NOVEL PHYTOANTICIPINS FROM *PRUNUS MAACKII*: POSSIBLE FACTORS IN DISEASE RESISTANCE

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Pathology – Doctor of Philosophy

2015

ABSTRACT

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Armillaria ostoyae, the cause of Armillaria root rot, is a major problem in cherry orchards in the northwest region of Michigan. One mechanism of resistance to pathogen invasion is the production of antifungal compounds. *Prunus maackii* was the only *Prunus* species tested that contained significant antifungal activity based on Thin Layer Chromatography bioassays. The purpose of this research was aimed at understanding the nature of resistance/defense found in *P. maackii* to Armillaria root rot. A series of additional bioassays were performed to examine the range of antimicrobial activity and the potency of the secondary metabolites contained in the periderm of *P. maackii.* Periderm-incorporated media was used to screen for growth inhibition of *Armillaria* species along with a group of non-host pathogens. *Prunus maackii* periderm amended-media was fungistatic at very low concentrations and also displayed fungitoxic properties. X-ray crystallography was used to determine the structures of two antifungal components of *P. maackii* periderm as 3,5,7-trihydroxy-6 methoxyflavone and 3,5,7-trihydroxy-6-methoxyflavanone. This is the first report of crystallization and antimicrobial activity for both compounds.

To my parents Joseph P. Kaniszewski and Kathi J. Kaniszewski;
There are no words that I could ever write that would hold a light to your brilliance, wisdom and values.

ACKNOWLEDGEMENTS

I could effortlessly fill countless pages expressing my appreciation to all of the wonderful people of the Plant, Soil and Microbial Sciences Department at Michigan State University. I am grateful beyond words to my mentor and dear friend Dr. Raymond Hammerschmidt for each and every opportunity and for simply believing in me. Thank you for investing your time and efforts into shaping my skillset and for allowing me to learn from your expertise, which is truly an understatement when referring to the physiology and biochemistry of resistance and disease. I will continue to think of you each time I draw out an aromatic compound, forever motivating me to do all that I can with your investment.

Thank you to my committee members for their time and direction. To Dr. Amy Iezzoni, I am thankful for the opportunity to learn from your plant breeding research and for the time that you've taken throughout the years to help me gain experience in your field. A special thank you to Dr. Frank Telewski for all of your insightful discussions, for the extra time that you've taken to help me achieve my goals and for your encouragement to pursue my dreams. Thank you to Dr. Richard Staples for your diligent efforts towards the identification of unknown samples, for teaching me new crystallization techniques and for the extra time that you have spent helping me to better understand critical concepts in x-ray crystallography.

I would like to express my most sincere gratitude and appreciation to my parents and sisters. To my mom, Kathi Jo (Russell), for your remarkable editing skills, for your constant push to achieve more and for your help overcoming obstacles of any kind and to my dad, Joseph Peter Kaniszewski, for always brainstorming with me - sharing your

creative and unique ideas, for imparting your self-taught organic gardening knowledge, and for your help collecting new samples for future *P. maackii* research. You have both made me who I am today – thank you for everything.

I would like to sincerely thank my sister Brynne for providing a place to live when first starting at MSU – I will remain forever grateful for your constant support, love and patience. To my sister Brooke, for always lifting my spirits and for the numerous lunches and care packages, making each day brighter and so much lovelier. Thank you to my brother-in-law Joel Buckley, my nephews and niece, Braden, Collin and Quinn for the immense joy that you have all brought to my life. A very special thanks to my grandparents, Phyllis and Howard Russell for infusing agriculture into my background with your farm in Fairgrove, MI and to Francis and Walter Kaniszewski for inspiring my curiosity in biology with every camping trip made to Grand Marais, MI. Thank you to my Aunt Lydia Maurer, for all of your uplifting words that helped me push through the subjects that I struggled with most and for helping me find my purpose in life as a teacher. To Leonard Russell, for taking such great care of the Russell farm and for his discussions on plant disease.

I am extremely grateful to Dr. Chris Vandervoort for your generous donation of time towards characterizing unknown samples using HPLC-MS and to Dr. Daniel Jones for additonal HPLC-MS analyses, Dr. Daniel Holmes and Kermit Johnson for NMR analyses, Audrey Sebolt for teaching me new skills and to Dr. Tyre Proffer for your help fostering new experimental ideas. To Shelley Redman, Linda Colon, Lee Duynslager, and Jeff Nobis who have all helped make East Lansing feel like a second home. I am appreciative to all of the lab members that have made this research project possible. I

owe tremendous thanks to current lab members Chris Behrens, Celeste Dmytryszyn and Michael Staples for their friendship and support.

To former lab members for their contributions on this project including Cory Outwater, Emmie Bishop, Janette Jacobs, Jordan Crandall, Nayara Marangoni, and Ryan Dickinson. Thank you to my fellow graduate students that have helped me throughout my academic journey – Helen Jiang, Nancy Dykema, Jane Jiang, Carolina Escobar, Saltanat Kurmanbekovna, Sandesh Dangi, and many others who will remain lifelong friends.

Finally, thank you to my husband Ryan Taylor – You truly know how to make each day count, which is evident by your sincere, thoughtful and honest character. Thank you for the weekends spent on campus, for helping me review test information and for always thinking of ways to make my life easier.

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ARMILLARIA ROOT ROT

Causal Agent.

Armillaria root rot is one of the most destructive diseases of trees, including many fruit tree species, in the temperate regions of the world (Worrall, 2004). This may be attributed to the broad host range of the pathogen, the need for resistant hosts, the lack of chemical control, and the longevity of the mycelium in the soil. Management of this disease in Michigan is critical because of the economic importance of fruit production.

Michigan was the leading state in cherry production at 11.6 million pounds in 2012 and 217.9 million pounds in 2013 (NASS, 2014). Pathogenic species in the Genus *Armillaria* have an extensive host range, including sour cherry, sweet cherry, peach, apple, and oak trees in Michigan. Since *Prunus* cerasus, tart cherry, is one the most common hosts for *Armillaria* and cherry production is in the northern part of Michigan where *Armillaria* is prevalent, there is a need for research on resistant root stock development. *Armillaria ostoyae* attacks both sour and sweet cherry and has been found to be widely distributed in Michigan, including Leelanau, Grand Traverse, Benzie, Manistee, Mason, and Oceana. *Armillaria mellea* has been reported on sour cherry and oak trees in Manistee and Oceana counties (Proffer et al, 1987). In addition to cherry and oak trees, peach and almond trees are also susceptible to root rot caused by *A. mellea* (Guillaumin et al., 1991). Peach trees have been found to be more susceptible to Armillaria root rot than apple trees because they succumb faster and more uniformly to infection caused by *Armillaria* compared to apple trees (Kable, 1974).

Hyphal walls of fungi are complex in chemical composition. For example, the walls of *A. mellea* consist of polysaccharides, proteins and lipids (Sánchez et al., 1990). Identification based on morphology is limited, due to the inconsistencies in the production of basidiocarps, which are transient and not produced on a regular basis (Baumgartner et al., 2011). Due to the limited morphological features, a molecular diagnostic approach has been employed for the identification of fungal pathogens, particularly those species that spread through the soil (Guillaumin, 1996). Some *Armillaria* species are closely related, including *A. ostoyae* and *A. gemina*. Polymorphism in the IGS region of *A. gemina* may provide evidence for common ancestry (Kim et al., 2006). Other species may coexist; *A. cepistipes* often coexists in the same geographic area as *A. ostoyae*, including Europe and North America (Prospero et al, 2004).

Signs and Symptoms.

The earliest symptoms of root rot infection by *A. mellea* include yellowing and browning of the foliage, reduction in growth and defoliation (Singh, 1980). Once the infection of the host has been established, this pathogen causes rot within the wood tissue, which leads to slow degeneration and eventual death of the host. Most *Armillaria* species are facultative necrotrophs. The parasitic phase involves the colonization and death of cambial tissue in roots resulting in necrosis beneath root bark. The fungus survives on dead tissue in the saprophytic phase making management difficult (Baumgartner et al., 2011).

The slow growth of *A. ostoyae* and the lack of symptoms is also problematic for management of the disease since the host species appear healthy for several years, post infection, and prior to a rapid decline. Although the hosts do not exhibit symptoms until the later stages of infection, the infected wood exhibits bioluminescence in the dark (Webster & Weber, 2010). Bioluminescence has been reported from mycelia of *Armillaria gallica, A. mellea, A. ostoyae, A. tabescens, A. calvescens, A. cepistipes, A. gemina, A. nabsnona*, and *A. sinapina* (Mihail, 2015).

Periderm formation, resinosis, compartmentalization, and callus formation occur in conifer roots in response to infection by *Armillaria* species (Robinson & Morrison, 2001). Conifer roots form traumatic resin canals (TRCs) in response to *A. ostoyae* colonizing the cambial tissues and can be used to determine the timing of infection. However, this is a non-specific response that can also be a result of a wide range of biotic and abiotic stimuli (Cruickshank et al., 2006).

In the field, rhizomorphs of the parasitic *Armillaria* species, *A. mellea, A. ostoyae* and *A. tabescens*, appear as mycelial fan-like ribbons. The more saprotrophic species, *A. calvescens, A. gallica* and *A. sinapine* have rhizomorphs that were more cylindrical, melanized cords (Mihail & Bruhn, 2005). It has been reported that *A. mellea* rhizomorph production is limited in the soil (Guillaumin et al., 1993). Rhizomorph development may be stimulated by micro-fungi that are commonly found in the forest soil and oak rhizosphere including *Penicillium lanosum, Penicillium notatum, Cylindrocarpon destructans, Penicillium spinulosum*, and *Nectria grammicospora* (Kwaśna et al., 2004). Growth of rhizomorphs in soil has been found to be optimal at 20°C. At temperatures

above 26°C, the rhizomorph apices are susceptible to drying out. Rhizomorph growth is also inhibited in heavy-textured and very moist soil (Rishbeth, 1978).

Hyphae from both *A. mellea* and *A. ostoyae* were able to directly penetrate the root bark of *Picea sitchensis* without prior wounding. Neither species produced rhizomorphs when root bark was not wounded. However, both species produced rhizomorphs when exposed to wounded tissues (Solla et al., 2002). Both *A. gallica* and *A. cepistipes* developed more rhizomorphs than *A. ostoyae*. Mushroom production however was greater in *A. ostoyae* compared to *A. gallica* and *A. cepistipes* (Legrand et al., 1996).

Dissemination & Spread of Pathogen.

Armillaria species produce basidia on the lining of the gills, which give rise to haploid spores with single nuclei (Ullrich & Anderson, 1988). Although it is thought that sexual basidiospores spores result in new infection, it has not been clearly demonstrated in a natural environment. However, it is clear that *Armillaria* species produce an abundance of mushrooms, which give rise to a significant amount of basidiospores costing the organism an immense amount of energy. The benefit of this energy cost of spore production needs to be further investigated (Worrall, 2004).

Recent research suggests that spore dispersal may play an important role in the colonization of new habitats. Spore dispersal in *A. mellea* was found to be spatially restricted. Homogeneity between separate sub-populations may indicate that long-distance spore dispersal occurs rarely (Travadon et al., 2012). The need for further research of the dissemination of distinct clones that are contained within a single

biological species would clarify the purpose of the energy costly process of mushroom and basidiospore production (Proffer et al., 1987).

In Basidiomycetes, basidiospores are involved in the primary infection process and are responsible for infection of new areas. The spread of vegetative fungal mycelia aids in secondary infection, allowing for the spread of those produced from the primary infection process (Garbelotto, 2004). Sexual reproduction in *Armillaria* species is regulated by two mating-type loci, containing multiple alleles. In order to be sexually compatible, single basidiospore isolates must have different alleles at both loci. Completion of the sexual cycle involves the formation of mushrooms that contain basidia lining the gills. Meiosis leads to the production of four haploid nuclei, each of which enters a basidiospore, completing the cycle. *Armillaria* species do not form asexual spores. Alternatively, they spread through the soil by growing as mycelia or rhizomorphs (Worrall, 2004). Pseudosclerotial plates aid in survival of the mycelium (Webster & Weber, 2010).

Armillaria species may spread through the soil either by rhizomorphs or by direct transfer of mycelium. Successful colonization of root tissues may require the oxidation of phenolics. The browning of tissues is a result of the fungus oxidizing host phenols. However, the spread of pathogen growth may be limited to tissues under stress (Wargo & Shaw, 1985). Rhizomorphs are formed from individual hyphae that together produce a cylindrical structure (Yafetto et al., 2009). Like roots, rhizomorphs have a cap that reduces friction between the fungal structure and the surrounding soil. Rhizomorph production and colonization capabilities may be influenced by the presence of other fungi. A mutually negative effect on colonization was detected when *A. cepistipes* and

A. ostoyae were simultaneously co-inoculated, which may be a result of interspecific competition. However, co-inoculation induced rhizomorph production in *A. cepistipes*, but had no effect on *A. ostoyae* (Prospero et al., 2006).

A single isolate of *Armillaria* species can colonize acres of land. In northern Michigan, a 33-acre genet of *A. bulbosa* was determined to be the largest living organism on earth. It is the secondary infection that is responsible for spreading among root systems of an entire forest for thousands of years (Garbelotto, 2004). A clone refers to all asexual derivatives from a common point of origin. In reference to mating in fungi, the term genet refers to one type of clone whose origin is associated with genetic exchange. Fungal genets include all derivatives of mitotic cell lineage originating in either fungi with dikaryotic or diploid mycelia or fungi with haploid mycelia. In fungi possessing haploid mycelia, genets include all vegetative derivatives of the union of two different gametic nuclei. In fungi with dikaryotic or diploid mycelia, the mating of two different gametic nuclei forms a genet (Anderson & Kohn, 1995).

Virulence & Pathogenicity.

Armillaria species vary in hosts and pathogenicity; *A. ostoyae* is primarily a pathogen of conifers, *A. mellea* primarily a pathogen of hardwoods and *A. gallica* occurs most commonly as a saprophyte (Kim et al., 2006). *Armillaria cepistipes* behaves similarly to *A. gallica*, which is considered a weak pathogen (Prospero et al, 2004). *Armillaria obscura* was found to be very pathogenic and *A. bulbosa* was only slightly pathogenic (Rishbeth, 1985). One-year-old almond trees were inoculated with *A. mellea, A. gallica* and *A. tabescens*. *Armillaria mellea* was found to be the most virulent

on this particular host and *A. tabescens* was the least virulent (Tsopelas & Tjamos, 1997).

The conditions of Douglas-fir and other conifer hosts were monitored after initial infection of *A. ostoyae*. *Armillaria ostoyae* caused death in Douglas-fir hosts seven years after infection. This root rot pathogen caused mortality in nine years for other coniferous species (Morrison, 2011). In Japan, *A. mellea, A. ostoyae, A. cepistipes*, and *A. tabescens* were classified as moderate to aggressive pathogens of conifers. *Armillaria tabescens* was rarely found compared to *A. mellea, A. ostoyae* and *A. cepistipes* (Hasegawa et al., 2011). *Armillaria cepistipes, A. gallica, A. mellea*, and *A. ostoyae* are also commonly found in forests of central and southern Europe. Both *A. mellea* and *A. ostoyae* were classified as highly pathogenic on conifers. *Armillaria cepistipes*, which mainly causes heart rot in conifers, has low virulence (Keča et al., 2009).

Melanization contributes to increased virulence, resistance to microbial attack and aids in survival of pathogens (Fogarty & Tobin, 1996). Melanin is produced from the oxidative polymerization of phenolic compounds, which usually result in dark brown or black pigments. Melanin pigmentation reduces the susceptibility of fungi to host defense mechanisms. Melanin also guards fungi from hydrolytic enzymes, UV radiation, heavy metals, abiotic stress, and toxic secondary metabolites (Taborda et al., 2007). Another factor influencing the pathogenicity of *Armillaria* species is their ability to produce rhizomorphs, which is one method of spreading the disease to another host.

There is a positive correlation between rhizomorph production and laccase activity (Worrall et al., 1986). Laccase activity is also involved in melanin production

(Fowler et al., 2011). Laccase activity has been reported in *Armillaria* species, *A. ostoyae, A. mellea, A. gallica*, and *A. cepistipes*. Other white-rot fungi are also known to have this phenol-oxidizing enzyme. Laccase also plays a role in lignin biodegradation and oxidation of antifungal phenols (Robene-Soustrade & Lung-Escarmant, 1997).

Branching patterns of the rhizomorphs may reflect the virulence of the species. Rhizomorphs of species from the southern hemisphere, *A. luteobubalina, A. fumosa, A. hinnulea, A. novae-zelandiae*, and *A. limonea* all branch dichotomously. Rhizomorphs from species of the northern hemisphere branch both monopodially and dichotomously. *Armillaria mellea, A. borealis* and *A. ostoyae* branched dichotomously. Monopodial branching was detected in *A. gallica, A. cepistipes, A. gemina, A. calvescens, A. sinapine*, and *A. nabsnona*. The northern dichotomously branching species were more virulent than species that branched monopodially (Morrison, 2004).

Management.

The presence of enzymes, including chitinase and β-1,3-glucanase contribute to the protective barrier found in healthy trees and may aid in resistance to pathogen invasion by lysis of hyphal walls of *A. mellea* (Wargo, 1975). The responses triggered by wounding may aid in the identification of anatomical traits indicating resistance or susceptibility to *A. ostoyae* (Cleary et al, 2012). Trichoderma and some nematodes have been found to be potential sources of biological control, but have shown limited success in a natural environment (Webster & Weber, 2010). *Trichoderma* species are known antagonists of some pathogenic fungi. Bark treated with *Trichoderma atroviride* can suppress Armillaria root rot. Strawberries with *T. atroviride* pre-treated mulch

showed significantly lower infection of Armillaria root rot than those with untreated mulch (Pellegrini et al., 2014).

Armillaria mellea is a pathogen of apple, walnut and kiwifruit. Mycelial growth of *A. mellea* was completely inhibited by the fungicide cyproconazole (Thomidis & Exadaktylou, 2012). The few additional chemicals known to inhibit *Armillaria* growth also include methyl bromide (phased out in 2005) and carbon disulphide, both chemicals were able to kill mycelia present in decayed trees to a soil depth of approximately 1 m (Baumgartner et al., 2011). Chloropicrin has also been used to eradicate inoculum from soil in orchards (Worrall, 200). Pinosylvin monothyl ether isolated from the fresh sapwood of *Pinus strobus* inhibited the growth of *A. ostoyae*. However, pines are still attacked in the field setting despite the presence of these inhibitors (Mwangi et al., 1990). Pinosylvin monothyl ether may potentially be used in management by utilizing these biological markers in breeding for resistance. Management must also be directed toward efficient detection and removal of diseased trees. Susceptible tree species should be avoided including Douglas-fir, Engelmann spruce and blue spruce (Worrall et al., 2004). Determining whether a pathogen spreads by either primary infection or secondary infection is critical in understanding the epidemiology of the pathogen and utilizing proper management techniques (Garbelotto, 2004).

Resistance.

Host susceptibility has been linked to low phenolic/sugar ratio among additional elements. Other factors include nutrition availability for the production of defense compounds and also the variation in chemical composition of the bark throughout the year. It has been reported that high glucose concentrations in hosts are correlated with susceptibility to Armillaria root rot. The selection of resistant species should be made for low sugar concentrations (Myszewski et al, 2002).

Western Larch tree roots form necrophylactic periderm with bands of phellem around lesions caused by *A. ostoyae*. This wound-response periderm may be responsible for the increased resistance detected in the western larch (Robinson & Morrison, 2001). Host enzymes, chitinase and glucanase may also be involved in host resistance. These enzymes are responsible for the lysis of fungal hyphal walls. Chitinase and β-1,3-glucanase were isolated from sugar maple (*Acer saccharum*), red oak (*Quercus rubra*), black oak (*Quercus velutina*), and white oak (*Quercus alba*) and were found to lyse hyphal walls of *A. mellea* (Wargo, 1975).

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LITERATURE REVIEW

ROLE OF SECONDARY METABOLITES IN PLANT DEFENSE

Primary Metabolism.

Plant primary metabolism refers to anabolic and catabolic processes that are required for cell viability. These processes include carbohydrate metabolism, the biosynthesis of amino acids and proteins, nucleotides, fatty acids, and lipids. Defense against pathogens requires increased demands for energy, carbon skeletons and reducing equivalents (Bolton, 2009). Primary metabolism provides the metabolic precursors that are used in secondary metabolism. Resource availability may play a role in the regulation of growth and defense-related metabolism. The shikimate pathway directly or indirectly produces all phenols with most classes using the aromatic amino acid phenylalanine as an intermediate (OßWALD ET AL., 2012).

Secondary Metabolism.

Plant secondary metabolism includes a diverse range of compounds, enzymes and mechanisms of gene regulation of metabolites. Secondary metabolism involves compounds that are not essential for primary metabolism, but are needed for host survival in the environment (Lattanzio et al., 2008). All cells in an organism possess genes for the synthesis of secondary metabolites, but only a limited number express them (Seigler, 1998).

These metabolites may be released only when the cells are broken. Compartmentalization protects uninfected cells from the toxic compounds used in defense against pathogens. For example, when peppers were challenged against *Colletotrichum coccodes*, the highest levels of total phenolic content were found in the bordering zone of healthy and infected tissue. This zone of phenolic content indicates synthesis in tissues surrounding the site of infection (Mikulic-Petkovsek et al., 2013). A broad variety of pathogens produce tyrosinase, which is a phenol oxidase that is responsible for the biosynthesis of melanin as a contributing factor of virulence (Taborda et al., 2008). This multifunctional enzyme is inhibited by taxifolin (Miyazawa & Tamura, 2007). The virulence of pathogens partly depends on the pathogens ability to overcome host defenses (Casadevall & Pirofski, 2001).

Phenolic compounds are defined as those with one or more hydroxyl groups(s) on an aromatic ring. Phenolic compounds and polyphenols (a polymeric phenol) include all products arising from the shikimate-phenylpropanoid pathway (Lattanzio & Cardinali, 2006). Plant phenolics may function as antioxidants by neutralizing free radicals. Some polyphenols are more easily oxidized than others because of the different types and locations of constituents. Flavonoids may possess properties for scavenging reactive oxygen species. The presence of an *ortho*-hydroxylation on the B-ring of the flavonoid molecule, free hydroxyl groups, a C2-C3 double bond in the C-ring, or the presence of a 3-hydroxyl group are indicative of antioxidant and antiradical capabilities (Burda & Oleszek, 2001).

The catechol-type B ring in the skeletal structure of a flavonoid is the antioxidant active moiety. Since flavonoids such as quercetin, rutin and (+)-catechin are known antioxidants and radical scavengers, they have also been linked to their inhibition of lipid oxidation. They possess antioxidant properties and also chelating properties of metal ions such as iron or copper, which are known to catalyze many biological processes, having the ability to inactivate enzymes and proteins (Le Nest et al., 2004).

Flavonoids.

Flavonoids are based on the skeleton structure containing two phenyl groups connected by a three-carbon bridge, $C_6 – C_3 – C_6$, and can be further classified based on substituents (Bano et al., 2013). This carbon bridge often cyclizes to give rise a third ring termed the C-ring (Cuyckens & Claeys, 2004). There is structural variation in the basic flavonoid skeleton due to the variation in hydroxylation, methoxylation, methylation, prenylation, benzylation, and glycosylation. Flavonoids include various groups such as chalcones, flavan-3-ols, isoflavonoids, flavanones, flavones, flavonols, catechins, and anthocyaninidins (Croteau & Lewis, 2000). Classes of flavonoids have been identified as both intermediates and as end products, including chalcones, flavanones, and flavan-3-ols. Other classes of flavonoids have only been identified as end products in the shikimate pathway, which include anthocyanidins (Cushnie & Lamb, 2005).

Phenylalanine ammonia-lyase (PAL) is the first enzyme involved in the biosynthesis of phenylpropanoid compounds. PAL activity can be regulated by feedback control and accumulation of precursors (Ju et al., 1995). Feedback inhibition plays a role in controlling the biosynthesis and metabolism of phenolic compounds. Intermediate products and end products of this pathway may repress or induce enzyme synthesis (Kosuge, 1969). Cinnamic acid inhibits the transcription of *CHS*, but p-coumaric acid stimulates the production of CHS. *PAL* activity can be negatively regulated by exogenous application of *trans*-cinnamic acid or by blocking downstream activity (Yin et al., 2012). *Trans*-cinnamic acid regulates *CHS* at the transcriptional level. Therefore, cinnamic acid may act as a regulator of phenylpropanoid biosynthetic genes (Mavandad et al., 1990). Chalcone synthase is part of the large superfamily of polyketide synthases. In the phenylpropanoid pathway of higher plants, chalcone synthase (CHS) is the first committed step of flavonoid biosynthesis. The CHS reaction products serve as the basic building blocks for secondary metabolism (Dare et al., 2013). Chalcones, $C_{15}H_{12}O$ have a linear C_3-chain connecting the two phenyl rings, containing a double bond. In comparison, the C_3-chain is saturated in dihydrochalcones. Pre-flavonoid precursors are derived from carbohydrate metabolism and are condensed by chalcone synthase (CHS) (Halbwirth, 2010). Chalconaringenin (2',4',6', 4-tetrahydroxychalcone) has been determined to be one of the main phenolic components in cherry tomatoes (Slimestad & Verheul, 2005). However, there is a significant decrease in chalconaringenin concentrations during postharvest ripening (Slimestad & Verheul, 2005).

The cyclization of chalcones involves the reaction of the meta-hydroxyl group with the α-carbon to produce aurones. Aurones, $C_{15}H_{10}O_2$ are responsible for yellow pigmentation in flowers (Vermerris & Nicholson, 2006) and are isomeric with flavones (Seigler, 1998). Aurones contain a 5 – membered C-ring, instead of a 6 – membered C-ring more commonly found in other flavonoids.

The final flavanone structure is formed when chalcone isomerase (CHI) produces (2*S*)-flavanones (Fowler & Koffas, 2009). Upon further enzymatic action, flavanones serve as the intermediate precursors to flavones, isoflavones, flavan 4-ols, and dihydroflavonols (Halbwirth, 2010). Isoflavones are synthesized from flavanones by the co-action of 2-hydroxyisoflavanone synthase (IFS) and a dehydratase (IDH). Alternatively, position 3 of flavanones can be hydroxylated to produce dihydroflavonols, which is catalyzed by 2-oxoglutarate-dependent dioxygenase (FHT) (Halbwirth, 2010). Flavanones contain various substitutions, e.g. hydroxy, methoxy, on the A- or B- ring. Flavanones can also be isolated from both above and belowground parts of the plant (Khan & Dangles, 2014).

Flavones have the backbone of 2-phenylchromen-4-one with a basic molecular formula of $C_{15}H_{10}O_{2,}$ which is also the formula for aurone and isoflavone structures. A C_2-C_3 double bond distinguishes flavones from flavanones ($C_{15}H_{12}O_2$); the flavones have a double bond at C_2-C_3. This conversion requires two types of enzymes, flavone synthase I (FNSI) and flavone synthase II (FNSII) (Halbwirth, 2010). This class of flavonoids is typically colorless-to-yellow and soluble in both water and ethanol (Singh et al., 2014). Flavones are a biologically active class of flavonoids exhibiting antioxidant,

antimicrobial and cytotoxic activities. Flavones and flavonols are structurally very similar and therefore flavonols may be referred to as 3-hydroxyflavones (Das et al., 2014).

Dihydroflavonols, $C_{15}H_{12}O_3$ are biosynthetic intermediates of flavones (Balza et al., 1988). *Trans*-dihydroflavonols can be synthesized from flavanones by flavanone 3-β-hydroxylase (F3-β-OH). Dihydroflavonols may be converted to flavonols by flavonol synthase (FLS) (Wilmouth et al., 2002). Flavonol synthase (FLS) catalyzes the introduction of a double bond between the C_2-C_3 positions of dihydroflavonols producing flavonols. Dihydroflavonols are also direct intermediates of leucoanthocyanidins. DFR reduces dihydroflavonols to leucoanthocyanidins, which serve as direct precursors of catechins and anthocyanidins (Halbwirth, 2010). The most common dihydroflavonols include dihydroquercetin, dihydrokaempferol and dihydromyricetin (Marais et al., 2005). Dihydroflavonols, such as taxifolin, have antioxidative and antifungal properties (Gong et al., 2009).

Anthocyanins are a group of biologically active compounds that are responsible for the pigmentation of many plant parts including fruits, vegetables and flowers. The production of anthocyanins requires the action of flavonoid-3-*O*- glucosyltransferase (UFGT) (Rahim et al., 2014). Other enzymes are required to synthesize anthocyanidins, which include chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), and finally anthocyanidin synthase (ANS) (Zhang et al., 2014).

The site of the biosynthesis of flavonoids remains elusive. It is believed that they are formed in the cytosol requiring an unknown flavonoid transport mechanism from the formation site to the site of accumulation (Halbwirth, 2010). Flavonoids are most

commonly present in glycosidic form and stored in vacuoles of stems, roots, flowers, and leaves (Cuyckens & Claeys, 2004).

Phenolic Compounds in the Genus *Prunus*.

Flavonols, flavanols, anthocyanins, isoflavones, and dihydrochalcones are commonly identified in Rosaceae (Ogah et al., 2014). In addition to flavonoids, coumarins have also been identified from *Prunus* species. Scopolin has been identified in the bark of *Prunus verecunda*, *P. cyclamina* and *P. maackii* and herniarin was reported in the bark of *P. mahaleb*. Herniarin has also been reported in the leaves of *P. mahaleb*, *P. pensylvanica* and *P. maximoqiczii* (Santamour & Riedel, 1994).

Flavonols that are found in sweet cherry (*Prunus avium* L.) and tart cherry (*Prunus cerasus* L.) are in higher concentrations compared to other rosaceous fruits. Glycosylated quercetins and kaempferols are two flavonols commonly identified in sweet and sour cherries (Ogah et al., 2014). Additionally, the flavonols myricetin 3-rutinoside, quercetin 3-rutinoside and kaempferol 3-rutinoside have been found in the ‘sweet heart’ and ‘sweet late’ sweet cherry cultivars of *P. avium*. All of which were significantly increased by oxalic acid (OA) treatment (Martínez-Esplá et al., 2014). Cherry fruits are also known to contain high amounts of anthocyanins, which contribute to both fruit color and antioxidant capacity. Six Michigan tart cherry (*Prunus cerasus* L.) selections were evaluated for their anthocyanin content; selection 25-14 (20) contained the highest amount followed by selection Tamaris and 27-10 (50), respectively. Montmorency cherries contained the lowest amount of anthocyanin content followed by Balaton (Siddiq et al., 2011).

Chlorogenic acid, neochlorogenic acid, (+)-catechin, and (-)-epicatechin are the major phenolic compounds found in apricot fruit, *Prunus armeniaca* (Schmitzer et al., 2011). The leaves of *Prunus persica* contain caffeic acid, chlorogenic acid, kaempferol, and quercetin (Kazan et al., 2014). Procyanidins, cyanidin 3-rutinoside and C-alkyl isoflavones have been recently identified in *Prunus domestica* (Khallouki et al., 2012). The fruit of *Prunus avium* have been found to be a rich source of neochlorogenic acid, *p*-coumaroylquinic acid and chlorogenic acid (Ballistreri et al., 2013). Kaempferol, naringenin and catechin have been isolated from almond *Prunus amygdalus* (Esfahlan et al., 2010). *Prunus mume* seed extracts contained 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid and 4-*O*-caffeoylquinic acid. The crude extract had antifungal properties (Xia et al., 2011). The wood of *P. campanulata* contains a system of flavanones including naringenin, aromadendrin, isosakuranetin, sakuranetin, pinocembrin, eriodictyol, taxifolin, prunin, among others (Hasegawa, 1958).

Plant Defense.

Plants have evolved defense mechanisms including physical barriers and chemical protection. Antifungal metabolites can either be preformed or induced after infection (Grager & Harborne, 1994). Phytoanticipins are antimicrobial compounds that are present prior to pathogen attack. These low molecular weight compounds may also be produced from pre-existing constituents (VanEtten et al., 1994). The presence of preformed antifungal compounds has been correlated with the resistance detected in young rockmelon fruit (*Cucumis melo* L) cv. ‘Colorado’. Levels of these compounds decrease as the fruit matures (Kumar & McConchie, 2010). The durability of *Larix*

leptolepis may be attributed to the toxic material found in the heartwood tissue including taxifolin, aromadendrin and quercetin (Sasaya et al., 1970).

Examples of phytoanticipins include avenacins, which are saponins found in roots of *Avena sativa* (Oat) and are responsible for resistance to *Gaeumannomyces graminis* var. *tritici*. However, oat plants are susceptible to *G. graminis* var. *avenae* due to the secretion of avenacinase, which detoxifies avenacins (Osbourn et al., 1994). Avenacin saponins also inhibit pathogen growth of *Blumeria graminis f. sp. hordei*, *Bipolaris oryzae* and *Magnaporthe oryzae* (Inagaki et al., 2013). The following phenolic compounds have been identified as phytoanticipins from various host species including gallic acid and catechin in *Acer* spp., phloroglucinol, salicin and saligenin in *Salix alba* var. *caerulea* and ellagitannins in *Castanea* and *Quercus* spp. (Pearce, 1996). The phenolic acid, protocatechuic acid, is correlated with maximum pigmentation in scales. Unpigmented scales contain little or no protocatechuic acid (Link et al., 1929). Catechol and protocatechuic acid appear to be the chief toxic chemicals found in the outer scales of pigmented onions (*Allium cepa*) and are thought to contribute to the resistance to onion smudge caused by *Colletotrichum circinans*. This type of resistance involving the accumulation of catechol is only found in pigmented onions and not in the susceptible white onions (Link & Walker, 1933).

Techniques for Phenolic Detection.

Thin Layer Chromatography (TLC) can be used to separate phenolic compounds from crude extracts. A small amount of the extract is applied to the bottom of the TLC plate, which is coated with either silica gel or cellulose. The capillary action of the solvent moves the extract to the top of the plate. The R_f-value can be calculated for each compound, which can be used for characterization and identification. This value is the ratio of distance the compound has migrated divided by the migration value of the solvent, with a maximum value of one (Vermerris & Nicholson, 2006).

Unknown compounds that are present together interfere with each other in chromatographs. HPLC-MS and PDA detection can be used to separate and quantify those with similar UV spectra (Justesen et al., 1998). High performance-liquid chromatography, including reversed-phase HPLC, can be used to quantify and purify individual compounds from a complex mixture (Temerdashev et al., 2011).

Individual compounds can be identified by their retention times and UV spectra. High performance-liquid chromatography coupled with diode array detection and electrospray ionization mass spectrometry (HPLC-DAD/ESI-MS) has been used to analyze the chemical properties of berries. Retention times and UV-visible spectra were important for the identification of anthocyanins from complex mixtures in berries (Chen et al., 2014). UV spectrophotometry can also be used to detect polymeric forms of some flavonoids, including dihydroquercetin (DHQ); absorption band at 328 nm corresponds to the monomeric form of DHQ and the 290 nm band was attributed to both monomeric and polymeric forms of DHQ (Vekshin, 2009). Determining flavonoid structures is challenging and requires the use of analytical tools. ESI-MS analyses of fragmentation

patterns for flavonols and flavan-3-ols are often analyzed in negative-ion mode because of increased sensitivity compared to that of the positive-ion mode (Crupi et al., 2014).

Mass spectrometry is a technique that can be employed for the analyses of flavonoids. The purpose of MS is to detect charged molecular ions and fragments separated according to their molecular masses. Fragmentation patterns provide molecular masses and provide structural and stereochemical information (Andersen & Markham, 2005). The negative ion mode is usually more sensitive and selective compared to the positive ion mode for crude plant phytochemical analyses (Shu et al., 2010).

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CHAPTER ONE

ANTIFUNGAL BIOCHEMICAL COMPARISON OF *PRUNUS* SPECIES

ABSTRACT

The periderm tissues from seventeen *Prunus* species and common *Prunus* rootstocks were screened for the ability to inhibit the growth of *Armillaria* species. Among the screened species and varieties, only *Prunus maackii* was able to significantly inhibit the growth of *Armillaria ostoyae*, which is the most detrimental cherry root rot pathogen in Michigan. The *P. maackii* extracts from periderm, cambium, root, and wood tissue were analyzed by bioassay guided Thin Layer Chromatography (TLC) techniques. Based on separation of extracts by TLC, the periderm from *P. maackii* appeared to have a much higher phenolic compound content in comparison to the other *Prunus* species screened based on the number and area of bands fluorescing and absorbing under the wavelengths of 365 nm. Since many phenolic compounds exhibit this behavior under UV, extracts were further characterized using chemical reagents, which included diazotized *p*-nitroaniline (DPN, phenol detecting reagent) and vanillin (a flavanol detecting reagent). Periderm and cambial tissues from both mature and young *P. maackii* trees were screened to examine the distribution of phenolic compounds as trees advance in age. The periderm tissue was incorporated into YMPG nutrient-medium to further screen for antimicrobial properties. The objective of this study was to partially characterize the compound(s) from the periderm of *P. maackii* that were inhibitory to *Armillaria* species.

Introduction

The genus *Prunus* contains more than 400 species, including stone fruits, almonds and ornamentals (Osman et al., 2012). Armillaria root rot, caused by *Armillaria ostoyae*, is a major problem in cherry orchards in the northwest region of Michigan, particularly on sour cherry, *Prunus cerasus*, variety Montmorency (Proffer and Jones, 1988). Since Montmorency constitutes >95% of tart cherry production in Michigan, it is important that long-term management solution such as host resistance is found. Aside from devastating cherry production in Michigan, Armillaria root rot caused by *A. mellea* is the second leading cause of peach tree mortality (Beckman and Pusey, 2001). Overall, Armillaria root disease is considered one of the most detrimental diseases of trees in the temperate regions of the world (Worrall, 2004). *Armillaria* species can be pathogenic, necrotrophic or mutualistic (Mihail, 2015). Fungicide drenches or fumigation treatments show little effects due to the difficulty of reaching the pathogens (Amiri & Schnabel, 2012).

It is the white mycelium that destroys the phloem and cambium of the host. White mycelial fans between the wood and bark grow saprotrophically after death of the host (Webster & Weber, 2007). Wounds serve as an entrance for several pathogens. Necrophylactic periderm forms in response to wounding and is more resistant to infection. This type of periderm has major structural components that include lignin and suberin (Briggs, 1986). These polymers aid in resistance to pathogens. Resistance is a result of the plant pathogens inability to degrade lignin and suberin, which serve as both a physical and chemical barrier to the diffusion of nutrients. Diffusion and physical

barriers concentrate antimicrobial compounds at the site of infection (Bostock & Stermer, 1989).

Rhizomorph production and the colonization of wood allow the pathogen to spread through the soil forming infection centers that can cover large areas (Szewczyk et al, 2014). Due to the devastating effects of this disease, finding a long-term solution is critical. Host resistance is one potential means of management. Preliminary screening using the colonization of *Armillaria* species on twig segments has suggested that *P. maackii* has the potential for resistance to *A. ostoyae*. *In vitro* work included the screening of twigs from various *Prunus* species. *Armillaria ostoyae* was able to completely colonize the cambial tissue of all *Prunus* spp. screened with the exception of *P. maackii* (Warnstrom, 2011). This reduced colonization of *A. ostoyae* on *P. maackii* twig segments may be a result of antimicrobial compounds contained in the periderm tissue.

Higher plants produce diverse chemical compounds with defensive properties against pathogens. These chemicals include various low-molecular-weight natural products (Dixon, 2001). Some of the most well studied defensive compounds are the phytoalexins, which are antimicrobial compounds that are synthesized after the plant tissue is exposed to pathogen infection occurring first at the attempted infection site within single host cells (Nicholson & Hammerschmidt, 1992). Phytoalexins are known to have defensive capabilities and can be either fungitoxic or fungistatic (Ingham, 1972).

