CHARACTERIZATION OF GROWTH CONDITIONS FOR PRODUCTION OF A LACCASE-LIKE PHENOLOXIDASE BY *AMYLOSTEREUM AREOLATUM*, A FUNGAL PATHOGEN OF PINES AND OTHER CONIFERS

by

JOHN MICHAEL BORDEAUX

(Under the Direction of Jeffrey F. D. Dean)

ABSTRACT

Amylostereum areolatum is a wood-degrading fungus vectored by the European woodwasp *Sirex noctilio* recently introduced into North America. Together they constitute a pathosystem capable of killing healthy conifers, particularly pine. The fungus kills liquid cultured cells of loblolly pine and can infect living seedlings. The fungus can be cultured on a described defined medium. *Amylostereum areolatum* produces a ca. 75 kDa laccase (multicopper oxidase) and conditions have been established for maximizing laccase production over 11 days. A three-step purification yields a single laccase activity band. The laccase is likely monomeric and oxidizes ABTS, catechol, 1, 8-diaminonaphthalene, guaiacol, hydroquinone, phloroglucinol, *p*-phenylenediamine, and syringaldazine. Its oxidation of resveratrol indicates a possible role in detoxification. Laccase activity is maximum at pH 4.0 and 30°C. It is apparently inhibited by *L*-cysteine, sodium azide and sodium chloride. SDS and EDTA activate the enzyme. Sodium azide has been demonstrated as a true inhibitor.

INDEX WORDS: *Amylostereum areolatum*, *Sirex noctilio*, *Pinus taeda*, laccase, phenoloxidase, resveratrol

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JOHN MICHAEL BORDEAUX

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JOHN MICHAEL BORDEAUX

Major Professor:	Jeffrey F. D. Dean
Committee:	Claiborne V. C. Glover III Scott Gold

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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CHAPTER 1: AN INTRODUCTION TO *AMYLOSTEREUM AREOLATUM*, A FUNGAL PATHOGEN OF PINES AND OTHER CONIFERS

Introduction

The European woodwasp *Sirex noctilio* is a major pest when introduced as an exotic into the commercial forest plantations of the southern hemisphere (Talbot 1977). *Sirex noctilio*, unlike other members of the Siricid wasps, attacks conifers exclusively (Talbot 1977). *Sirex noctilio* has typically appeared as a major pest in countries where the pines themselves have been exotics, planted to bolster the forest industry (Ciesla 2003). The success of an invasive species is based upon its aggressiveness in competition for resources and the lack of natural predation that would occur in its native range. *Sirex noctilio* has targeted Monterey pine (*Pinus radiata*) in New Zealand, Australia, South Africa, Uruguay, and Argentina, and attacks loblolly pine (*Pinus taeda*) in Brazil (Borchert 2006). In 1954, 30% of a 600,000 acre *P. radiata* forest in New Zealand was lost to *S. noctilio* attack (Coutts and Dolezal 1966). The insect was discovered in Uruguay in 1980; by then it had already spread over all of that nation's pine plantations (Ciesla 2003). Five million Monterey pines were killed in an outbreak of the insect in Australia between 1987 and 1989 (Ciesla 2003). The woodwasp was discovered in Brazil in 1998; where infestations were reported, 60% tree mortality occurred (2006). If no action is taken, potential losses in the southern United States (from 10-50% mortality) are estimated at $1.9 to $11 billion total, based on present value (APHIS 2007). A current map of *S. noctilio* detection since first discovered in the U.S. reveals slow but sobering progress of the infestation (Figure 1).

Sirex noctilio

Sirex noctilio, the European horn-tailed woodwasp, is an insect pest of conifers recently introduced into the United States (Dunkle 2005; Borchert 2006; Dunkle 2006; Dunkle 2006). Females of the species attack dead or dying *Pinus* species in its native range (Europe, Asia, and northern Africa), where it has little if any economic impact (Coutts and Dolezal 1966; Borchert 2006). The relative importance of *S. noctilio* to forest health in Europe may be measured by the fact that it is sometimes neglected entirely in discussions of European forest pests (Ciesla 2003).

Sirex noctilio is an insect in the order Hymenoptera, suborder Symphyta, superfamily Siricoidea, family Siricidae (Gauld et al. 1990). *S. noctilio* attacks are in fact the act of oviposition: female insects drill one to five small holes in the host tree through the bark into the cambium, injecting a phytotoxic mucus containing arthrospores of the fungal pathogen, *Amylostereum areolatum,* and sometimes an egg, into the holes (Coutts and Dolezal 1966; Madden 1968; Coutts 1969).

The female wasp is well-adapted for depositing these materials simultaneously: the egg is at the opposite end of the reproductive tract from the ovipositor and must pass through a common oviduct; both the source organs of the fungal pathogen's oidia (the mycangia) and the mucus or venom secretions (mucus gland and Dufour's gland) feed into this oviduct. Historical controversy concerning woodwasp larval diets notwithstanding, it is clear the larvae are at least partially mycophagous and ingest fungal mycelium and fungus-altered wood fragments (Francke-Grosmann 1939; Buchner 1965; Coutts and Dolezal 1969).

Amylostereum areolatum

Amylostereum areolatum is a fungus of the Basidiomycota, order Agaricales, family Thelephoraceae. It may be more familiar as a member of the earlier artificial order Aphyllophorales. At least one author characterizes it as "corticioid" based on basidiocarp

morphology (Graham 1967; Buchanan 2001). Clamp connections, a definitive basidiomycete character, were reported visible at high magnification (1750x) (Boros 1968). A white-rot fungus, *A. areolatum* is capable of breaking down cellulose, hemicellulose and, significantly, the chemically-resistant polymer lignin, though white-rot fungi vary in both the relative proportions of the compounds broken down as well as rate of degradation (Campbell 1932; Buchanan 2001). The term "white-rot" derives from the ability of these fungi to leave degraded wood with a bleached or whitened appearance, owing to the oxidative effects of the fungal extracellular enzymes. This bleaching potential has been harnessed in the decolorization of industrial effluent and wastewater containing dyes (Wesenberg et al. 2003).

Wood is principally composed of the biopolymers cellulose, hemicellulose, and lignin (Martínez et al. 2005). Lignin, an amorphous polymer of guaiacyl, syringyl, and *p*-hydroxyphenyl propanoid subunits, confers both mechanical strength and chemical resistance to wood (Martínez et al. 2005). White-rot fungi begin their degradation of wood carbohydrates (cellulose, hemicellulose, amylose, pectin) and lignin with oxidation and hydrolysis reactions (Campbell 1932). As lignin does not have bonds susceptible to hydrolysis, an oxidase initiates lignin fragmentation (Hatakka 2001). Any wood-degrading fungal enzymes must be extracellular to reach their intended substrate (Morozova et al. 2007).

