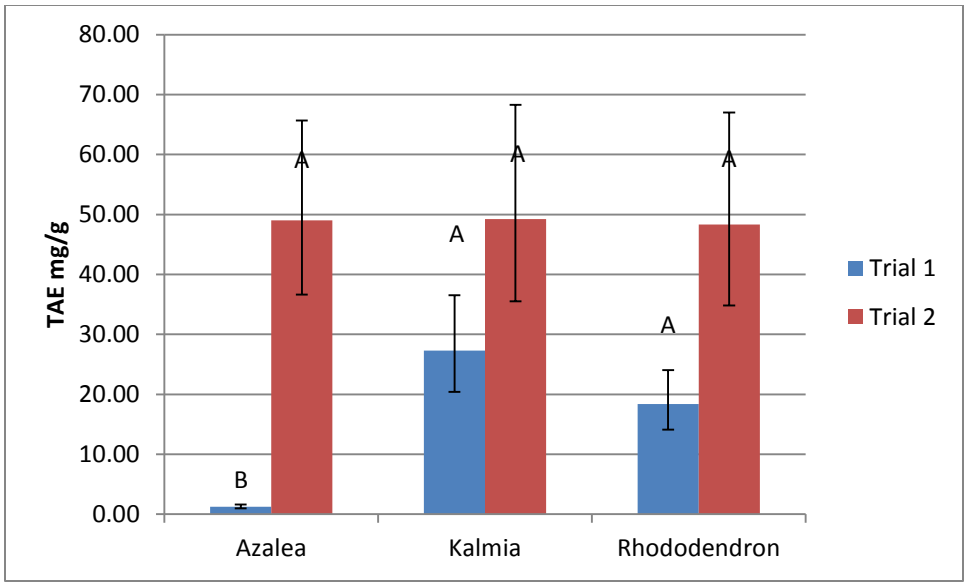

Figure 3. 1. Total Phenolics two way ANOVA trial by cultivar from Folin-Denis assay in Tannic Acid Equivalents (TAE). Each observation is the mean and standard error. Bars with the same letter are not significantly different at the 95% confidence level

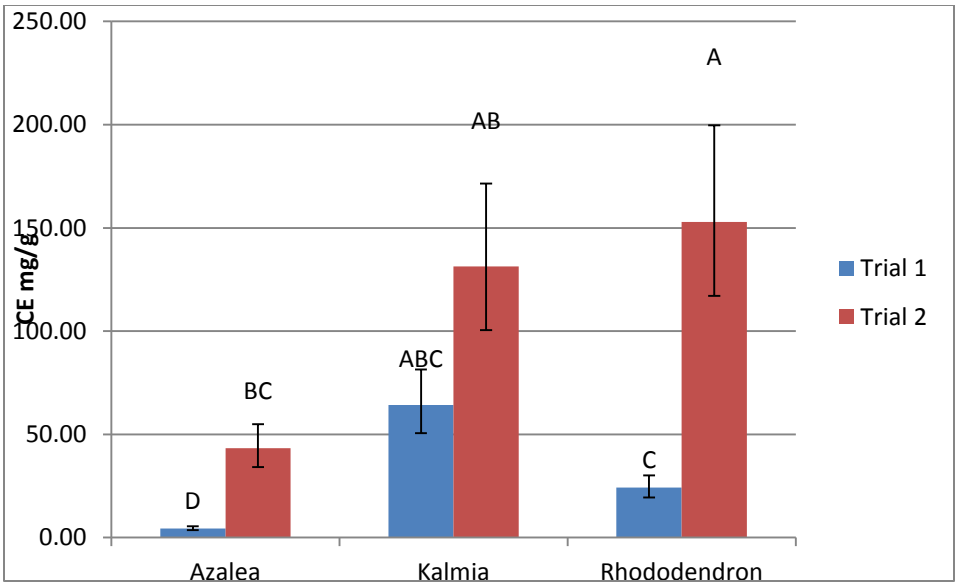

Figure 3.2. Condensed tannin two way ANOVA trial by cultivar from vanillin assay in (+) catechin equivalents (CE) Each observation is the mean and standard error. Bars with the same letter are not significantly different at the 95% confidence level