Phytoalexins accumulate in quantities that exceed the concentration needed to inhibit pathogen growth (Ribera & Zuñiga, 2012). Piceatannol was found to inhibit the growth of *Colletotrichum falcatum*. This phytoalexin was only synthesized as a response to pathogen infection, therefore uninfected sugarcane stalks (*Saccharum* spp.) did not contain piceatannol or it was found in very low levels (Brinker & Seigler, 1991). Camalexin is a nitrogen and sulfur containing phytoalexin from *Arabidopsis thaliana* and *Camalina sativus*, which is synthesized in response to various plant pathogens (Glawischnig, 2007). Young rice (*Oryza sativa* L.) seedlings produce the diterpene momilactone B as a phytoalexin and as a potential allelochemical to inhibit the growth of neighboring plants (Kato-Noguchi et al., 2002). Additional examples of phytoalexins include medicarpin from alfalfa, *Medicago sativa*, and pisatin from peas, *Pisum sativum* (Iriti & Faoro, 2009).

Phytoanticipins are preformed antimicrobial compounds that are constitutively present or produced in response to pathogen elicitors from preexisting precursors (VanEtten et al., 1994). Some phytoanticipins include saponins, cyanogenic glycosides and glucosinolates (Piasecka et al., 2015). Antifungal glucosinolate derivatives are found in significant quantities localized in the roots of Brassicaceae members (Schreiner & Koide, 1993). The avenacins are antifungal saponins found in oat roots and determine resistance to the take-all disease of wheat pathogen *Gaeumannomyces graminis* var. *tritici*. This soil pathogen is able to infect wheat but not oats. However, *Gaeumannomyces graminis* var. *avenae* produces avenacinase, which detoxifies avenacin and therefore can infect both cereals (Osbourn et al., 1994). Phytoalexins require induced transcriptional and/or translational activities while phytoanticipins are

constitutively present or produced from preexisting constituents (González-Lamothe et al., 2009).

The objectives of this research include screening a variety of *Prunus* species for antifungal activity in the periderm. Thin Layer Chromatography (TLC) bioassays will be used to examine the biological activity and complexity of the antifungal component(s) in the crude periderm extract of *P. maackii*. TLC bioassays will also be used to screen for heritable antifungal compounds in *P. maackii* hybrids. DPN and vanillin reagents will be used for the comparison of phenolic content in various tissues from young and mature *P. maackii* trees and in other *Prunus* species.

Materials and Methods

Young (< 5 years) *P. maackii* were purchased from Nature Hills Nursery, Omaha, NE. Trees were planted in pots, using Baccto® premium potting mix, located in the MSU greenhouse courtyard. Mature *Prunus* twigs, from *P. maackii*, *P. mahaleb* and *P. serotina* were collected at the NW Michigan Horticulture Research Station near Traverse City, MI and stored at -20˚C until used for analysis. Mature twigs were ground using a chipper-shredder to produce mulch. The periderm was removed from the mulch for samples of wood. Dr. Amy Iezzoni and Audrey Sebolt of the MSU Department of Horticulture provided the following materials for testing: MSU test rootstock samples of Cass, Clare, Lake, and Clinton. Samples of current cherry rootstocks include Mazzard, mahaleb and 148-1 (Gisela 6). Breeding selection samples, having *P. maackii* in their ancestry include R1 (1), R2 (1), 25 11 (15), PO1 (01), P11 (2), P12 (2), and 26e 20 (50).

The 14 samples used are shown with their assigned numbers and other identifiers (Table 1.1), which are alternative names mainly related to the field location the samples were collected from. Periderm, cambial and root tissues were also collected from a mature *P. maackii* tree on Michigan State University's campus, accession number 97P028*02, samples were kindly provided by Dr. Frank Telewski. Three extraction methods were evaluated to determine the best method for extracting the antifungal compounds in different *Prunus* species.

Table 1.1. Samples used to screen for *Armillaria* growth inhibition

Number	NAME	OTHER IDENTIFIER (mainly field location)
1	Cass	
2	Clare	
3	Lake	
4	Clinton	
5	Mazzard	NY54
6	mahaleb	
7	R1(1)	24 3 (1)
8	R2(1)	24 2 (1)
9	25 11 (15)	MB 2 (6)
10	P01(01)	MB 6 (30)
11	P11 (2)	MB 6 (50)
12	P12 (2)	MB 7 (15)
13	26e 20 (50)	26 B(13)
14	148-1 (Gisela 6)	MB 2 (29-33)

1-4 = MSU test rootstock
5,6 and 14 = Current cherry rootstocks
7-13 = Breeding selections that have *P. maackii* in their ancestry

Method 1: The periderm was removed from the twigs and macerated with a razor blade. One half gram tissue samples were placed in clean vials containing 12.5 mL of 80% acetone. The tissue was allowed to extract for three days at room temperature. After the initial extraction, tissues were rinsed twice with 5 mL of 100% acetone. All of the acetone extracts for each sample were combined for further analysis. The acetone extracts were concentrated to dryness by rotary evaporation at 37.2˚C. The residue remaining after evaporation of the solvent was dissolved in 2.5 mL of 80% acetone yielding a final concentration of 0.20 g tissue/mL. The samples were stored at -20˚C.

Method 2: The periderm was removed from the twigs and macerated with a razor blade. One half g tissue samples were added to 62.5 mL of water in 125 mL flasks. These samples were autoclaved for 35 minutes and then allowed to cool to room temperature. Once cooled, the tissue was filtered with Whatman #1 filter paper and was rinsed with 20 mL of water (2 x 10 mL rinses). The extracts were stored at -20˚C prior to lyophilization. The lyophilized material was dissolved in 80% acetone to a final concentration of 0.10 g of tissue per mL.

Method 3: The periderm was removed from the twigs and macerated by a razor blade. Samples containing 0.50 g of tissue were placed into vials containing 20 mL of distilled water. The vials were place in a water bath at 99˚C for 2 hours. The water extracts were filtered and transferred to clean storage containers and an additional 10mL of distilled water was added back to the vials containing periderm tissue. The vials were placed back into the water bath for an additional hour to collect any additional

compounds still contained in the periderm. The remaining supernatant was combined with the initial extraction and transferred to a separate container. The extracts were stored at -20˚C and then subjected to lyophilization. The lyophilized material was dissolved in 80% acetone to a final concentration of 0.10 g of tissue per mL.

Thin Layer Chromatography (TLC) was used to separate compounds from a mixture. TLC plates (20 x 20 cm) were purchased from Analtech. A small amount of the extract is applied approximately 2 cm from the bottom of a TLC plate, which is a glass plate coated with either cellulose or silica gel. Cellulose plates were used for polar compounds and developed in water. Silica plates were used for non-polar compounds and were developed in 90:10 chloroform – methanol (v/v). The TLC plate is placed in a glass container with approximately 100 mL of solvent. A zone was carved out at the 12 cm mark to allow a separation length of 10 cm. Plates were allowed to air dry in a fume hood and then were placed in a vacuum desiccator overnight for removal of any residual solvent. Acetone (0.20 g/mL), autoclave (0.10 g/mL) and hot water (0.10 g/mL) extracts were used for TLC analyses. Since acetone was found to be the most efficient solvent for extraction, it was used for extraction in all additional assays unless otherwise specified.

To create a TLC bioassay, within 24 hours of development, the plates were evenly sprayed with a dense spore and mycelia suspension of *Cladosporium cucumerinum* in ½ strength potato-dextrose broth and then placed in an incubation chamber for three days. The darkly pigmented hyphae of *C. cucumerinum* covered the entire surface of the TLC plate with the exception of areas containing antifungal compounds (Klarman & Sanford, 1968).

For phenolic compound detection, a ChromaDoc-It TLC imaging system with a Digi 105 color camera (12-megapixel) was used to take pictures of TLC plates under UV light (365 nm) and under white light to record bioassay results. Phenolic compounds were also detected on TLC plates using two reagents: diazotized *p*-nitroaniline (DPN) assay (Somaroo et al., 1973) and the vanillin assay (Sarkar & Howarth, 1976). The vanillin reaction used to assay for tannin content in Sorghum grain was found to be extremely temperature dependent (Price et al., 1978). This assay is semi-specific to a range of both monomeric and polymeric flavanols, dihydrochalcones and anthocyanins (Sarkar & Howarth, 1976). Additionally, in some cases this assay showed reactivity with some non-flavanol compounds including phenolic acids, such as cinnamic acid and flavonols including kaempferol and myricetin (Sun et al., 1998). Diazotized *p*-nitroaniline (DPN) is one of the various spray reagents used for the detection of phenolic compounds including syringic, *p*-coumaric and sinapic acids (Somaroo et al., 1973).

Twigs (approx. 2 cm in diameter) were collected for periderm extraction. The periderm was removed with a razor blade and was ground using a Krups® coffee grinder. The periderm tissue was added to the YMPG medium prior to autoclaving. After autoclaving, the periderm amended-medium was poured into the petri plates (150 x 15 mm) and then inoculated with plugs (5mm) of *A. ostoyae*. Plugs were taken from the margin of actively growing cultures. The cultures were grown for three weeks and then the radial growth (in cm) was measured and recorded. The ChromaDoc-It TLC imaging system was also used to take pictures of the Petri dishes containing *Armillaria* growth. This system contains a white light setting, which was used while taking pictures of

pathogen growth on media. The radial growth of pathogens was recorded, using the ruler tool in Photoshop. Measurements were analyzed using Tukey's HSD in the statistical computing program, R (R Core Team, 2014).

Results

Both cellulose and silica gel TLC plates were used to screen for antifungal compounds. TLC analyses of the extract (0.20 g/mL) from *P. maackii*, *P. serotina* and *P. mahaleb* were used to determine preliminary information on the presence and chemical nature of the antifungal compounds. The best band resolution was detected when extracts were applied to silica gel TLC plates and developed in a chloroform 9:10 v/v solvent (Figure 1.1).

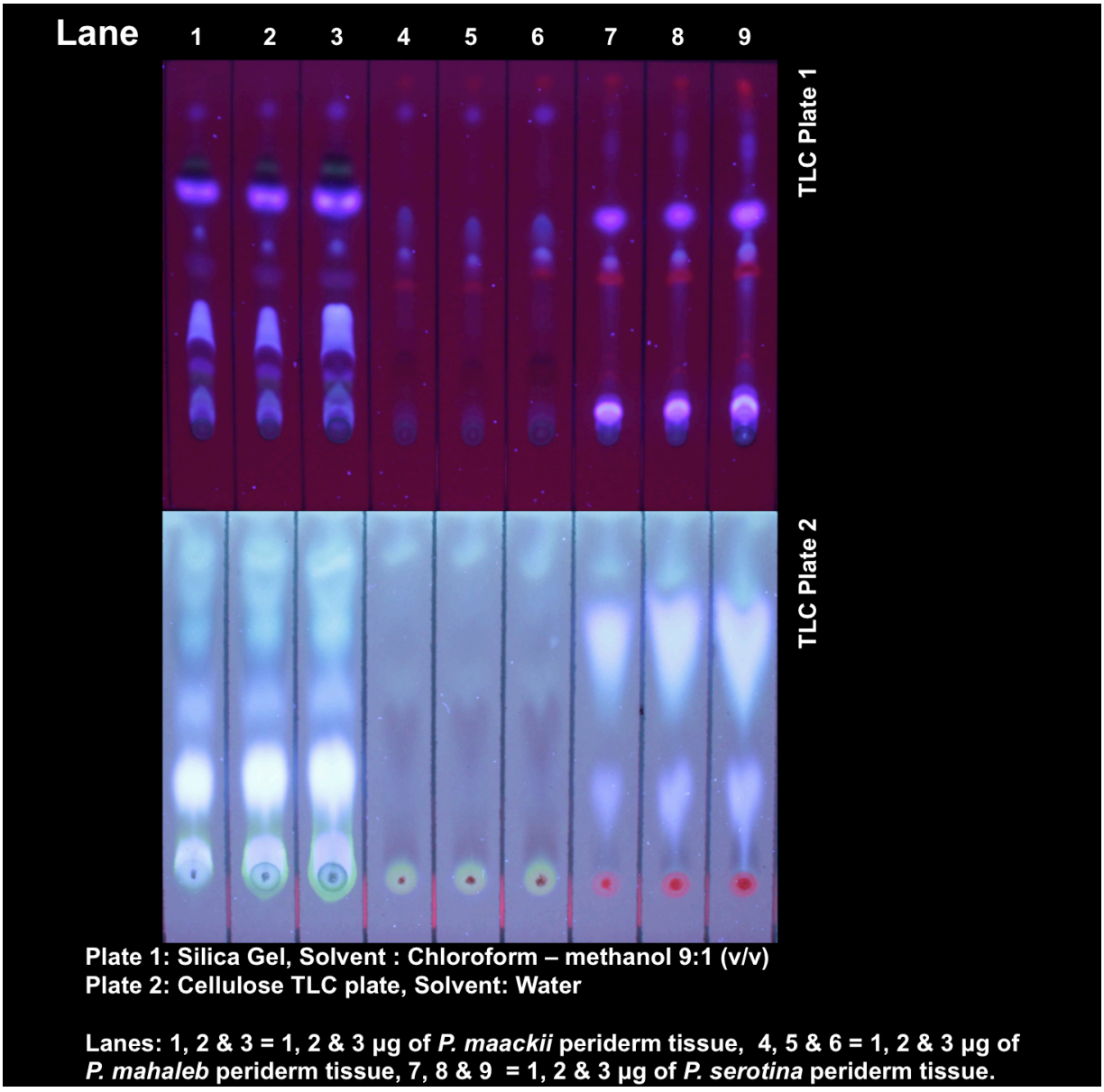

Figure 1.1. Separation of *P. maackii*, *P. serotina* and *P. mahaleb* samples visualized with UV (365 nm). Samples were developed on silica TLC plates using chloroform - methanol 9:1 (v/v) (top) and on cellulose TLC plates using water as the solvent (bottom).

When extracts were applied to cellulose plates and developed in distilled water, the unknown compounds appeared as streaks instead of individual bands. For both plates shown in Figure 1.1, lanes 1, 2 & 3 contain 1, 2 & 3µg equivalent of *P. maackii* periderm tissue (respectively). Lanes 4, 5 & 6 contain 1, 2 & 3µg of *P. mahaleb* periderm tissue, and lanes 7, 8 & 9 contain 1, 2 & 3µg of *P. serotina* periderm tissue. The R_f value can be used to characterize unknown compounds, based on their migration in various solvents.

The extracts were first examined under ultraviolet light (365 nm). The unknown compounds can be characterized based on their R_f values and on their ability to absorb or fluoresce UV light. After the extracts are visualized on TLC plates under UV light, TLC bioassays can be done for the characterization of biological activity. TLC plates are sprayed with a spore and mycelia suspension of *C. cucumerinum* and any antifungal compounds present are visualized as white areas on the darkly pigmented TLC plate when the bioassay is complete.

For both Figures 1.2 & 1.3, lanes 1-9 contain *P. maackii* periderm tissue. Lanes 1-3 are samples from the acetone extraction, lanes 4-6 are samples from the hot water extraction and lanes 7-9 are samples from the autoclave hot water extraction. The same order follows for extraction types in lanes 10-18 containing *P. serotina* periderm tissue and lanes 10-18 containing extracts from *P. mahaleb*. For both cellulose and silica gel TLC plates – solvent systems, *P. maackii* was the only species shown to have significant antifungal activity.

TLC analyses of extracts revealed that *P. maackii* was the only species that contained significant amounts of antifungal compounds as determined by bioassays. The separation with water of *P. maackii* periderm extract applied to a cellulose plate revealed three zones of antifungal activity, as shown by the white areas against a dark background of fungal mycelia and spores (Figure 1.2). The bioassay results show that antimicrobial compounds migrate together when applied to a cellulose plate. In the acetone extracts, the major zone was found between R_f values of 0.59 and 0.83 with a smaller zone at the origin (.07). Separation of extracts prepared with hot water or by autoclaving also showed zones of inhibition. However, the upper zone was found over a smaller R_f range. Additionally, the activity at the origin was reduced in hot H_2O and autoclaved H_2O extracts.

The extracts were also applied to silica gel TLC plates and were developed in a $CHCl_3$: MeOH (v/v) solvent. The more nonpolar solvent revealed several zones of inhibition in the periderm extract from *P. maackii* (Figure 1.3). Similar to the results using cellulose plates, the activity was greatest in the acetone extracts. The antifungal compounds seem to be correlated with the unknown compounds that absorb purple under UV (365 nm) with R_f values of 0.08, 0.51, 0.74, and 0.90. The separation of acetone extracts from *P. serotina* on silica gel showed a small amount of activity at an R_f of 0.80. The separation of *P. mahaleb* extracts on silica gel showed compounds with antifungal activity with R_f values of 0.64 and 0.85. However, the activity was much less than what was observed with *P. maackii*.

TLC results indicate that all three-extraction methods were able to extract compounds from *Prunus* periderm tissues. However, increased white areas in the lanes containing acetone extracts suggest that this solvent was the most efficient in antifungal compound extraction. The zones of antifungal activity were smaller when periderm tissue was extracted with hot H_2O and autoclave H_2O, possibly because the hot water and autoclave extraction procedures may have destroyed some of the compounds.

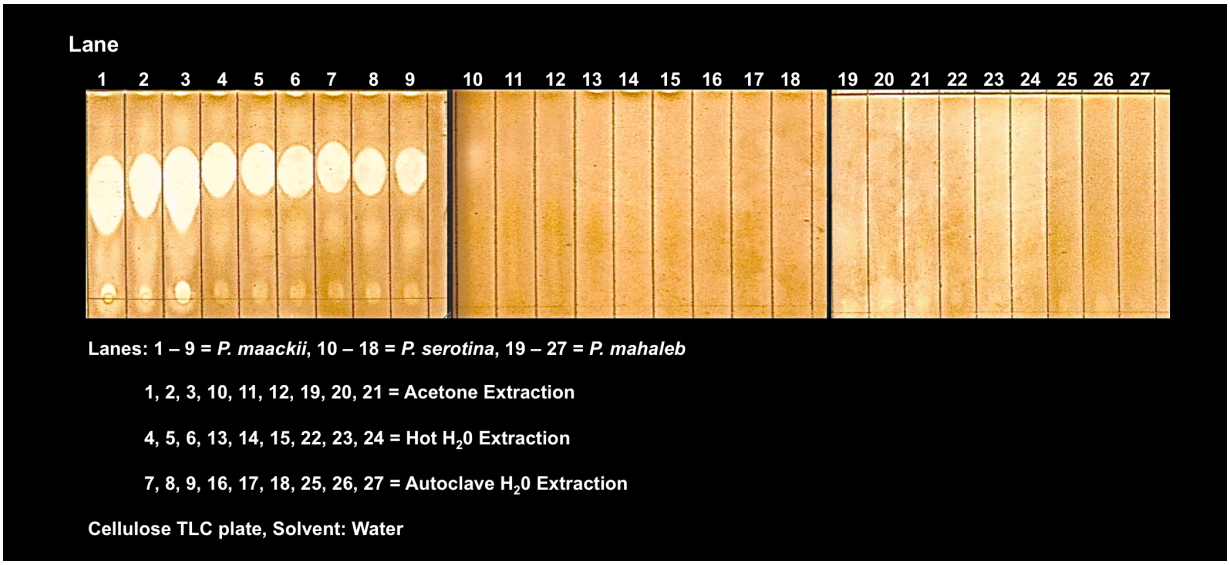

Figure 1.2. The periderm extracts from *P. maackii, P. serotina* and *P. mahaleb* were developed on cellulose TLC plates, in a water solvent and sprayed with *C. cucumerinum*. Four microliters of acetone extract (0.20g/mL) was used and eight microliters of hot water extract (0.10g/mL) and autoclave extract (0.10g/mL) were applied to the TLC plate.

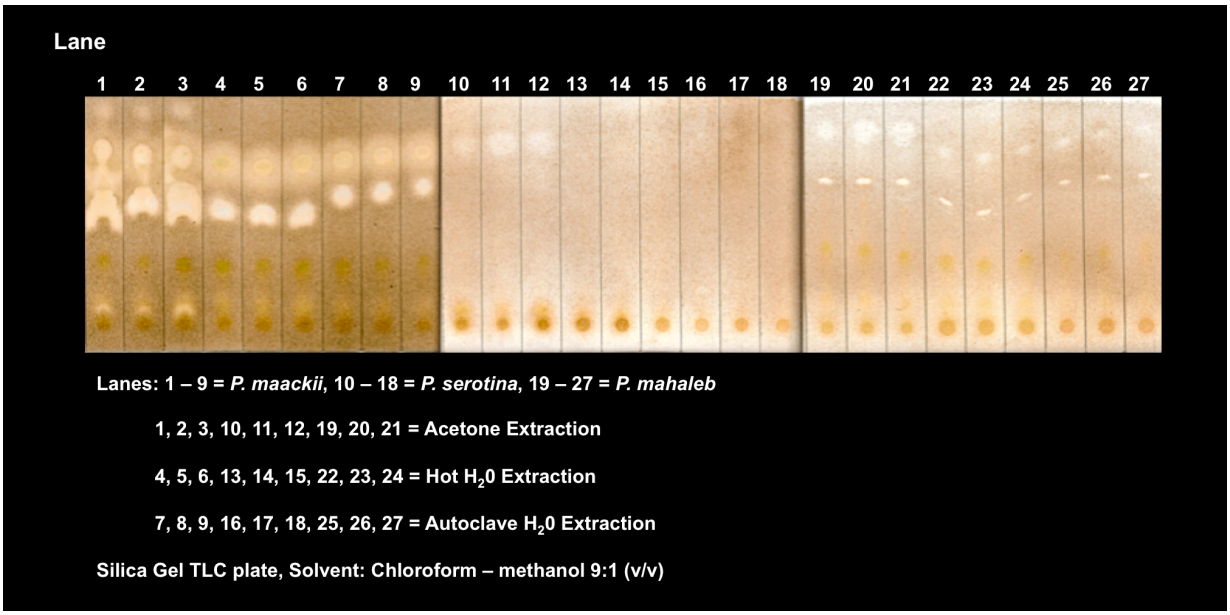

Figure 1.3. The extracts from *P. maackii, P. serotina* and *P. mahaleb* were applied to silica TLC plates, developed in chloroform – methanol (9:1 v/v) and sprayed with *C. cucumerinum*. Four microliters of acetone extract (0.20g/mL), eight microliters of hot water extract (0.10g/mL) and autoclave extract (0.10g/mL) were applied to the TLC plate.

In order to determine if the antifungal properties were related to the age of the tree, extracts from five young trees were screened for antifungal compounds and were compared with mature *P. maackii* trees. Periderm and cambial tissues were removed from twigs collected from young and mature trees for the comparison of chemical content. Samples from both age groups showed significant antifungal activity. The young trees were not exposed to any soil containing *Armillaria* spp., suggesting the presence of phytoanticipins, which are constitutively present antimicrobial compounds. However, trees may have been exposed to other microbes naturally present in the growing environment.

Dr. Amy Iezzoni, and Audrey Sebolt, kindly provided samples of the *P. maackii* x Clare hybrid seedling, which is part of the sweet and sour cherry breeding program. The periderm crude extract of *P. maackii* was compared to a *P. maackii* x Clare hybrid seedling to see if any antifungal compounds were inherited in the cross (Figure 1.4). Crude extracts were first visualized under UV light (365 nm) and later sprayed with *C. cucumerinum* for TLC bioassay results. Lane 1 shows the UV detection (365 nm) of compounds contained in the crude extract of *P. maackii* periderm tissue. Lane 3 shows the bioassay results of the same compounds in lane 1. Lane 2 contains periderm from the hybrid *P. maackii* x Clare. Lane 4 shows the bioassay results of lane 2. Based on TLC bioassay results, the antifungal compounds did not appear to be inherited in this particular hybrid seedling. The periderm of *P. maackii* (PM) contained significant antifungal activity and the *P. maackii* x Clare hybrid seedling (PM x) did not show antifungal activity.

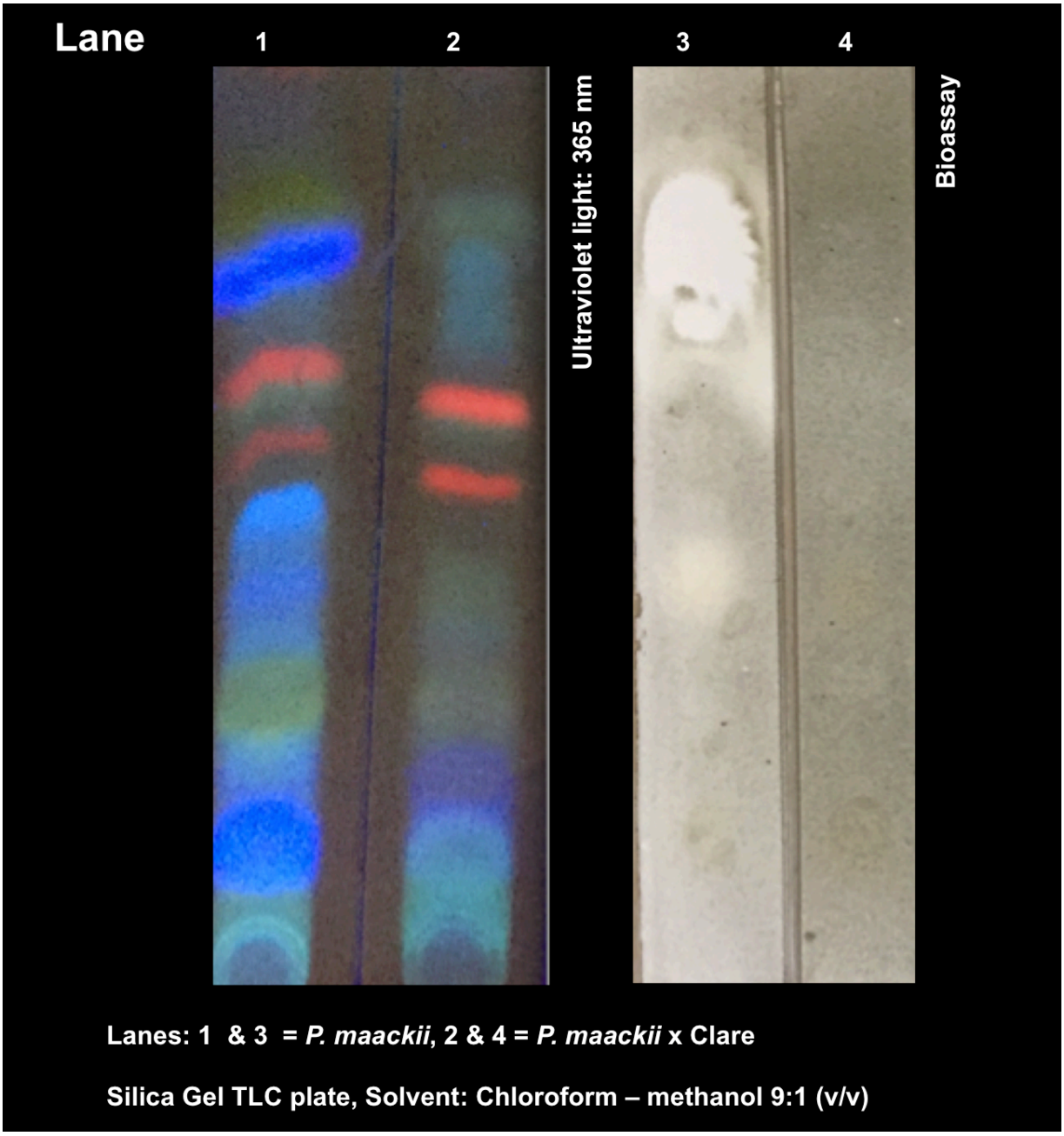

Figure 1.4. UV detection (365 nm) and TLC bioassay results for the crude periderm extract of *P. maackii* (PM) and *P. maackii* x Clare hybrid seedling (PM x). All samples were 0.20g/mL, 10 µL were applied to each lane. Silica TLC plates were used and developed in chloroform – methanol (9:1 v/v).

It is critical that the source antimicrobial activity is also located in the roots as well. The periderm tissue from roots was examined to detect whether the inhibition was also found in the periderm below ground. Samples were collected from roots approximately 6 inches below ground. Antimicrobial activity was greater in the above ground periderm, but the roots did exhibit antifungal activity. This is significant since *Armillaria* tends not to do well in the above ground parts of trees. The fact that the antimicrobial compounds are present in the above ground periderm, suggests that this would make it difficult for the pathogen to cause disease above the soil line. The phenolic content from the shoot periderm (P), cambium (C), root periderm (R) and wood (W) were compared under UV light (365 nm) and then sprayed with *C. cucumerinum* (Figure 1.5). Lanes 1-4 contain different older tissues collected from mature *P. maackii* trees; lane 1 contains periderm, lane 2 contains cambial extract, lane 3 contains root extract and wood extract was applied to lane 4. Antifungal compounds were detected in all tissues screened.

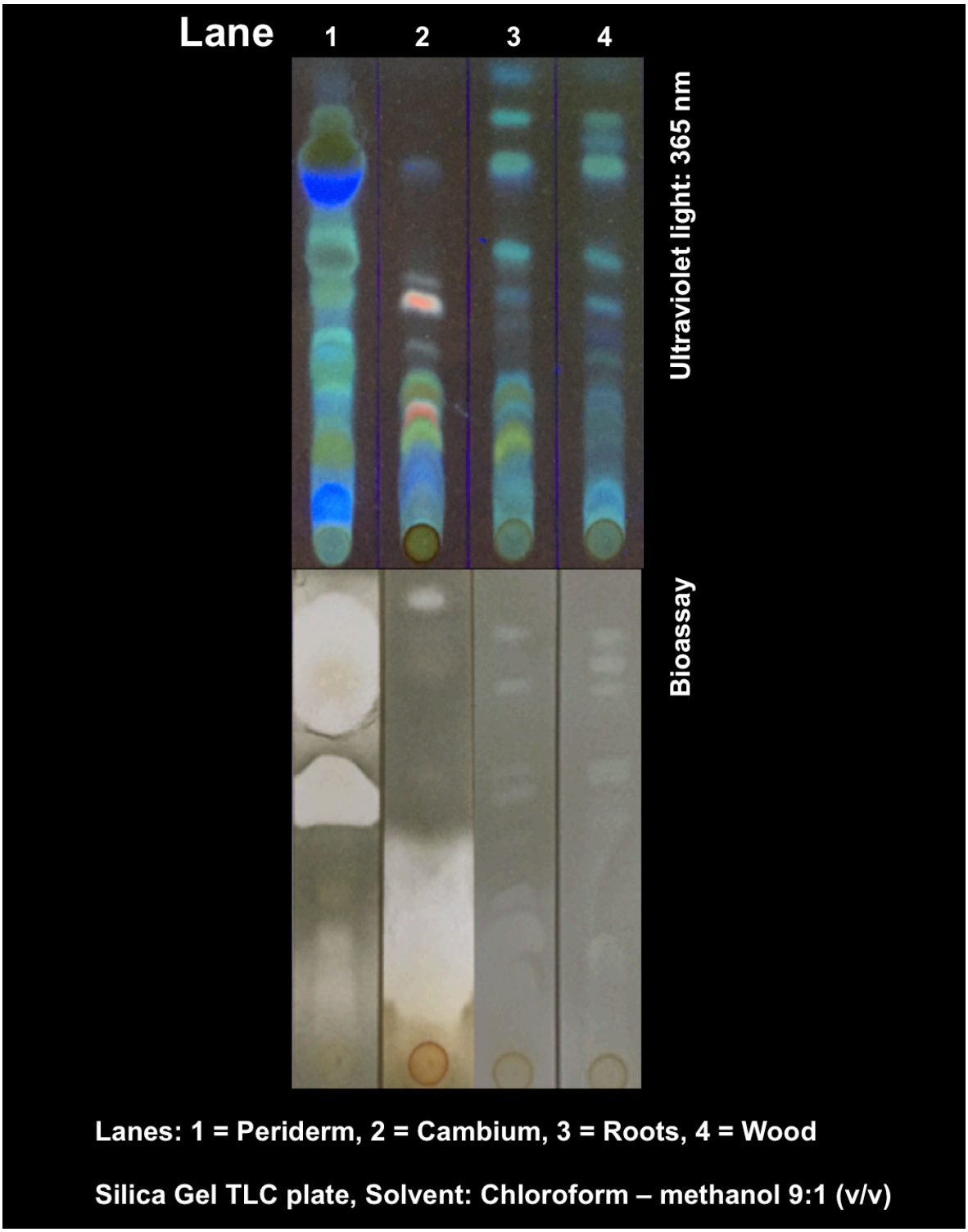

Figure 1.5. UV detection (365 nm) and TLC bioassay of *P. maackii* periderm tissue (P), cambial tissue (C), periderm from roots (R), and wood (W). All samples were 0.20g/mL, 10 µL were applied to each lane. Silica TLC plates were used and developed in chloroform – methanol (9:1 v/v).

For phenolic compound detection, the crude extracts from *P. maackii* periderm were chemically compared to extracts from *P. serotina* and *P. mahaleb* (negative controls). Crude extracts from *P. serotina* (lanes 1, 4 & 7), *P. mahaleb* (lanes 2,5 & 8) and *P. maackii* (lanes 3, 6 and 9) were applied to silica gel TLC plates and developed in a chloroform-methanol 9:1 v/v solvent (Figure 1.6). Lanes 1-3 of the TLC plate were sprayed with diazotized *p*-nitroaniline (DPN) reagent, lanes 4-6 were sprayed with a vanillin reagent and lanes 7-9 were sprayed with *C. cucumerinum* for the detection of antifungal compounds.

Based on the number of bands, the DPN reagent revealed that *P. maackii* contained higher phenolic content compared to the negative controls, *P. serotina and P. mahaleb*. The DPN reagent detected four bands in *P. serotina* extract. The first band was detected directly above the origin, R_f 0.12, the second band at R_f 0.23, third at R_f 0.38, and a very faint band at R_f 0.66. The vanillin reagent only detected one band in the *P. serotina* extract at R_f 0.12. Seven bands from the *P. mahaleb* extract were detected using the DPN assay with the following R_f values 0.12, 0.34, 0. 37, 0.40, 0.44, 0.50, and 0.65. The vanillin reagent detected six of the seven bands with R_f values 0.12, 0.34, 0.40, 0.44, 0.50, and 0.65. The DPN assay detected eleven bands in the periderm extract of *P. maackii* with R_f values 0.09, 0.13, 0.33, 0.44, 0.51, 0.55, 0.62, 0.68, 0.75, 0.89, and 0.95 and the vanillin assay detected eight bands at 0.13, 0.33, 0.44, 0.51, 0.62, 0.68, 0.89, and 0.95.

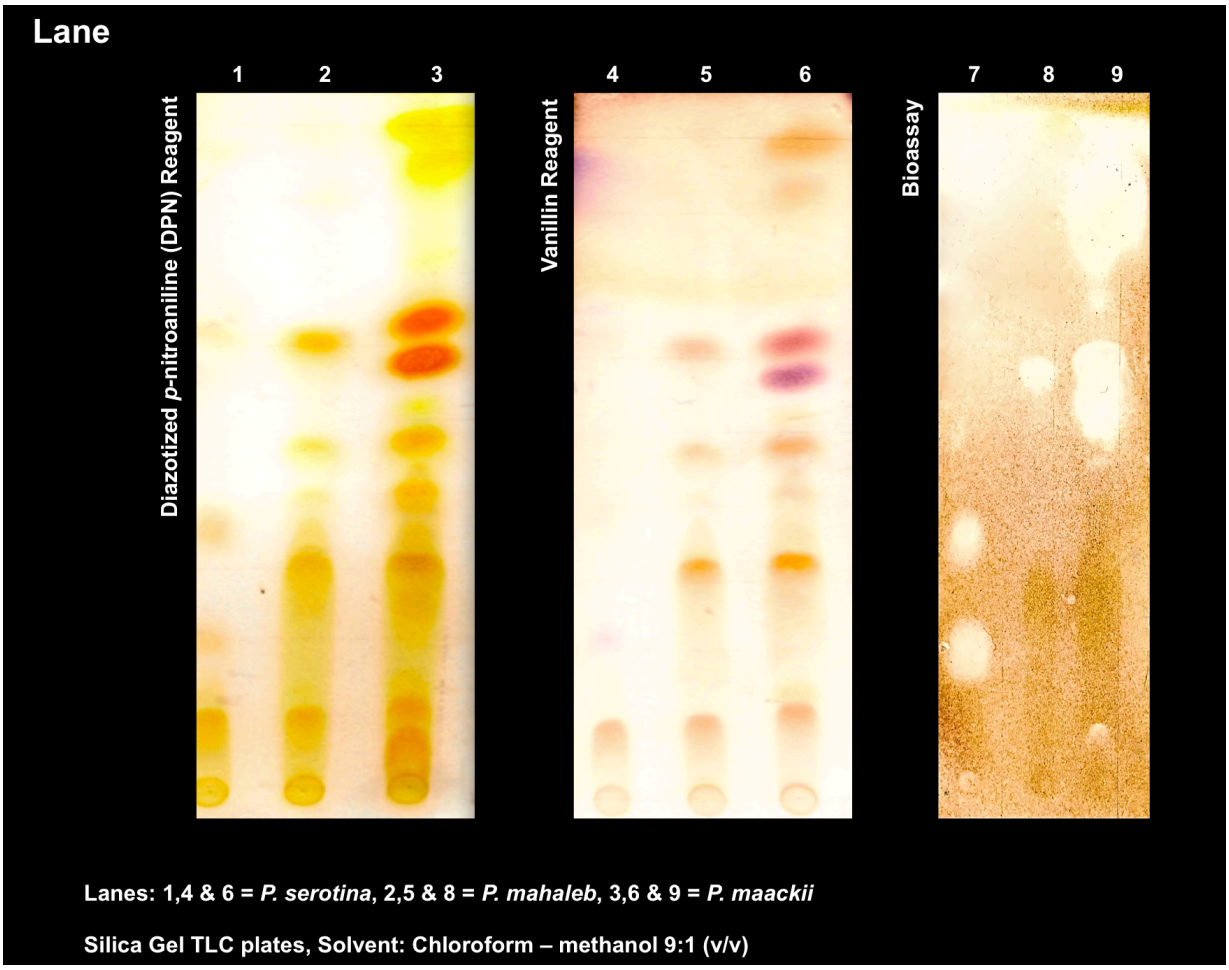

Figure 1.6. Extracts from *P. serotina, P. mahaleb* and *P. maackii* were compared. Extracts were applied to a silica TLC plate (0.20 g/mL), developed in chloroform – methanol (9:1), sprayed with the DPN reagent (left), vanillin reagent (middle) and *C. cucumerinum* (right).

Periderm and cambial extracts from young trees and from older tissues collected from mature trees were compared under UV light (365 nm) in lanes 1,2,5, & 6, and by using a vanillin spray reagent in lanes 3,4,7, & 8 (Figure 1.7). The number of compounds, spot intensity and R_f values were compared. In both mature and young tree samples, there were more bands in the periderm, detected under UV light and by the vanillin reagent, compared to cambial tissues. The extract from older tissues collected from mature trees contained higher phenolic content in cambial tissues than the young trees based on the number of bands detected under UV (365 nm).