The *S. noctilio/A. areolatum* Pathosystem

The lifecycles of the insect and its fungal symbiont are straightforward and are illustrated in Figure 2. As adults, males are first to emerge from conifer hosts and fly into the uppermost branches of the trees, swarming in a mating frenzy. Females may or may not mate with males before laying eggs. *Sirex* is facultatively parthenogenetic: unfertilized females lay eggs that produce only males; fertilized eggs result in males or females (Rawlings 1953; Morgan 1968). The female probes potential host trees with her ovipositor to determine suitability of the tree for oviposition. Preference is shown to trees previously stressed by suppression, drought,

overstocking, windthrow, or earlier *Sirex* attack (Gilmour 1965; Coutts and Dolezal 1966; Madden 1968; Madden 1977; Hurley et al. 2007). Once a tree is selected, the female drills with her ovipositor one to several centimeters into the wood, and any egg that is deposited passes through this channel into the hole, along with mucosal venom and arthrospores of *A. areolatum* (Coutts and Dolezal 1969; Gaut 1969).

The suborder Symphyta charge the larval stage of the insect life cycle with principal responsibility for obtaining nutrition (Gauld et al. 1990). The thriving fungus provides a primary source of nutrition for the woodwasp larvae. The fungus is directly introduced into its tree host (thus spared the trouble of penetrating a tree's formidable outer defense, its bark). The larvae tunnel in the tree for one to two years, apparently feeding and tunneling in fungus-altered wood exclusively (Vasiliauskas 1999), until they emerge as mature wasps. Emerging, mature females now carry oidia of the fungus in their mycangia (specialized organs in which the fungal arthrospores are stored in the female woodwasp). Adult insects do not feed (Madden 1975; Zondag and Nuttall 1977). Neither male nor female wasps survive long after mating and oviposition, and females can often be found on tree trunks in the act of oviposition.

The fungus is thus propagated clonally, and evidence suggests *A. areolatum* has come to rely on clonal propagation. *Amylostereum areolatum* rarely produces fruiting bodies in the wild in its native range. Though it has shown an ability to produce basidiocarps in the laboratory, *A. areolatum* fruiting bodies have not been seen at all in Southern hemisphere introductions of *S. noctilio* (Talbot 1977; Thomsen and Koch 1999; Slippers et al. 2001).

As little as one week following attack, the wood inside the oviposition tunnels has dried considerably. Tunnels are begun in sapwood, which is not normally dry. As early as two weeks after *Sirex* attack, diameter growth of trees is slowed or stopped, temporarily or permanently depending upon extent of wasp attack and susceptibility of the tree. Visible symptoms include the bending of needles at the fascicle sheath, chlorosis or browning of the needles, and in warmer climes, resin exuding from oviposition holes. Needles may brown all at once or

progressively from oldest to youngest. In the most serious cases, trees die within weeks to several months (Rawlings 1948; Coutts and Dolezal 1965). The initial effects of woodwasp attacks on conifer hosts *in vivo* appear related to senescence and altered water relations in the tree, initially laid to the fungus cutting off water supply to the crown, but later understood to be caused mainly by wasp mucus conditioning. "Conditioning" refers to the weakening of tree resistance to *A. areolatum* prior to the fungus becoming well-established. Conditioning is proposed to be the consequence of interrupted photosynthate transport from leaf to stem (starch accumulation in leaves, with a consequent starch deficit in the stem), with subsequent early browning and dropping of needles, resulting in tree stress (Rawlings 1948; Coutts and Dolezal 1965; Coutts 1969; Hart 1981).

Partial or complete recovery of *Sirex*-attacked trees is possible. Pines have bark as a physical barrier against fungal attack, but *Amylostereum* is inserted into the host tree beneath the bark. In this case the pine can exude resin as a second physical barrier to the invasion by insect and fungus. Early research showed that pines that have a high resin pressure were more resistant to *Sirex* attack and the insects were less successful. *Sirex* females sense resin pressure and moisture when drilling boreholes, via sensillae located on the ovipositor. They avoid, or abandon ovipositing into trees with high resin pressure or high moisture content (Madden 1968; Morgan 1968). Female woodwasps may simply die as a result of becoming glued in place when resin pressure is high, and eggs and larvae in oviposition holes flooded by resin are unlikely to survive (Coutts and Dolezal 1965; Zondag and Nuttall 1977),

Polyphenols, which can have fungicidal or fungistatic properties, are a second line of defense produced by trees against internal assault. Their composition and quantity change in response to fungal attack, and it is likely they are produced *in situ* as a direct response to fungal challenge (Coutts and Dolezal 1966; Hillis and Inoue 1968; Hart 1981). Stilbenes fall into this category of compounds (pinosylvin, pinosylvin monomethyl ether, resveratrol), and are considered phytoalexins, defensive compounds actively produced by plants in response to

infective insult (Coutts and Dolezal 1966; Hemingway et al. 1976; Zondag and Nuttall 1977; Hart 1981; Lange et al. 1994). The differential production of these secondary plant metabolites may be a factor in the inherent resistance of some trees to fungal infection. In *Pinus radiata* they act both to restrict ingress of *A. areolatum*, and to deter its growth. Pinosylvin has been demonstrated as a fungistat against another white-rot pine pathogen, *Heterobasidion annosum* (form. *Fomes annosus*) (Hart 1981).

The Model System Approach to Studying Tree Diseases

The most straightforward means to investigate the *Sirex/Pinus/Amylostereum* pathosystem would be to use living trees. This is neither cost-effective nor practical as achieving statistical power would require many dozens of trees and resources of time and money not available to this project. Additionally, since *A. areolatum* is an invasive pathogen that has not spread to the southeastern United States yet, there would be an unacceptable risk involved with using mature trees in the open environment. The use of model systems offers the advantages of minimal cost, manageability by a single researcher in a laboratory setting, and relatively fast results. The goal of this research project, to more fully understand the mechanisms of pathogenesis in *Sirex/Amylostereum*, is achievable using model systems. Model systems may yield results which can be generalized to populations of trees in the field and can be of predictive value when applied to the target population.

A useful experimental model for *S. noctilio* attack in pines must take into account the age and condition of the wasp's target trees. *Sirex* attacks older trees in preference to younger ones. Grown trees offer the geometry necessary for the woodwasp to complete its lifecycle. *Sirex noctilio* is known to have attacked young plantations of four-year-old trees (Morgan 1968), as well as trees from 18 to 34 years of age (Madden 1975). Using seedlings as a model system presents the drawback of comparing full grown potential host trees to trees of an age that would never be attacked. Nonetheless, seedlings offer a model of response closer to full grown trees

in comparison with tissue-cultured cells. Representing a relatively vulnerable state of maturity, seedlings offer a lowered threshold of resistance and response to pathosystem challenge in general. *Pinus strobus* seedlings have been used to model resistance to *Cronartium ribicola* (fungal pathogen) in grown trees (Smith et al. 2006). Five-year old *Pinus nigra* seedlings have been used to study *Sphaeropsis sapinea* pathogenicity, a disease most common in much older trees (Bonello and Blodgett 2003).