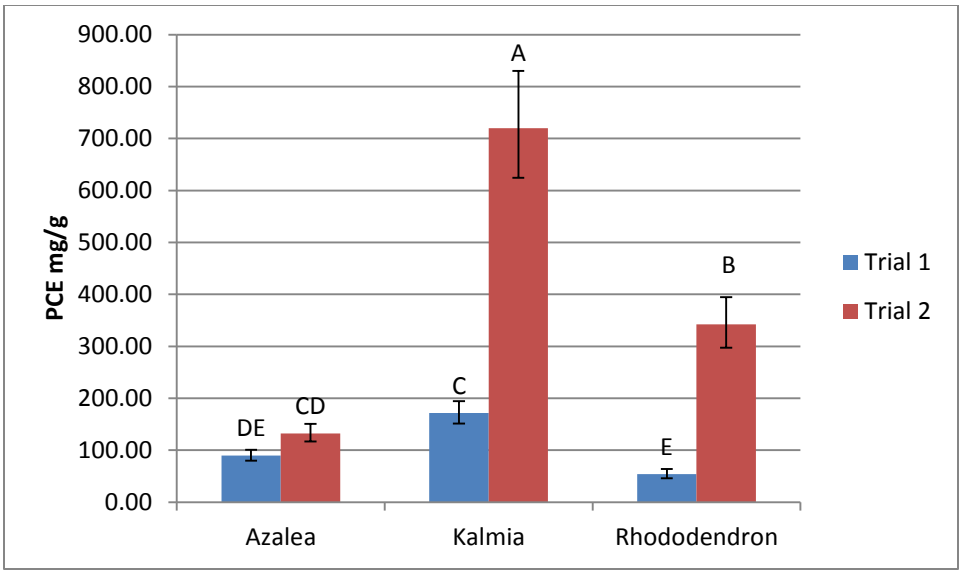

Figure 3.3. Condensed tannin two way ANOVA trial by cultivar from acid butanol assay in Procyanadin C1 equivalents (PCE). Each observation is the mean and standard error. Bars with the same letter are not significantly different at the 95% confidence level.

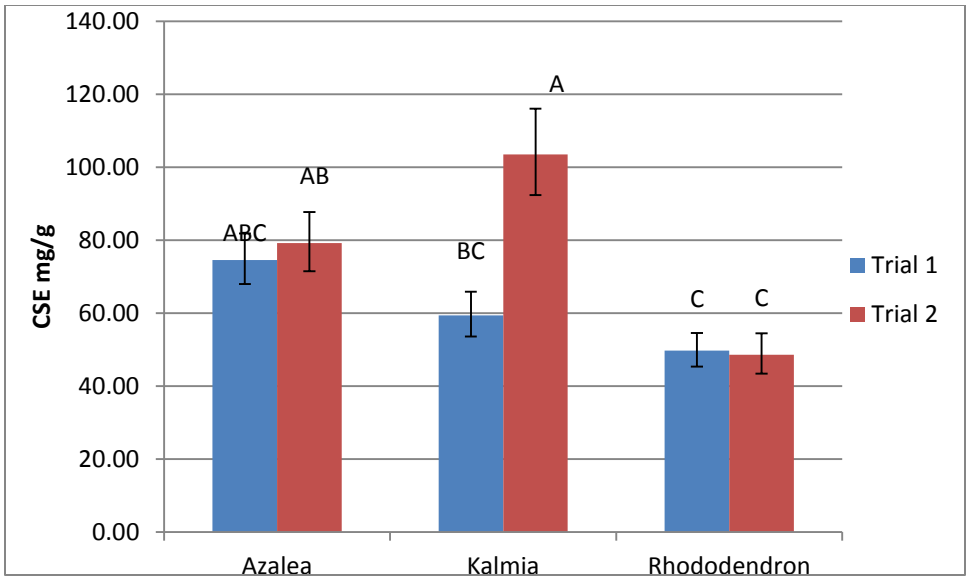

Figure 3.4. Sterol two way ANOVA trial by cultivar in Cholesterol equivalents (CSE) Each observation is the mean and standard error. Bars with the same letter are not significantly different at the 95% confidence level.

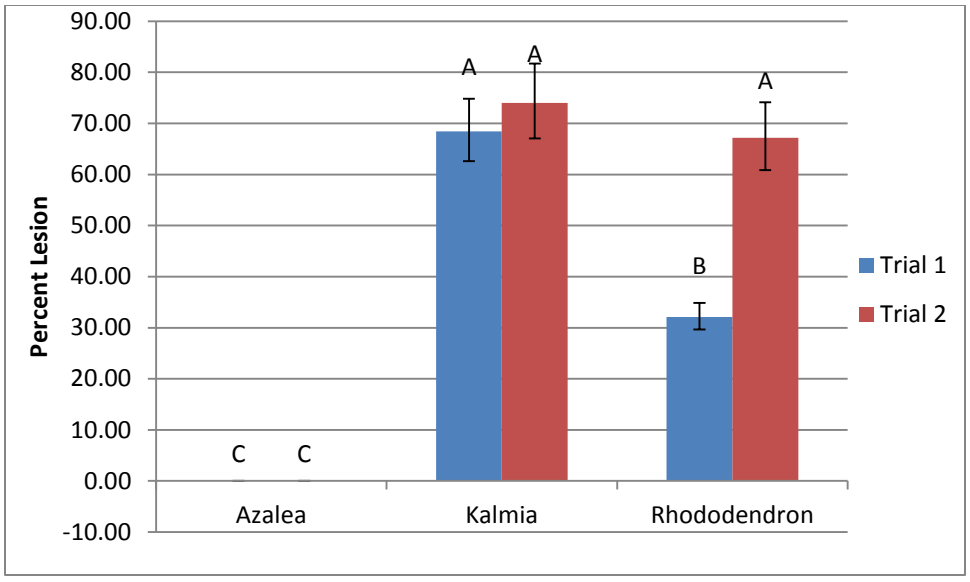

Figure 3.5. Susceptibility two way ANOVA trial by cultivar calculated by Percent Lesion. Each observation is the mean and standard error. Bars with the same letter are not significantly different at the 95% confidence level.