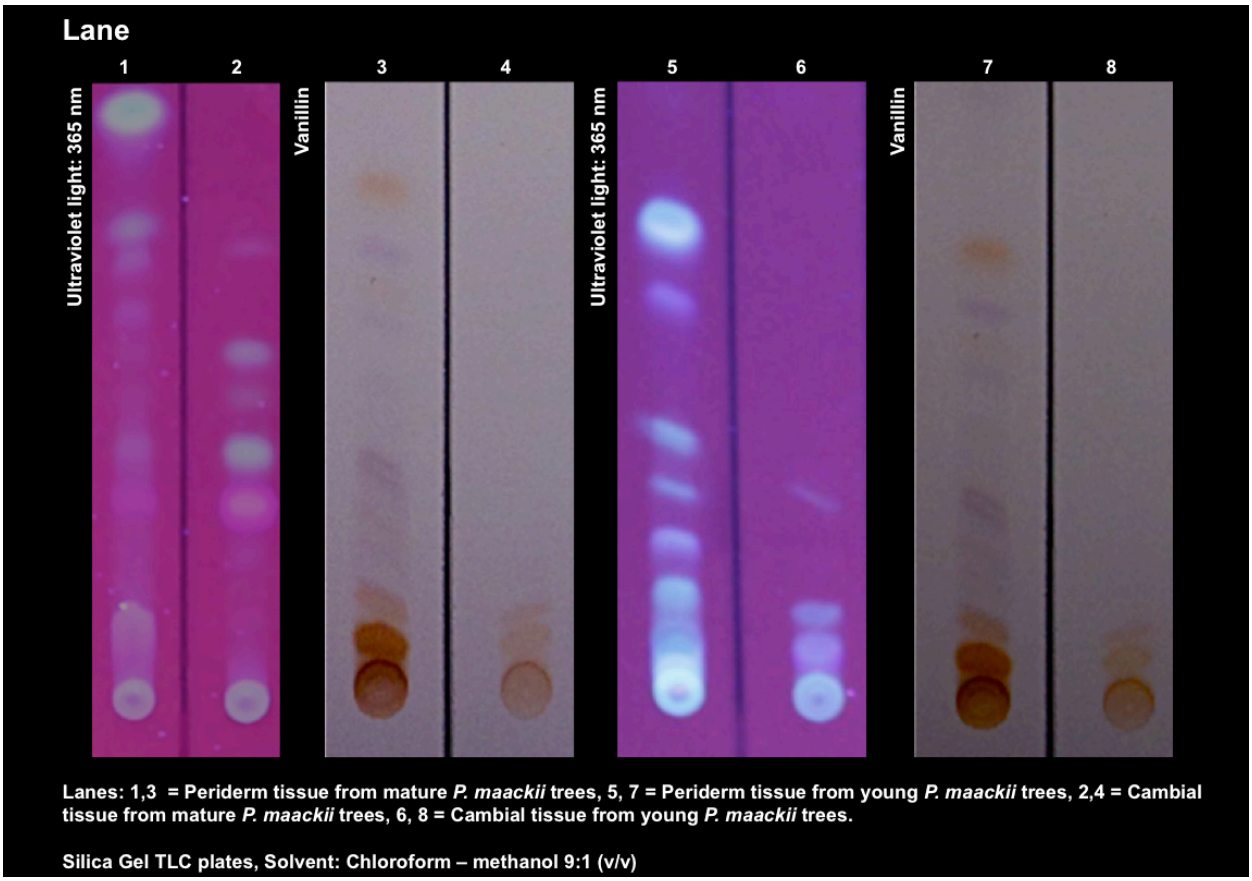

Figure 1.7. UV detection (365 nm) and vanillin reagent comparison of periderm tissue from mature and young *P. maackii* trees. Crude extracts were applied to silica TLC plates (0.20 g/mL) and were developed in chloroform-methanol (9:1 v/v).

TLC bioassays are not a practical option for growth inhibition screening of *Armillaria* species because of slow mycelial growth. Assays to test the inhibitory effects of the periderm and other tissues were conducted by incorporating plant tissues into culture media. Periderm incorporated bioassays (left to right) using tissue from *P. maackii, P. serotina* and *P. mahaleb* were compared to the control samples that contained only YMPG nutrient-medium. The radial mycelial growth was recorded from all samples after three weeks of incubation. Complete growth inhibition was only detected when *P. maackii* periderm tissue was added to the YMPG (10g/L YMPG), while extracts from the other species did not inhibit growth (Figure 1.8).

Additional samples of *Prunus* species and rootstocks, provided by Dr. Iezzoni and Audrey Sebolt, were screened for their ability to inhibit the growth of *A. ostoyae*. Periderm from an additional 13 rootstocks plus *P. mahlaleb* (negative control) and *P. maackii* (positive control) were screened for antifungal properties. At the concentration of 10 g/L YMPG, only periderm from *P. maackii* inhibited growth of *A. ostoyae* (Figure 1.9). To ensure that the other rootstocks didn't have the potential to inhibit, the amount of periderm in the media was increased to 17 g/L YMPG and cultures were grown for two weeks. Even with additional periderm tissue added to the samples, only the periderm from *P. maackii* was able to completely inhibit the growth of *A. ostoyae* (Figure 1.10). Crystals appeared only on the surface of *P. maackii* periderm-amended medium, approximately two weeks after preparation (discussed in later chapters).

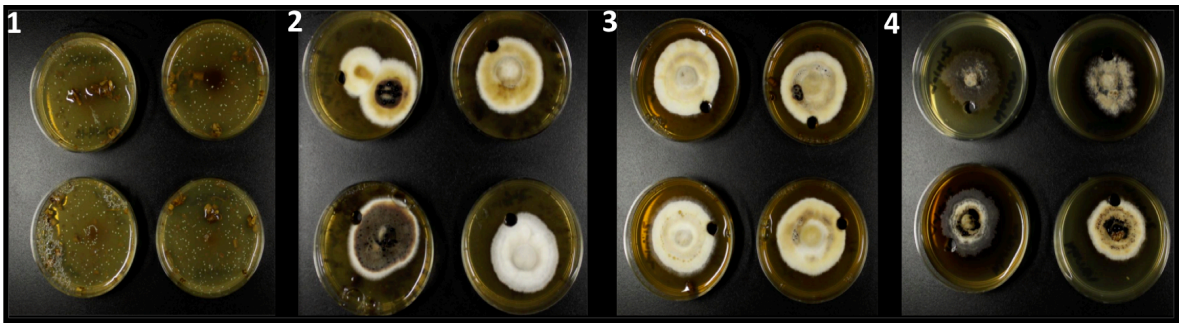

Figure 1.8. The growth of *A. ostoyae* on periderm-amended media (10 g/L YMPG) of *P. maackii* (1), *P. serotina* (2) and *P. mahaleb* (3). The control growth is shown on YMPG nutrient-medium (4). Cultures were grown for 3 wk.

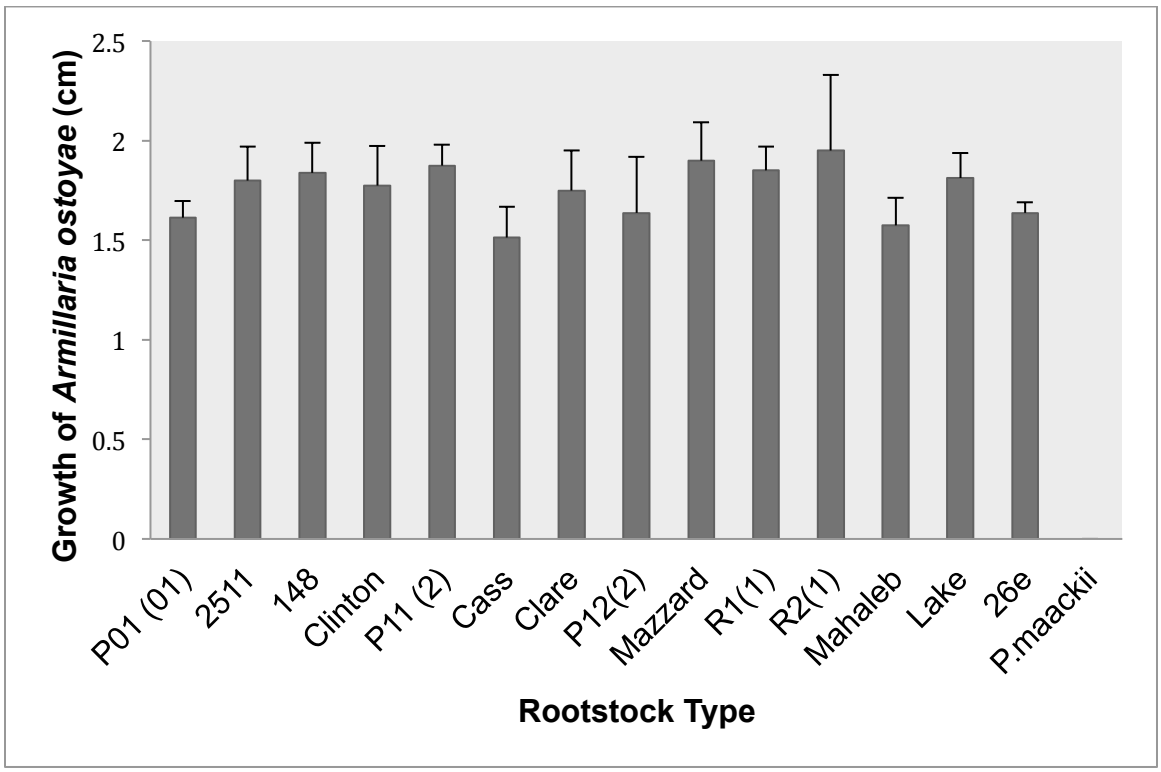

Figure 1.9. Survey of *Prunus* species and rootstocks (10 g periderm tissue/L YMPG) for their ability to inhibit the growth of *A. ostoyae*. Samples were grown for 3 wk.

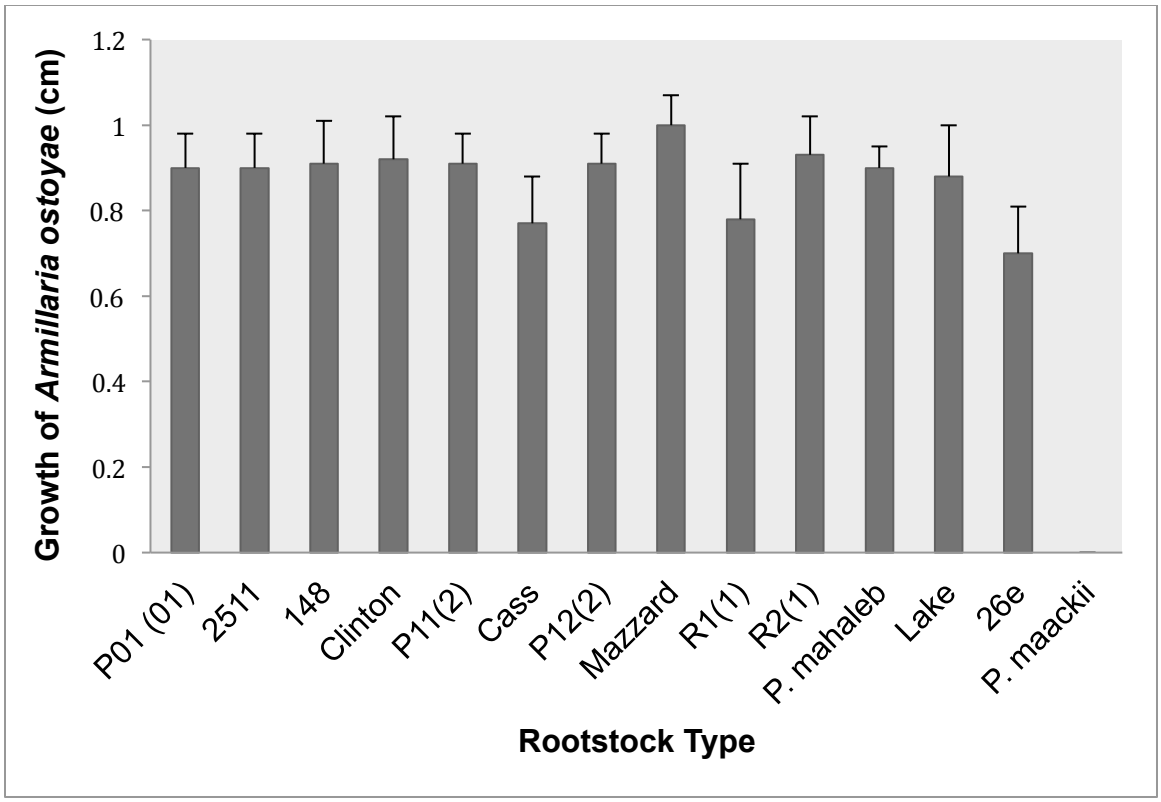

Figure 1.10. Survey of *Prunus* species and rootstocks (17 g/L YMPG) for their ability to inhibit the growth of *A. ostoyae*. Samples were grown for 2 wk.

Discussion

Armillaria species can infect over 700 species of plants including fruit, nut and vine crops. Armillaria root rot was previously controlled by soil fumigation with methyl bromide (Larue et al., 1962), but it was phased out because of its harmful effects on the environment. Finding a resistant species is critical for proper management of this disease (Soltantoyeh et al., 2014). Pears are considered to be immune or highly resistant to *Armillaria* infection. Thus, it is recommended that pear may serve as an alternative crop to replant in infected sites (Dalili et al., 2010). However pears are susceptible to infection caused by *Erwinia amylovora* in states that have significant fire blight pressure. Crop rotation is often utilized to reduce pathogen inoculum, but this means of control is not practical for forest or fruit trees. The removal of infected roots and stumps is one management strategy that successfully reduces inoculum levels (Morrison et al., 2014). Additionally, the orchard may be left fallow for a number of years to allow for the breakdown of wood and roots, which may harbor the pathogen.

The periderm serves as a structural barrier and varies in texture, color and thickness depending on the species (Dickison, 2000). New periderm tissue is produced each year, which accumulates as trees mature. Periderm tissue is non-living, compared to the living vascular and cambial tissue. In both young and mature trees, the accumulation of additional layers results in thicker periderm tissue, which may contribute to increased concentrations of tannins, phenolics, waxy substances, etc. Periderm tissue from several *Prunus* species and rootstocks were screened for phenolic compounds, which can range from single-aromatic rings to complex tannins. Phenolic

compounds are often involved in host defense against UV radiation and plant pathogens (Ferretti et al., 2010). The periderm of *P. maackii* contained a greater number of UV- fluorescing and absorbing bands. Vanillin and DPN reagents were used to further characterize these compounds as phenolics. These reagents also detected the greatest number of bands in the periderm extract from *P. maackii* compared to extract from *P. mahaleb* and *P. serotina*.

The incorporation of periderm tissue into culture media was used to screen for growth inhibition of *A. ostoyae*. It is likely that the antifungal compounds detected in the TLC bioassay are extracted from the periderm during the autoclaving process and contribute to antimicrobial activity. Complete growth inhibition of *A. ostoyae* was detected at 10 g /L YMPG. Crystals appeared on the surface of the *P. maackii* periderm-amended medium approximately two weeks after the date of preparation.. Results suggest that the extract slightly decreases in antimicrobial activity as the periderm-amended medium ages, indicating that some antifungal compounds crystallize out of the medium.

Plants need to balance the costs and benefits of defense responses in the absence of enemies because employing these mechanisms is costly to the host (Thaler et al, 2012). Future research could include examining *P. maackii* as a model host system for the balance of constitutively present antifungal compounds with any inducible phytoalexins present.

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CHAPTER TWO

IDENTIFICATION OF FLAVONOIDS FROM *PRUNUS MAACKII* TISSUES

ABSTRACT

Prunus maackii was identified as a potential source of resistance to *Armillaria* species. Previous research using Thin Layer Chromatography (TLC) bioassays detected the presence of antifungal compounds that are most likely phenolic in *P. maackii* periderm extract. *Prunus maackii* periderm collected from both young trees and older tissues from mature trees completely inhibited the growth of *A. ostoyae* when added to the YMPG nutrient-medium. *Prunus maackii* contained twice the amount of dried extract compared to *P. mahaleb* and *P. serotina*. High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) was used to characterize the unknown compounds by comparing molecular masses and retention times. Dry-column chromatography was used to separate and purify the crude extract. X-ray crystallography was used to identify the unknown compounds. Alnusin and two stereoisomers of alnustinol were identified from the crude periderm extract and were found to be antifungal, possibly contributing to the broad-spectrum antimicrobial activity of the *P. maackii* periderm extract. Dihydrowogonin was identified from the crude extract of *P. maackii* leaves. These flavonoids are unique compared to those commonly found in other *Prunus* species because of the absence of substituents on the B-ring.

Introduction

Phytoanticipins are preformed compounds that create the first chemical barrier in some host-microbe interactions. Distinction between phytoalexins and phytoanticipins is based on when they are produced. Phytoanticipins are constitutively present whereas phytoalexins accumulate after exposure to pathogens (VanEtten et al., 1994). Phytoalexins are synthesized as a result of attempted pathogen infection. Phytoanticipins may be stored in vacuoles in their glucosylated forms, which increases solubility and protects the host from the toxic effects of the aglycone. The aglycones are released through hydrolysis by β-glucosidases (Morant et al., 2008).

Research reported in the previous chapter demonstrated the presence of antifungal activity through bioassays using periderm extracts prepared with 80% acetone, hot water and with water used for extraction during the autoclaving process. The antifungal compounds were partially characterized and determined to be phenols, most likely flavonoids. Among the *Prunus* species and rootstocks screened, *P. maackii* was the only *Prunus* species that showed significant antifungal activity.

There are various chemical forms of phytoanticipins, which include terpenoids, dienes and phenolic compounds (Aharoni et al., 2005). Phytoanticipins also include saponins, cyanogenic glycosides and glucosinolates (Iriti & Faoro, 2009). The seeds of *Prunus spinosa* contain cyanogenic glycosides including amygdalin, prunasin and sambunigrin (Kumarasamy et al., 2003). Avenacins, terpenoid sapnonins, have potent antifungal activity and have been shown to play a role in resistance of oats to certain varieties of the *Gaumanannomyces graminis*, which is often referred to as the take all fungus.

Take-all is a disease of cereal plant roots. Evidence supporting the role of avenacins in host defense comes from surveys of *Avena* spp. that show little variation in avenacin content with exception of a diploid species (*Avena longiglumis*), which lacks avenacin A-1. This species is significantly more susceptible to infection by *G. graminis* than other *Avena* spp. (Osbourn, 2003). Avenacin B-1, also absent or present in trace-amounts in *A. longiglumis*, was found to be of secondary importance compared to avenacin A-1 in terms of aiding in resistance (Osbourn et al.,1994). Saponin-deficient mutant varieties of *A. strigosa* were unable to produce β- amyrin, which is required for the production of avenacins (Trojanowska et al., 2001). Oat species that lack the saponin avenacin are more susceptible to the take-all fungus *Gaumanannomyces graminis* var. *tritici* (Osbourn et al., 1994).

Avocado fruit contains long-chain dienes that suppress *Colletotrichum gloeosporioides* growth in the epidermis of young fruits (Shin et al., 2014). When avocado fruits were inoculated with *C. gloeosporioides*, the concentration of the preformed diene, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene, was nearly doubled. Mechanical wounding also enhanced the concentration of the diene in the peel and flesh of avocado. However, wounding only enhanced concentrations over a 24 h period compared to 3-4 days with inoculated fruits (Prusky et al., 1990). The concentration of antifungal dienes decrease as the fruit matures (Prusky et al., 1983). Disease resistance in strawberry cv. Elsanta fruit and flowers against *Botrytis cinerea* is also dependent on concentrations of preformed antifungal compounds (Terry et al., 2004).

Additional examples of phytoanticipins include prenylated genistein, which may function as a phytoanticipin in lupin seedling development (Dixon & Ferreira, 2002). Andean lupin seeds (*L. mutabilis*) are a potential source of isoflavones, including genistein (Ranilla et al., 2009). Constitutive antifungal resorcinols in mango fruit (*Mangifera indica*) inhibit the development of black spot by *Alternaria alternata* (Adikaram et al., 2010). In response to peeling, mango fruits contained 5-substituted resorcinols in the outer layer of flesh in similar concentrations found in susceptible ripened peels. Fruits that were peeled or ripened contained approximately half the amount of resorcinols compared to the concentration found in resistant unripened peels (Droby et al., 1987).

Objectives of this research include the isolation of phytoanticipins present in *P. maackii* periderm and leaves. Suitable single crystals collected from purified fractions will be used for x-ray crystallography analyses, which will provide structure identification. These structures will be used to further elucidate the unique phenylpropanoid pathway found in this ornamental species.

Materials and Methods

Young (< 5 years) *P. maackii* trees were purchased from Nature Hills Nursery, Omaha, NE, and planted in large pots, using Baccto® premium potting mix, and grown in the MSU greenhouse courtyard. As needed, periderm and cambial tissues were removed from the young twigs and were used for chemical analyses. Mature *Prunus* twigs (2-3 cm in diameter) were collected at the NW Michigan Horticulture Research Station near Traverse City, MI and stored (-20˚C) until used for analysis. Older periderm tissue was also collected from a mature *P. maackii* tree, accession number 97P028*02, on Michigan State University's campus, samples were kindly provided by Dr. Frank Telewski. The periderm was removed from the twigs and macerated with a razor blade. Thirty grams of periderm tissue were collected from *P. maackii, P. mahaleb* and *P. serotina* and placed in 500 mL of 80% acetone.

These samples were allowed to extract for three days at room temperature. After the initial extraction, the tissue was rinsed twice with 100 mL of 100% acetone. All of the acetone extracts for each sample were combined and concentrated to near dryness by rotary evaporation at 37˚C. The samples were further concentrated to complete dryness by lyophilization. In dry weight, 4.5 grams of extract were collected from the thirty grams of *P. maackii* periderm tissue, 1.92 grams from *P. serotina* and 1.72 grams from *P. mahaleb*. Samples were stored in a freezer (-20˚C) until needed.

Leaves were collected and extracted on June 4th, 2013. Three grams of fresh leaves were collected from *P. maackii* and placed in 100 mL of 80% acetone. Green buds were collected and extracted on June 5th, 2015. These samples were allowed to

extract for three days at room temperature. The extract was filtered using Whatman™ grade 1 filter paper and the solvent was evaporated using a rotary evaporator. Crystals were formed upon evaporation and were removed from the crude extract. The crude extract was re-suspended in 8 mL of 80% methanol. Samples were stored at -20˚C.

High-performance liquid chromatography coupled with mass spectrometry (HPLC-MS) requires a very small amount of analyte. Samples contained approximately 10 mg periderm tissue/mL 80% methanol and 10µL was used for analyses. Solvent A was 0.1% Formic Acid (FA) in H_2O and Solvent B was acetonitrile. Run time was set to 12 minutes. An Ascentis ® Express 10 cm C18 column was used for analyses.

Dry column-chromatography was used to separate individual compounds in the crude periderm extract. Silica gel was purchased from Jade Scientific Inc. and was used to pack the column for the collection and purification of compounds. Six grams of crude extract were dissolved in methanol and mixed with 10 grams silica gel. The column was made from nylon tubing purchased from Sorbtech and packed with silica gel. The tubing was 4 cm in diameter and approximately 12 cm long. Glass wool was purchased from Alltech® and was placed in the bottom of the column, with holes punched through the surface to allow air to escape as the solvent moved through the dry silica gel. Sand was placed on the top of the column so that the solvent wouldn't disrupt the silica gel as it was added. Chloroform: methanol, 90:10 (v/v) was used as the solvent. A hand-held UV light (Spectroline, Westbury) was used to detect and locate the different UV absorbing bands from the periderm extract contained in the column. Based on UV detection (365 nm), the column was cut into sections according to different fluorescing/absorbing bands. Each isolated fraction from the column was placed in 80%

methanol for the re-elution of compounds. The silica gel was centrifuged out of the samples using a Beckman Coulter™ Microfuge® 22R centrifuge.

All fractions from the column, in addition to crude extract collected from the leaves, were concentrated to dryness by the use of a rotary-evaporator (KDscientific, Holliston). The dried powder product from the crude extract of leaves and individual fractions from the periderm extract were dissolved in 1 mL of chloroform (J.T. Baker, Austin) contained in scintillation vials (Sigma-Aldrich Co., St. Louis). One drop of toluene (J.T. Baker, Austin) was added to the samples prior to capping the scintillation vials, giving an approximate concentration of 95% chloroform. Crystals were formed after 3 days of slow-evaporation. Dr. Richard Staples, Michigan State University Department of Chemistry and Chemical Biology, provided x-ray crystallography analyses.

Thin Layer Chromatography assays were used to confirm the antimicrobial activity of three fractions collected using dry-column chromatography and identified using x-ray crystallography. Five microliters of fractions were applied to a silica TLC-plate and developed with a chloroform-methanol, 90:10 (v/v) solvent. Within 24 hours of developing the TLC plates, the plates were evenly sprayed with a dense fungal suspension of *Cladosporium cucumerinum* in ½ strength potato-dextrose broth and then placed in a moist incubation chamber for three days to allow fungal growth.

Results

Preliminary HPLC-MS analyses of the crude extract from older periderm tissues collected from a mature *P. maackii* tree, detected over five different compounds present, with flavonoid structural characteristics. The peaks and shoulders can be used to identify the class of the unknown compound. Dihydroflavonols, flavanones and isoflavones can be readily distinguished from flavones and flavonols by their UV spectra due to having little or no conjugation between the A- and B- rings.

In methanol, dihydroflavonols show a major peak in the range of 270 – 295 for band II, with only a shoulder or low intensity peak for band I (Mabry et al., 1970). An unknown compound with a molecular weight of 301, in negative ion mode, was in much higher concentration than the rest of the other unknown compounds (Figure 2.1). This unknown compound, 301 m/z, was present in samples from both young trees (<5 years old) and more mature trees (>12 years) with a retention time of 4.6 minutes (Figure 2.2).

MS/MS analysis of the 301m/z peak produced 225.05, 213.06, 168.00, 197.06, 183.03, and 242.05 m/z fragmentation, indicating how the flavonoid structure was broken apart (Figure 2.3). The UV spectrum of the unknown compound with the 301-m/z peak is also characteristic to that of flavonoids, having a major peak at 293.9 nm with a shoulder peak in the region of 340-360 nm (Figure 2.4).

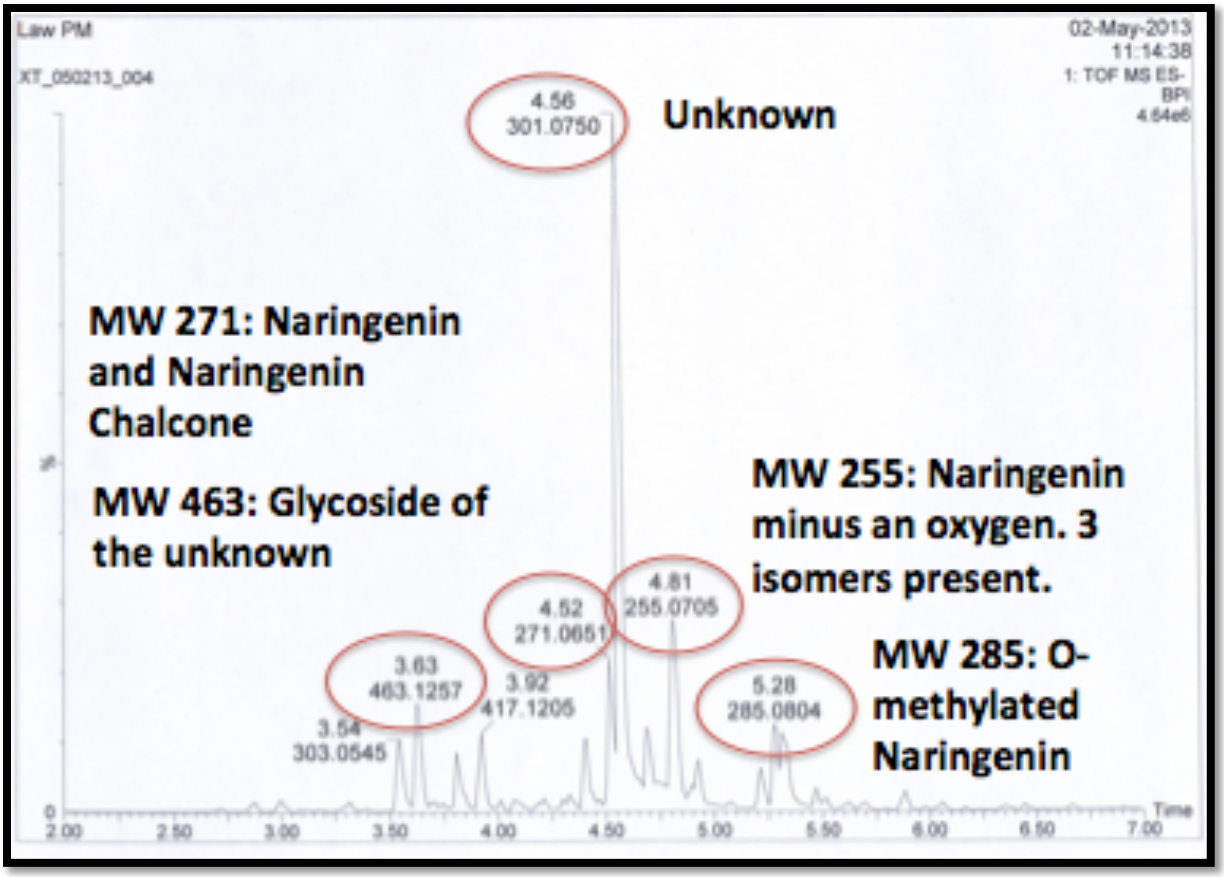

Figure 2.1. HPLC-MS analysis of periderm extract from older tissues collected from a mature *P. maackii* tree. Potential names were assigned based on retention times and masses.

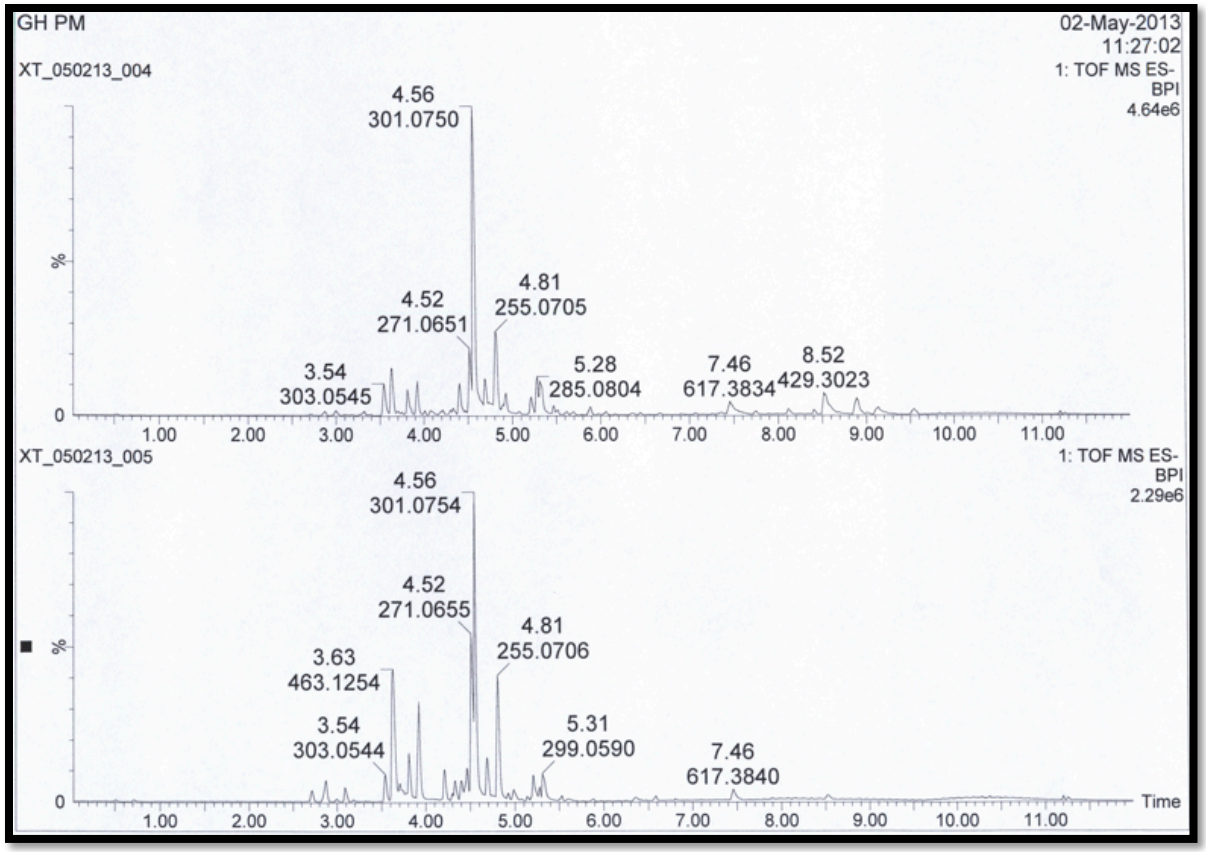

Figure 2.2. HPLC-MS comparison of crude periderm extract; samples were collected from a young *P. maackii* tree (top) and older tissues were collected from a mature tree (bottom).

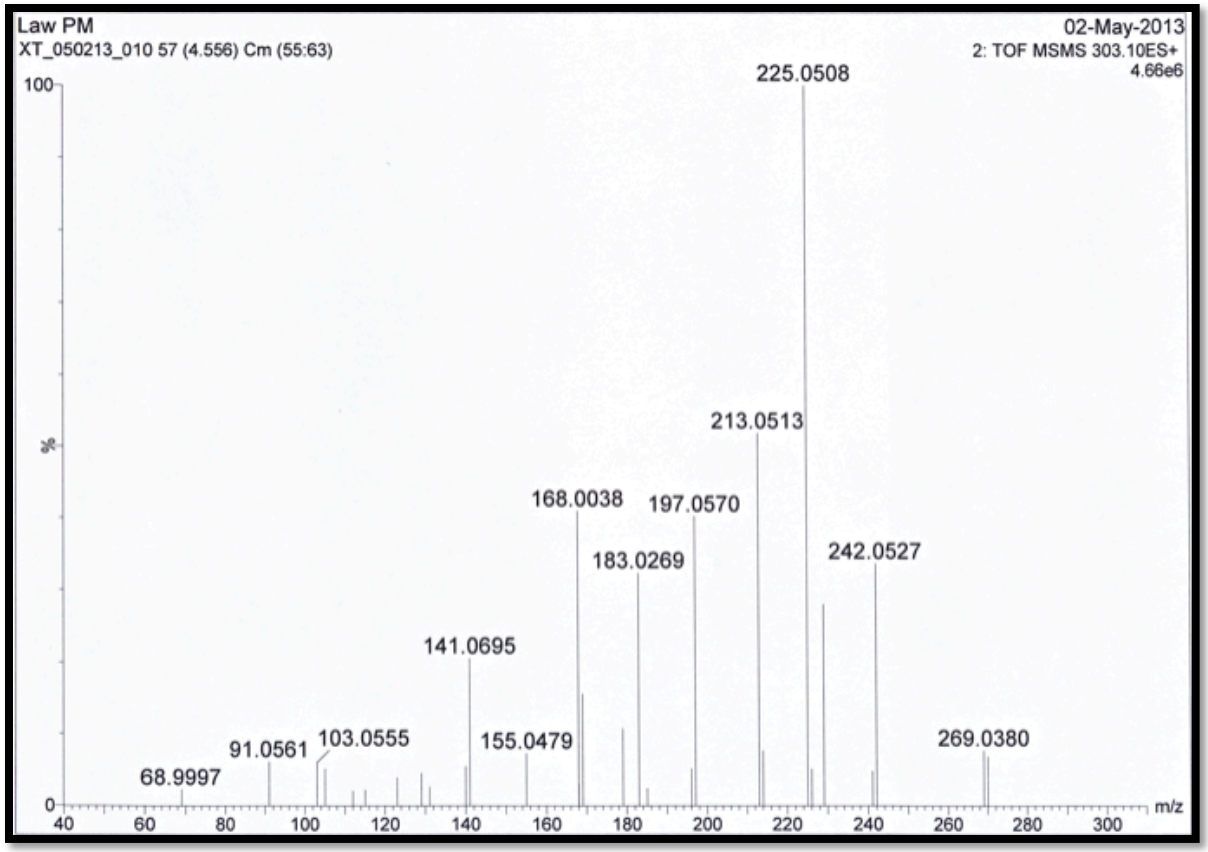

Figure 2.3. MS/MS of unknown compound with 301 m/z from periderm tissue of *P. maackii*.

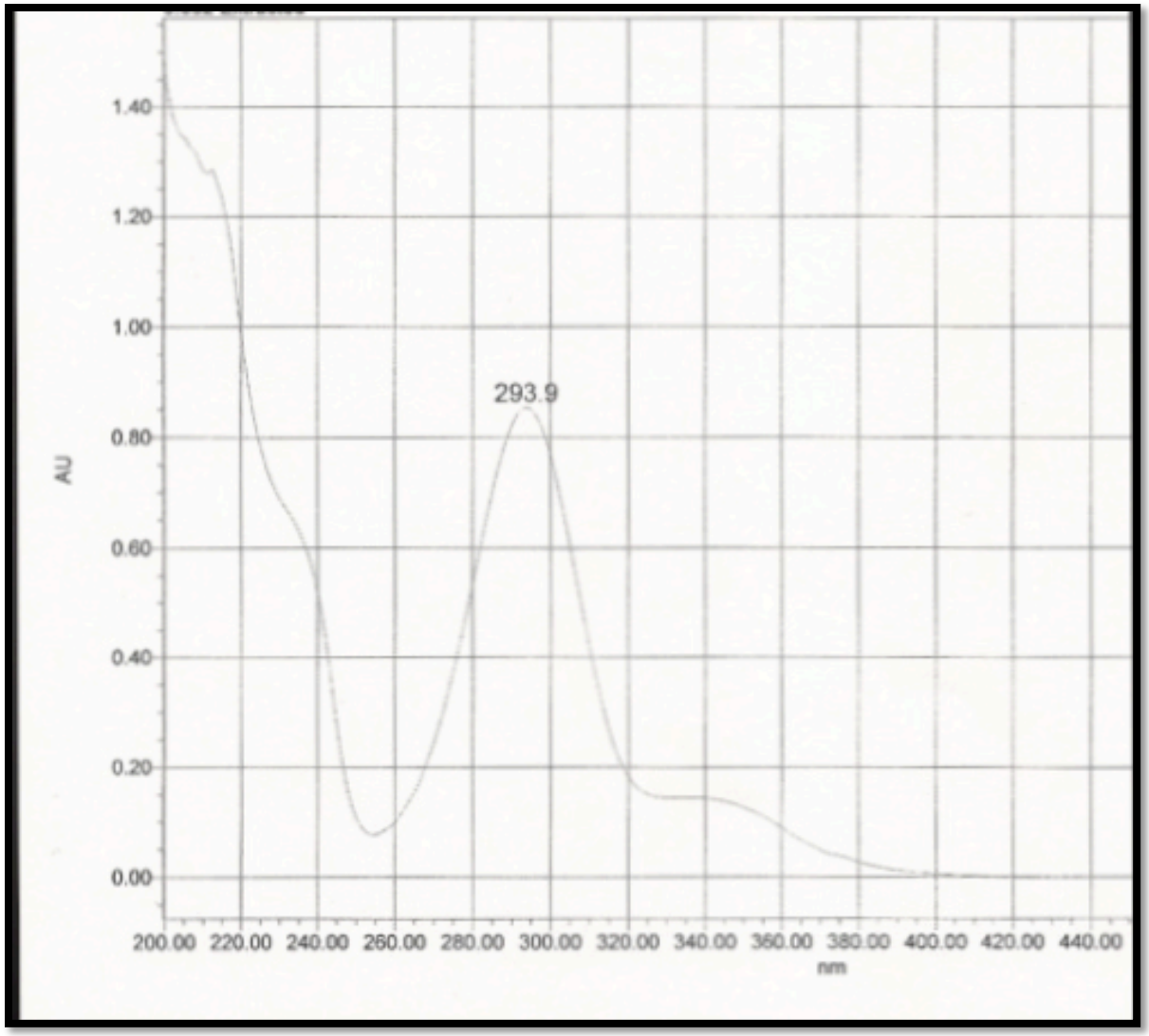

Figure 2.4. The UV spectrum of the unknown compound with 301 m/z from the periderm tissue of *P. maackii*.

Based on UV detection (365 nm) and polarity, three semi-purified fractions were collected from the 6 g of crude *P. maackii* periderm extract. Dry-column chromatography was used to separate out the three fractions from the crude periderm extract. All fractions collected from the periderm were re-eluted from the silica gel with 80% methanol and TLC analysis was used to confirm the purity of fractions based on the number of bands present under UV light.