The use of model systems also eliminates the influence of variability in the growing environment. Environmental factors correlating with higher susceptibility to wasp attack include abiotic stresses such as stand overcrowding, suppression (trees in understory starved of light), tree internal temperature, low internal moisture content, drought followed by heavy rainfall, wind damage, and insect injury in the tree (Rawlings 1948; Jamieson 1957; Gilmour 1965; Graham 1967; Coutts 1968; Talbot 1977; Ciesla 2003; Hurley et al. 2007). The laboratory environment allows for control of the environmental factors. If environmental influences are investigated in the laboratory, variables can be introduced independently. Genetic factors enhancing susceptibility to attack include low resin pressure and differential production of fungistatic/fungicidal phenolic compounds (Hillis and Inoue 1968; Talbot 1977). These serve to undermine general tree vigor. Model systems can also control the genetic component of response variability by selection of the population to be tested.

As a model system for tree response, callus can provide inexpensive and easily-manipulable experimental units. Callus tissue is a mass of proliferative, undifferentiated or unspecialized plant cells that can be cultivated *in vitro*. In this project callus tissues were produced from loblolly pine needles and cambium for testing with *A. areolatum* mycelium and secretion products. Similar cell-suspension culture models have been established previously for *P. taeda*, *P. radiata*, *P. sylvestris*, and *P. banksiana* (Campbell and Ellis 1992; Eberhardt et al. 1993; Lange et al. 1994; Hotter 1998). *P. sylvestris* callus has been used to measure elicited responses (accumulation of stilbenes) to *Lophodermium seditiosum*, a fungal pathogen

(Teasdale 1984; Lange et al. 1994). *P. banksiana* callus was used to characterize cell-wall phenolic response to an ectomycorrhizal fungal elicitor (Campbell and Ellis 1992). It is reasonable to expect that responses to *A. areolatum* can also be elicited from *P. taeda* callus cell cultures.

Laccase

Extracellular phenoloxidase enzyme production has been reported in a wide variety of white-rot fungi and virtually all white-rot fungi show such activity (Hatakka 1994; Hatakka 2001; Morozova et al. 2007). Two of these phenoloxidases, laccase and peroxidase, are the most important enzymes involved in the breakdown of lignin (Hatakka 1994; Morozova et al. 2007). Both require a specific oxygen-containing species as a cosubstrate (Ullrich and Hofrichter 2007).

The reaction typically catalyzed by laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) is shown in Figure 3 (the substrate may be *ortho*- or *para*-; *para*- is shown here) (Moss 2008). Laccases play an important role in lignolytic systems (Morozova et al. 2007). Because their active sites consist of four copper ions, laccases are sometimes called “multicopper oxidases.” One of these coppers is a Type-1, which gives a strong absorbance in visible light, resulting in the other common appellation for laccases, “blue copper oxidases” (Morozova et al. 2007). Laccase uses molecular oxygen as the oxidant. While the laccase that acts to degrade lignin is extracellular, intracellular laccases have been described and correlate with separate physiological functions (Zhu et al. 2001). Laccase is characterized by its broad specificity for substrates, phenolic and otherwise (Morozova et al. 2007). Plant laccases have been proposed to be involved in synthesis of lignin (Dean and Eriksson 1994). Fungal laccases, on the other hand, frequently appear involved in degradation of lignin (Bertrand et al. 2002). Fungal laccases are usually extracellular, have a wide substrate range, are monomeric, and have a range of glycosylation of 10-25% (Baldrian 2006). One sampling of white-rot fungal laccases gave a range of molecular weights from 53-64 kDa; among fungi generally the median laccase size

among 115 species was 66 kDa, and the range for all laccases is 50-130 kDa (Hatakka 1994; Baldrian 2006; Morozova et al. 2007). Fungal laccases, in addition to their oxidation function in degrading lignin, play a role in detoxifying defense compounds (plants as well as other fungi) as well as in pathogenesis (Rigling et al. 1989; Choi et al. 1992; Sbaghi et al. 1996; Saparrat et al. 2002; Schouten et al. 2002; Zhu and Williamson 2004; Morozova et al. 2007; Velazquez-Cedeno et al. 2007).

Peroxidases (EC 1.11.1) are heme-based enzymes that oxidize their substrates using hydrogen peroxide as the oxidant. Two main groups of peroxidases produced by wood-degrading fungi are manganese peroxidase (MnP) (EC 1.11.1.13) and lignin peroxidase (LiP) (EC 1.11.1.14). MnP oxidizes Mn^{2+} to Mn^{3+} in the presence of H_2O_2 (Moss 2008). The oxidized manganese then abstracts an electron and a proton from the O-group in a substrate phenol ring. The resulting free radical causes the breakdown of the molecule. The characteristic reaction is shown in Figure 3. Like MnPs, LiPs also remove electrons, but from non-phenolic portions of lignin. Again, resulting free radicals cause the fragmentation of the oxidized compound (Hatakka 1994; Moss 2008). The reaction catalyzed by lignin peroxidase is shown in Figure 3.

To be precise, the use of the term “laccase” should be clarified. “Laccase” is a term used to commonly describe a phenoloxidase characterized by multiple copper centers and the ability to oxidize hydroquinone with the uptake of oxygen. The name for an enzyme should describe the substrate for which the enzyme is specific. As most “laccases” are generally quite non-specific for substrates, the term is used here as is commonly used. To name the enzyme without ambiguity it is necessary to understand what the target substrate(s) is for *A. areolatum*, a question out of the scope of this study. Hereafter in this document, the word “laccase” in reference to the phenoloxidase purified from *A. areolatum* should be taken to mean “laccase-like” to be as precise as possible.

Except for a single general reference to “laccase positive” (Baxter et al. 1995) there have been no previous reports of peroxidase or laccase production by *A. areolatum*. However, genes for both lignin peroxidase and manganese peroxidase have been identified in this fungus, indicating the presence of a suite of phenoloxidase enzymes [53]. Additionally, recent work has demonstrated genetic relationships between the extracellular phenoloxidases of *Heterobasidion annosum* and those of *Amylostereum* and other white-rot fungi (Maijala et al. 2003). These relationships suggest a common mechanism of the lignin-degrading mechanisms of the pathosystems and possible commonalities in the pathosystem. *H. annosum* and *A. areolatum* are both destructive white-rot fungal pine pathogens, and discovering common mechanisms of pathogenesis between them may offer common targets for controlling them.

Conclusion

The threat of *S. noctilio* to North American pines in general and loblolly pine in particular likely will not be resolved through any single means. A multi-pronged approach to protecting trees of commercial and recreational value will most likely take into consideration management practices, insecticides, biocontrol methods, and the development of resistant trees. To develop resistant trees, genetically-suitable source material must be identified. *P. taeda* responses elicited by *A. areolatum* have been identified, and a system for measuring them reliably has been developed. Successful methods for *A. areolatum* resistance screening may find further utility as the basis for similar resistance assays to combined *S. noctilio* /*A. areolatum* insult in *P. taeda.*