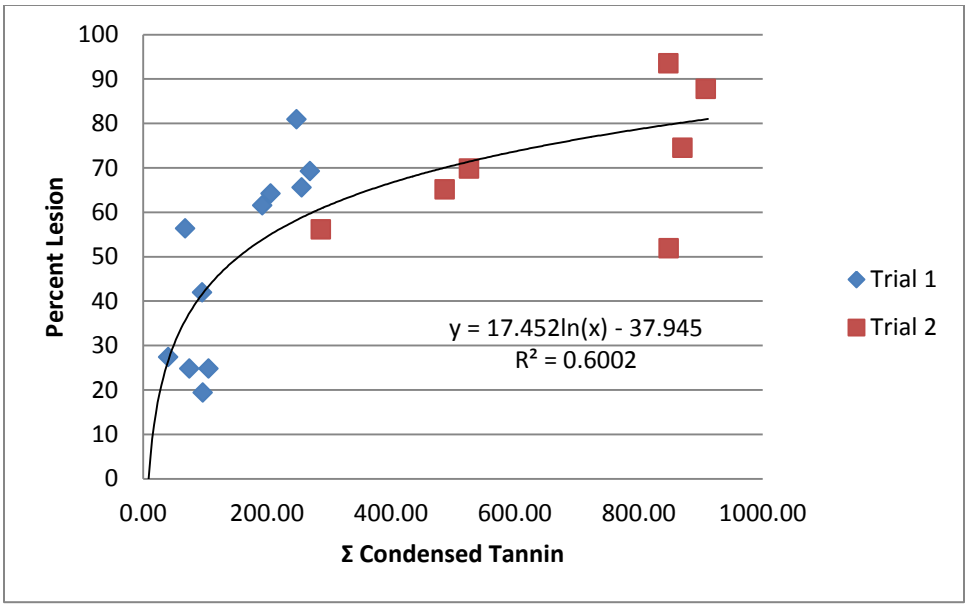

Figure 3.6. Correlation between Percent Lesion size and sum of condensed tannin measured by acid butanol assay and vanillin assay using ls-means of each cultivar

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CHAPTER 4

DISSCUSION

This research was conducted to gain an understanding of the role plant tannins may play in plant susceptibility to *Phytophthora ramorum*. Three aspects of this interaction were explored including *P. ramorum* elicitin, leaf sterols and leaf tannin content.

Chapter 2 describes the effect of sterols, tannins and ground foliage on *P. ramorum* growth, sporulation, 'functional' elicitin production and elicitin gene expression. Ground leaf tissue, sterols, and tannin-enriched extracts were obtained from three different plant species (California bay laurel, California black oak, and Oregon white oak) in order to evaluate the effect of differing sterol/tannin contents on *P. ramorum* growth *in vitro*. For all three species, high levels of foliage inhibited *P. ramorum* growth and sporulation, with a greater inhibition for the two oak samples. *P. ramorum* growth and sporulation was also inhibited by either phytosterols or tannin-enriched extracts, but by differing mechanisms. For example, high levels of sterols diminished elicitin gene expression; whereas the tannin-enriched extract decreased in the amount of 'functional' or ELISA-detectable elicitin, but not gene expression. Significant positive correlations were observed between the amount of functional elicitin and *P. ramorum* growth and sporulation, suggesting a possible role for elicitin-sterol- tannin complexes in *P. ramorum* growth and sporulation in foliage.

Chapter 3 describes the relationship seen between leaf susceptibility and foliar tannin or sterol contents. Several cultivars of evergreens, Rhododendron, Kalmia and Azalea, were assessed for constitutive tannin contents, sterol contents, and leaf susceptibility to *P. ramorum*. Significant differences were seen between the trials and species for tannin contents. Tannin content and susceptibility was higher for trial 2 than trial 1; however, across all treatment combinations a significant positive correlation was observed between tannin content and *P. ramorum* susceptibility in compatible hosts. We hypothesize

elicitin-sterol-tannin complexes may have a role in the intensity of a hypersensitive-like response in susceptible plant hosts.

This research has shown that tannins can reduce the amount of 'functional' elicitin produced by *P. ramorum* and in vitro this can reduce growth and sporulation; however, *in planta* these declines appear to be offset by a reduction in host defense responses leading to increased *P. ramorum* colonization in plant tissues with high tannin contents.

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