Compound X was the most non-polar compound present in the silica gel; compound Y was the second most non-polar compound present. All semi-purified fractions were submitted for X-ray crystallography analyses, but only three samples contained suitable single crystals; 0.39 g of compound X from fraction 4 and 0.28 g of compound Y from fraction 3. The third sample from fraction 2 was identified, as a stereoisomer of compound Y. A modified version of the bioassay was done to see if any antifungal activity is present in any of the isolated fractions. All fractions collected were only partly purified. Fraction 2, 3 and 4 showed antimicrobial activity to *C. cucumerinum* (Figure 2.5).

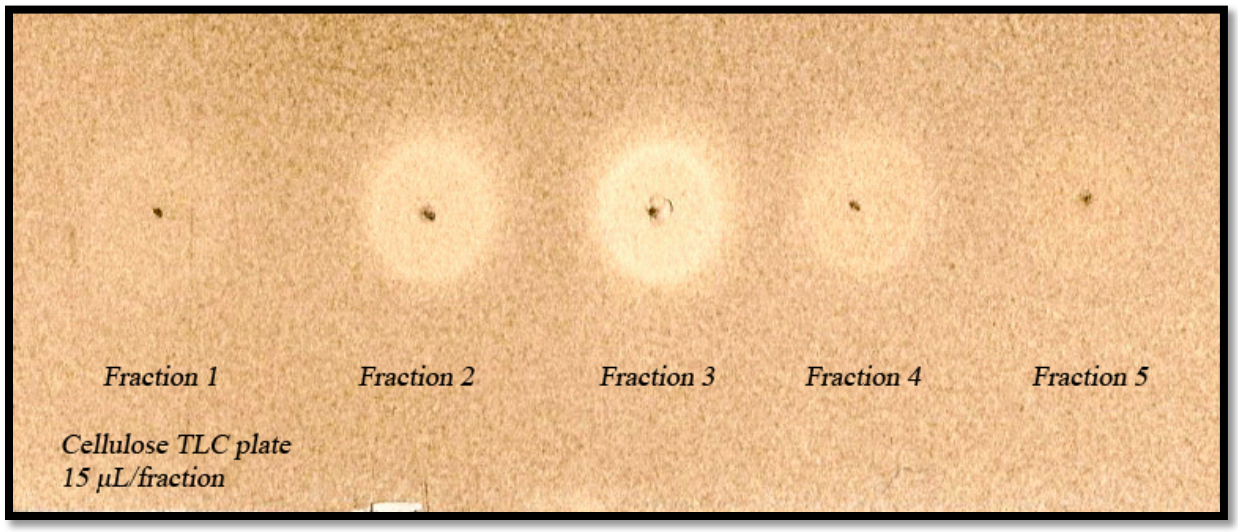

Figure 2.5. Modified bioassay of isolated fractions from the periderm of *P. maackii*.

X-ray crystallography identified the unknown compounds as 3,5,7-trihydroxy-6-methoxyflavone (alnusin) and 3,5,7-trihydroxy-6-methoxyflavanone (alnustinol). Lane 1 contains semi-purified alnusin and alnustinol. Lane 2 has more purified alnustinol, compared to lane 1. Lane 3 contains mostly alnusin, with small amounts of alnustinol (Figure 2.6).

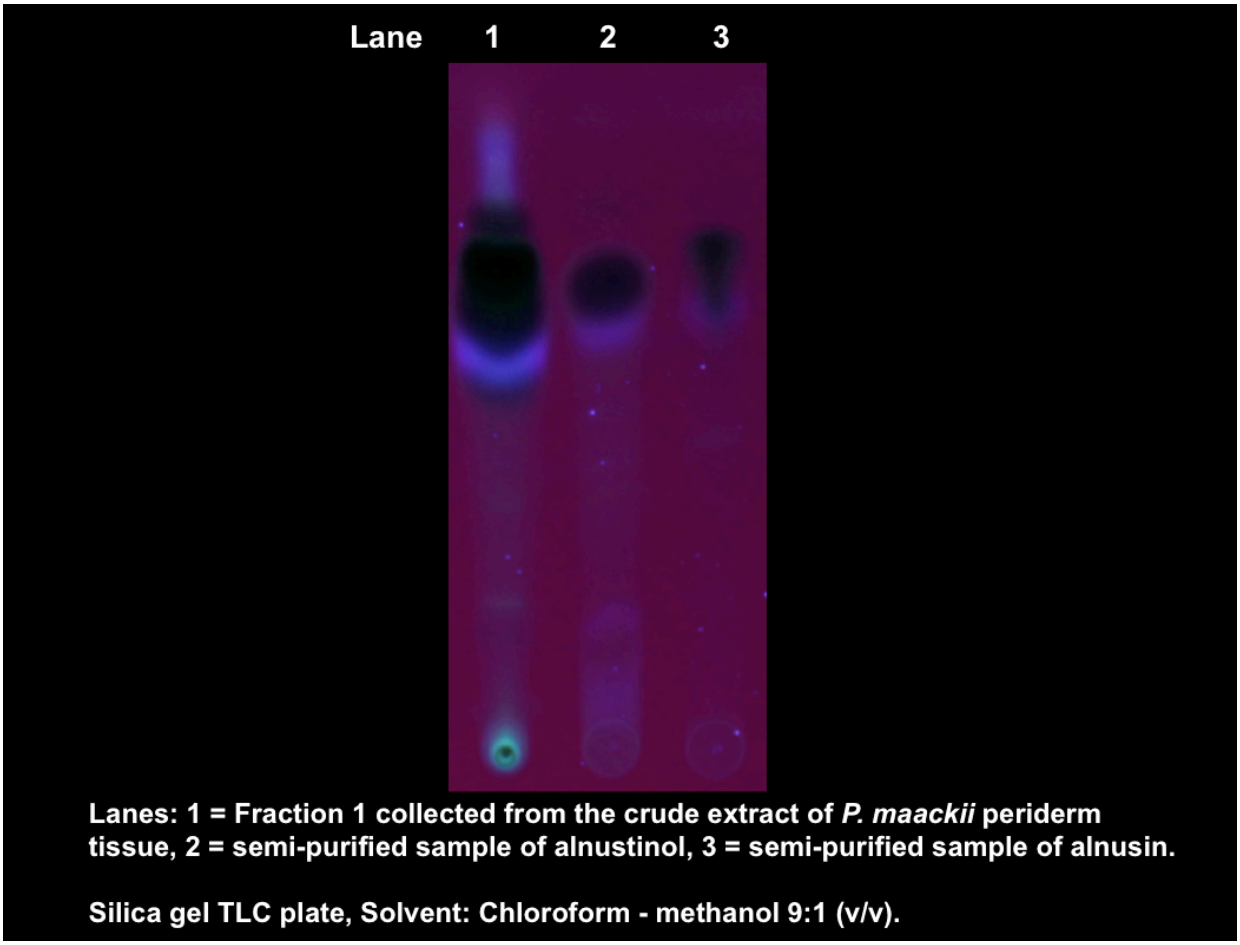

Figure 2.6. UV detection (365 nm) used to determine the purity of three biologically active fractions 2, 3 and 4 collected from crude *P. maackii* periderm extract.

3,5,7-trihydroxy-6-methoxyflavone (alnusin) and 3,5,7-trihydroxy-6-methoxyflavanone (alnustinol) were obtained during the *P. maackii* periderm extraction process. A single crystal was used for the identification of 3,5,7-trihydroxy-6-methoxyflavone, The common name for this flavonoid is alnusin (Figure 2.7). An additional crystal was used to identify 3,5,7-trihydroxy-6-methoxyflavanone, which is commonly called alnustinol (Figure 2.8). The crystal used to identify alnustinol also showed potential hydrogen bonding properties (Figure 2.9). Although the only difference between the two compounds in Figure 2.7 and Figure 2.8 is a double bond contained in

the C-ring, the two crystals used for identification have different shapes. A third crystal was used to identify a stereoisomer of 3,5,7-trihydroxy-6-methoxyflavanone (Figure 2.10) and its potential hydrogen bonding properties (Figure 2.11).

Crystal Data for 3,5,7-trihydroxy-6-methoxyflavone (Compound X): $C_{16}H_{12}O_6$, M =300.26, triclinic, a = 7.2248(14) Å, b = 7.3142(14) Å, c = 12.588(2) Å, α = 84.755(2)°, β = 79.644(2)°, γ = 82.434(2)°, V = 647.1(2) Å3, T = 173.15, space group P-1 (no. 2), Z = 2, μ(MoKα) = 0.119, 5928 reflections measured, 2396 unique (Rint = 0.0445) which were used in all calculations. The final wR2 was 0.0948 (all data) and R1 was 0.0462 (>2sigma(I)).

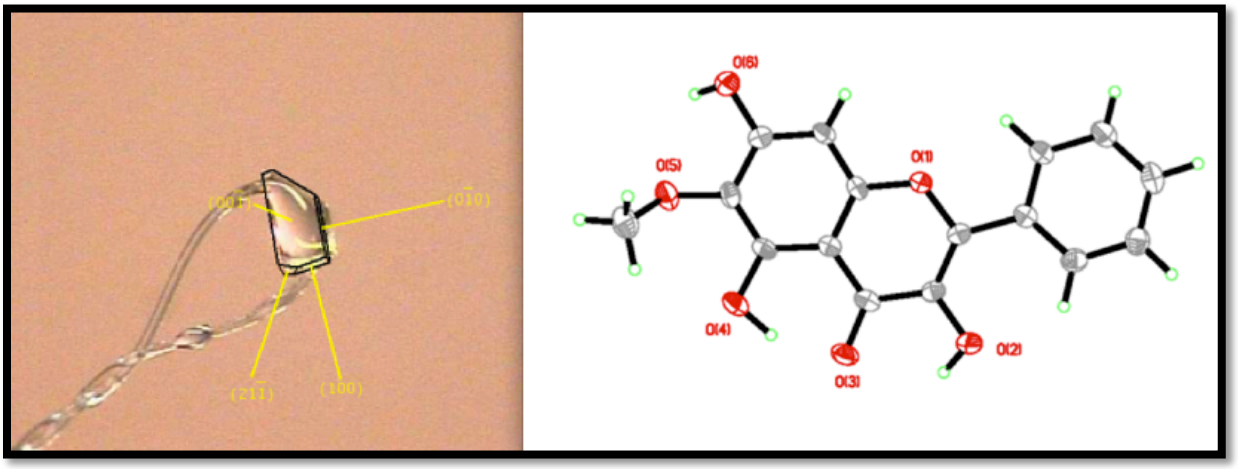

Figure 2.7. Single crystal used for x-ray crystallography analysis and the corresponding structure of 3,5,7-trihydroxy-6-methoxyflavone.

Crystal Data for 3,5,7-trihydroxy-6-methoxyflavanone (Compound Y): $C_{16}H_{14}O_6$, M =302.27, monoclinic, a = 13.751(2) Å, b = 5.4210(8) Å, c = 36.666(6) Å, β = 100.204(2)°, V = 2690.0(7) $Å^3$, T = 173.15, space group C2 (no. 5), Z = 8, μ(MoKα) = 0.115, 11109 reflections measured, 4681 unique (R_{int} = 0.0559) which were used in all calculations. The final wR_2 was 0.0948 (all data) and R_1 was 0.0513 (I > 2\s(I)).

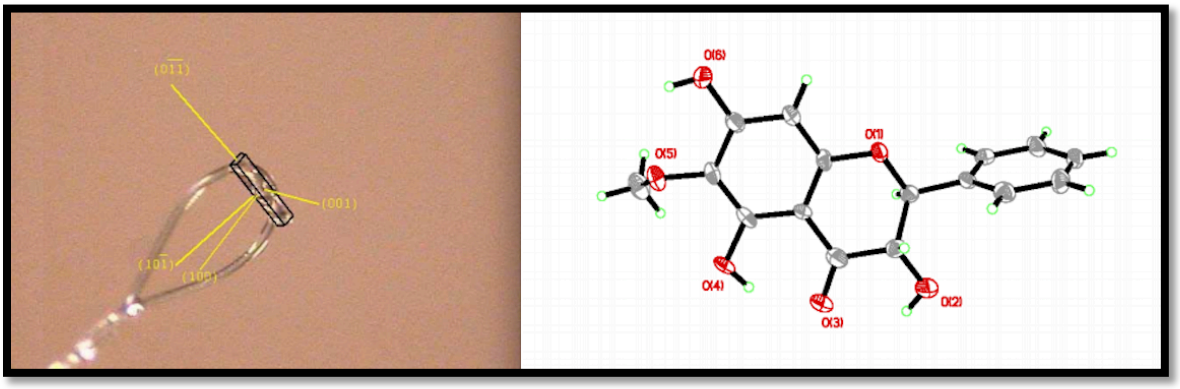

Figure 2.8. Single crystal used for x-ray crystallography analysis and the corresponding structure of 3,5,7-trihydroxy-6-methoxyflavanone.

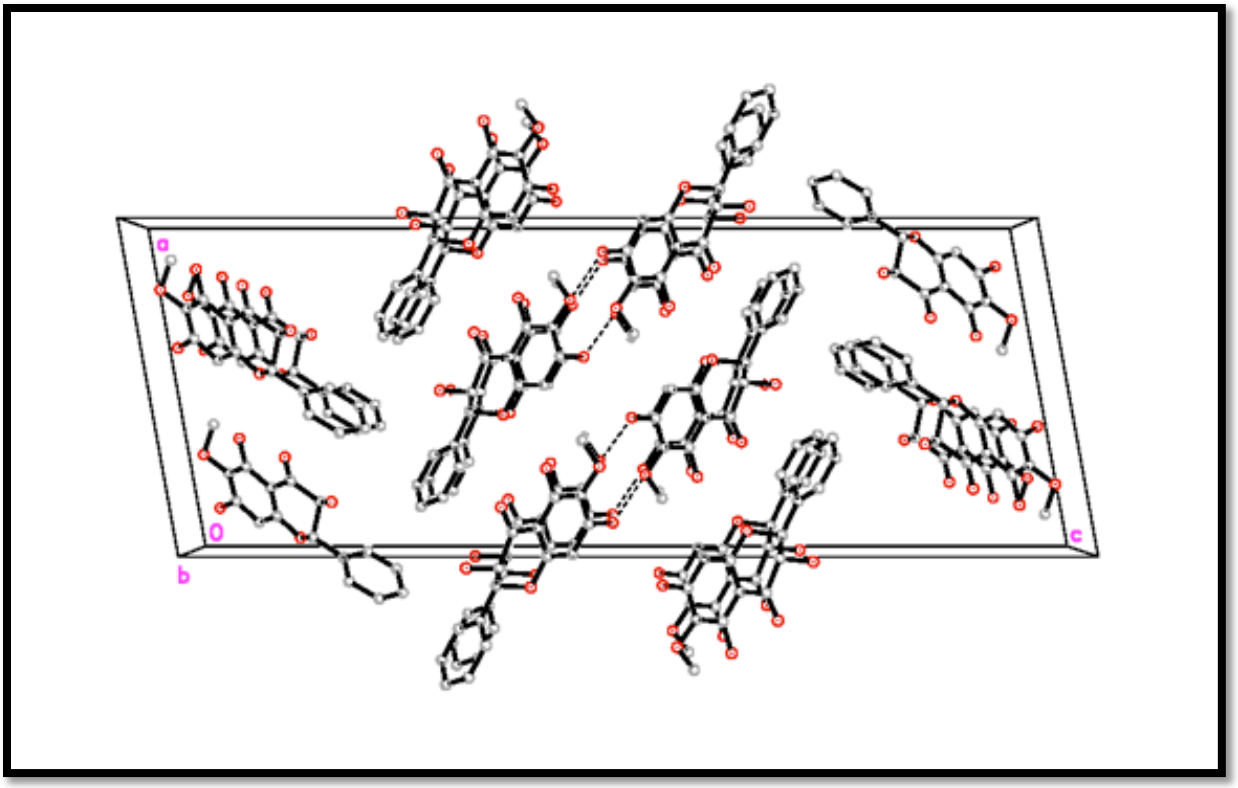

Figure 2.9. Potential hydrogen bonding of 3,5,7-trihydroxy-6-methoxyflavanone identified using x-ray crystallography.

Crystal Data for 3,5,7-trihydroxy-6-methoxyflavanone (stereoisomer of Y):

$C_{16}H_{16}O_7$, M =320.29, triclinic, a = 7.4815(8) Å, b = 9.7111(11) Å, c = 10.6107(12) Å, α = 76.6330(10)°, β = 72.8300(10)°, γ = 87.6590(10)°, V = 716.28(14) Å3 , T = 173.15, space group P-1 (no. 2), Z = 2, μ(MoKα) = 0.118, 11802 reflections measured, 2613 unique (Rint = 0.0309) which were used in all calculations. The final wR2 was 0.2258 (all data) and R1 was 0.1079 (>2sigma(I)).

One single crystal was also used for the identification of a stereoisomer of 3,5,7-trihydroxy-6-methoxyflavanone (Figure 2.10). The only difference between the compounds in Figure 2.8 and 2.10 is disorder at the C1 and C2 atom positions and a water molecule of co-crystallization from the solvent. Thus, the bonds at C1 and C2 are the same in both stereoisomers, but have different configurations. Despite being stereoisomers, the crystals used for identification appeared differently when compared.

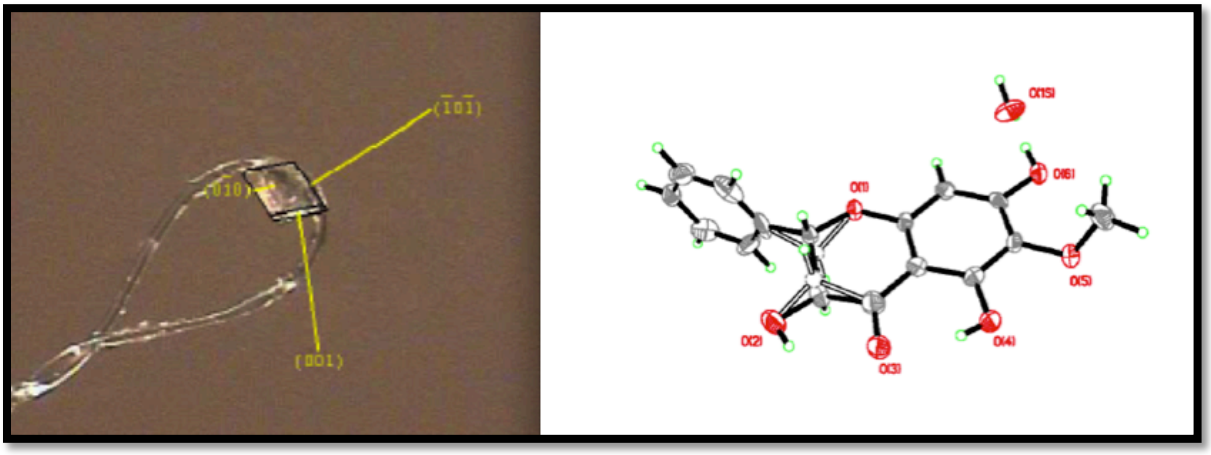

Figure 2.10. Single crystal used for x-ray crystallography analysis and the corresponding structure of the stereoisomer, 3,5,7-trihydroxy-6-methoxyflavanone.

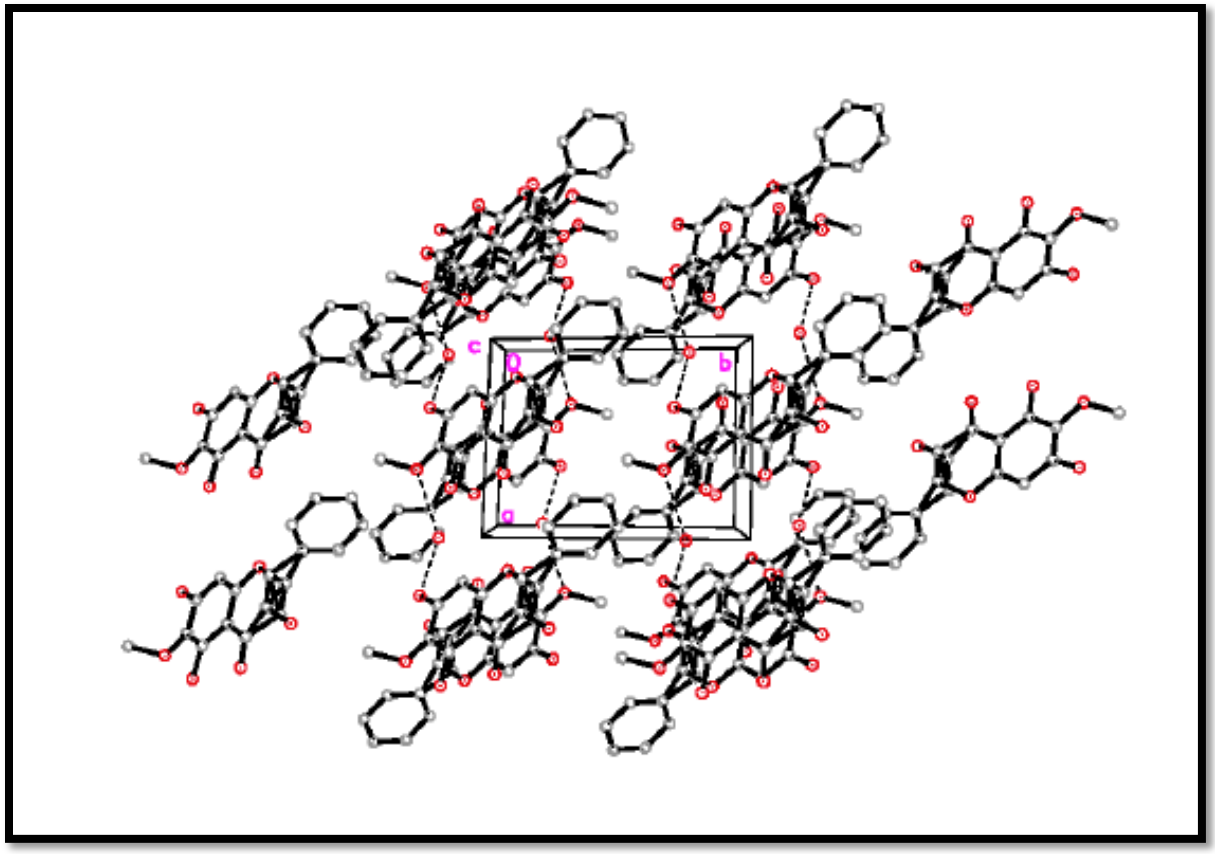

Figure 2.11. Potential hydrogen bonding of 3,5,7-trihydroxy-6-methoxyflavanone (stereoisomer) identified using x-ray crystallography.

Crystals were also formed when leaves were placed in 80% acetone and extracted for 3 or more days. The crystals were collected from the crude extract of *P. maackii leaves* and one suitable crystal was isolated and used for the identification of (*S*)-5,7-dihydroxy-8-methoxy-2-phenyl chroman-4-one (Figure 2.12). This flavonoid is commonly called dihydrowogonin. Hydrogen-bonding properties were also identified in dihydrowogonin molecules (Figure 2.13).

Crystal Data for (S)-5,7-dihydroxy-8-methoxy-2-phenyl chroman-4-one: $C_{17.75}H_{16}O_5$, M =309.30, monoclinic, a = 9.74520(10) Å, b = 23.7471(3) Å, c = 13.3595(2) Å, β = 104.4600(10)°, V = 2993.72(7) $Å^3$, T = 172.99, space group $P2_1$ (no. 4), Z = 8, μ() = 0.835, 23919 reflections measured, 9001 unique (R_{int} = 0.0496) which were used in all calculations. The final wR_2 was 0.1824 (all data) and R_1 was 0.0609 (I > 2\s(I)).

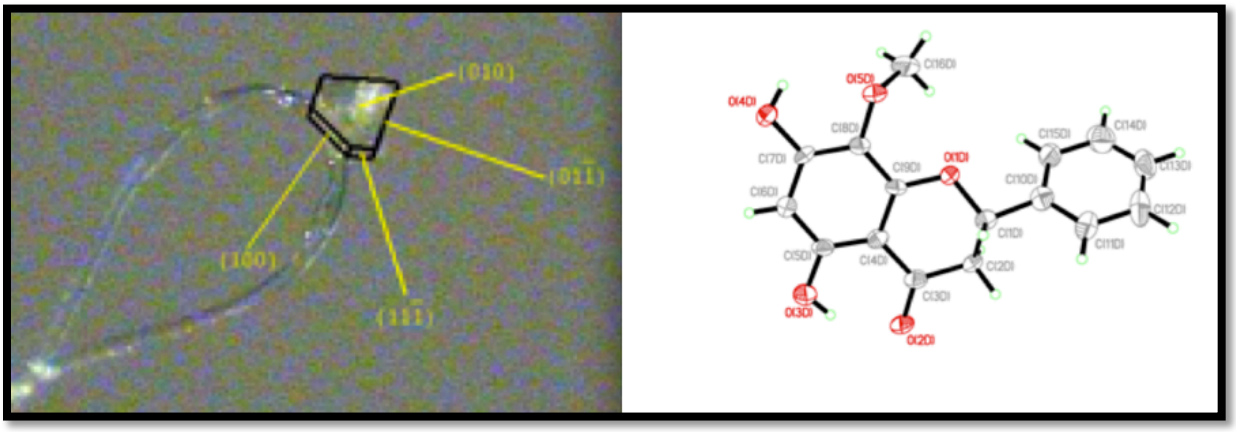

Figure 2.12. Single crystal used for x-ray crystallography analysis and the corresponding structure of (*S*)-5,7-dihydroxy-8-methoxy-2-phenyl chroman-4-one.

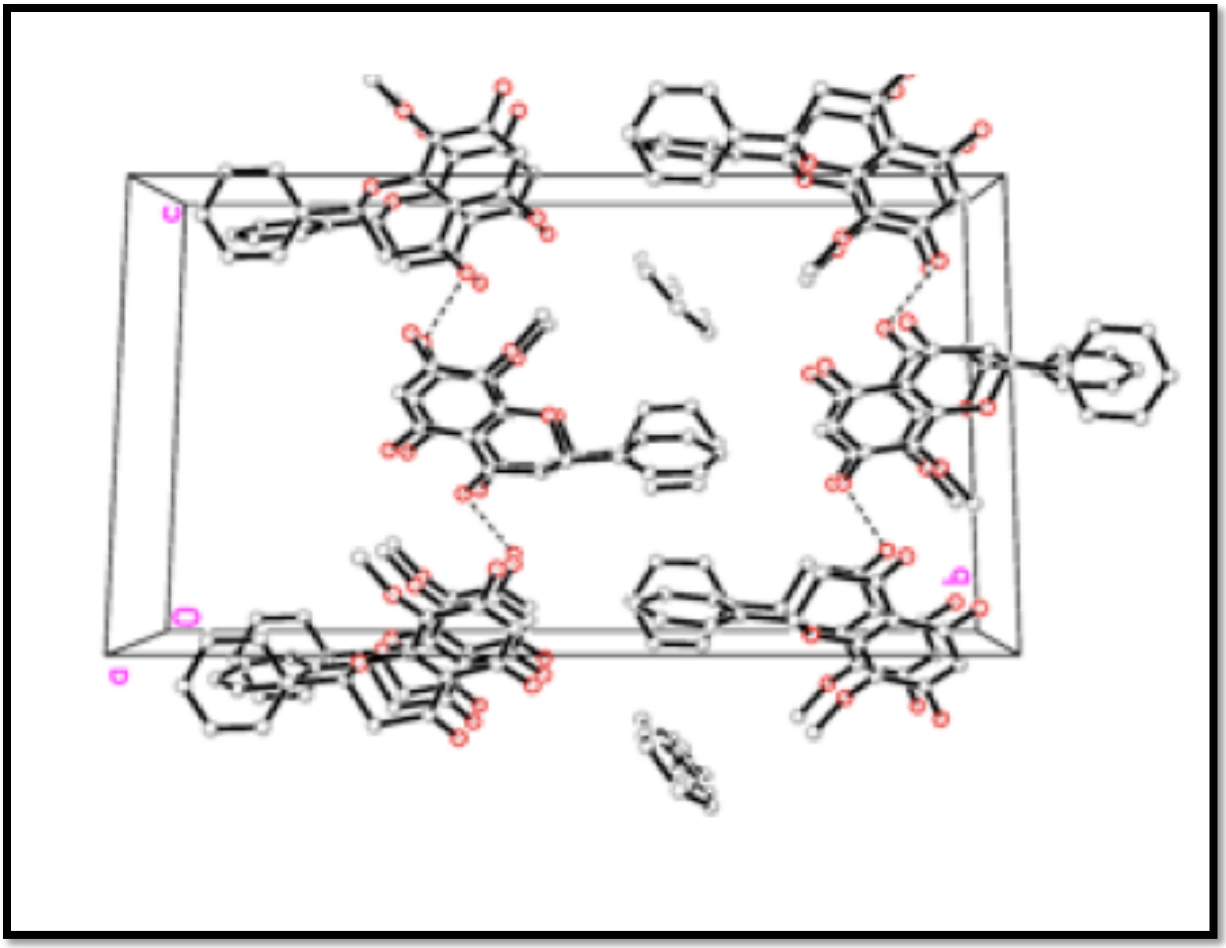

Figure 2.13. Potential hydrogen bonding of (*S*)-5,7-dihydroxy-8-methoxy-2-phenyl chroman-4-one identified using x-ray crystallography.

X-ray crystallography identified the structures of two unknown compounds from crude *P. maackii* periderm extract. Samples containing alnusin (compound X) and alnustinol (compound Y) were analyzed by TLC and were screened for antifungal activity. Samples of alnusin and alnustinol were compared under UV light (365 nm). Based on the presence of additional bands, the samples containing alnusin and alnustinol were only partly purified. Both of these fractions contained more alnusin, but the latter sample named alnustinol contained both alnusin and alnustinol in more comparable amounts.

After The TLC plates were compared under UV light, the same plates were used for the bioassay detection of antifungal compounds. TLC plates were sprayed with a dense *C. cucumerinum* spore and mycelial suspension. Both flavonoids showed antifungal activity; white areas on the TLC plate are a result of growth inhibition of *C. cucumerinum*, indicating the presence of antifungal compounds (Figure 2.14).

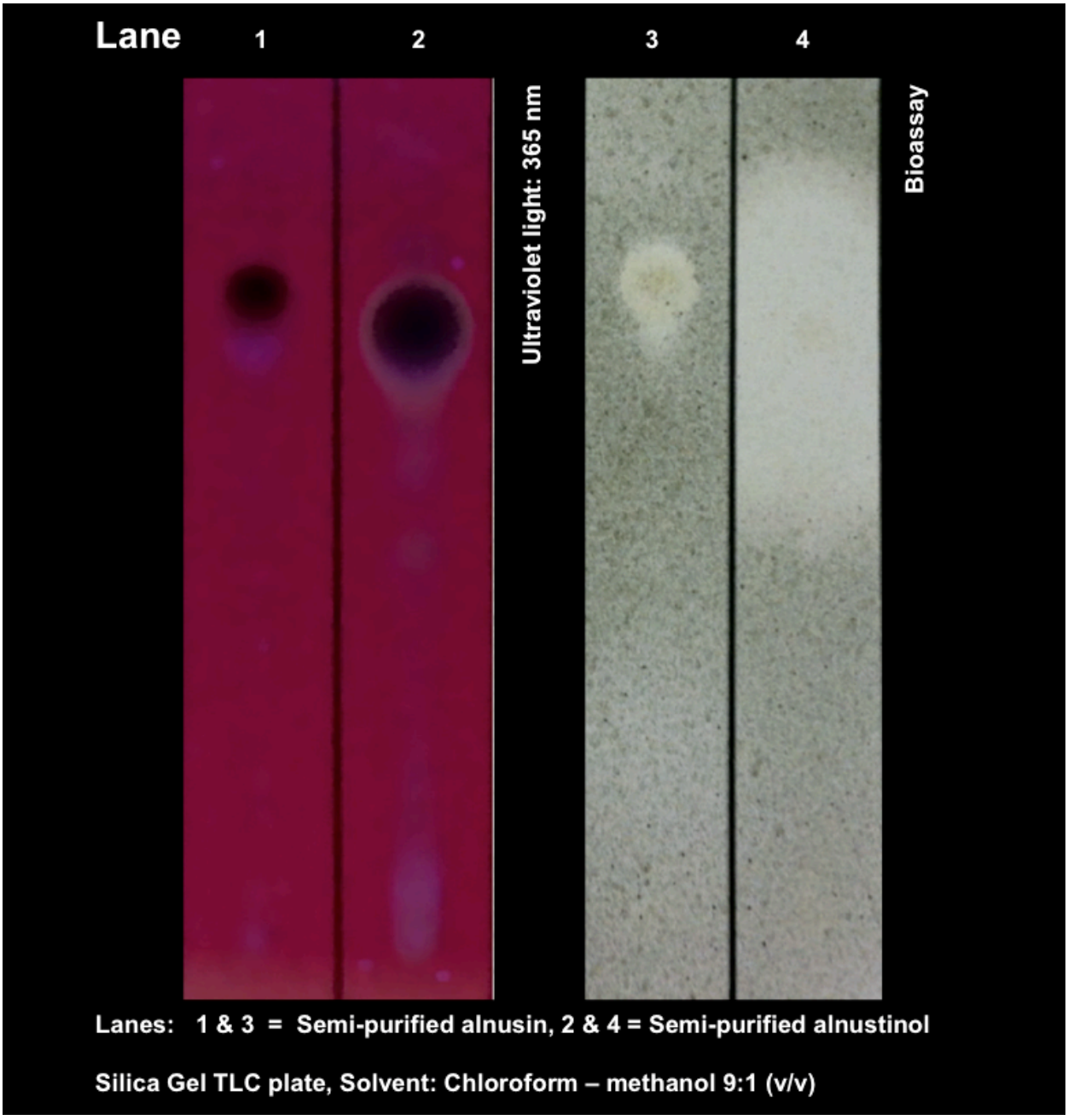

Figure 2.14. UV detection (365 nm) and bioassay of semi-purified fractions both containing alnusin (195mg/mL 80% MeOH), approximately 80% pure on left lane; right lane contains approximately 40% alnustinol (140mg/mL 80% MeOH).

To visualize which compounds crystallized, the crystals collected during the extraction process were dissolved in 100% methanol for TLC analysis. Based on UV light (365 nm) detection, most all of the compounds contained in the crude *P. maackii* periderm extract were capable of forming crystals. The crystals that were collected during the extraction process (lane 1) were compared in Figure 2.15 to the compounds present in the crude periderm extract (lane 2). Bioassay results indicated that the antimicrobial activity was significant in both samples of crystal extract (lane 3) and crude periderm extract (lane 4) (Figure 2.15).

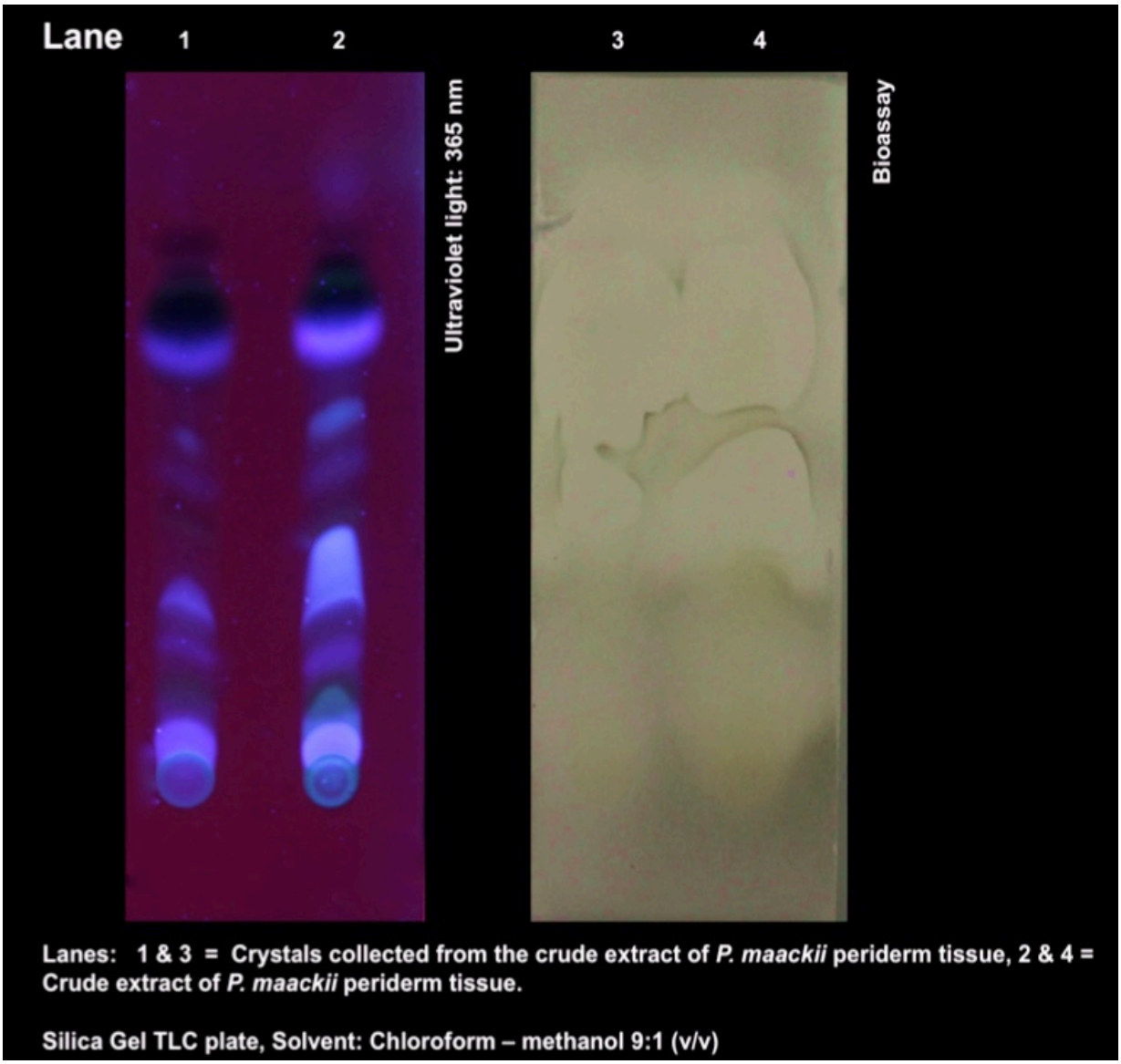

Figure 2.15. Most of the compounds, including antimicrobial metabolites, present in the crude periderm extract of *P. maackii* were able to form crystals.

The bands of compounds under UV light (365 nm) are shown in the top TLC plate with the bioassay results shown in the bottom plate in Figure 2.16. A black and white filter was applied to the bioassay to better visualize the antifungal regions. Methanol extract from the crystals was applied in lane 1, crude leaf extract was applied in lane 2 and the crude extract of *P. maackii* buds was applied in lane 3. All three samples contained antifungal compounds, which were detectable under long-wave UV light. The extract from *P. maackii* leaves appeared to contain antifungal compounds with higher R_f values, indicating that some of the compounds are considerably non-polar.