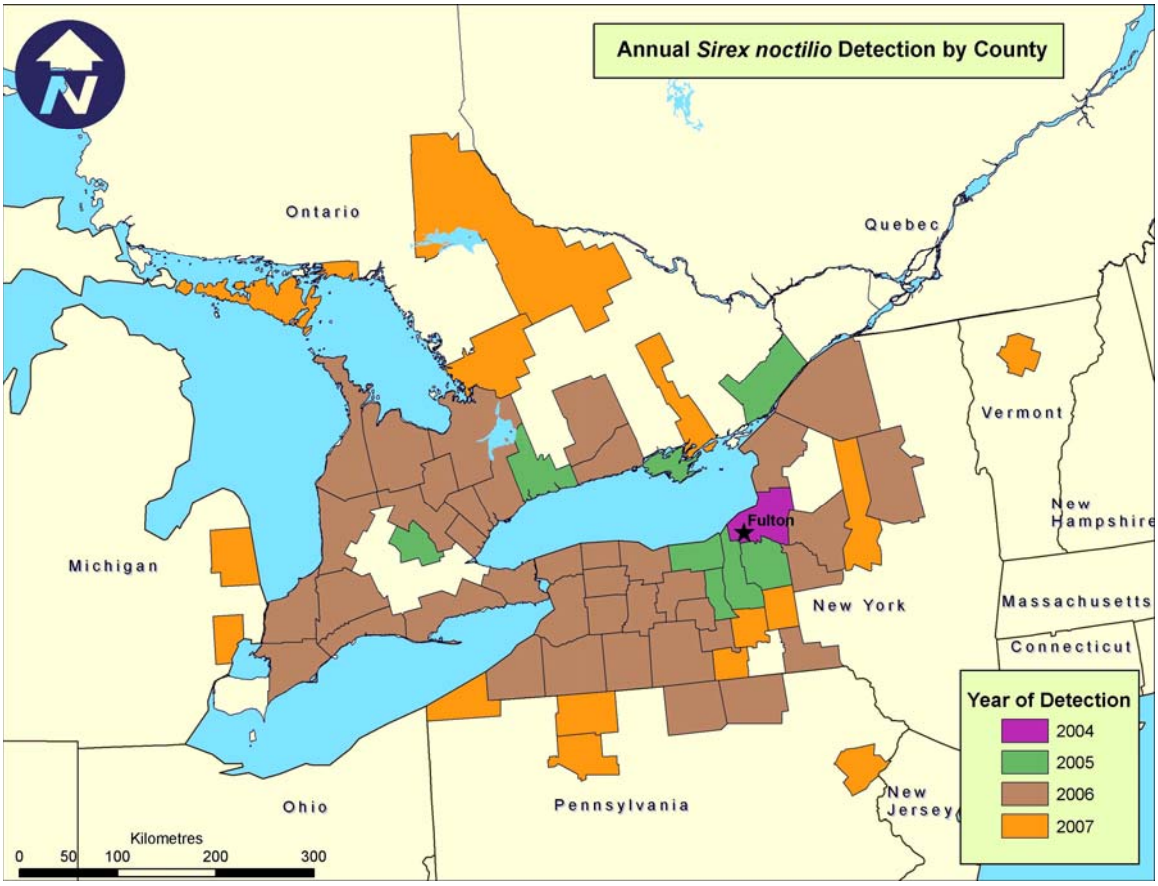


Figure 1: Annual *Sirex noctilio* Detection by County, North America, April 2008. Map shows detection based upon trapping and identification of woodwasps. (Peter de Groot, Canadian Forest Service).

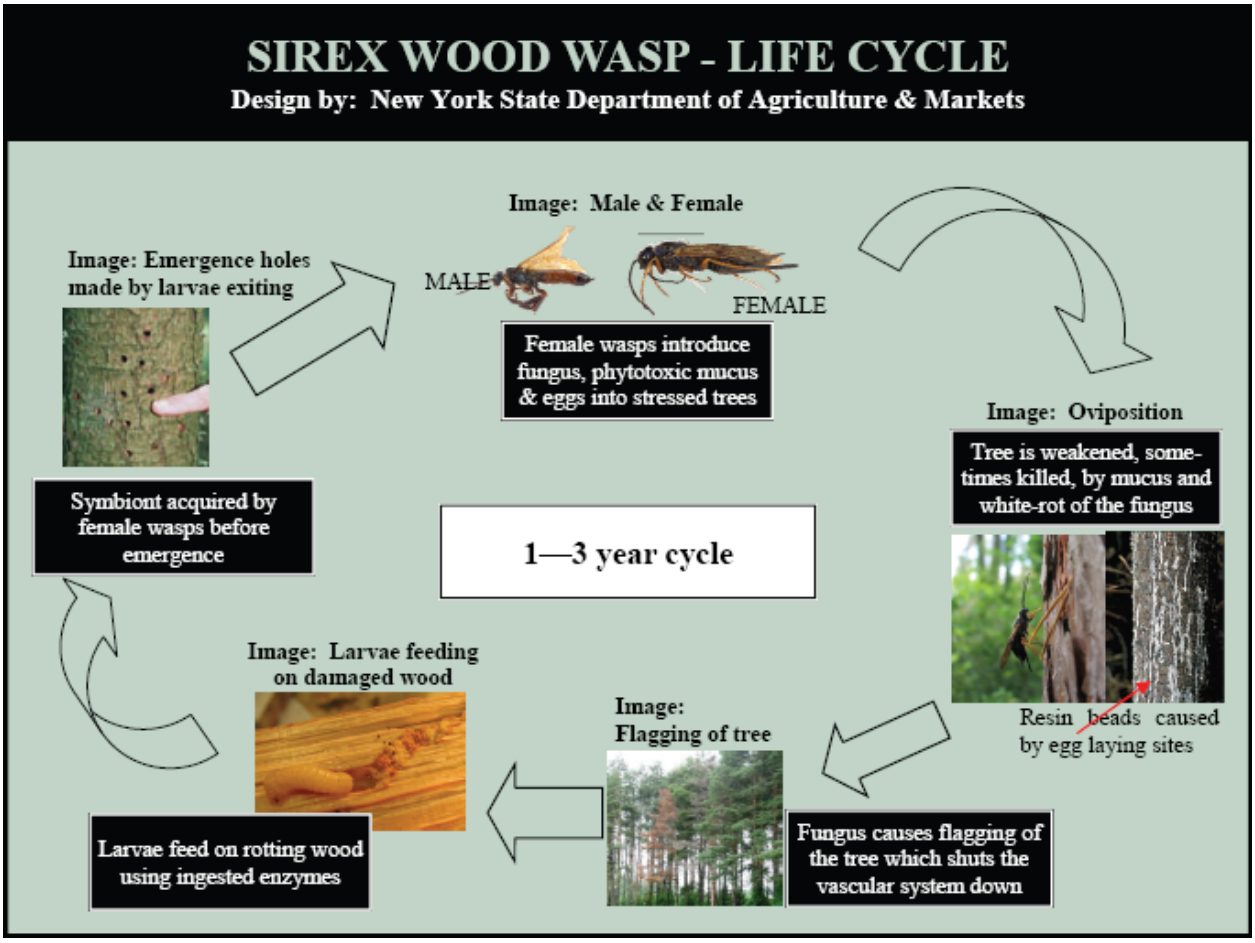
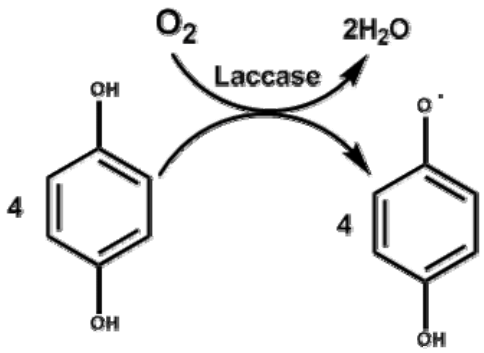
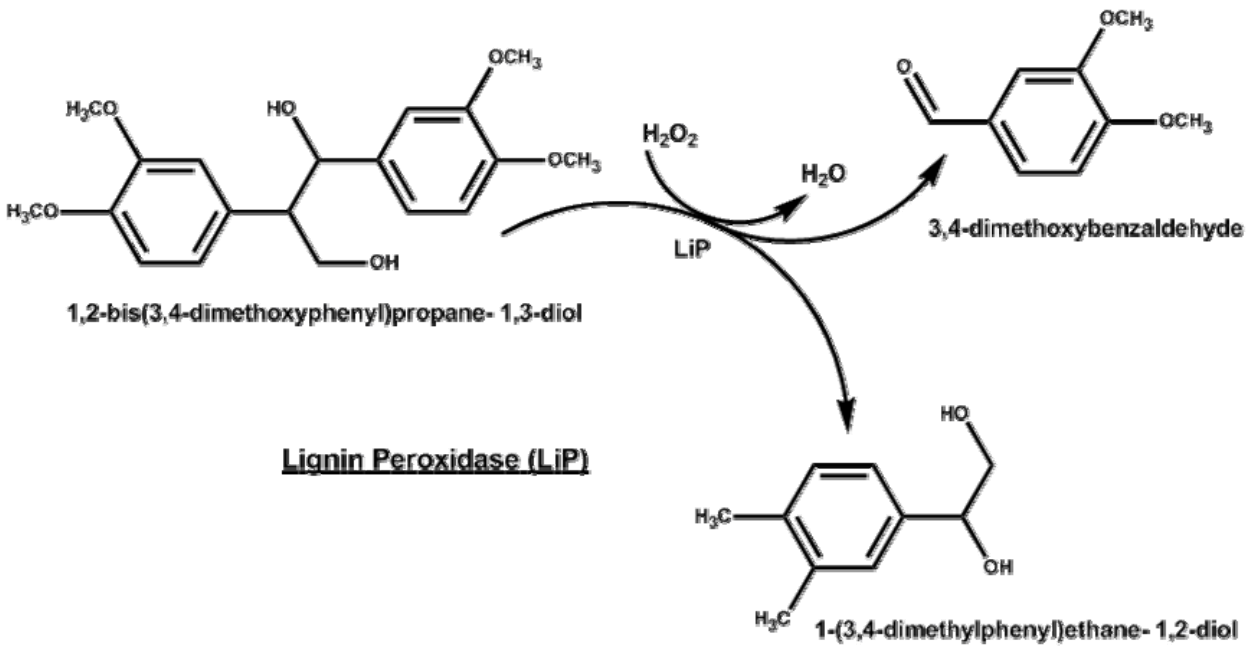