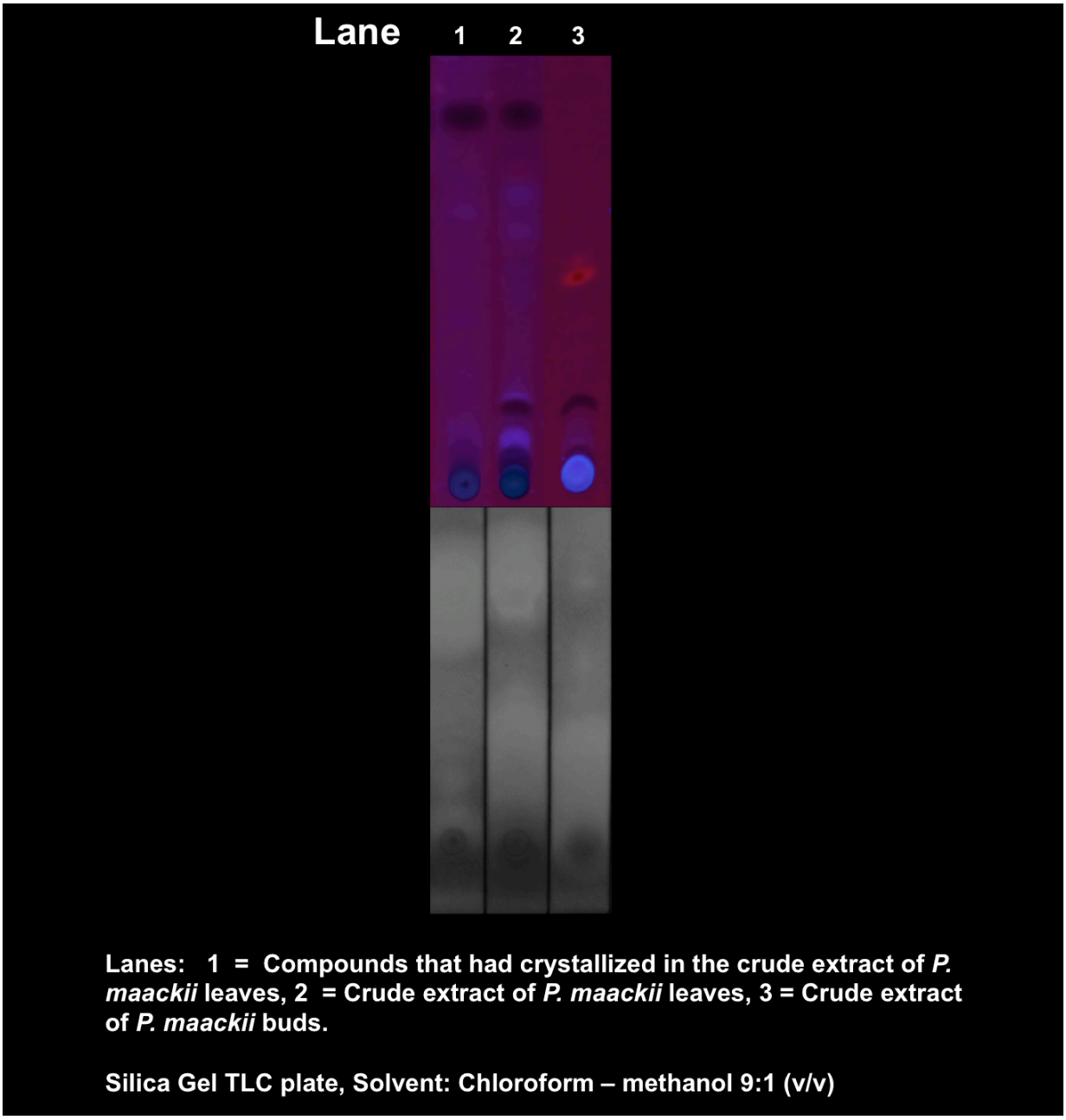

Figure 2.16. Antimicrobial compounds present in the leaves and buds of *P. maackii*.

Some of the compounds took on crystalline form while in the 80% acetone crude extract of *P. maackii* leaves. The crystals were dissolved in methanol and applied in 5, 10 and 15 µL in the left 3 lanes and 5, 10 and 15 µL of crude extract from leaves was applied in the right three lanes of the TLC plate shown in Figure 2.17. Dihydrowogonin would be expected to have similar chemical properties to that of alnusin and alnustinol because of their similar substituent patterns. Flavanones with a 5-OH group lacking B-ring hydroxyl groups have been documented to appear deep purple under UV (Mabry et al.,1970). Further purification of the extract from leaves is needed to identify the exact R_f value and to determine if there is any biological activity of dihydrowogonin. The expected R_f value (shown in red box) is based on migration values similar to alnusin and alnustinol.

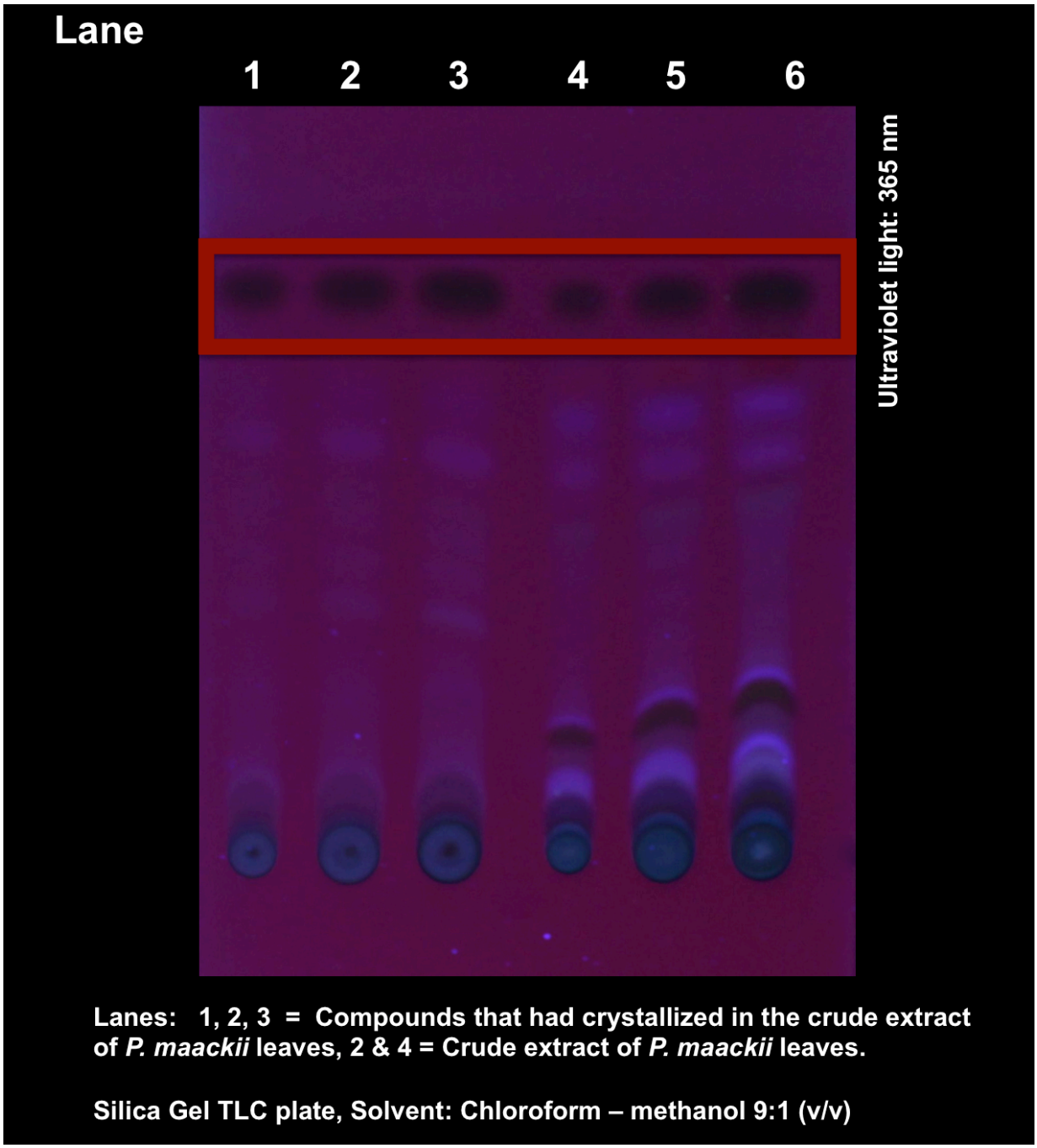

Figure 2.17. 5, 10 and 15 µL of crystal extract containing dihydrowogonin (left) compared under UV light (365 nm) to the crude extract (right) of *P. maackii* leaves (40mg/mL 80% acetone). Estimated R_f value of dihydrowogonin is in the red box.

Discussion

This is the first report of alnusin and alnustinol in the genus *Prunus*. This is also the first report of crystallization and antimicrobial activity for both compounds. Alnusin and alnustinol were first isolated from the male flower of *Alnus sieboldiana*, but not from the closely related species *Alnus pendula* (Suga et al., 1972). Both compounds were identified later from the leaf and stem resin produced by *Baccharis bigelovii* (Arriaga-Giner, 1986) and *Chromolaena chaslaea*. Alnusin has been identified in trace amounts in *Gnaphalium microcephalum* and from the leaf exudates of *Anaphalis margaritacea* (Wollenweber et al., 1996). Alnustinol has also been reported in *Cassinia quinquefaria* (Wollenweber, 1993). Alnusin and alnustinol are somewhat unique with 6-methoxylation and lack of B- ring substitution (Asakawa, 1971).

Numerous flavonoids and other phenolic compounds have been identified in various tissues of *Prunus* species. The leaves of *Prunus persica* contain various phenolic compounds including prunasin and amygdalin and the bark was reported to contain persicogenin, naringenin, aromadendrin, and eriodictyol (Backheet et al., 2003). Genistin, prunin, isosakuranin, chrysin-7-glucoside, and dihydrowogonin-7-glucoside were isolated from a *Prunus* heterograft, *Prunus avium* as the scion and *Prunus cerasus* as the rootstock. These compounds were found to move from their original compartments and were concentrated in the scion, which may result in slight or delayed incompatibility (Feucht & Treutter, 1991). Dihydrowogonin has been isolated from *Adenostoma sparsifoliaum* and from the heartwood and bud exudate of *Prunus* species (Wollenweber, 1996).

Prunetin 5-glucoside, tectochrysin 5-glucoside and pinostrobin 5-*β*-D-glucoside distinguish *P. cerasus* from *P. avium*. Genistein 5- glucoside (Geibel et al., 1990), apigenin 5- glucoside, genkwanin 5- glucoside, and neosakuranin are minor components of both species (Geibel & Feucht, 1991). *Prunus cerasus* is more resistant to the perennial canker pathogen *Cytospora persoonii*, in comparison to the susceptibility of *P. avium*. Flavonoid aglycones are present in trace amounts in unwounded bark of *Prunus* spp. suggesting that hydrolysis of flavonoid aglycones from their glucosides may be involved in resistance. Stock solutions of naringenin (2.5 mM) and chrysin (1.0 – 2.5 mM) inhibited the growth of *C. persoonii* (Geibel, 1995).

Two stereoisomers of alnustinol were identified in the periderm of *P. maackii*. The biological significance of stereoisomers needs to be further investigated. Stereoisomers have the same chemical bonds, but different configurations, and cannot be interconverted without breaking covalent bonds (Nelson et al., 2008). Hydrogen bonding involves electrostatic interactions between electronegative atoms, which involve weaker interactions compared to covalent bonding. Further research is needed to determine if hydrogen bonding has any influence on the biological activity of flavonoids.

Phenols that form intermolecular hydrogen bonds are typically solid at room temperature (Vermerris & Nicholson, 2007), which allows for crystalline solid samples. Based on comparing experimental data, the periderm amended-medium from *P. maackii* (24+ hours) that contained crystals on the surface had slightly less antimicrobial activity compared to freshly made medium that did not have crystals. These results

suggest that the compounds crystallized out of the agar, which could result in a loss of available compound.

The primary metabolite phenylalanine serves as a precursor to flavonoids found in plant tissues including dihydroflavonols, flavones, flavanones, and flavonols. Phenylalanine ammonia lyase (PAL) is found in the parenchyma cells of many plants and catalyzes the first step of flavonoid biosynthesis (Seigler, 2012).
Chalcone isomerase requires naringenin chalcone as a substrate for the formation of the flavanone naringenin, which leads to the formation of dihydroflavonols, flavonols and flavan-3,4-diols (Figure 2.19). Additional examples of possible flavonoids that contain B-ring substituents include kaempferol (flavonols), apigenin (flavone) and dihydroquercetin (dihydroflavonols). The biosynthesis of most flavonoids involves *p*-coumaric acid derivatives that are hydroxylated at the 4'-position of the B-ring. Those that lack this hydroxylation pattern on the B-ring are comparatively rare (Seigler, 2012). The possible phenylpropanoid pathway that may be found in *P. maackii* likely involves a variety of secondary metabolites lacking B-ring substituents. The lack of B-ring substituents is one unique characteristic of the flavonoids contained in *P. maackii* periderm tissue indicating that it is likely that the phenylpropanoid pathway includes derivatives from cinnamic acid (Figure 2.18). The absence of B-ring substituents in flavonoids, from crude *P. maackii* periderm extract, may influence antimicrobial properties.

The biosynthetic pathway of flavonoids lacking B-ring substituents, including alnusin and alnustinol, is predicted to involve pinobanksin and pinocembrin derivatives with a cinnamic acid origin (Figure 2.20). These particular flavonoids have two hydroxylations on the A-ring, without B-ring substitutions, and therefore are not derived from the common *p*-coumaric acid precursor and are considered comparatively rare. Cinnamic acid and cinnamoyl-CoA give rise to flavonoids lacking B-ring substitutions. Pinocembrin is a known antifungal flavanone found in leaf glands of cottonwood (Shain & Miller, 1982). Pinobanksin also lacks B-ring substituents and may be an additional precursor in the biosynthesis of alnusin and alnustinol. Additional identification of structures in the crude extract would give insight to which genes and enzymes are required to synthesize antimicrobial flavonoids.

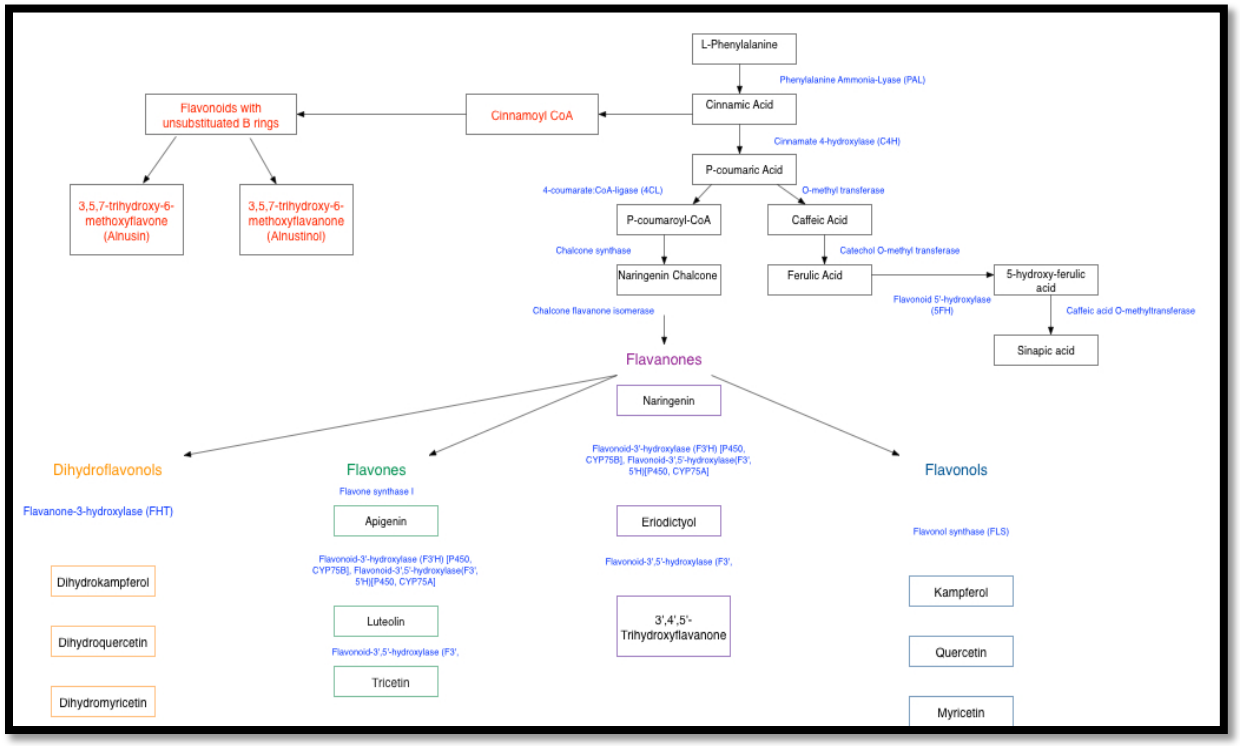

Figure 2.18. Diagram of the possible phenylpropanoid pathway found in *P. maackii.*

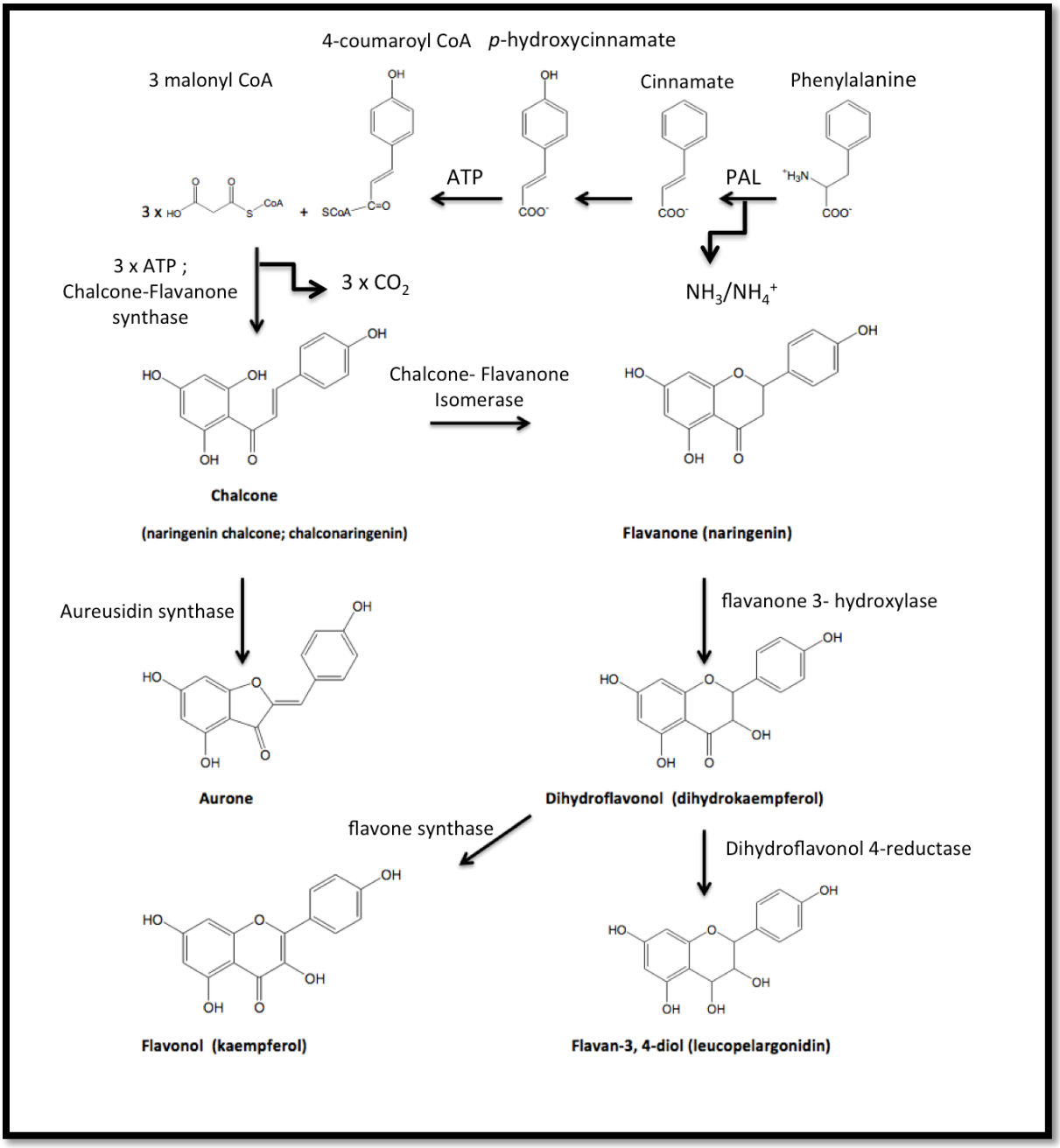

Figure 2.19. Diagram of possible biosynthetic pathways of flavonoids containing B-ring substituents.

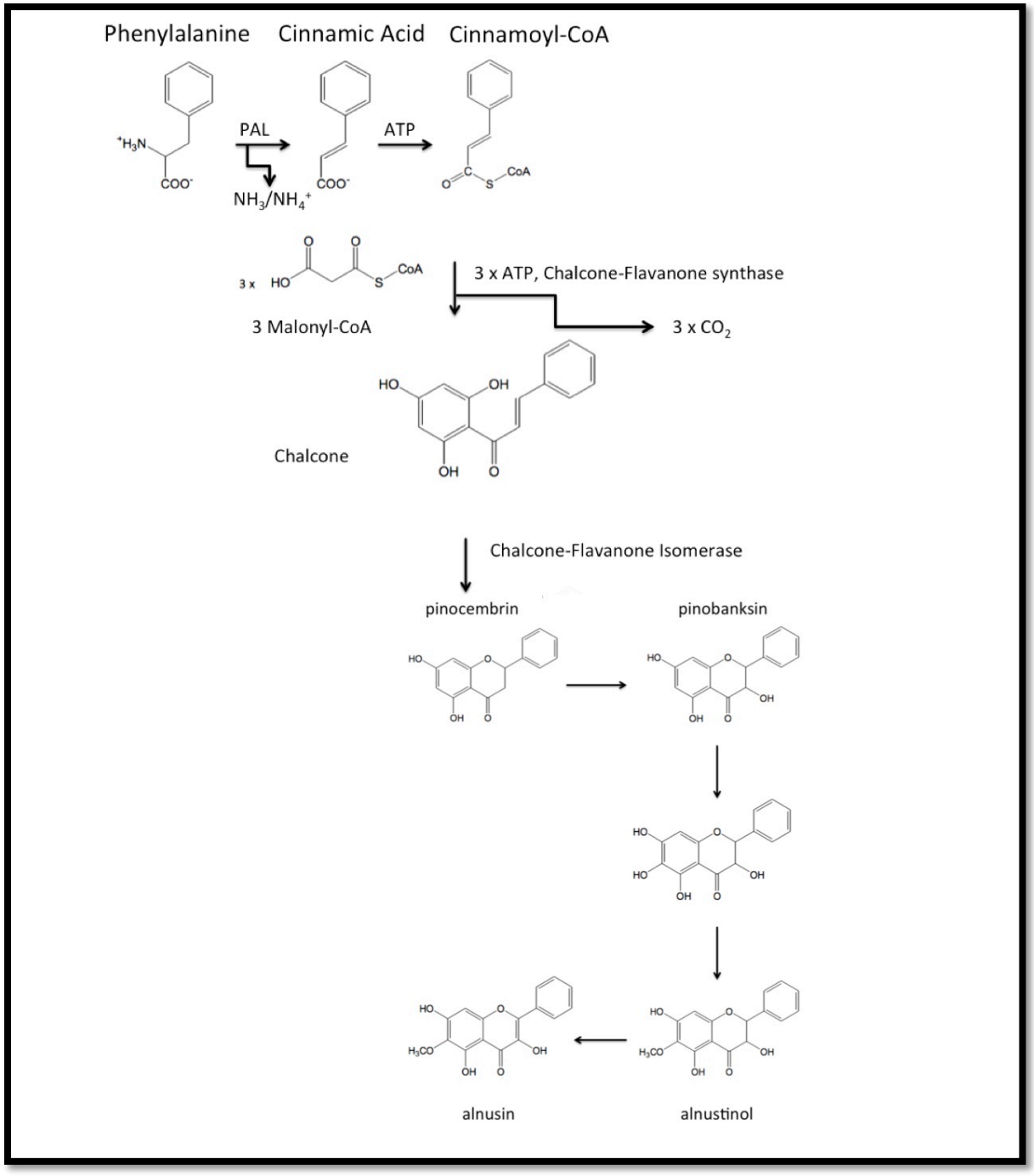

Figure 2.20. Diagram of the possible biosynthesis of alnustinol and alnusin.

The primary goal of future research will be to elucidate the gene expression correlated with cinnamoyl-CoA-ligase, flavonoid-6-hydroxylases and flavonoid-6-methoxylases. Additionally, further characterization of enzymes is needed. Enzymatic modifications are responsible for the conversion of different classes of flavonoids. The correlation of gene expression and enzymes such as flavone synthase, flavanol synthase, flavanone reductase, and dioxygenase will help elucidate the general biosynthetic pathway of flavonoids found in *P. maackii*. Based on TLC bioassays, there are several other antifungal compounds present in various *P. maackii* tissues that remain unidentified.

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CHAPTER THREE

THE ANTIMICROBIAL SPECTRUM OF PERIDERM TISSUE FROM *PRUNUS MAACKII*

ABSTRACT

The periderm of *Prunus maackii* contains compounds that are inhibitory to the growth of *Armillaria ostoyae*. Periderm from young trees and older tissues collected from mature trees were screened for antimicrobial properties. To determine if this activity is effective against other plant pathogens, additional *Armillaria* species, other true fungi and one Oomycete were tested. Yeast-malt-peptone-glucose (YMPG) medium supplemented with *P. maackii* periderm was used to test for antifungal activity against *A. mellea, A. gemina* and *A. gallica* in addition to *A. ostoyae*. Growth of all *Armillaria* species screened was inhibited. The periderm was also antimicrobial to several other plant pathogens including *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. A series of bioassays were performed to examine the range and potency of antimicrobial activity in the periderm. A dose-response of *P. maackii* periderm was examined to further characterize the threshold of antifungal activity. The plugs containing mycelia of *A. ostoyae* (warren) previously grown on Petri dishes containing periderm were transferred to plain YMPG-nutrient medium to determine whether the periderm amended-medium was fungistatic or fungitoxic. These tests indicated that the medium was fungistatic at a concentration of 12 g periderm/L YMPG and displayed fungitoxic properties. Preliminary bioassays using leaves and mulch from *P. maackii* also detected antimicrobial properties.

Introduction

Plants have developed a variety of defense mechanisms in response to pathogens. Potential pathogens may trigger the biosynthesis of antimicrobial compounds, pathogenesis-related (PR) proteins, and reactive oxygen species, among other defense responses (Ahuja et al., 2012). Antimicrobial compounds that are constitutively present, or produced from pre-existing precursors, are known as phytoanticipins (VanEtten et al., 1994). *R*-genes play a role in the mediation of disease resistance levels by controlling the amount of phytoalexins produced and the speed at which they accumulate (Hammerschmidt, 1999). Phytoalexins are low molecular weight antimicrobial compounds that accumulate in response to pathogen infection (Kuć, 1995). Phytoalexins must be present at the right time and place to be effective (Hammerschmidt, 2011).

The plant family Rosaceae is composed of approximately 90 genera and 3,000 species (Potter et al., 2007) and includes apricot, sweet & sour cherry, almond, apple, blackberry, rose, and strawberry (Hummer & Janick, 2009). Plants belonging to the family Rosaceae have been studied extensively using targeted analytical assays, to investigate the secondary metabolites and corresponding pathways (Shulaev et al., 2008). Flavonols are found in high concentrations in cherry, such as quercetins and kaempferols (Ogah et al., 2014). Herniarin has been identified in the leaves of *P. mahaleb, P. pensylvanica, P. maximowiczii*, and *P. maackii*. Herniarin has also been identified in the bark of *P. mahaleb* and *P. pensylvanica*. The bark of *P. verecunda*, *P. cyclamina* and *P. maackii* contained scopolin (Santamour & Riedel, 1994). Flavonoids identified from the bark of *P. cerasus* include naringenin, prunin, sakuranetin, sakuranin,

prunetin, and tectochrysin, all of which slowed mycelial growth of *Cytospora persoonii* at 1 mM concentration (Geibel, 1995). Isosakuranetin, dihydrokaempferide and naringenin have been isolated from the heartwood extract of *P. domestica* (Parmar et al., 1992). The wood of *P. verecunda* contained genistein, prunetin, pinocembrin, and taxifolin (Hasegawa & Shirato, 1957). Eriodictyol, genkwanin and sakuranin have been isolated from *P. donarium* var. *spontanea* (Hasegawa & Shirato, 1955).

Scopoletin, herniarin, taxifolin, and naringenin are some phenolics that have been classified as antimicrobial. The isoflavones genistein is antifungal and also inhibits soybean lipase and peroxidase activity. The flavanone pinocembrin is antimicrobial to *Candida albicans*, *Saccharomyces cerevisiae* and *Cryptococcus neoformans* at concentrations of .0001 mg/ml - .003 mg/ml and to *Bacillus subtilis* at concentrations of .003 mg/ml. The flavanone eriodictyol has antibacterial activity against *Pseudomonas maltophilia* and *Enterobacter cloacae* (Harborne & Baxter, 1993). An infection site caused by *Cochliobolus heterostrophus* on resistant maize cultivar B73 Htrhm results in the accumulation of anthocyanin pigments in the margin area of lesions. This accumulation of anthocyanins is absent in the susceptible genotype (Ht) and therefore may play a role in microbial defense (Nicholson & Wood, 2001).

The objectives of this research include further characterizing the antifungal compounds found in various *P. maackii* tissues. Samples of periderm tissue were taken from young trees and older tissues were collected from mature trees. These samples will be screened for constitutively present antimicrobial compounds to further support the presence of phytoanticipins. Screening other *Armillaria* species in addition to non-host pathogens will further characterize the spectrum of antimicrobial properties. The

threshold of fungistatic and fungitoxic activity will be characterized through dose response experiments. Petri dishes containing *P. maackii* mulch and leaves in YMPG nutrient medium were inoculated with non-host pathogens as a preliminary screen for additional antimicrobial compounds.

Materials and Methods

Twigs from young (< 5 years) *P. maackii* trees, purchased from Nature Hills Nursery, Omaha, NE, were harvested as needed from potted trees (Baccto® premium potting mix) grown in the MSU greenhouse courtyard. Twigs (approx. 4 cm in diameter) from *P. maackii, P. serotina* and *P. mahaleb* were collected for periderm extraction from the NW Michigan Horticulture Research Station near Traverse City, MI. Twigs collected from mature trees were ground using a chipper shredder to produce mulch. Older periderm tissue was also collected from a mature *P. maackii* tree on Michigan State University's campus, accession number 97P028*02. Samples were kindly provided by Dr. Frank Telewski. Leaves were collected from young *P. maackii* trees on June 4th, 2013 and were stored at -20˚C.

Periderm tissue was removed with a razor blade from *P. maackii* branches. Periderm and leaves were ground to a fine powder using a Krups® coffee grinder. Mature twigs were ground using a chipper-shredder to produce mulch. These tissue types were added to the YMPG medium with a final concentration of 12 g of tissue/L of YMPG. This periderm-amended medium was autoclaved at standard time (15 minutes/L). The medium was poured into the Petri dishes (60 x 15 mm) and then

inoculated with *Armillaria* species and non-host pathogens, within 24 hours of preparation, with a 4.8 mm cork borer. The cultures were allowed to grow for three weeks for *Armillaria* species and one week for non-host pathogens. Radial growth, in cm, was measured and recorded.

To further characterize the antifungal activity present in the periderm tissue, this bioassay used samples containing decreased amounts of periderm. The first dose-response experiment had samples containing an initial 25%, 50% and 75% reduction in grams of *P. maackii* periderm per L of YMPG. The second dose-response experiment starts with 12 g periderm/L YMPG and ends with 2 g periderm/L YMPG, consisting of 0.5-gram/L YMPG increments. A third dose-response experiment starts with 12 g/L YMPG and ends with 0.4 g/L YMPG. Petri dishes containing *P. maackii* periderm-amended media were inoculated with *A. ostoyae*. Data were analyzed using Tukey's HSD test, $p = 0.05$.

Armillaria species used for inoculation for growth inhibition screening are shown in Table 3.1. The 35-5 isolate of *A. gemina* was collected from Camel's Hump, VT. The ISRA-5 isolate was collected from a hardwood forest in Ionia County, MI. The collection information for the Kloody T1T isolate of *A. gallica* is undetermined. Three isolates of *A. mellea* were used for inoculation. The M. Thomas isolate was found on plum in Van Buren County, MI. The 97-1 isolate was found on red maple in Provincetown, MA. The third *A. mellea* isolate, 49-5, was collected in the Boston area from an undetermined host. All three isolates of *A. ostoyae* were isolated from tart cherry trees in the Grand Traverse area. The 4-58 isolate was from Grand Traverse County, MI and both CT3T1 and warren originating from Leelanau County, MI.

Media containing periderm from *P. maackii* was inoculated with isolates from four species of *Armillaria: Armillaria ostoyae, A. gemina, A. mellea,* and *A. gallica*. This experiment was repeated three times. Data were analyzed using Tukey's HSD test, p = 0.05.

Table 3.1. *Armillaria* species used to evaluate the antimicrobial activity of *P. maackii* periderm

Species	Isolate ID	Host	Geo. Origin
A. gemina	35-5	Undetermined	Camel's Hump, VT
A. gallica	ISRA-5	Hardwood Forest	Ionia County, MI
	Kloody T1T	Undetermined	Undetermined
A. mellea	M. Thomas	Plum	Van Buren County, MI
	97-1	Red Maple	Provincetown, MA
	49-5	Undetermined	Boston Area
A. ostoyae	4-58	Tart Cherry	Grand Traverse Co, MI
	CT3T1	Tart Cherry	Leelanau County, MI
	Warren	Tart Cherry	Grand Traverse Co, MI

Fungistatic and fungitoxic assays were designed to determine the toxicity of the *P. maackii* periderm-amended medium. The medium containing periderm (12 g/L YMPG) was prepared and inoculated with *Armillaria* species. The cultures were grown for three weeks and the radial growth was recorded. Once the radial growth data were collected, the plugs used to inoculate the periderm amended-medium were transferred to plain YMPG medium. This would determine whether the compounds in *P. maackii* periderm tissue are fungistatic (pathogen is viable) or if it they are fungitoxic (pathogen

is non-viable). This experiment was repeated three times, 10 Petri dishes/species. Data were analyzed using Tukey's HSD test, $p = 0.05$.

The antifungal content was compared between samples from young and mature trees. The young trees were purchased from Nature Hills Nursery, Omaha, NE and immediately planted in Baccto® Premium potting mix. Periderm tissue was collected from five young trees. For each tree, 13 Petri dishes containing periderm-amended medium were inoculated with *A. ostoyae*. This experiment was done once. Data were analyzed using Tukey's HSD test, $p = 0.05$.

Twigs were collected from mature *Prunus* trees grown at the NW Michigan Horticulture Research Station near Traverse City, MI and stored at -20˚C until used for analysis. *Prunus maackii* periderm samples were collected from seven different trees and screened for antimicrobial properties. For each mature tree sample, 18 Petri dishes containing periderm-amended medium were inoculated with *A. ostoyae*. This experiment was done once. Data were analyzed using Tukey's HSD test, $p = 0.05$.

To examine the spectrum of antifungal activity, the periderm-incorporated media was inoculated with several plant pathogens from three different phyla: Basidiomycota, Ascomycota and Oomycota (Table 3.2). The non-host pathogens used to further characterize antimicrobial activity include *Sclerotinia sclerotiorum, Botrytis cinerea, Rhizoctonia solani,* and *Phytophthora capsici.* All of these pathogens have a broad host range. *Cladosporium cucumerinum* and *Colletotrichum orbiculare* are pathogens of cucumber. *Fusarium sambucinum, F. Sambucinum* TBZ-resistant and *Alternaria solani* are pathogens of potato and *Cochliobolus carbonum* is a pathogen of Maize.

Periderm from *P. maackii* was incorporated into YMPG medium (12 grams/L of YMPG) and inoculated with *P. capsici, S. sclerotiorum, F. sambucinum, F. sambucinum* (TBZ-resistant), *R. solani, C. orbiculare,* and *C. cucumerinum*. This experiment was repeated three times, 17 Petri dishes/species were inoculated. Data were analyzed using Tukey's HSD test, $p = 0.05$.

Table 3.2. Pathogens of non-*Prunus* species used to evaluate antimicrobial activity of *P. maackii* periderm.

Phylum	Species	Host
Ascomycota	*Cladosporium cucumerinum*	Cucumber
	Colletotrichum orbiculare	Cucumber
	Fusarium sambucinum	Potato
	F. sambucinum TBZ resistant	Potato
	Sclerotinia sclerotiorum	Broad Host Range
	Alternaria solani	Potato
	Botrytis cinerea	Broad Host Range
	Cochliobolus carbonum	Maize
Basidiomycota	*Rhizoctonia solani*	Broad Host Range
Stramenopiles	*Phytophthora capsici*	Broad Host Range

The ChromaDoc-It TLC imaging system was employed for recording the bioassay results for Petri dishes containing inoculated periderm-amended media. This system contains white light, which was used to take pictures of mycelial growth. Radial growth was recorded, using Photoshop, and analyzed by Tukey's HSD, $p = .05$, in the statistical computing program, R (R Core Team, 2014).

Results

To determine the threshold of antifungal activity, the standard screening concentration for *Armillaria* growth inhibition was reduced to dilute samples containing 6g/L and 3g/L (Figure 3.1). The growth of *A. ostoyae* on media containing the different concentrations of periderm tissue was compared to growth on plain YMPG nutrient medium (control growth). Even at a dilute concentration of 3g/L, the periderm-amended medium was able to significantly inhibit the mycelial growth of *A. ostoyae*. All concentrations were able to significantly inhibit the growth of *A. ostoyae*. Complete growth inhibition was detected at 12 g /L YMPG and significant inhibition was detected at 6 g/L YMPG. Since the 3 g /L still inhibited the growth of *A. ostoyae*, periderm concentrations were reduced further to better identify the threshold of inhibition.

The second dose-response experiment started with the standard concentration of 12 g /L of YMPG and was diluted by 0.50 gram/L, ending with a final concentration of 2 grams/L of YMPG. All of the plugs containing *Armillaria* mycelia were transferred to YMPG medium to see if they were viable after being exposed to *P. maackii* periderm. The medium containing high concentrations of *P. maackii* periderm had fungitoxic properties.

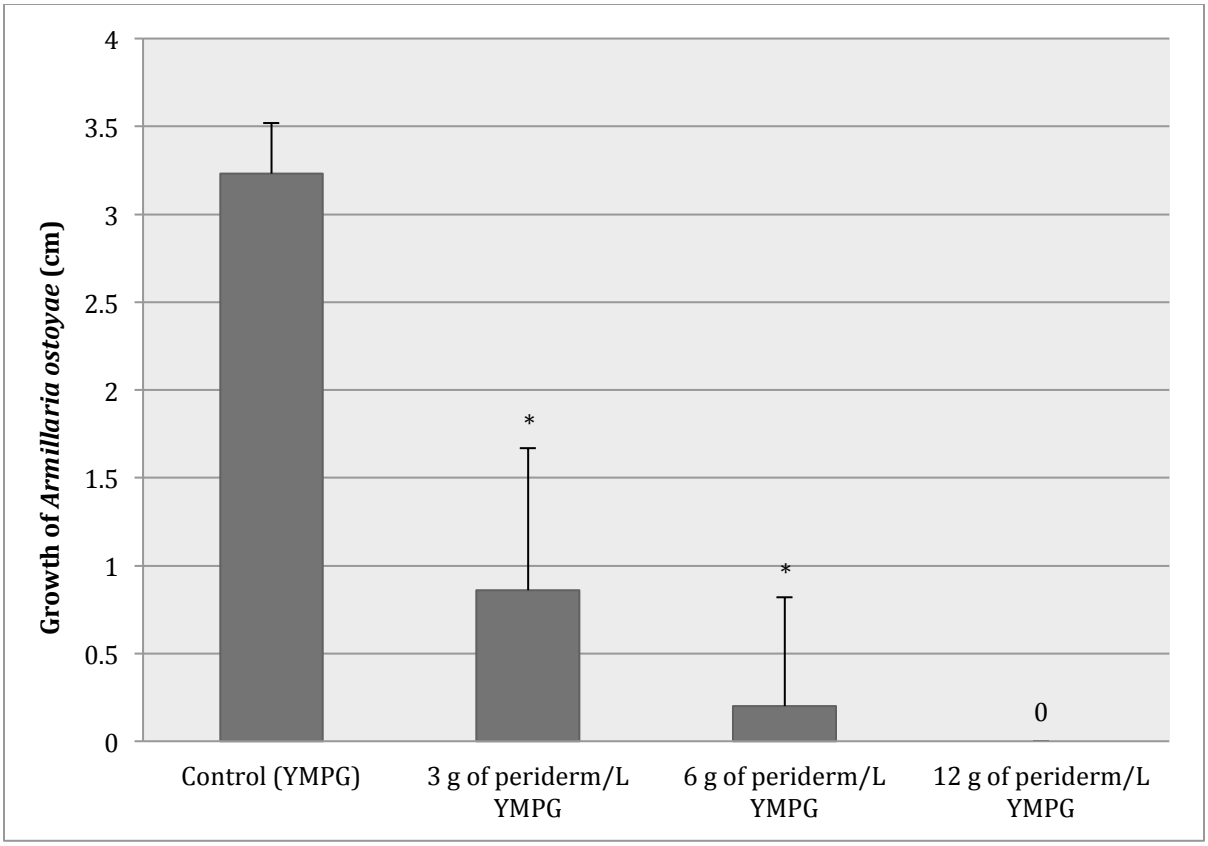

Figure 3.1. Dose-response of *P. maackii* periderm on the growth of *A. ostoyae* (warren isolate).