Figure 2: *Sirex noctilio* Life Cycle. Prepared by the New York Department of Agriculture and Markets (NYDAM 2008).



Laccase (1, 4-benzenedioloxidoreductase)

$2Mn(II) + 2H^+ + H_2O_2 \rightarrow 2\ Mn\ (III) + 2H_2O$ **Manganese Peroxidase (MnP)**



Lignin Peroxidase (LiP)

Figure 3: Reactions Catalyzed by Phenoloxidases: Laccase, Manganese Peroxidase, and Lignin Peroxidase. These are the phenoloxidase reaction types expected in white-rot basidiomycetes (wood-degrading fungi).

CHAPTER 2: GROWTH OF *AMYLOSTEREUM AREOLATUM* ON A DEFINED NUTRIENT MEDIUM, AND CHARACTERIZATION OF FUNGUS/PINE INTERACTIONS

Introduction

Amylostereum areolatum is a wasp-vectored fungal pathogen that uses pines and other conifers as its host. The fungus is a white-rot basidiomycete, degrading both cellulose and lignin in its host trees. For the following studies it was important to establish optimal solid and liquid culture conditions for fungal growth and enzyme production. A variety of growth media for maintenance of *A. areolatum* have been described in the literature, including glucose agar, malt extract agar, potato dextrose agar, neutral Dox + yeast liquid medium, corn meal agar, and blocks of wood (Talbot 1964; King 1966; Coutts 1969; Kile and Turnbull 1974; Thomsen and Harding 1996). However, no defined growth media for *A. areolatum* have been described. Defined media will allow precise manipulation of components for nutrition studies and for optimizing enzyme production.

As wood-degrading fungi, white-rot basidiomycetes have the capacity to degrade cellulose, hemicellulose, and lignin (Müller 1934; Francke-Grosmann 1939; Martínez et al. 2005). The primary wall in plant cells is composed of cellulose, hemicellulose and pectin (Isaac 1992). The breakdown of these polysaccharides depends upon production by the fungus of cellulases, hemicellulases and pectinases, and likely many other hydrolytic enzymes. These carbohydrates are readily useable and are likely the primary source of carbon nutrition for the fungus.

As the primary cell wall is broken down, these simpler carbohydrates run short. The secondary cell walls also contain cellulose and hemicellulose. But another major component of this secondary cell wall is a digestion-recalcitrant phenolic biopolymer called lignin. To fully

exploit available cell wall polysaccharides, fungi must attack the secondary cell wall. Removal of lignin allows access to the hemicellulose contained in the plant cell wall. Under such conditions of carbon deficit (starvation), white-rot fungi shift to production of enzymes capable of degrading the secondary cell wall. Fungi produce phenoloxidase enzymes to degrade lignin. Lignin is also concentrated in the middle lamella of plant cells and always presents a barrier to cell-to-cell fungal movement.

The best characterized of the phenoloxidases include manganese peroxidase, lignin peroxidase, and laccase. It is possible to manipulate the culture medium for white-rot fungi to induce higher production of these enzymes. The most important and widely-used inducer of fungal laccase in liquid culture is 2, 5-xylidine (Figure 4). It is a laccase substrate, and has demonstrated induction of fungal laccase production as much as 160-fold (Rogalski et al. 1991; Eggert et al. 1996; Leonowicz et al. 2001; Bertrand et al. 2002).

Detection of specific phenoloxidase activities in living tissue or media can be problematic. Living systems typically contain a large and heterogeneous mixture of enzymes, as well as a variety of small and large molecules have inherent chemical properties. A reagent for phenoloxidase detection must be unaffected by the matrix and specific for the enzyme being tested. Syringaldazine is a reagent with these characteristics (Harkin and Obst 1973; Harkin et al. 1974). The insoluble oxidation product of syringaldazine is a deep purple, readily distinguishable from many biological matrices. To be specific for laccase, the material tested must be free of hydrogen peroxide; otherwise, syringaldazine may also be oxidized by peroxidase. Guaiacol is also a known substrate for phenoloxidase enzymes (Dean and Eriksson 1994). Its oxidation product is dark red or maroon in color and is easy to see in solid culture. ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) has been used as an artificial substrate for both laccase and peroxidase activity (Sterjiades et al. 1993). It has a high

molar absorptivity and a dark green oxidation product (readily detectable) and is useful at the low pH levels found in some fungal liquid cultures.