Table 3.3. Dose-response of *P. maackii* periderm-amended media on the growth and viability of *A. ostoyae* (warren isolate).

Concentration of periderm-amended medium (g/L YMPG)	Average Radial Growth (cm)	Average Viability Percentage (%)
12.00 - 10.00	< 0.50	30
9.80 - 8.00	< 0.50	25
7.80 - 6.00	< 0.50	57
5.80 - 4.00	< 0.50	47
3.80 - 2.60	< 0.50	95
2.40 - 0.60	< 2.50	100
0.40 - 0.20	< or = 3.00	100
Control	3.2	100

To further examine the dose-response of *P. maackii* periderm-amended medium, additional concentrations were screened for the ability to inhibit the growth of *A. ostoyae* (Table 3.3). When YMPG contained 2.60-12.00 g of periderm tissue/L YMPG, the average radial growth of *A. ostoyae* was less than 0.50 cm after three weeks of growth. The threshold of antifungal activity to *A. ostoyae* appears to be around 2.40-2.60 g of periderm tissue/L YMPG.

There was a significant decrease in antifungal activity in periderm-amended media containing less than 2.60g/L YMPG. Media containing 0.40-0.20g/L YMPG had *Armillaria* average radial growth slightly less than that of the control growth on plain YMPG medium. The strength of fungitoxic activity decreased as periderm tissue concentrations decreased. At standard screening concentration, the periderm-amended media was fungitoxic to most *Armillaria* samples, resulting in plugs containing non-viable mycelia.

Percentages of viability were recorded when plugs were transferred to plain YMPG medium. There was an increase in *A. ostoyae* mycelial growth on media containing 2.40 – .60 g of periderm tissue /L YMPG. When the periderm-amended media was diluted to 0.40 – 0.20 g/L, the growth was similar to that of the control. The periderm amended-media was fungitoxic to approximately 70% of the plugs containing *Armillaria* mycelia when grown on media containing 12.00 – 8.00 g/L YMPG. The fungitoxic properties of the periderm decreased to approximately 40% of *A. ostoyae* plugs when inoculated on media containing 7.80 – 4.00 g of periderm/L YMPG. The fungitoxic threshold was detected when plugs containing *Armillaria* mycelia were grown on media containing 3.80 – 2. 60g/L YMPG. This concentration was only fungitoxic to

5% of the isolates; however, fungistatic properties are still detected at this concentration. The average radial mycelial growth of *A. ostoyae* increased when grown on media containing 2.60 - 0.60 g of periderm tissue/L YMPG concentrations.

An additional dose-response experiment was setup to further characterize the threshold of antifungal activity. Similar to the previous dose-response experiment, this experiment started with the standard screening concentration of 12g/L and consisted of 2g incremental decreases, but ends with 0.4g/L YMPG (Figure 3.2). Complete inhibition of *A. ostoyae* growth was detected when the pathogen was grown on periderm-amended media containing 12 – 6.0g/L YMPG, with an exception of a few samples showing very slight growth (less than approximately 0.25 cm).

The growth of *A. ostoyae* was less than 0.25 cm when grown on medium containing 4.0 g/L YMPG. There was significant growth inhibition when the pathogen was grown on medium containing 2.0 g/L, however, average radial growth for this concentration exceeded 1 cm, which is still significantly inhibited compared to control growth. Growth of *A. ostoyae* was comparable to control growth on YMPG when the periderm-amended media contained less than 0.40g/L YMPG. Thus, the threshold for antifungal activity in this dose-response experiment is expected to be between 2.0 – 0.40 g/L YMPG. Complete inhibition was detected when the pathogen was grown on media containing increased amounts of periderm tissue (12 g/L – 4.0 g/L) with the exception of a few plugs of *A. ostoyae* that showed slight viability.

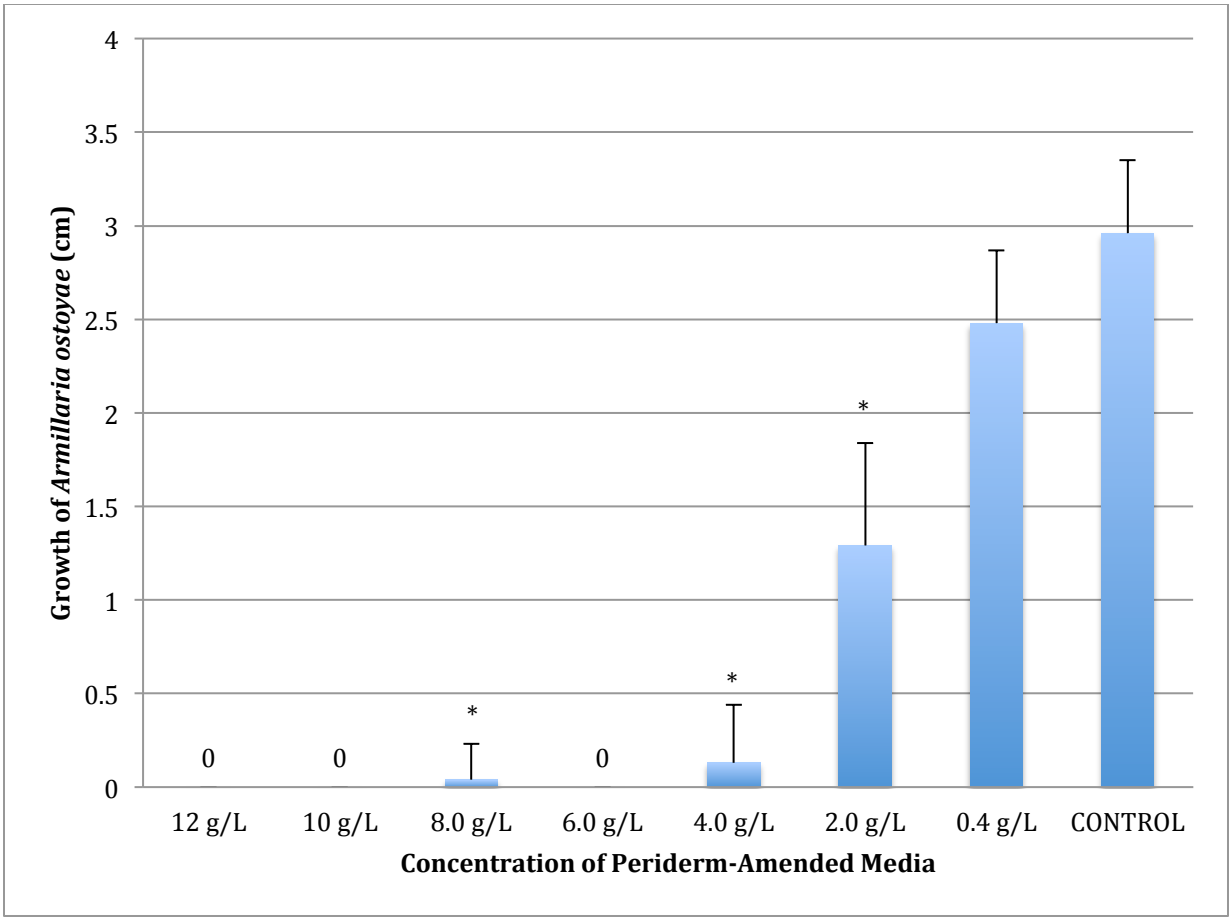

Figure 3.2. Dose-response (12 g – 0.4 g/L) of *P. maackii* periderm on the growth of *A. ostoyae (*warren isolate). Tukey's HSD, $p = 0.05$. * = Significantly different from the control.

To further test the spectrum of antifungal activity, samples containing periderm-incorporated YMPG were inoculated with additional *Armillaria* species. Preliminary screening for the ability of three *Prunus* species to inhibit growth of *A. ostoyae*, warren isolate, showed that *P. maackii* was the only antifungal species (Figure 3.3). This preliminary screen was done using older periderm-amended media. As previously discussed, the age of the media slightly affects toxicity. Since pathogen growth inhibition was only detected when isolates were grown on *P. maackii* periderm-amended media, additional experiments only used *P. maackii* as a positive control and

plain YMPG nutrient-medium as a negative control. Additional screening included 3 isolates of *A. ostoyae*, 1 isolate of *A. gemina*, 3 isolates of *A. mellea*, and 2 isolates of *A. gallica*.

Armillaria ostoyae isolates grown on periderm-amended medium (12 g/L YMPG) were compared to those grown on plain YMPG nutrient-medium. Complete growth inhibition of all *A. ostoyae* isolates was detected when grown on *P. maackii* periderm-amended medium (Figure 3.4). The periderm-amended media was able to significantly inhibit 4-58, CT3T1 and warren isolates of *A. ostoyae*. The growth of *A. gemina*, isolate 35-5, was significantly inhibited when grown on *P. maackii* periderm-amended medium (Figure 3.5). The majority of the samples from all three isolates of *A. mellea* were completely inhibited, with the exception of a few samples showing slight growth. The periderm-amended medium completely inhibited 100% of the samples of isolate 97-1 (Figure 3.6). Most samples of *A. gallica*, isolates ISRA-2 and Kloody T1T, were completely inhibited when grown on the medium containing periderm from *P. maackii* (Figure 3.7). Most plugs containing the isolates were not able to establish any growth on medium containing *P. maackii* periderm.

All *Armillaria* isolates screened were sensitive to the *P. maackii* periderm – amended medium. Isolates were screened for viability after exposure to the periderm. *Armillaria mellea* was the most sensitive to the *P. maackii* periderm-amended medium. The periderm tissue from *P. serotina* stimulated the growth of *A. mellea* and *A. gallica* and periderm from *P. mahaleb* stimulated the growth of *A. ostoyae*, *A. gemina* and *A. gallica*. These results were taken from isolates grown on media that was within a week old. Fresh media was used in Figures 3.4 – 3.7.

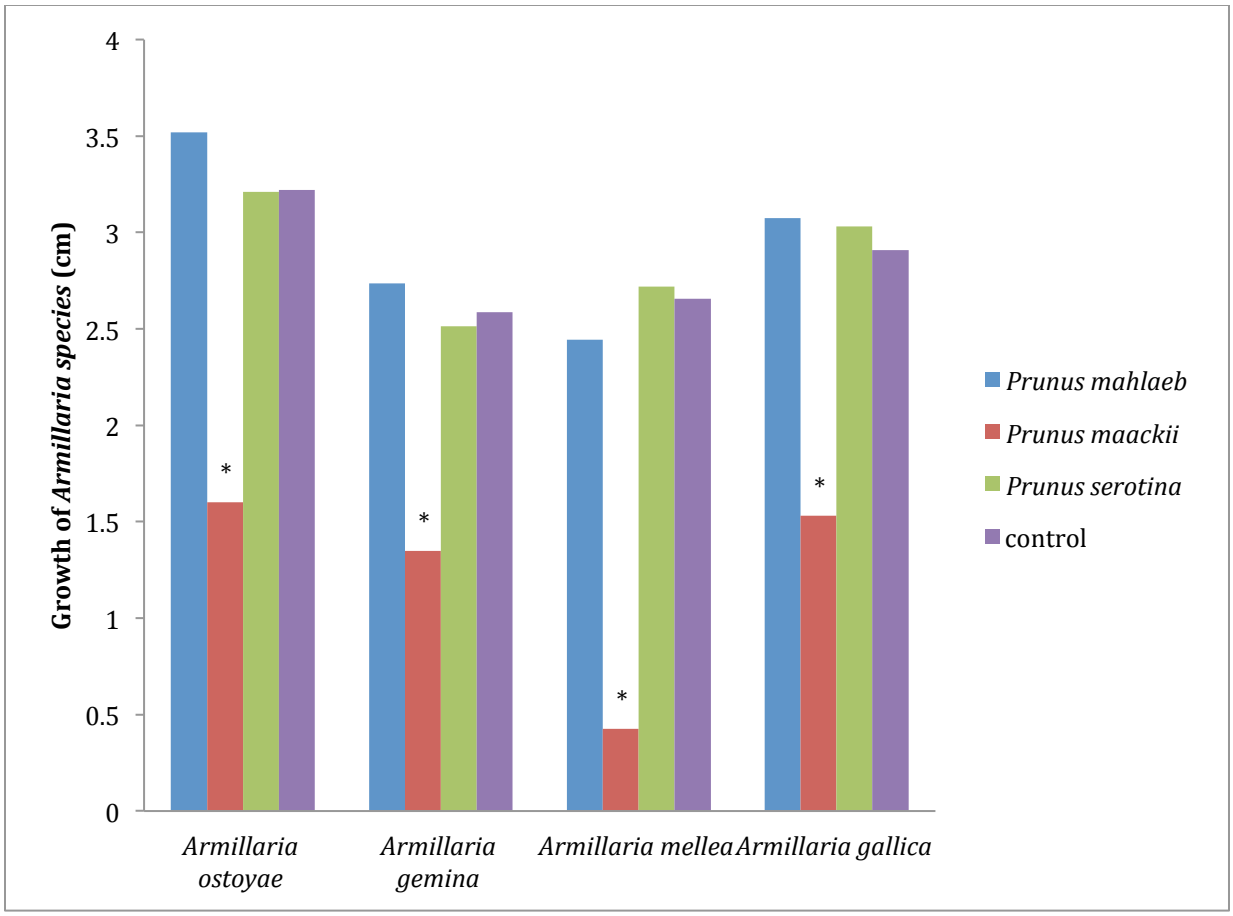

Figure 3.3. The growth of *A. ostoyae* (warren), *A. gemina* (35-5), *A. mellea* (49-5), and *A. gallica* (ISRA-5) on periderm-amended media (12 g/L YMPG). * = Significantly different from the control. Tukey's HSD, $p = 0.05$.

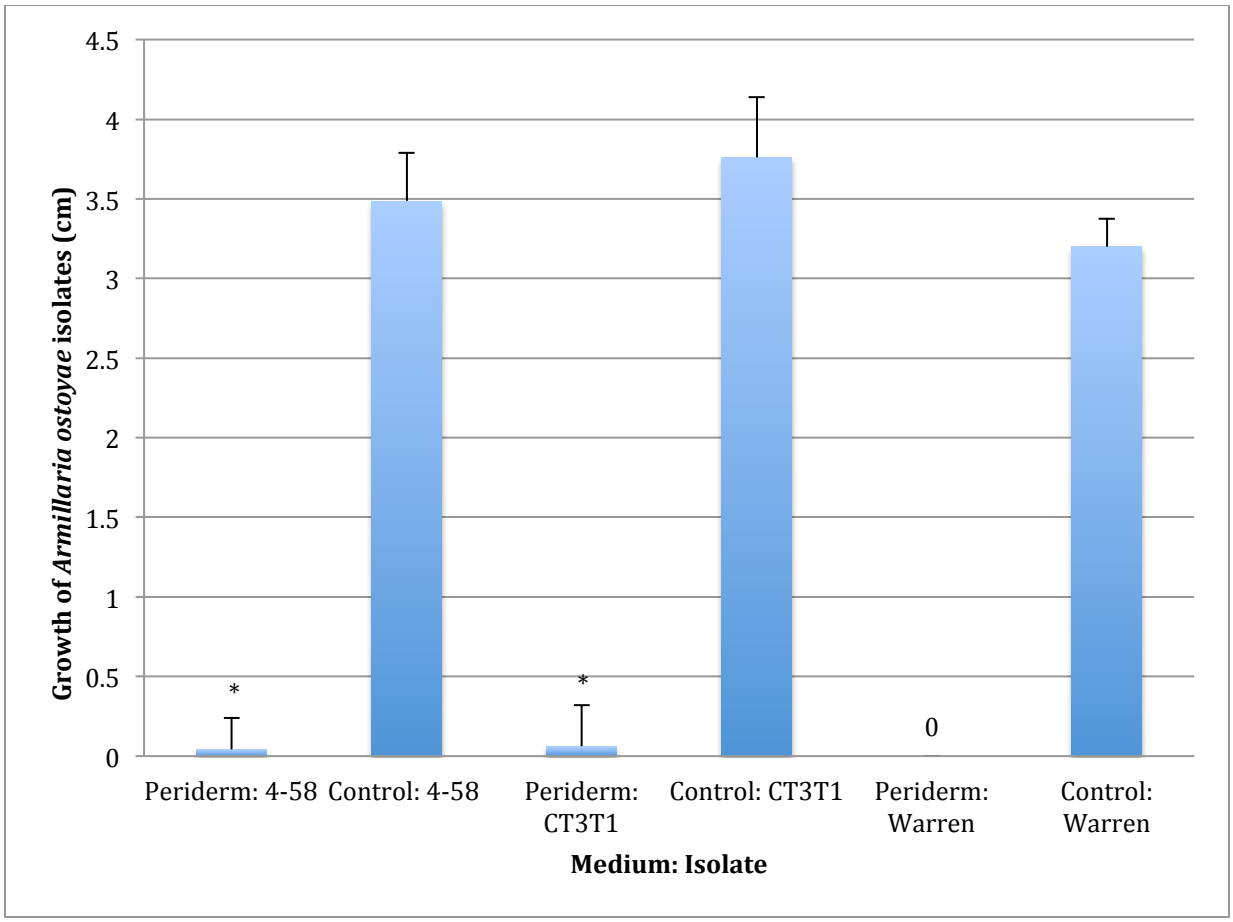

Figure 3.4. The growth of *A. ostoyae* isolates on *P. maackii* periderm-amended medium (12 g/L YMPG). * = Significantly different from the control. Tukey's HSD, $p = 0.05$.

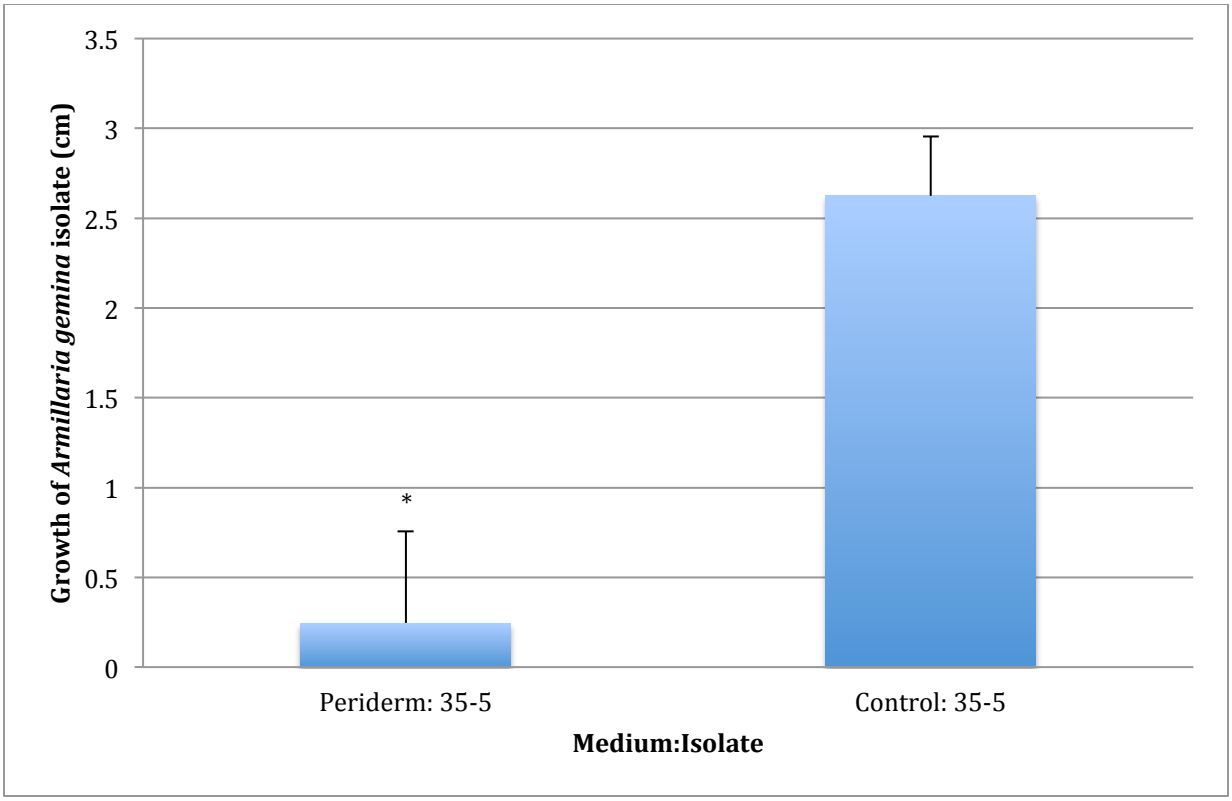

Figure 3.5. The growth of one isolate of *A. gemina* on *P. maackii* periderm-amended medium (12 g/L YMPG). * = Significantly different from the control. Tukey's HSD, p = 0.05.

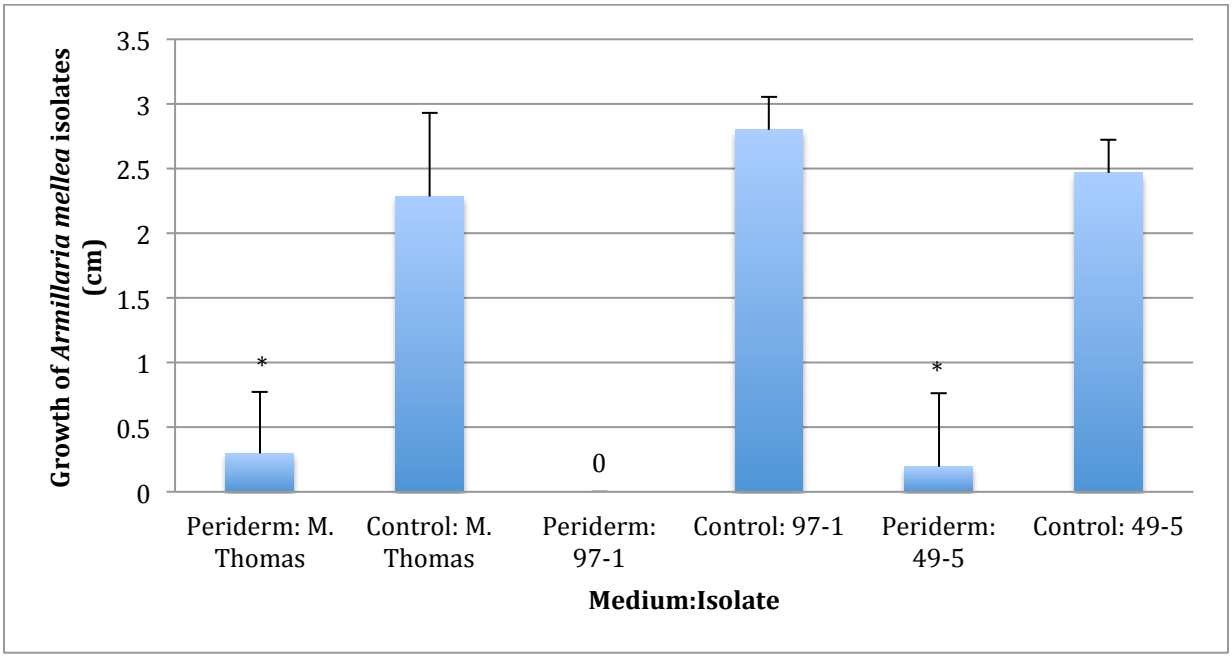

Figure 3.6. The growth of *A. mellea* isolates on *P. maackii* periderm-amended medium (12 g/L YMPG). * = Significantly different from the control. Tukey's HSD, p = 0.05.

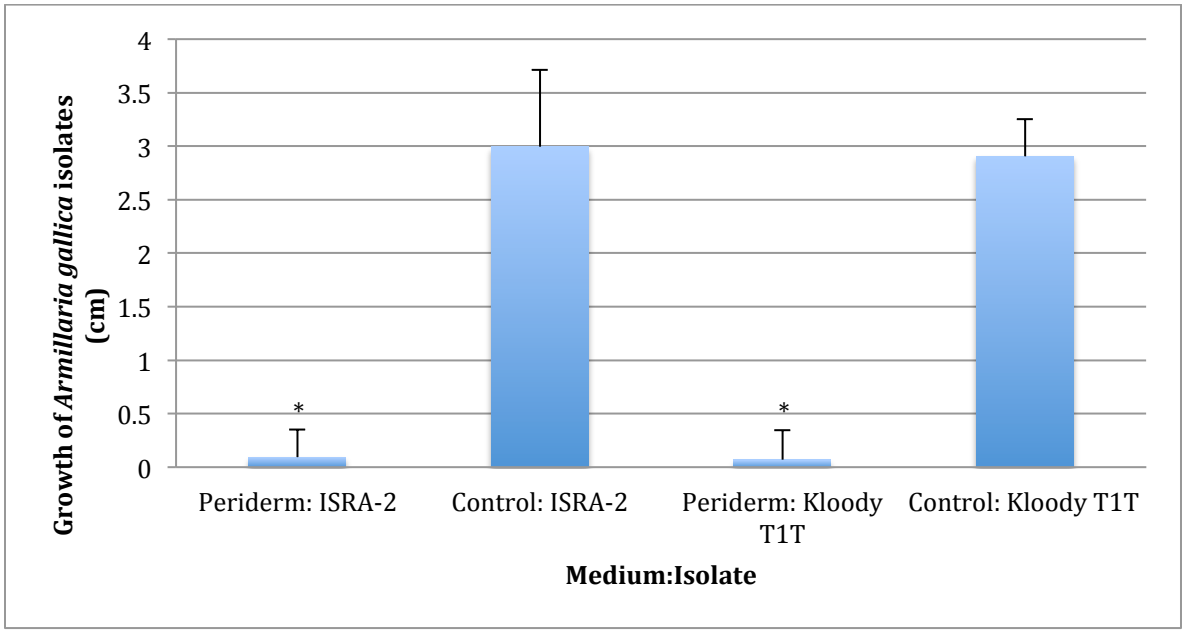

Figure 3.7. The growth of *A. gallica* isolates on *P. maackii* periderm-amended medium (12 g/L YMPG). * = Significantly different from the control. Tukey's HSD, $p = 0.05$.

Results suggest that older media was found to be less fungitoxic and therefore periderm-amended media should be inoculated within one day of preparation. More plugs containing mycelia were able to colonize the older periderm-amended media, thus the average radial growth is slightly higher when isolates are grown on media that is older than 24 hours. However, even when grown on media older than 24 h, the growth of *A. ostoyae* is significantly inhibited compared to control growth on YMPG. The slight decrease in antifungal activity could be partly attributed to the crystal formation on the surface of the media (Figure 3.8). This could be a possible indication that some of the antimicrobial compounds have crystallized out of the media resulting in decreased toxicity due to less chemicals coming into contact with plugs containing mycelia.

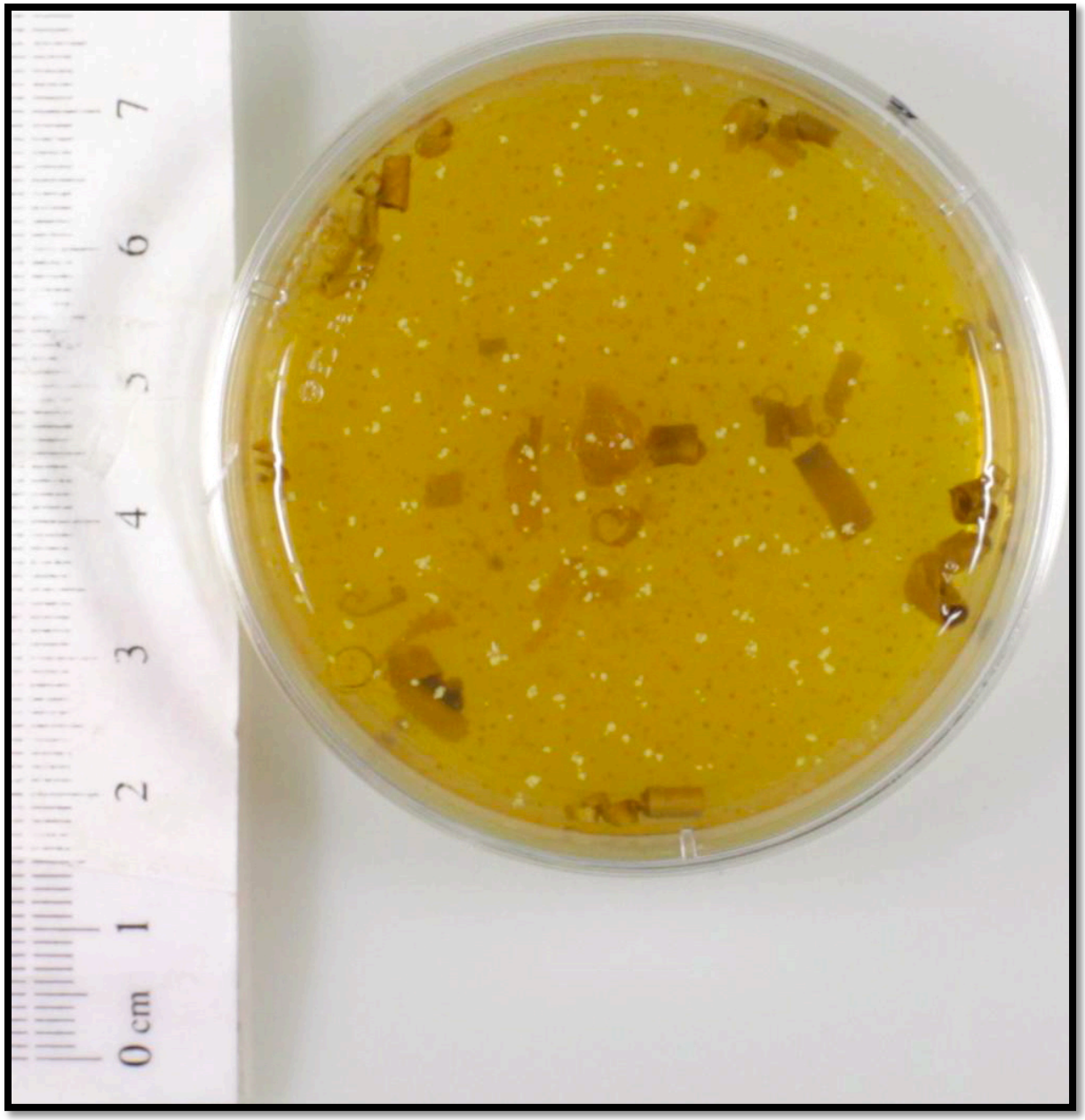

Figure 3.8. Crystal formation on the surface of *P. maackii* periderm-amended medium.

To determine whether the periderm-amended media was fungistatic or fungitoxic, all plugs containing *Armillaria* isolates were transferred from Petri dishes containing periderm tissue to those with only YMPG nutrient-medium. The figures below show the growth of transferred plugs of *A. ostoyae, A. gemina, A. mellea*, and *A. gallica*. The toxic effects of the periderm were shown to significantly inhibit the growth of all *Armillaria* species screened, even after being transferred to YMPG nutrient-medium.

The growth of *A. ostoyae* isolates was recorded when isolates 4-58, CT3T1 and warren were transferred from *P. maackii* periderm-amended media to YMPG (Figure 3.9). Some species were able to establish more growth than others. The periderm-amended medium was fungitoxic to 100% of samples containing mycelia of isolate 4-58. It was also fungitoxic to most samples of CT3T1 and warren, with the exception of a few samples/isolate. The growth of all three isolates of *A. ostoyae* was significantly inhibited after being transferred to YMPG medium. The growth of *A. gemina*, isolate 35-5, when transferred from periderm-amended medium to plain YMPG medium remained significantly inhibited (Figure 3.10).

The growth of *A. mellea* isolates M. Thomas, 97-1 and 49-5, transferred from the periderm-amended medium onto YMPG nutrient-medium was recorded. All three isolates were significantly inhibited after being transferred from media containing periderm tissue (Figure 3.11). The periderm-amended medium was fungitoxic to samples including those of M. Thomas and 49-5. However, there were a few samples that were able to re-establish growth when transferred to plain YMPG, showing average radial growth between 0.5 – 1 cm. The medium was fungitoxic to 100% of the 97-1

samples. The *P. maackii* periderm-incorporated medium was 100% fungitoxic to isolate 97-1 of *A. mellea.*

The periderm-amended media was fungitoxic to some plugs containing mycelia of Kloody T1T and ISRA-1. The samples that established growth were still significantly inhibited compared to samples that had not been exposed to *P. maackii* periderm. The growth of *A. gallica* isolates, ISRA-5 and Kloody T1T, was inhibited even when transferred from periderm-amended medium to YMPG medium (Figure 3.12). The Kloody T1T isolate was more sensitive to the periderm-incorporated media compared to ISRA-5 isolate.

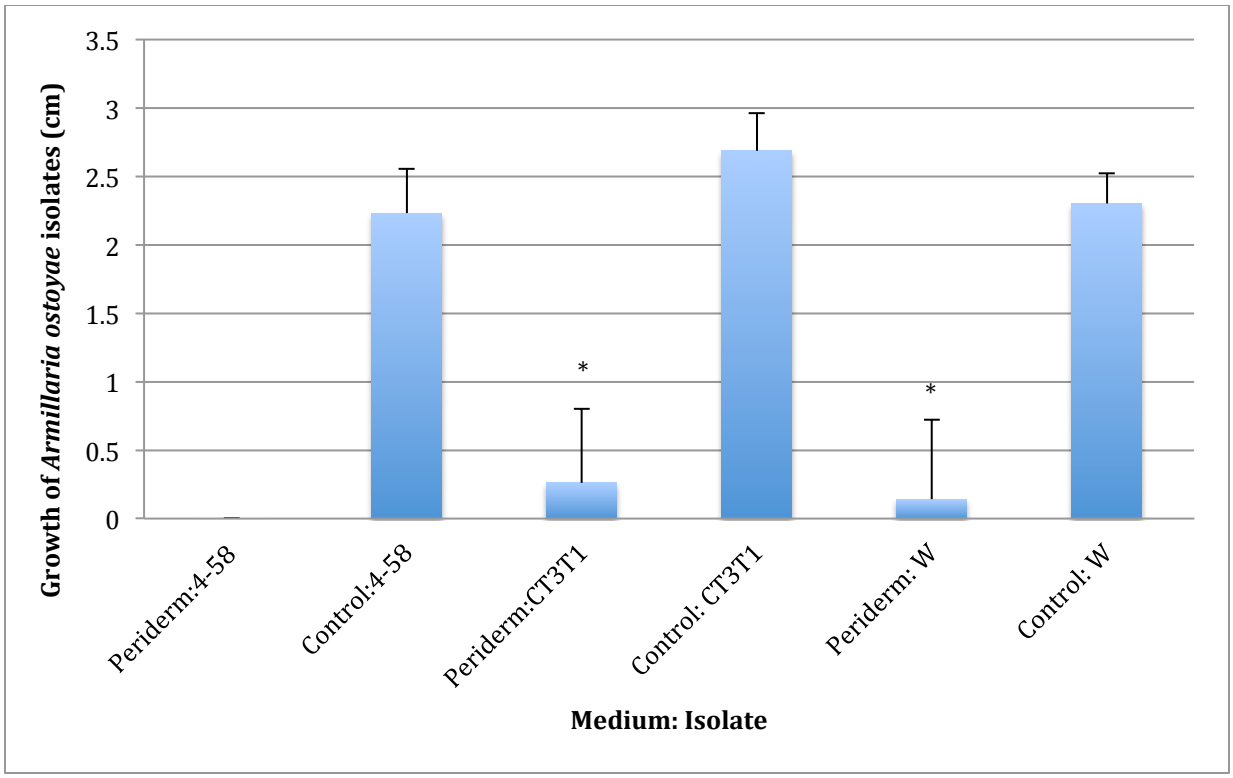

Figure 3.9. Growth of *A. ostoyae* isolates when transferred from *P. maackii* periderm-amended medium to plain YMPG nutrient-medium. * = Significantly different from the control. Tukey's HSD, $p = 0.05$.

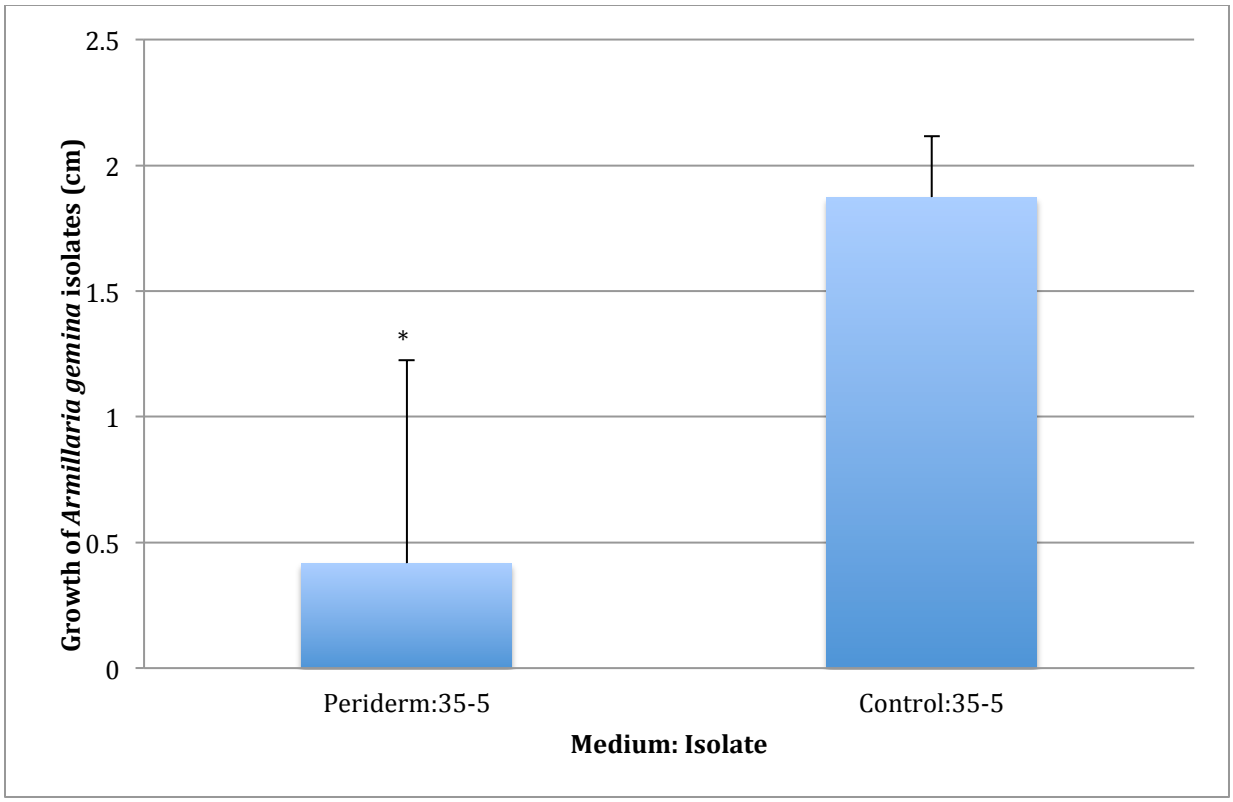

Figure 3.10. Growth of *A. gemina*, isolate 35-5, when transferred from *P. maackii* periderm-amended medium to plain YMPG nutrient-medium. * = Significantly different from the control. Tukey's HSD, $p = 0.05$.

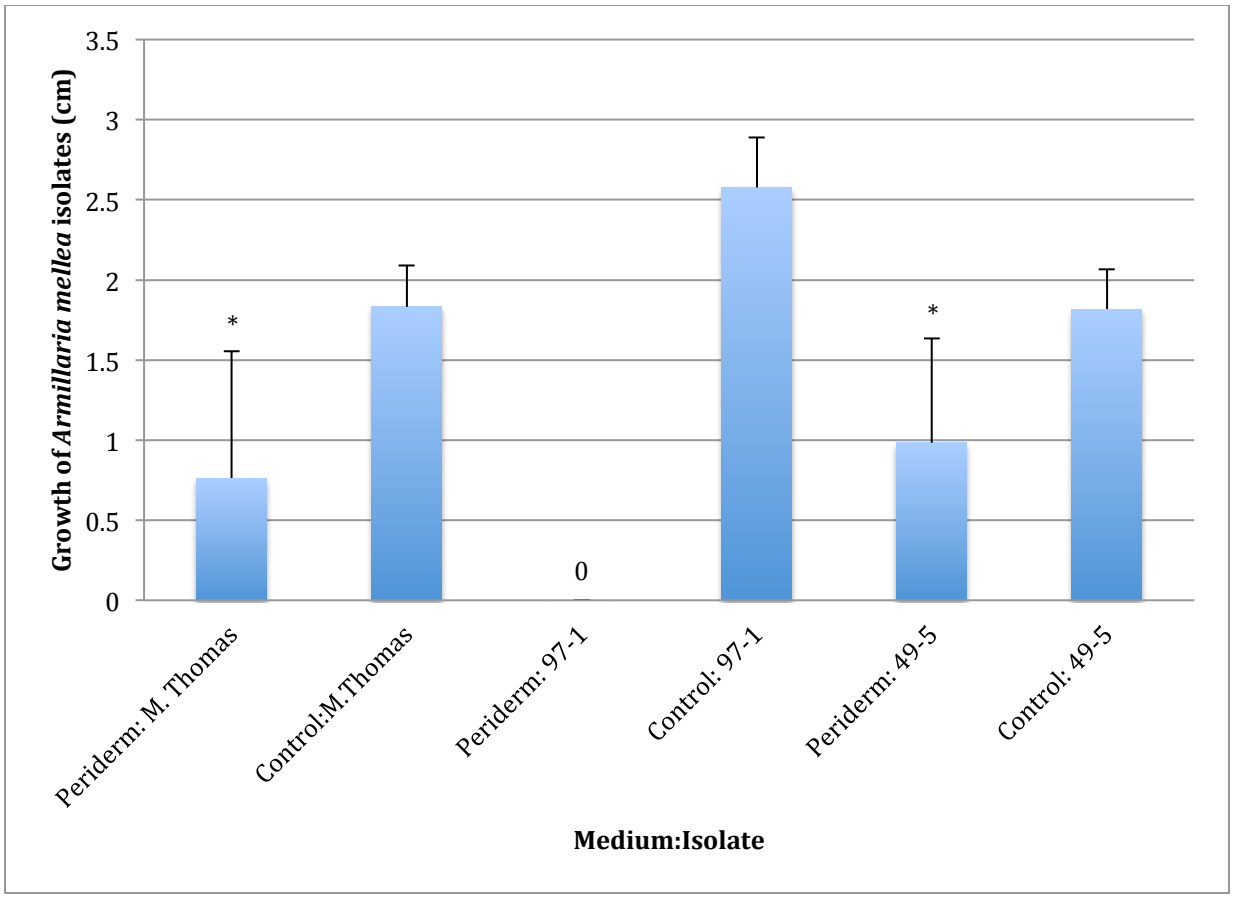

Figure 3.11. Growth of *A. mellea* isolates when transferred from *P. maackii* periderm-amended medium to plain YMPG nutrient-medium. * = Significantly different from the control. Tukey's HSD, $p = 0.05$.

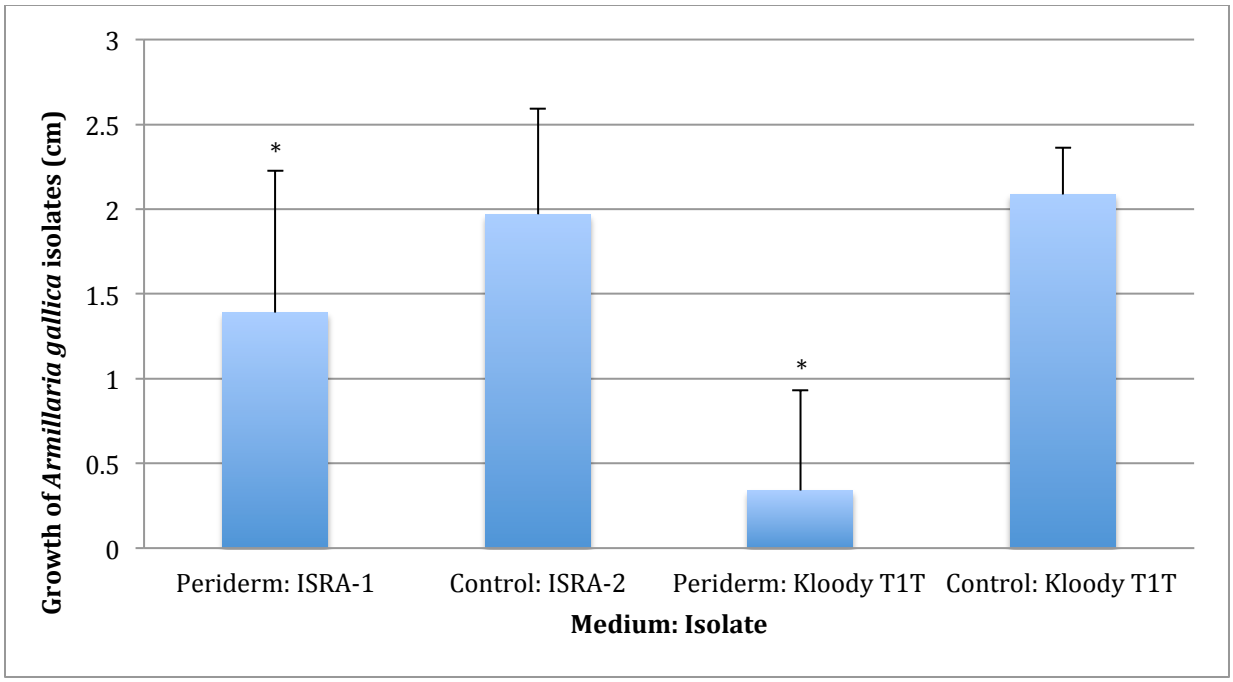

Figure 3.12. Growth of *A. gallica* isolates when transferred from *P. maackii* periderm-amended medium to plain YMPG nutrient-medium. * = Significantly different from the control. Tukey's HSD, $p = 0.05$.

Thus, growth inhibition of *Armillaria* species was maintained even after being removed from the periderm-amended medium. The fungitoxicity varied among isolates of *Armillaria* spp. The medium containing *P. maackii* periderm was fungitoxic to a percentage of all isolates.

Older periderm tissues were collected from several mature *P. maackii* trees and were screened for antifungal activity against *A. ostoyae*. All of the trees screened were able to significantly inhibit pathogen growth. After this screen was done, it was found that the periderm-amended media needed to be inoculated within 24 hours of preparation. The media used to screen for antifungal activity in young and mature trees was approximately one week old.

Five young (< 5 years old) *P. maackii* trees were screened for their ability to inhibit the growth of *A. ostoyae* (Figure 3.13). The periderm-amended media was older than 24 hours when used. Older media (over 24 h) was also used to screen older tissues collected from eight mature (>12 years) *P. maackii* trees. Despite the slight decrease in toxicity due to the age of the media, all *P. maackii* trees screened were able to significantly inhibit the growth of *A. ostoyae* (Figure 3.14). The antifungal compounds were also confirmed using TLC and HPLC-MS analyses. Samples from the same mature trees were later used for fungistatic and fungitoxic bioassays. When inoculation is done within 24 hours of preparation, the growth of *A. ostoyae* is completely inhibited.

Armillaria ostoyae growth is shown on media containing periderm from young (A) and mature (B) *P. maackii* trees and on plain YMPG medium (C) (Figure 3.15). This assay was also done using older media. The top three Petri dishes in the A group (periderm from young trees) show slight viability of *A. ostoyae*. When these experiments were repeated with fresh media there was an increase in fungitoxic activity, most plugs showing no growth at all. Pictures of samples A, B and C were taken after 3 weeks of growth. Crystals appeared on the surface of the periderm from mature trees. There is also a slight difference in the color of the media. Crystals later appeared on the surface of media containing young periderm; perhaps this is an indication of a difference in concentration of some yellow colored flavonoids.

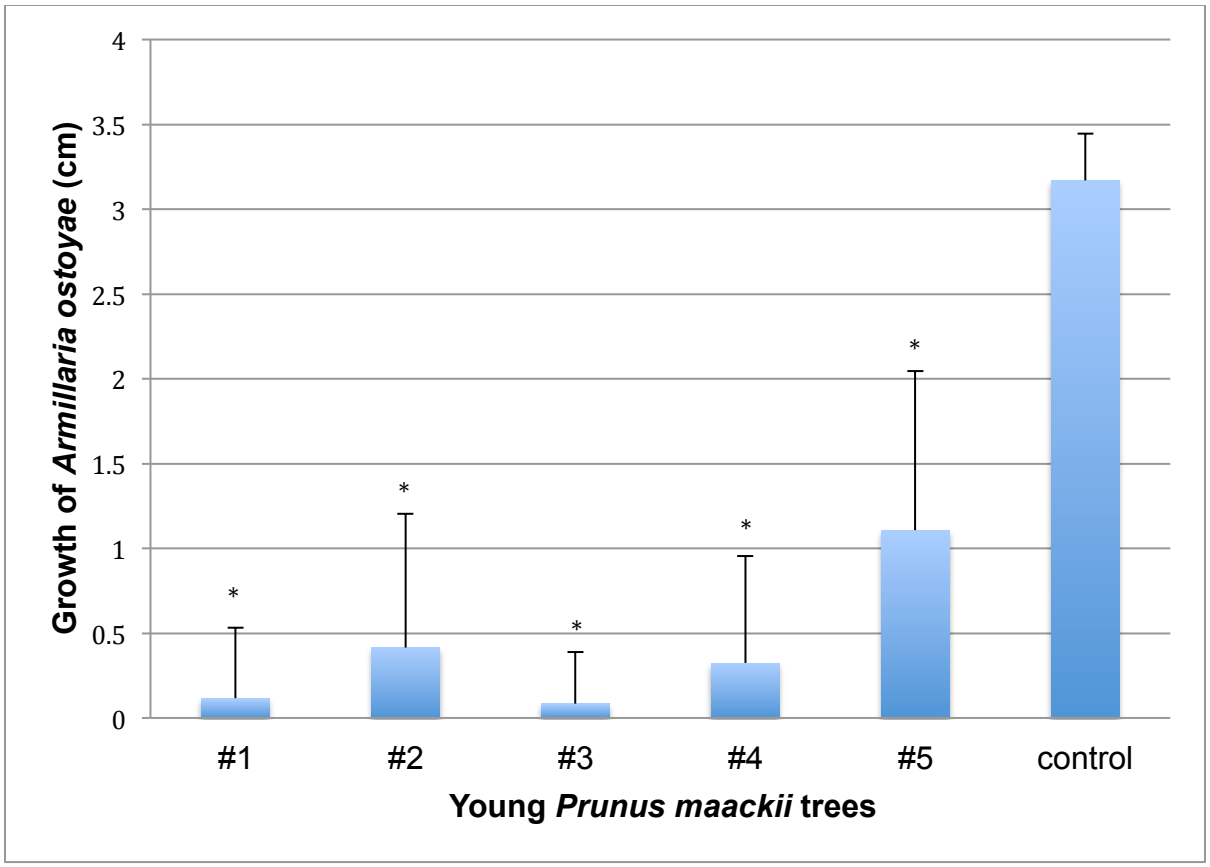

Figure 3.13. Periderm tissues from five young *P. maackii* trees were screened for their ability to inhibit the growth of *A. ostoyae*. All trees were able to significantly inhibit pathogen growth. Tukey's HSD, $p = 0.05$.

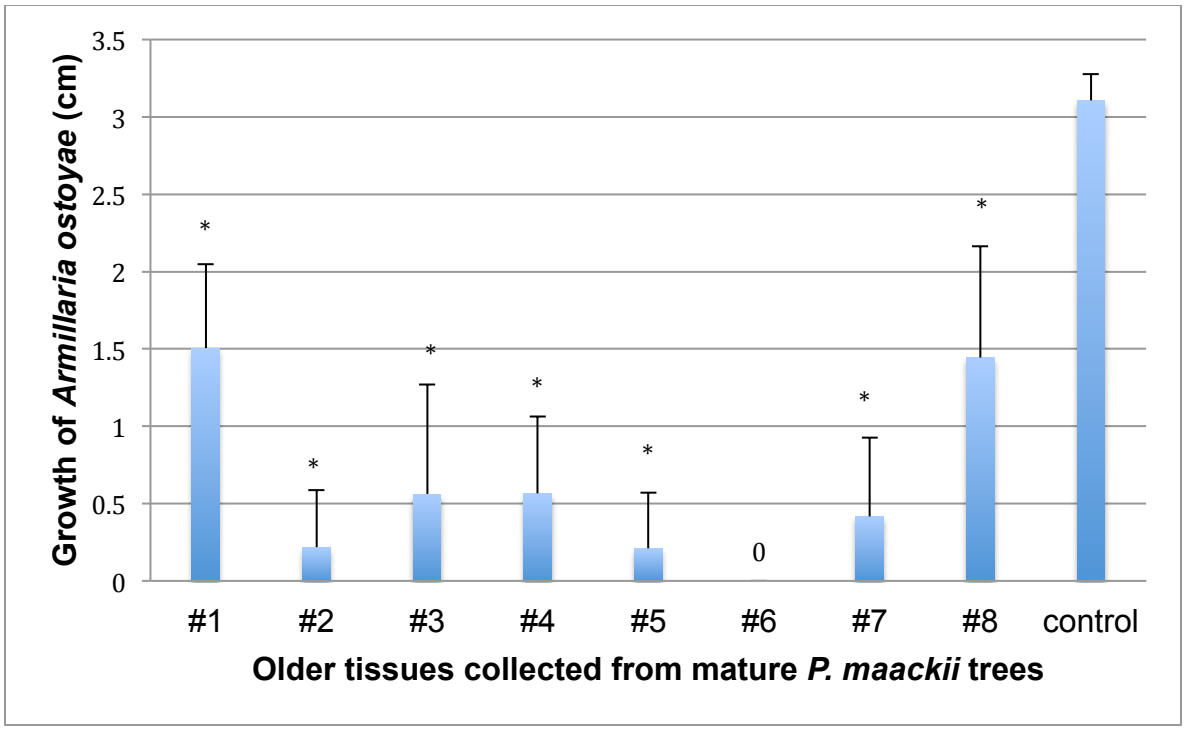

Figure 3.14. Screening for antifungal activity in older tissues collected from mature *P. maackii* trees. All trees were able to significantly inhibit pathogen growth. Tukey's HSD, $p = 0.05$.

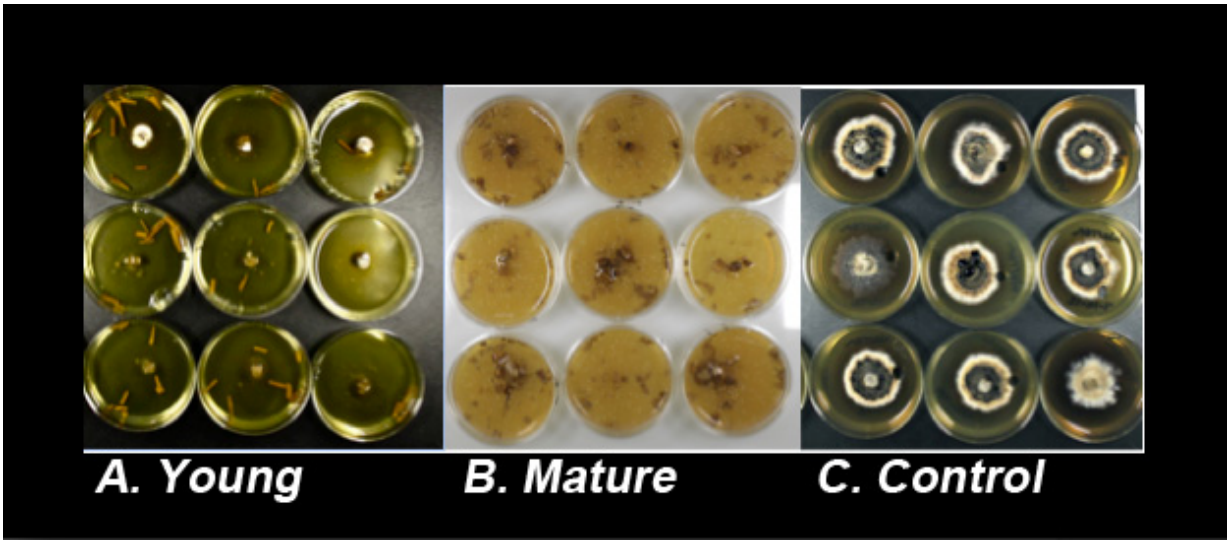

Figure 3.15. Periderm-amended media from both young and mature *P. maackii* trees were able to significantly inhibit *A. ostoyae* growth. Samples were grown for three weeks. Tukey's HSD, $p = 0.05$.

To further classify the toxicity of the *P. maackii* periderm, non-host pathogens were used for inoculation. Additional antimicrobial screening used *P. maackii* periderm-incorporated media inoculated with four Ascomycetes: *Cladosporium cucumerinum*, *Colletotrichum orbiculare*, *Fusarium sambucinum*, and *Sclerotinia sclerotiorum*, one Basidiomycete, *Rhizoctonia solani* and one Ooymcete, *Phytophthora capsici*. The periderm-amended media significantly inhibited all pathogens screened (Figure 3.16). Complete inhibition was detected in samples containing *P. capsici, S. sclerotiorum, C. cucumerinum*, and *C. orbiculare*. Slight growth was detected in samples containing plugs of mycelia from *F. sambucinum, F. sambucinum* TBZ-resistant and *A. solani*. *Rhizoctonia solani* and B*. cinerea* were the most tolerant, colonizing the greatest areas among species screened, however samples were still significantly inhibited when compared to control growth.

The amount of inhibition was not equal among the different species screened. Although the periderm-amended media was able to significantly inhibit all pathogens screened, *R. solani* and *B. cinerea* appeared to be the most tolerant. The periderm-amended media completely inhibited the growth of *P. capsici*, *S. sclerotiorum*, *C. cucumerinum*, and *C. orbiculare*. Significant growth inhibition was detected in all other species used for screening, which include *A. solani, F. sambucinum* (wild-type) and thiabendazole (TBZ)- resistant isolates. TBZ-resistant isolates of *F. sambucinum* are those resistant to the fungicide (TBZ). For comparison, salicylaldehyde when applied as a volatile is known to inhibit TBZ-resistant *F. sambucinum* at gas levels of 20 µg/mL or lower. Media containing cinnamaldehyde and thymol (0.1% v/v) also inhibits growth of TBZ-resistant isolates (Vaughn & Spencer, 1994).

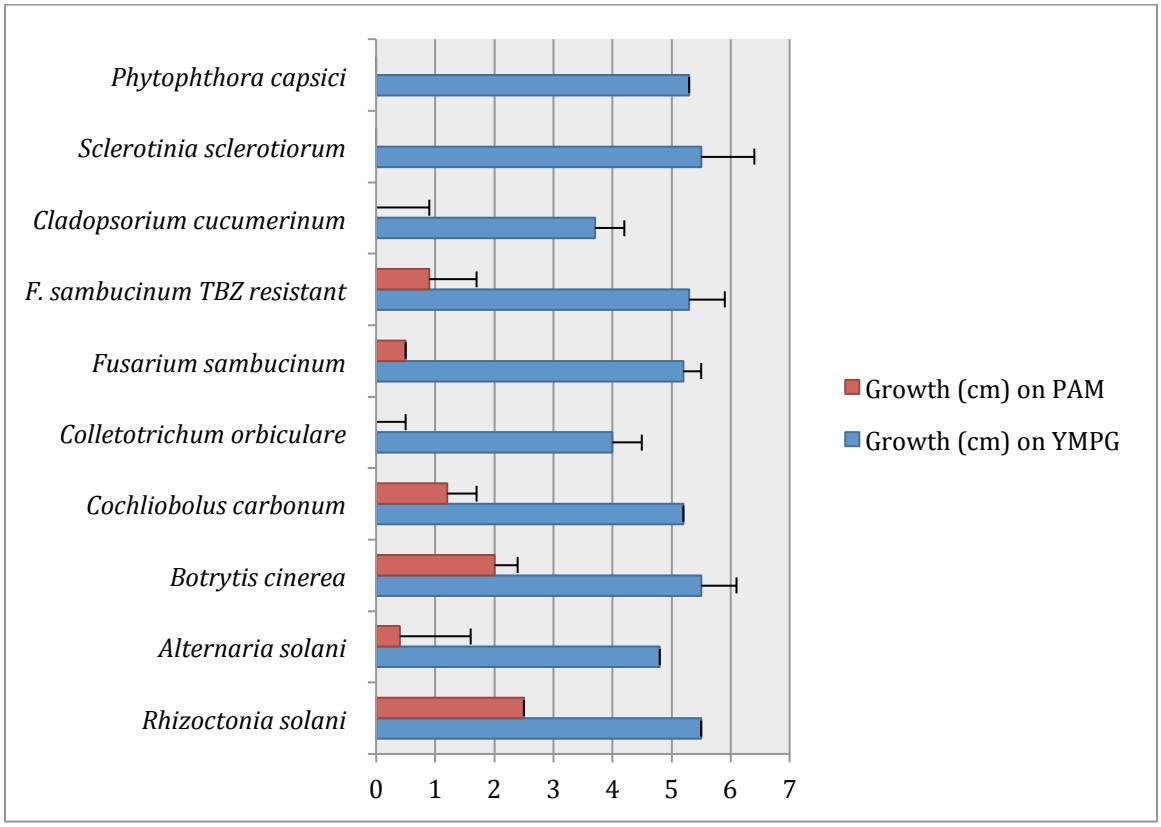

Figure 3.16. The periderm-amended medium from *P. maackii* was inoculated with several non-host pathogens. Isolates were grown for two weeks. Tukey's HSD test, p = 0.05.

Discussion

Phytoanticipins play a role in host resistance in many different host-pathogen interactions. The peel of an unripe avocado fruit contains the preformed antifungal *cis, cis*-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene, which is toxic to *Colletotrichum gloeosporioides*. The concentration of this diene decreases as fruits mature (Prusky et al., 1983).

Additionally, catechol in onion, epicatechin in avocado, chlorogenic acid (widely distributed), caffeic acid (widely distributed), 3-hydroxyacetophenone in carnations, 5-pentadecyl resorcinol in mango, and pinocembrin in cottonwood are all known phenolic phytoanticipins (Hammerschmidt & Hollosy, 2008). The phenolic composition changes seasonally in phloem tissues of sweet cherry, *Prunus avium* L. (Schwalb & Feucht, 1999). The first rise of flavanols in the phloem tissues of sweet cherry occur at the end of winter with transiently increased temperatures. Just before bud breaking in early spring, a second increase in flavanols was observed. Flavanol maximum occurred during mid-July and coincided with decline of shoot growth. The fourth flavanol peak in shoot phloem was observed during the fall season when there is a back flow of carbohydrates from the leaves (Schwalb & Feucht, 1999). For this reason, future research should include sampling of *P. maackii* tissues each month to examine any seasonal effect on phenolic concentration.

The viability of *A. ostoyae* isolates increased as periderm concentration decreased. There were constitutively present antifungal compounds in media made from all trees. It was found that the periderm-amended media should be inoculated

within 24 hours of preparation. To further examine the spectrum of antimicrobial activity, non-host pathogens were used to inoculate *P. maackii* periderm-amended media. The periderm was fungistatic to all pathogens tested. Mulch was made from the twigs of *P. maackii* to screen for antimicrobial activity in the periderm, cambial and wood tissues.

The mulch extract was able to significantly inhibit *A. solani, C. orbiculare, C. carbonum*, and *C. cucumerinum* and completely inhibit one *Trichoderma* spp. Leaves were also screened for antimicrobial activity. Preliminary data shows that the leaf-amended media completely inhibited the growth of *C. orbiculare* and *C. cucumerinum*.

Samples containing periderm from young trees and older periderm tissues from mature *P. maackii* trees had antimicrobial activity. The periderm tissue showed the most broad-spectrum inhibition to both *Armillaria* species and non-*Prunus* pathogens compared to other tissues screened. These antimicrobial compounds are likely phytoanticipins since they are consistently extracted from all extracts. The periderm extract was fungitoxic to a percentage of all *Armillaria* isolates. As the periderm concentrations decreased, growth and viability of the isolates increased.

In addition to serving as a resistant rootstock, *P. maackii* may also provide a unique phenylpropanoid pathway involving the biosynthesis of flavonoids lacking B-ring substituents. The structure of the antimicrobial compounds can provide valuable information for plant engineering. This ornamental species could serve as a model organism for the production of phytoanticipins. With this information, the phenylpropanoid pathway in other plants could be manipulated to produce more of these constitutively present antimicrobial compounds.

In conclusion, *P. maackii* periderm tissue is antimicrobial to a wide variety of pathogens. Assays using periderm-amended media show that compounds in the periderm tissue are fungitoxic. Certain enzymes can provide valuable information for the engineering of resistant crops. For example, genistein serves as a common precursor in phytoalexin and phytoanticipin biosynthesis in legumes. Isoflavone synthase (IFS) is a key enzyme in isoflavone production and can be utilized for engineering purposes. When chalcone isomerase is overexpressed in *Arabidopsis*, the expression of IFS leads to a 3-fold increase in flavonols (Dixon & Ferreira, 2002). This type of enzymatic research would provide needed information in regard to flavonoid biosynthesis in *P. maackii*. Future research should also include the characterization and identification of all unknown compounds from all tissue extracts, including those tissues that have not yet been examined including fruit and heartwood tissues.

APPENDIX

APPENDIX

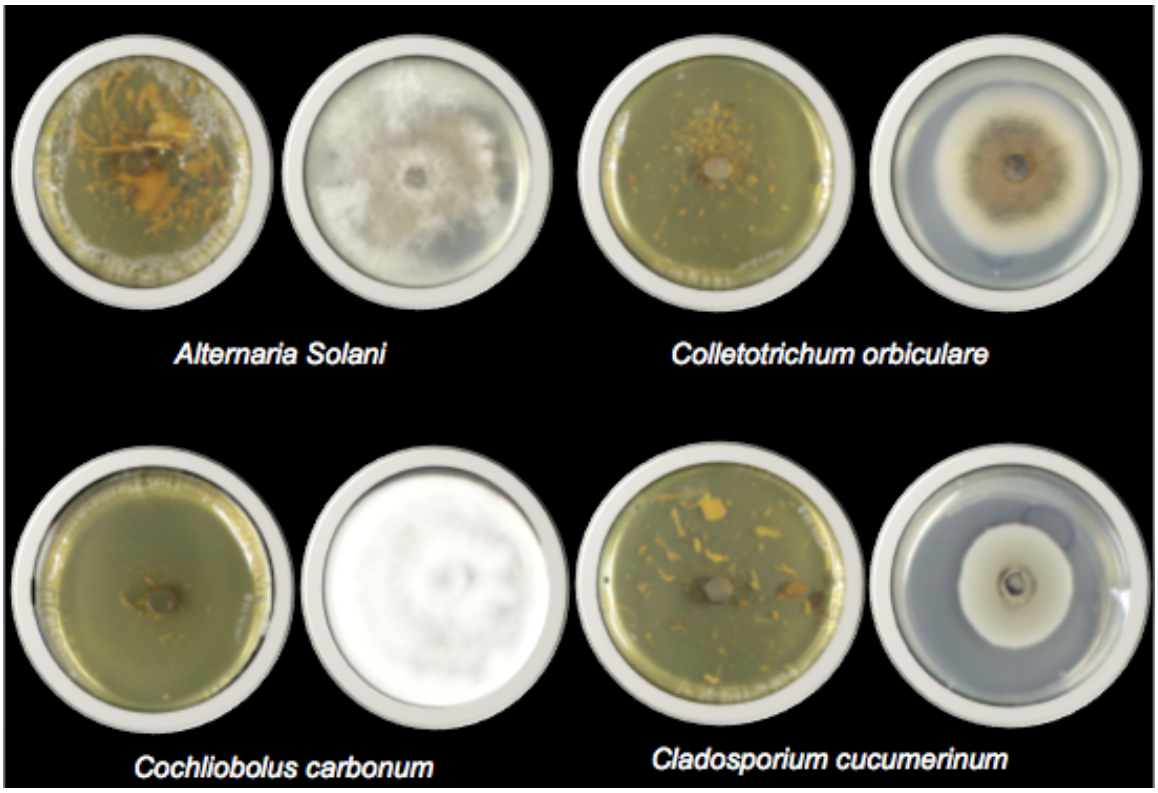

Figure A.1. Growth comparison of *A. solani, C. orbiculare, C. carbonum*, and *C. cucumerinum* on *P. maackii* mulch-amended medium and YMPG nutrient-medium.

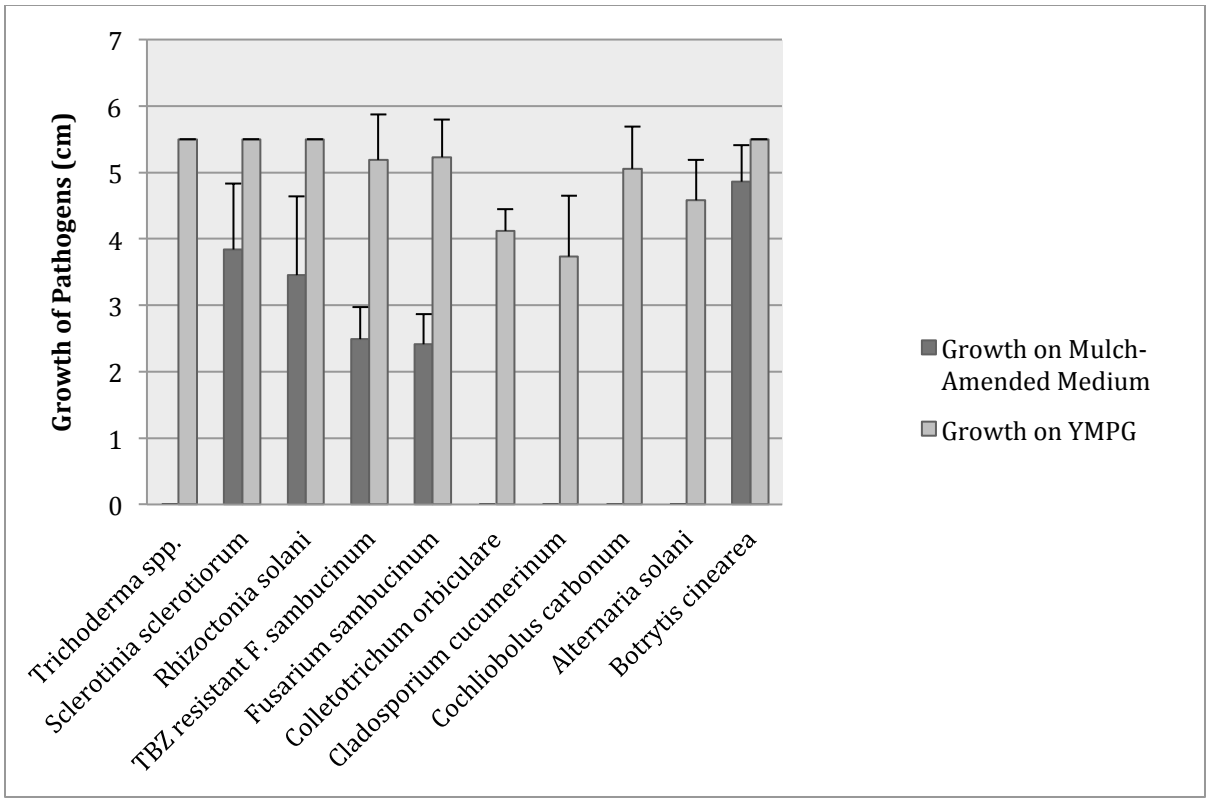

Figure A.2. Growth of non-host pathogens on *P. maackii* mulch-amended medium.

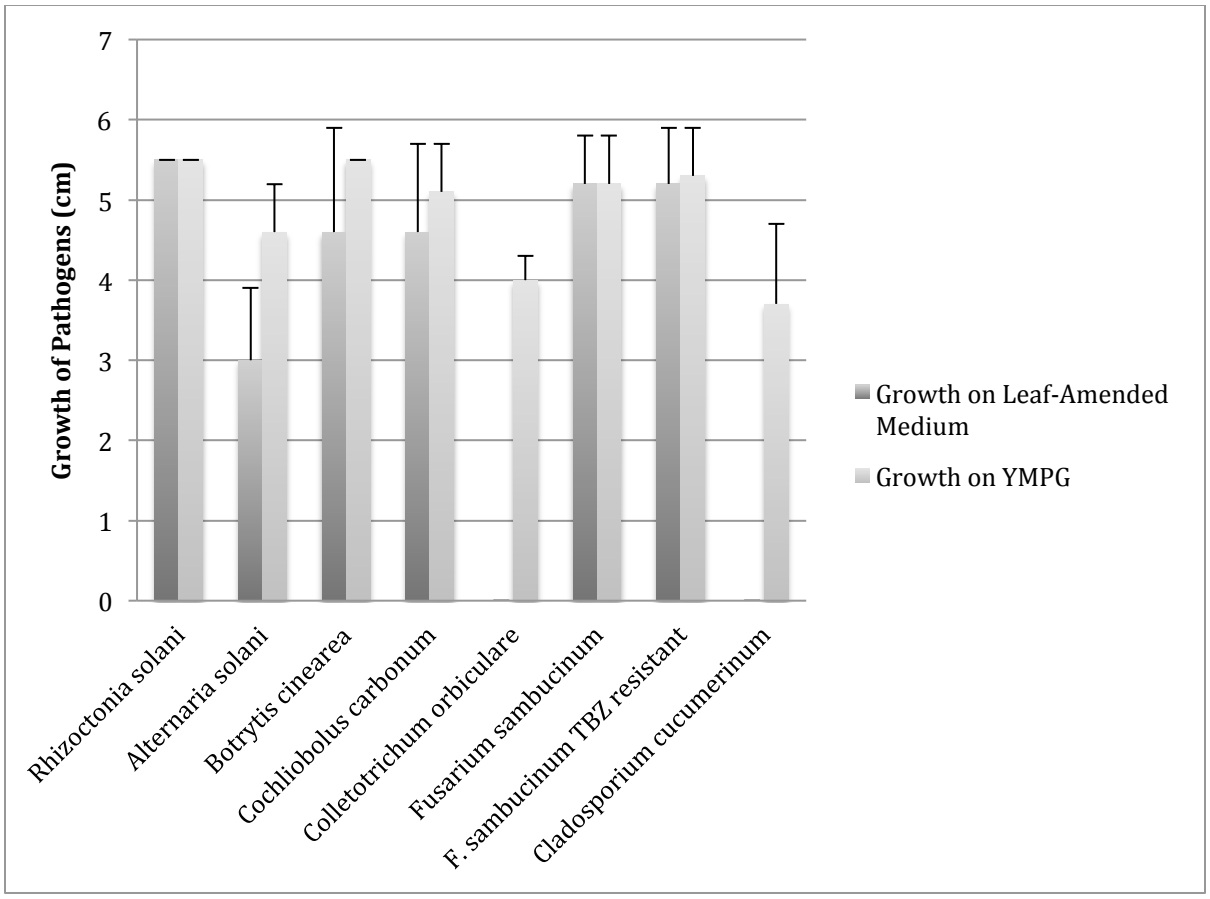

Figure A.3. Growth of non-host pathogens on *P. maackii* leaf-amended medium.

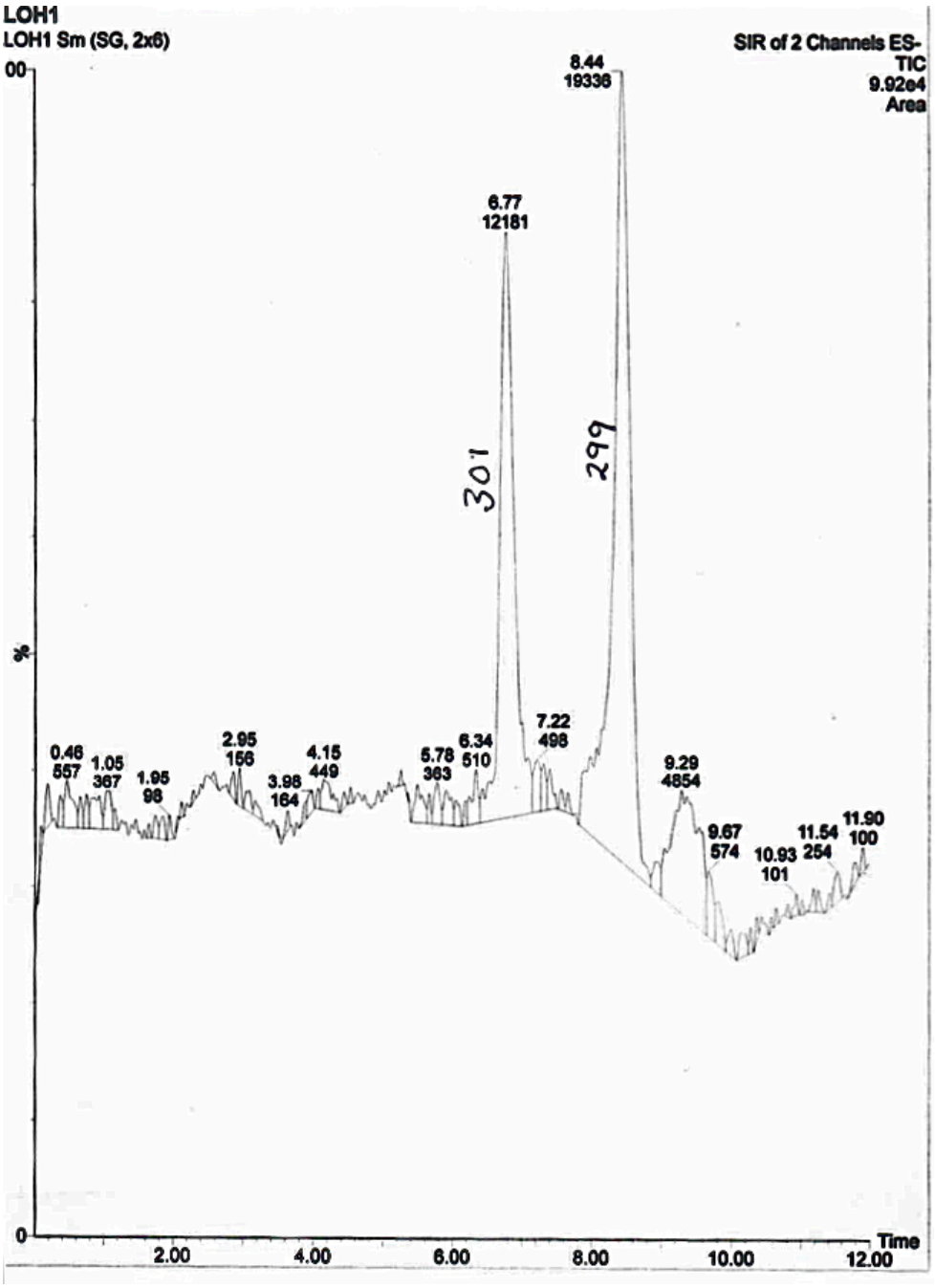

Figure A.4. HPLC chromatogram of the semi-purified fraction collected from *P. maackii* periderm tissue using dry-column chromatography. Alnustinol showed a retention time of approximately 6.77 (301 ion) and alnusin with a retention time of approximately 8.44 (299 ion) in negative ion mode.