To study the fungus/pine interactions it is also necessary to identify a host plant system that can be manipulated under controlled conditions. Mature pine trees are large and difficult to manipulate as experimental units. Model systems, such as pine seedlings, or plant cells grown in culture are more amenable to small-scale, confined investigations of fungal/pine interactions. Pine seedlings have been used by other investigators as model systems for fungal challenge (Preisig-Mueller et al. 1999; Bonello and Blodgett 2003; Smith et al. 2006). *Pinus* seedlings were employed in an early *Sirex* study of fungal infection (Vaartaja and King 1964). *Pinus taeda* seedlings can be generated in a relatively short time (6-12 months), making them readily available and replaceable. Maintaining large quantities of identically-grown seedling experimental units eliminates many of the environmental and abiotic variables always present in a complete ecosystem.

Pinus taeda tissue explants can be used to generate callus tissue, non-photosynthesizing undifferentiated cells. These cells can be grown in large quantities on solid or in liquid medium (Brown and Lawrence 1968; Brown 1990; Caponetti 1996). Liquid suspension cultures of pine cells have been used to explore several types of pine response to fungal challenge: accumulation of cell wall phenolics, accumulation of free phenolics, ethylene and chitinase production, lignification, and oxidative burst (Campbell and Ellis 1992; Eberhardt et al. 1993; Lange et al. 1994; Popp et al. 1996; Hotter 1998; Shein et al. 2003).

With optimally-grown *A. areolatum* and *P. taeda* tissue available, it is possible to study interactions between them. That a fungal symbiont likely overcomes plant host defense is well-supported (Dowd 1992). Literature examples of fungal enzymes involved in overcoming host defense include the detoxification of legume genistein by *Armillaria mellea* (Curir et al. 2006), detoxification of grapevine resveratrol by *Botrytis cinerea* (Sbaghi et al. 1996), production of laccase-derived, fungus-protective melanin by *Cryptococcus neoformans* in human hosts (Zhu

and Williamson 2004), and the hypovirulence of laccase-less mutants of *Cryphonectria parasitica* in chestnut (Rigling et al. 1989).

Materials and Methods

Components for the preparation of defined media were supplied by J. T. Baker, (Phillipsburg, NJ), Fisher Scientific (Pittsburgh, PA), Fluka (St. Louis, MO), Gibco (Carlsbad, CA), Research Organics (Cleveland, OH), Research Products Intl. (Prospect, IL), and Sigma-Aldrich (St. Louis, MO). Catalog numbers are listed alongside the chemicals in the medium preparation tables. 2, 5-xylidine (D0668) was supplied by TCI America (Portland, OR). Potato dextrose broth (PDB, 254920) was supplied by Becton-Dickinson (Franklin Lakes, NJ). Potato dextrose agar (PDA, P2182) was supplied by Sigma-Aldrich (St. Louis, MO). Malt agar (R453891) was supplied by Remel (Lenexa, KS). Phytagar (10675-023) was supplied by Gibco (Invitrogen, Carlsbad, CA).

***A. areolatum* Culture Conditions and Induction of Laccase Activity**

Amylostereum areolatum (strain IFO-9221 ATCC# 22251) was provided by Dr. David Williams (USDA/APHIS Center for Plant Health Science and Technology, OTIS ANGB, MA USA). The fungus was serially propagated by placing a five millimeter plug of solid medium with active mycelium top face down on fresh potato dextrose or malt agar medium. For long-term storage, two-centimeter slivers of sterile *P. taeda* seedling cambium or two-centimeter toothpick fragments were placed at the periphery of a PDA plate, and a one cm^3 agar plug from an active *A. areolatum* culture was placed in the center. Plates were sealed, stored in darkness, and allowed to grow for two months. At two months the wood fragments, which had been overgrown with *A. areolatum* mycelium, were placed into individual 1.5 ml centrifuge tubes and stored at -20°C or -80°C. Fresh cultures were prepared from these fragments at regular intervals throughout the study.

Defined solid and liquid media were based on those described by Pointing, with supplements suggested by Levin (Pointing et al. 2000; Levin and Forchiassin 2001). The glucose level was based on that for potato dextrose broth (20 g/L), and the nitrogen level according to Pointing (4 g/L ammonium tartrate). Copper levels were increased to 0.04 mM to provide sufficient copper for maximum laccase production. The final medium composition is described in Table 1.

Fungal growth in defined medium was measured at pH 3.5, 4.5, 5.5, 6.5, and 7.5. For each pH, 400 ml of defined medium was adjusted to a final value, medium was placed in 50 ml into 250 ml flasks to yield five replicates at each pH. Flasks were autoclaved after aliquoting. For each set of five flasks, three received a five mm agar plug with *A. areolatum,* one flask received a fungus-free agar plug, and the fifth flask received no addition. Flasks were incubated in darkness at 24°C without shaking (still cultures). After 26 days, dry weights for the mycelial mats were measured, pH of the culture filtrate was assessed, and the culture filtrates were stored frozen for later analysis of secreted enzyme activity.

To determine optimum growth temperature for the fungus on malt agar (1.5% w/v), plates were inoculated with a five mm plug of *A. areolatum* taken from fungus culture at least 15 days old. Plugs were placed mycelium-side down at the center of each plate. Plates were sealed and placed in darkness at 15°, 20°, 24°, 28°, 32°, or 37°C. Plates were replicated five times at each temperature. Growth, measured as diameter of the fungal colony edge-to-edge in millimeters, was recorded at three-day intervals for up to 24 days.

To determine the upper temperature limit for fungal growth and survival, three solid defined medium plates were inoculated with five millimeter agar plugs from a ten-day old *A. areolatum* culture. A fourth plate with a sterile agar plug placed in the center served as a control. After 12 days at 24°C, the four plates were moved to a 42°C chamber. After five days at 42°C, one of the plates was returned to 24°C. At ten days, a second plate was returned to 24°C. At 14 days a third plate and the control were returned to 24°C. In a second experiment,

ten-day old plated fungal cultures were shifted to 37°C for seven days, then returned to permissive (24°C) temperature conditions and monitored for diameter growth.