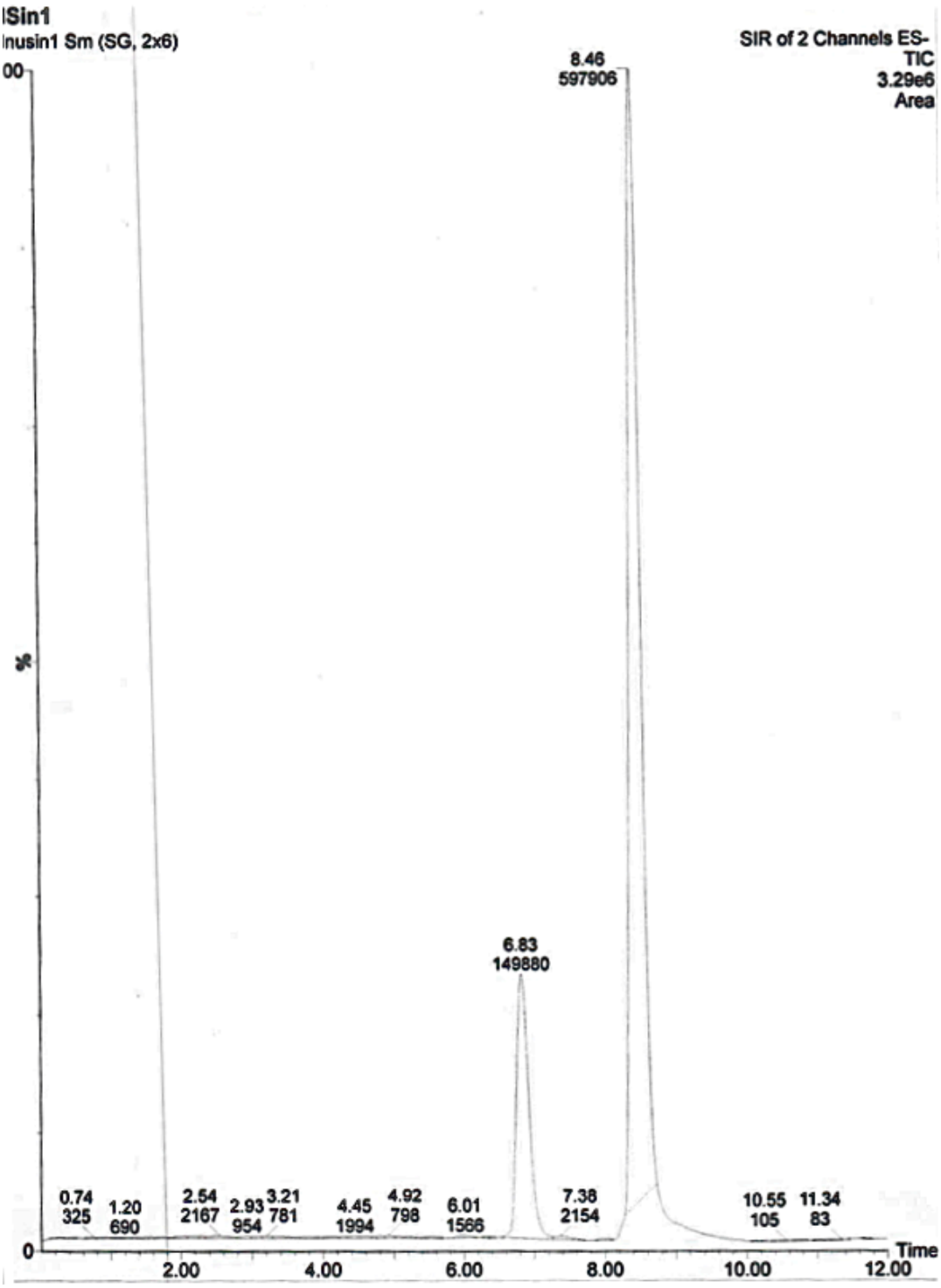

Figure A.5. HPLC chromatogram of the semi-purified fraction collected from *P. maackii* periderm tissue containing alnustinol with a retention time of approximately 6.83 and alnusin (nearly 80% pure) with a retention time of approximately 8.46 (299 ion) in negative ion mode.

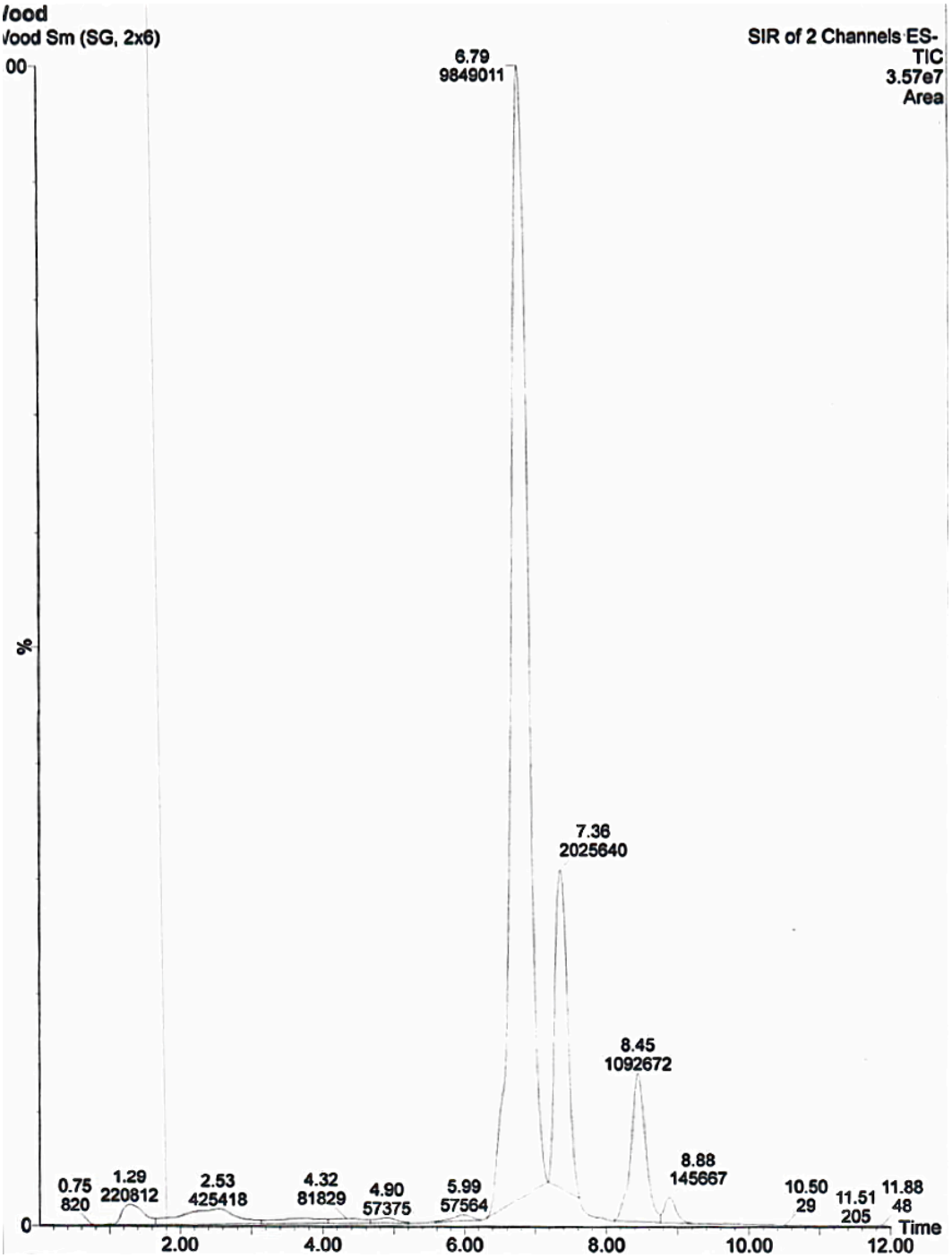

Figure A.6. Crude extract of *P. maackii* wood tissue containing alnustinol with a retention time of approximately 6.79 and alnusin with a retention time of approximately 8.45 in negative ion mode.

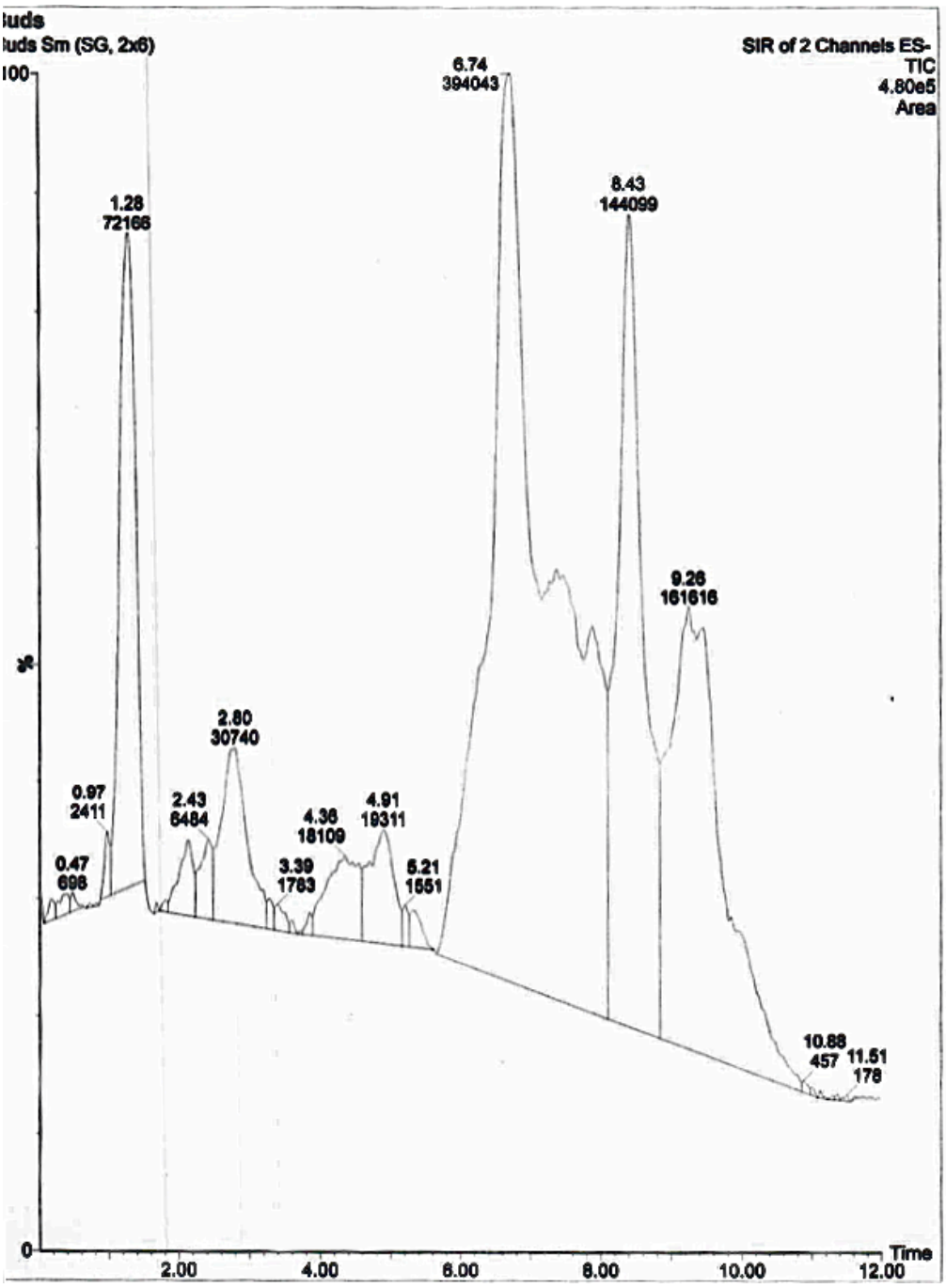

Figure A.7. Crude extract of *P. maackii* bud tissue containing alnustinol with a retention time of approximately 6.74 and alnusin with a retention time of approximately 8.43 in negative ion mode.

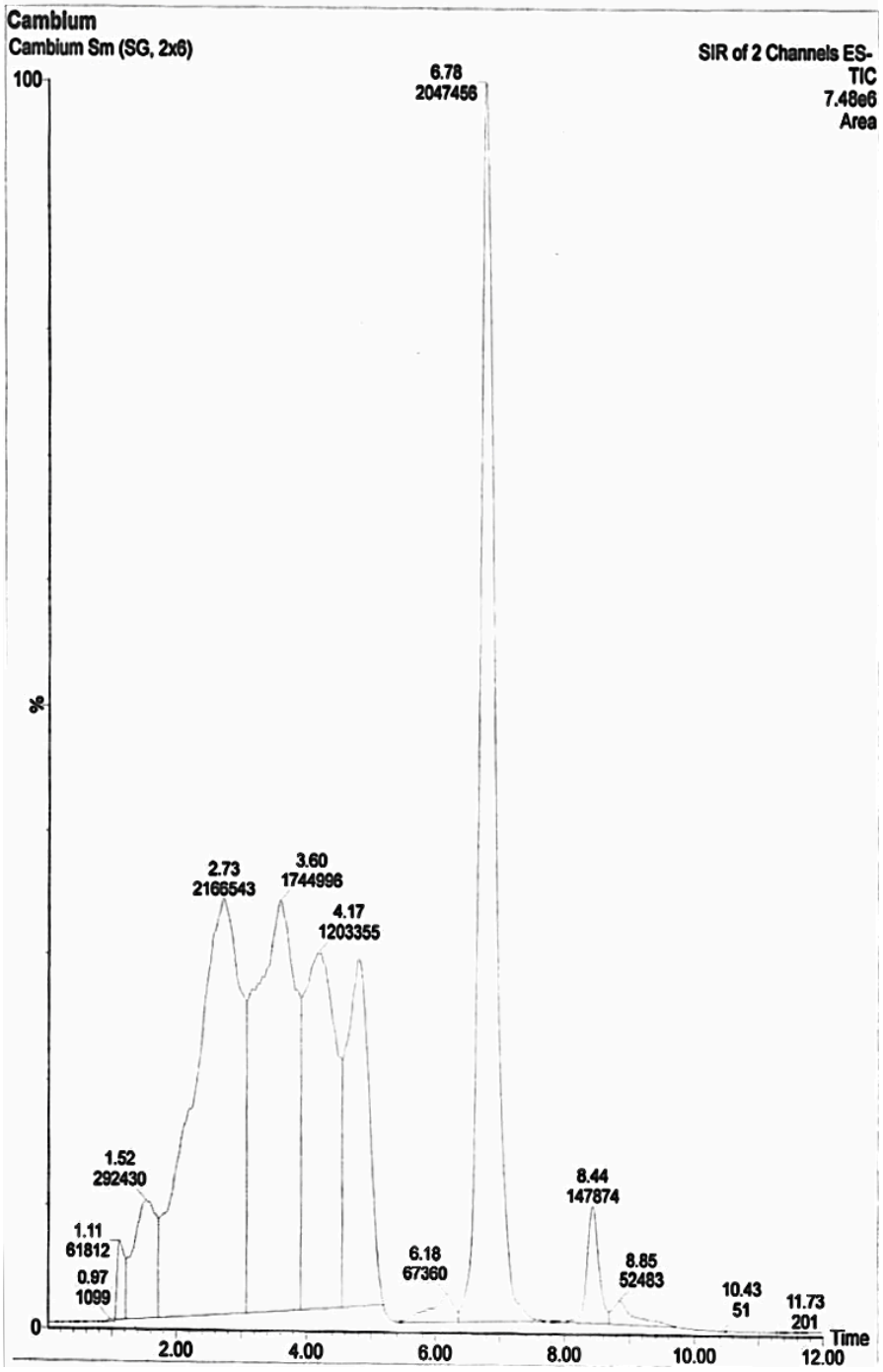

Figure A.8. Crude extract of *P. maackii* cambial tissue containing alnustinol with a retention time of approximately 6.78 and alnusin with a retention time of approximately 8.44 in negative ion mode.

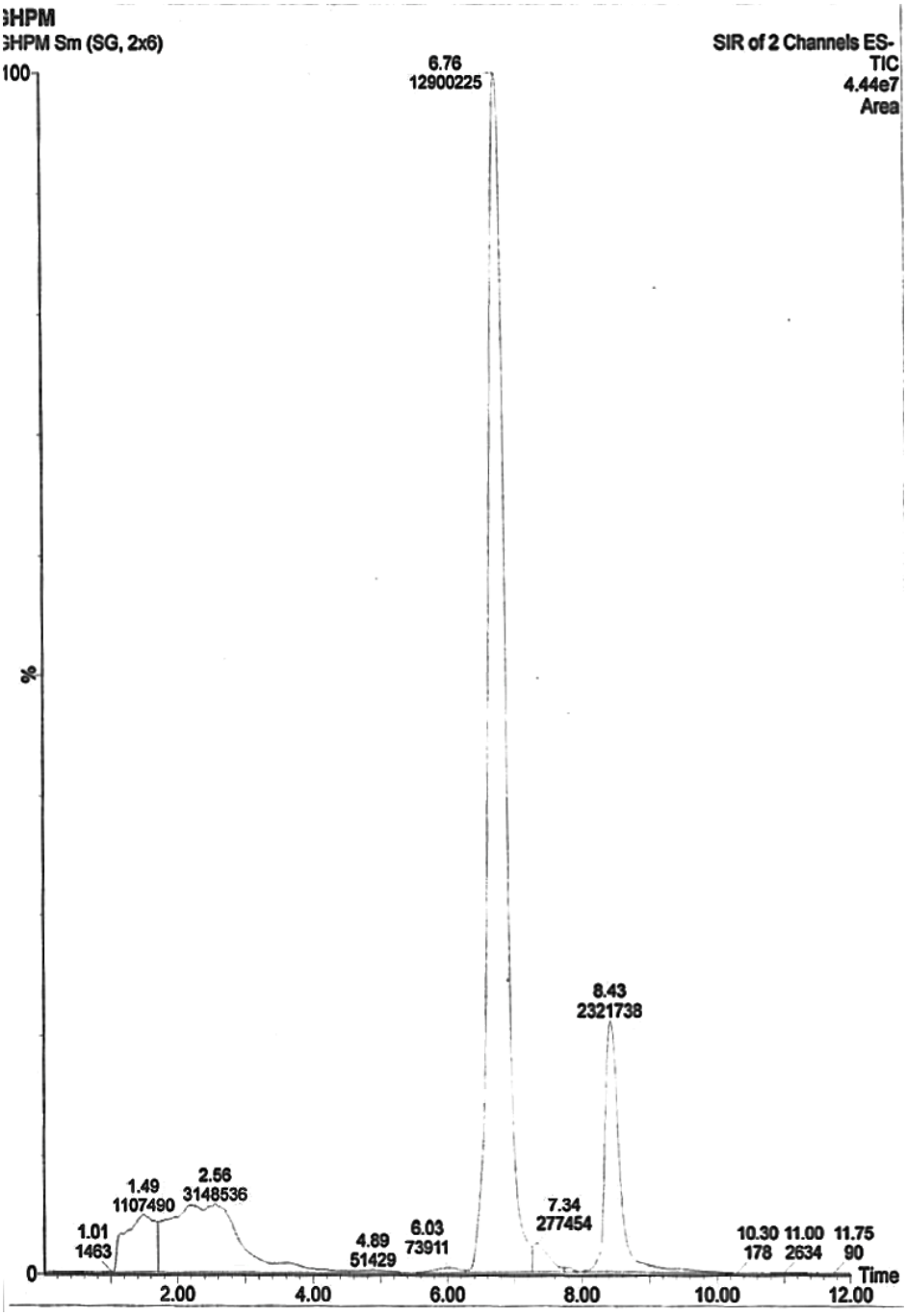

Figure A.9. Crude extract of *P. maackii* periderm tissue containing alnustinol with a retention time of approximately 6.76 and alnusin with a retention time of approximately 8.43 in negative ion mode.

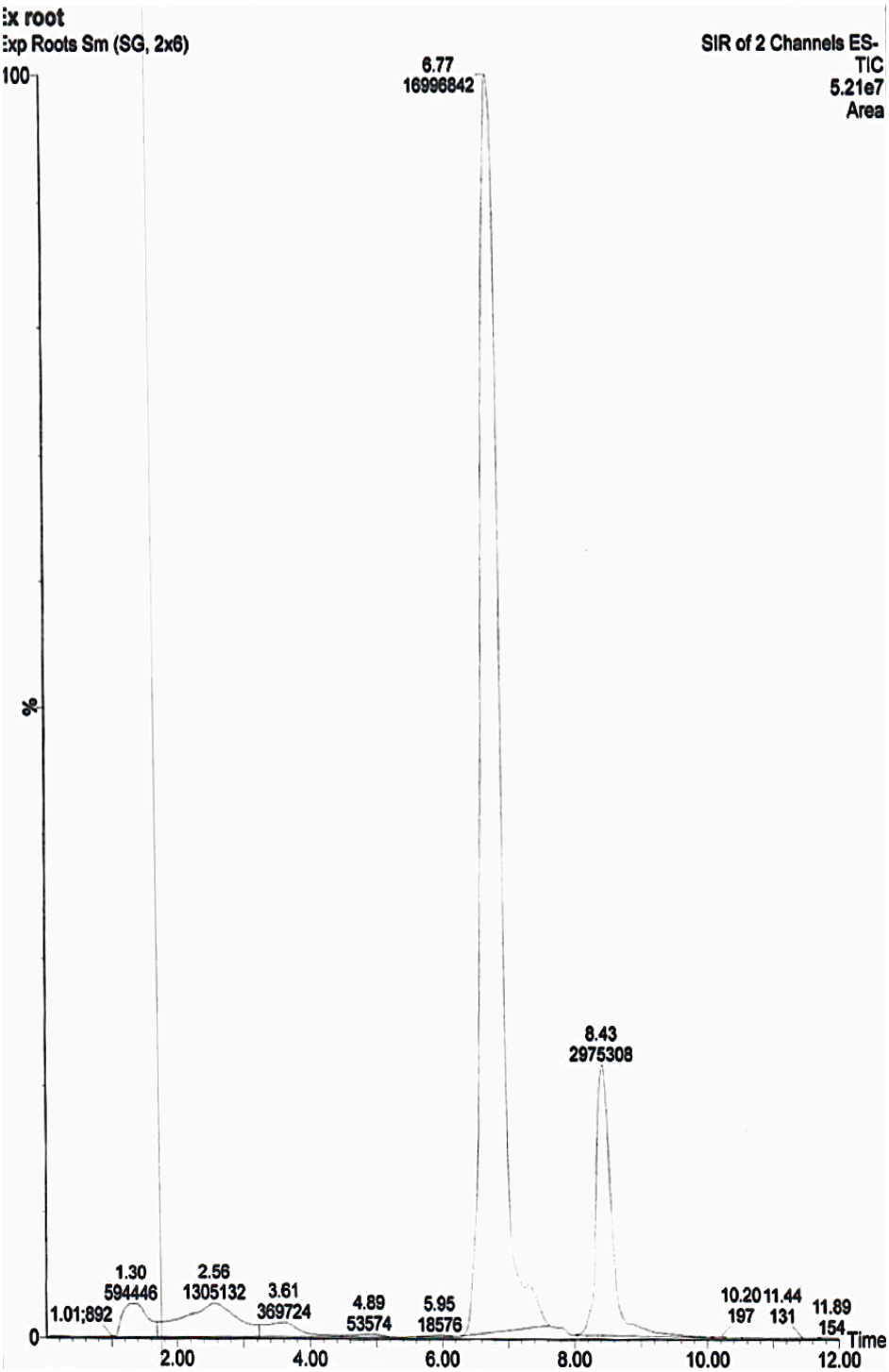

Figure A.10. Crude extract of *P. maackii* exposed root tissue containing alnustinol with a retention time of approximately 6.77 and alnusin with a retention time of approximately 8.43 in negative ion mode.

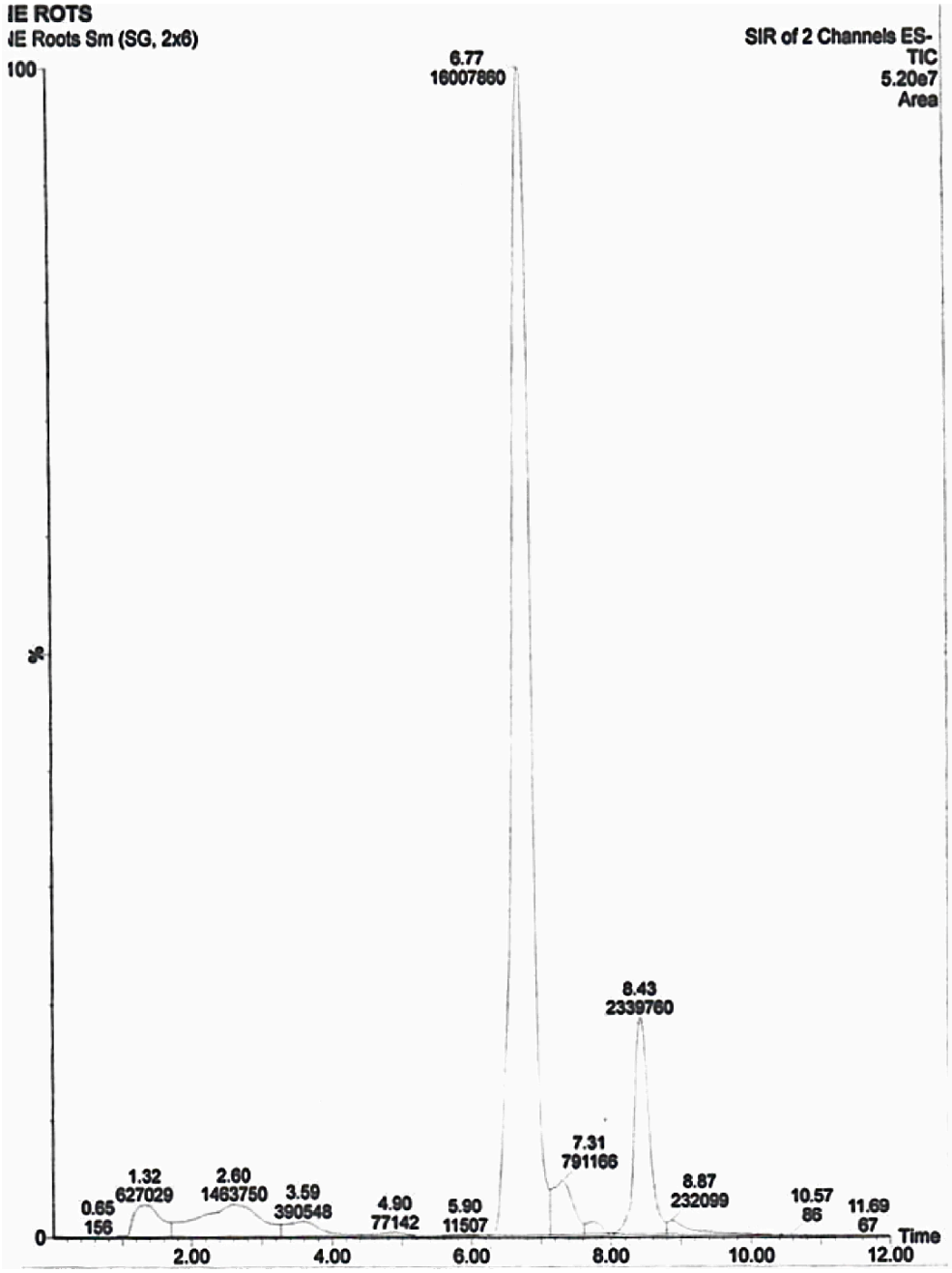

Figure A.11. Crude extract of *P. maackii* non-exposed root tissue containing alnustinol with a retention time of approximately 6.77 and alnusin with a retention time of approximately 8.43 in negative ion mode.

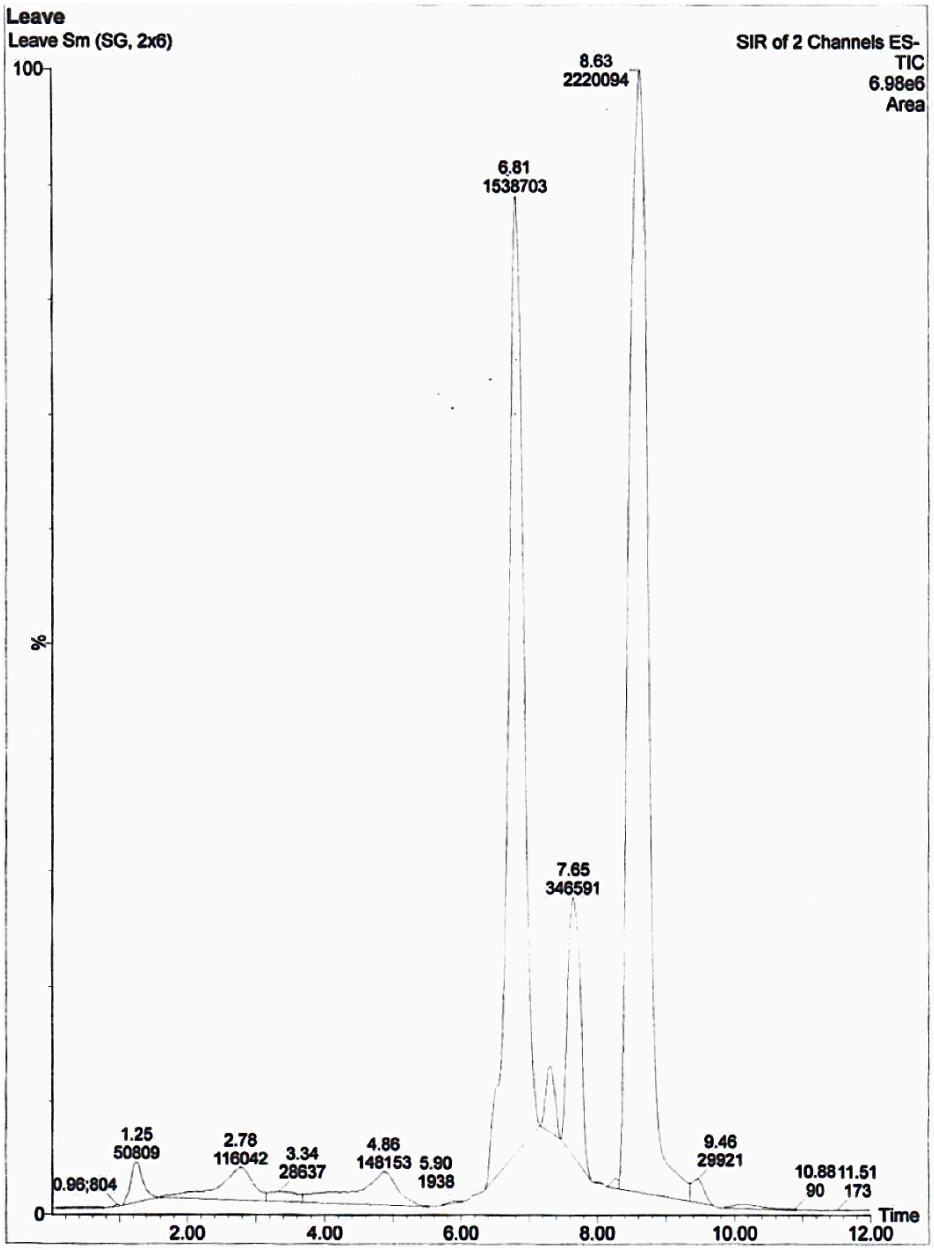

Figure A.12. Crude extract of *P. maackii* leaves containing alnustinol with a retention time of approximately 6.81 and alnusin with a retention time of approximately 8.63 in negative ion mode.

TLC Bioassay Protocol

1. 20 - 60 µL of sample extracts (.20g periderm/5mL 80% acetone) are applied approximately 1 cm from the bottom of a TLC plate (typically 20 x 20 cm; Silica gel G). Plates are scored to stop solvent migration, using the pointed end of a metal spatula, at approximately 12 cm from the bottom of the plate.

2. The TLC plates are developed with chloroform-methanol 90:10 (v/v).

3. TLC plates are photographed under long-wave (365 nm) or short-wave (254 nm) ultraviolet light, if UV absorbing/fluorescing compounds are thought to be present.

4. Plugs taken from the outer region of mycelial growth from an established culture on PDA can be used to start cultures of *Cladosporium cucumerinum*. Alternatively, cultures can also be started using a loop of mycelia and spores that are spread onto the PDA plate. Cultures are grown on PDA for approximately 7-11 days prior to use.

5. Add half-strength potato-dextrose broth (PDB). Full strength requires the suspension of 24 g HiMedia® premade PDB powder/L distilled water and only 12 g/L is required to make half-strength PDB. The mycelia and spores can be

scraped from the surface of the medium contained in the Petri dishes using a plastic spatula. The suspension is filtered through 2 layers of cheesecloth.

6. Humid chambers are created using two large baking dishes of the same size, the dishes should be large enough to contain a 20 x 20 cm TLC plate allowing for extra space to keep the edges of the top and bottom dishes away from the plate. One end of the baking dish, towards the top of the TLC plate (labeling section), is propped up by approximately 2 inches to avoid accumulation of moisture droplets. The other dish can be used as the cover (see below).

7. Distilled water should be brought to a boil prior to adding. Enough hot water should be added to cover ¾ of the bottom-baking dish, leaving the propped end water-free. After adding water, the top-baking dish is used as a cover. The inside cover should be wiped down 3-4 times, removing all moisture, prior to spraying plate.

8. TLC plates are sprayed with a suspension of *C. cucumerinum* spores (usually 3 – 3.5 x 10^6 spores/ml) in half-strength potato-dextrose broth.

9. Small beakers, 50 – 100mL, can be inverted to keep the plate above water (4 beakers/plate).

10. After the plates are sprayed with the *C. cucumerinum* suspension, they are immediately placed in the humid chamber. Masking tape should be used to completely seal the edges where the inverted baking dish (used as the top) is placed around the edge of the bottom-baking dish, sealing the two dishes together creating a chamber.

11. Plates should remain in the chamber for 3 days.

12. White areas on a grey-black background of fungal spores and mycelia on the TLC plate indicate antifungal compounds.

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LITERATURE CITED

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CHAPTER FOUR

PRUNUS MAACKII FUTURE RESEARCH

Knowledge of the common flavonoids found in *Prunus* and the identification of unique compounds, including non-substituted B-ring flavonoids, will help identify common precursors along with unique ones. The extraction of flavonoids is contingent on the type of plant and the biologically active compounds contained in the tissues (Khoddami et al., 2013). An HPLC/MS/MS machine, with a large fraction-collecting column, may be used to isolate the individual compounds based on retention-times and their UV-spectra. Once this is done, the individual compounds can be submitted for NMR and MS/MS analyses. Depending on each compounds crystallization properties, single crystal analyses may be employed as well.

In addition to the identification of unknown compounds, tissues also need to be examined for possible precursors. The chalcone isomerase (CHI) enzyme further synthesizes naringenin from a chalcone. Naringenin is the core flavonoid structure (flavanone) that leads to the production of other commonly found flavonoids by enzymatic modification e.g., flavones, isoflavonoids and flavanones (McNulty et al., 2009).

Thus, initial enzyme assays should include those commonly found in the flavonoid biosynthetic pathways, e.g., PAL, CHS/CHI and FHT assays (Halbwirth et al., 2009). For example, *PAL* gene expression would be screened *in vitro* because the PAL enzyme is a regulator of phenolic biosynthesis (Pina and Errea, 2008). The expression of this enzyme is critical because it represents the conversion of a primary metabolite

(phenylalanine) into a secondary metabolite (cinnamate)(Halbwirth et al., 2009). *O*-methyltransferases (OMTs) are particularly interesting from a plant pathology aspect. This class of enzymes is involved in the production of lignin precursors and the biosynthesis of phytoalexins. Expression of these enzymes has been shown to increase upon pathogen invasion. Gene expression can be examined by RNA blot analysis. In addition, the Southern blot analysis can be carried out upon DNA preparation (Suelves and Puigdomenech, 1998).

Since alnusin and alnustinol are somewhat unique with the 6-methoxylation and lack of B-ring substitution, it is likely that these flavonoids are synthesized from cinnamate, via 4-hydroxycinnamate. For this reason, unique enzymes will likely be found. One precursor could be pinocembrin, which is an antifungal compound found in the leaf glands of cottonwood (Shain & Miller, 1982). This particular flavonoid has two hydroxylations without B-ring substitutions. Pinocembrin is structurally similar to the commonly found flavonoids, e.g. naringenin and eriodictyol, but it is not synthesized from *p*-coumaric acid, but rather cinnamic acid. This compound can also be converted to pinobanksin by hydroxylation of the ketone. Thus, it is likely that pinobanksin is also present in the crude extract.

Enzymatic modifications are responsible for the conversion of flavanones (such as pinocembrin) to dihydroflavonols (such as pinobanksin). Many other products are produced from enzymatic reactions, which include tannins, flavonols, flavan-3-ols, and proanthocyanidins. The correlation of gene expression and enzymes such as flavone synthase, flavanol synthase, flavanone reductase, and dioxygenase will help elucidate the general biosynthesis of flavonoids found in *P. maackii*. Alnusin and alnustinol are

found in high concentration in *P. maackii* periderm tissue, and either absent or in very low concentration in other *Prunus* spp., therefore the primary goal will be to elucidate the gene expression correlated with cinnamyl-CoA-ligase, 6 hydroxylases and 6-methoxylases.

The expression of different genes should be compared among those commonly found in susceptible spp. and compared to the potentially resistant *P. maackii*. Once the biosynthetic flavonoid backbone enzymes have been identified, these enzymes should be compared to those found in *P. mahaleb* and *P. serotina*. The differences in enzyme activity and specificity will be of great use to identify the regulation of secondary metabolites with unique antimicrobial actvity.

Since fungicide drenches and fumigation treatments show little effects against *Armillaria* species (Amiri & Schnabel, 2012), finding a resistant rootstock is critical. It has been reported that pears are somewhat resistant (Dalili et al., 2010) and can serve as an alternative crop. However this is not an option in regions where fire blight is prevalent. It is possible that *P. maackii* can serve as a resistant rootstock. The cost and benefits of employing secondary metabolite biosynthesis in the absence of pathogens is important in host fitness (Thaler et al, 2012), therefore this should be investigated in *P. maackii* to give insight on the balance of phytoanticipin production.

The ultimate goal of *P. maackii* research is to identify the antifungal compounds and to potentially use this ornamental species as a resistant rootstock. The recent identification of two antifungal compounds suggests that flavonoids without B-ring substituents may contribute to the strong antifungal activity detected in the crude *P.*

maackii periderm extract. This species may contain a model pathway for the engineering of resistant *Prunus* species.

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