Defined media prepared at the optimal pH for fungal growth (pH 6.5) was used for the assessment of C/N ratio effects on fungal growth and lignolytic enzyme production. Five replicate flasks were prepared for each of four conditions: normal carbon and nitrogen (20 g/L glucose and 4 g/L ammonium tartrate), normal carbon, low nitrogen (20 g/L glucose and 0.4 g/L ammonium tartrate), low carbon, normal nitrogen (2 g/L glucose and 4 g/L ammonium tartrate), and low carbon, low nitrogen (2 g/L glucose and 0.4 g/L ammonium tartrate). For each set of five flasks containing 50 ml medium each, three received a five mm agar plug of *A. areolatum*, one received a sterile agar plug, and one received nothing. Flasks were incubated in darkness at 23°C without shaking (still cultures). At 30 days mycelium was collected by filtration through Whatman 934-AH glass fiber filters and oven dry weight was obtained. For fungal culture filtrates, final pH was measured, and the filtrate was then reserved for later activity testing. Growth curves for the defined (basal) fungal medium at optimum pH and temperature were obtained as follows. Flasks (250ml Erlenmeyer) containing 50ml basal medium were inoculated with eight mm plugs of *A. areolatum* mycelium then placed in darkness at 24°C with no shaking. Two flasks were removed every two days, and dry weights were determined for the mycelial mat. Liquid culture filtrates were tested immediately for optical density by running spectral scans from 221-749 nm on four microliter aliquots in triplicate (Nanodrop UV/VIS spectrophotometer, Thermo/Fisher, Pittsburgh, PA). The remainder of the filtrate was stored at -80°C and later tested for phenoloxidase activity over the time course of the experiment in microplate assays. For all microplate assays of laccase activity, the following reaction mixture was used:

30µl 100mM sodium acetate, pH 5.0

10µl 0.01mg/ml catalase (0.001mg/ml final concentration)

10µl test solution

50µl 0.03% ABTS

The assays followed this order of addition as well. Typical assays scanned once every thirty minutes for a total of 8 hours. Generally the 6-hour reading was used. Eight hours of data were collected each time to ensure the reaction profile indicated the reaction had gone to completion. The instrument used was the BioTek FL600 fluorescence plate reader with a 405nm filter. Early, highly dilute culture filtrates required lengthy oxidation periods, even with a high molar absorptivity substrate such as ABTS.

Attempts to induce laccase production by *A. areolatum* in liquid culture were carried out as follows. Erlenmeyer flasks (250ml) containing 50ml sterile potato dextrose broth (PDB) were inoculated with eight mm plugs of *A. areolatum* on solid medium. Flasks were incubated in the dark at 24°C, and 200µl aliquots were removed from each flask every two days. On day 13, the inducer, 2, 5-xylidine, was added to 0, 15, and 30 µM in triplicate. Samples were taken immediately before and after induction and every 12 hours thereafter for 21 days.

Enzyme Activity

Pilot experiments were carried out to detect phenoloxidase activity in solid and liquid fungal culture. To test for laccase activity in liquid culture, one ml of 2.3 mM ABTS was added to two small glass vials. To one vial was added 200 µl of two-week-old sterile-filtered liquid culture of *A. areolatum* grown in PDB, and to the second was added 200 µl PDB unexposed to fungus. Vials were incubated at ambient temperature overnight (18 hours). The flask exposed to spent fungal medium had changed to dark green, characteristic of ABTS oxidation, while the PDB-only exposed flask had not changed color (Figure 10).

To test for laccase activity in solid fungal culture, a paper disc was placed onto the growing edge of a 14-day-old *A. areolatum* colony on defined medium. To the disc was added 10 µl of 0.1% syringaldazine in ethanol. The discs were observed for color change. Syringaldazine (0.1%) was also applied directly to an *A. areolatum* colony of the same age, and the plate was observed for color change.

Fungal Mutagenesis Target Tissue Production

One way to demonstrate that laccase is directly involved in pathogenesis, is to show that a mutant fungal strain that doesn't produce active laccase is incapable of establishing itself in the host tree. One method of producing such mutants is dependent upon isolating single-nuclear hyphae (homokaryons) and irradiating them. Three methods were tested for production of *A. areolatum* mutagenesis target tissue. In the first method, fungal cultures on solid medium were placed at 37°C for at least two weeks to induce formation of arthrospores by heat-stress. At the end of the stress period, plates were placed atop magnetic stir plates, and one to two milliliter aliquots of Ringer's saline were added to the plates prior to stirring. After five minutes the liquid was recovered, the solution serial diluted, and plated onto potato dextrose agar plates. Plates were sealed with two layers of Parafilm (SPI Supplies, West Chester, PA) and stored at 25°C in darkness for 40 days and monitored daily for production of single colonies.

In the second target tissue production method, *A. areolatum* mycelium was scraped from 1 month and 5 month old malt agar plates. Mycelia from the two plates were combined and half of the mixture was added to one milliliter of 0.9% saline, and the other half to one milliliter 100 mM sodium citrate/200 mM sodium phosphate, pH 5. After vortexing and sonication for 1 hour, dilutions of each suspension (1:10, 1:100, and 1:1000) were made in their respective buffers, and 200 µl aliquots of each dilution were spread onto separate malt agar plates. Plates were allowed to dry in a sterile hood and then were sealed prior to incubation in the dark at 24°C. Plates were monitored daily for production of single colonies.

A third method of mutagenesis target tissue production attempted was dispersal of fungal mycelium homogenate onto solid medium. Three flasks containing 200 ml of defined medium were each inoculated with one eight millimeter plug of *A. areolatum* from a colony grown on solid medium. These flasks were sealed and stored in darkness at 24°C. After eight weeks the mycelial mats were combined along with the remaining liquid from one of the flasks and ground using a tissue homogenizer (PowerGen 700). The homogenate was reground on ice using a small-bore tissue grinder (Omni TH) for 20 minutes, and then diluted 10 and 100 times with Ringer's saline. Aliquots (200µl) were inoculated onto solid defined medium plates, sealed and incubated in the dark at 24°C. Plates were monitored daily for single colony production.

P. taeda Plant Tissue Cultivation

Pinus taeda (loblolly pine) seeds were soaked in a one percent hydrogen peroxide solution at 4°C for four days to stimulate germination (Barnett and McLemore 1967). Seeds were then drained and planted in commercial potting soil. At six weeks the seedlings were transferred to 164 ml growth cones (Ray Leach Cone-Tainer, Canby, OR), where they could develop substantial tap roots. Seedlings were thereafter maintained in growth chambers under a 16 hours light/8 hours dark diurnal cycle. Plants were watered every two to three days. At two week intervals seedlings were fertilized with a solution of 14.5 g MiracleGro (Scotts, Marysville, OH) and 1.2 g chelated iron (Becker-Underwood, Ames, IA) in four liters of water.

Tissue explants from needle and cambial tissues from loblolly pine seedlings were sterilized by soaking in a solution of 10-20% commercial bleach, adding several drops of commercial liquid detergent as a surfactant (Caponetti 1996). Surface-sterilized tissues were plated onto a solid inductive medium described by Eberhardt (Table 2) (Eberhardt et al. 1993). After six weeks when callus had begun to form at cut sites, callus was excised with a sterile scalpel and transferred to fresh medium. After several weeks on solid medium, callus tissue

was introduced into Eberhardt liquid medium (omitting agar). Liquid cultures were maintained under constant light at 25°C with 120 rpm orbital shaking. Serial transfers of three milliliters of cells to 47 ml of fresh liquid medium were made every three to five weeks to maintain cultures. Lines derived from separate source trees were kept separate.

Interactions of *A. areolatum* with *P. taeda* Tissues

To develop a preliminary understanding of pine seedling defense responses, the response of pine seedlings to a yeast cell wall elicitor preparation was tested (Brownleader et al. 1997). To prepare elicitor, 200 g of yeast extract were dissolved in 400 ml water and precipitated overnight twice with 1600 ml ethanol. This extraction was performed three times. Precipitate was collected by centrifugation and dried under vacuum. A 0.5 mg/ml filter-sterilized aqueous solution of the precipitate was used as elicitor. For seedling experiments, six month old seedlings were challenged with elicitor solution delivered into the cambium via three milliliter syringe, or by direct addition to a decapitation wound one centimeter below the apical meristem. Seedlings were subsequently returned to standard growth conditions and monitored for noticeable changes. A more concentrated elicitor preparation (5 mg/ml) was tested on decapitation wounds of other seedlings.

The infection of *P. taeda* seedlings with *A. areolatum* fungus was carried out as follows. Live ten month old seedlings were decapitated at the apical meristem. To prepare inoculant, mycelial mats of three, eight-week-old unstirred liquid cultures of *A. areolatum* in defined medium were ground with a tissue homogenizer, and the suspension stored for up to three months at 4°C. *Amylostereum areolatum* fungal homogenate was applied to the wounded seedling surface. The surface was then deeply injured with an 18-gauge needle, and the homogenate reapplied.

A control was prepared by decapitating a seedling and applying nothing. A second control was prepared by inoculating a decapitated seedling with a twice-sterilized fungal

homogenate (fungal homogenate was autoclaved twice at 131°C for 30 minutes each time). A third control was prepared by decapitating a seedling and applying a sterile-filtered (0.22 μm filter) fungal homogenate. A fourth control consisted of a seedling left completely unaltered. This set of five seedlings (unaltered, decapitated, live fungus, sterilized fungus, sterile-filtered culture medium) was replicated three times for each experiment, and the experiment was repeated a week later and again two weeks later for a total of three experiments and 45 seedlings. Quality of the inoculating material was assured by adding a quantity of the inoculating tissue to malt agar plates and observing subsequent fungal growth. The live fungus was the only inoculant which produced live mycelium.

The seedlings were kept afterward in a 24-26°C growth chamber with a light/dark cycle of 16/8 hours. They were watered two to three times each week and fertilized every two weeks with 14.5 g MiracleGro and 1.2 g chelated iron per four liters of water. Each week the seedlings were photographed and monitored for a total of 19, 20, and 21 weeks (begun at one-week intervals). The seedlings were observed carefully for signs of seedling mortality (curling of needles, fading to a gray-green or brown in needles, drying of needle tissue).

For *in vitro* fungus/pine challenge, it was necessary to establish a medium capable of supporting both tissues simultaneously. To test the ability of callus to grow on known fungal medium, aliquots of *P. taeda* needle cell-suspension culture (two milliliters) were inoculated onto malt agar or potato dextrose agar plates. To test the ability of fungus to grow on the pine inductive medium, *A. areolatum* agar plugs were placed on Eberhardt solid medium.

Pine callus tissue was challenged by *A. areolatum* in solid medium as follows. Three plates of Eberhardt medium were spread with 0.5 ml pine cell-suspension culture over half of each surface. When dry, an agar plug of *A. areolatum* hyphae was placed onto the opposite side of each plate. Plates were sealed, protected from light, and incubated at 25°C for 30 days. Plates were observed for callus death as indicated by browning and cessation of growth.

Results

A. areolatum Culture Conditions and Induction of Laccase Activity

Amylostereum areolatum was successfully cultured on potato dextrose agar, malt agar, and a defined medium based on that described by Pointing (Pointing et al. 2000). *Amylostereum areolatum* can also be grown, albeit poorly and with slow diameter growth, on the Eberhardt medium for pine callus tissue. Pine wood fragments and toothpicks overgrown with *A. areolatum* and stored for as long as 14 months at -20° and -80°C were successfully recultured by retrieval and placing onto malt agar.

Dry weight production of fungal mycelium was maximal at pH 6.5 in defined medium (Figure 6). Production of laccase activity on a volume basis was also greatest at pH 6.5 but was very nearly as high at pH 4.5 (Figure 5). Production of laccase activity on a per mass basis was maximum at pH 7.5. Peroxidase production on a per volume basis or a mass basis was maximal at pH 5.5 (Figure 6). Note that the activity assays here were performed only once, and the data should thus be considered preliminary.

Amylostereum areolatum growth was maximal at 24°C, but it grows very well from 20° to 28°C (Figure 7). Growth of *A. areolatum* on solid culture was completely checked after 5 days at 42°C or 7 days at 37°C. In either case growth of the fungus did not resume after return to permissive temperatures. Five days at 42°C and seven days at 37°C can be considered kill conditions.

Yield of laccase activity on a per-volume medium basis was maximal when carbon and nitrogen were at normal levels (Figure 8). On a per-unit fungal mass basis, laccase activity was highest under the low carbon/high nitrogen conditions, but a nearly equivalent level of production occurred under low carbon/low nitrogen conditions (Figure 9). Peroxidase activity was not detected when carbon levels were normal. Peroxidase activity recovery was maximized when both nitrogen and carbon were limiting (Figure 10). These assays were performed only once, and the data presented here should be considered preliminary.

Application of a known laccase inducer, 2, 5-xylidine, to liquid cultures of *A. areolatum* did not reproducibly increase laccase production under the growth conditions described here.

Enzyme Activity

Pilot experiments to test for phenoloxidase activity in liquid and solid medium gave encouraging results. A sample of 2-week-old *A. areolatum* spent liquid medium added to a buffered ABTS solution gave the characteristic dark green color of ABTS oxidation. Controls (medium unexposed to fungus, added to buffered ABTS solution) showed no color change. The color change occurred in the absence of hydrogen peroxide (catalase added), suggesting that an O_2-dependent phenoloxidase was responsible. Overnight exposure was required to fully oxidize the ABTS, indicating that the activity was present but not at high levels (Figure 11).

On solid medium, a 14 day old *A. areolatum* colony exposed to syringaldazine at its growing edge showed oxidation of the substrate within 10 minutes, growing more intense by 20 minutes (color was dark purple at its edge) (Figure 12). An autooxidation control (disc with syringaldazine added on a sterile plate) did not display a similar color change. The color change was obvious in a positive control (horseradish peroxidase and hydrogen peroxide were added to a paper disc). Syringaldazine applied directly to a separate *A. areolatum* culture of equivalent age developed the same purple color within eight minutes of application This coloration clearly delineated the growing edge of the culture after 24 minutes.

Fungal Mutagenesis Target Tissue Production

In order to show that laccase plays a role in pathogenesis, a mutant strain of *A. areolatum* not producing laccase should show reduced virulence in trees. While CFUs could not be achieved here, it has been done in a similar system (Eggert et al. 1997). To achieve this end, it is necessary to establish individual colony forming units (CFUs) and irradiate them, then screen them on selective medium. Individual CFUs were not produced in any of the

experiments. In cases where growth occurred, mycelium grew together as a mat. The important result of these experiments was the production of a useful fungal inoculant type (a fungal homogenate) that was used in subsequent laccase production experiments (Chapter 3).

P. taeda Plant Tissue Cultivation

P. taeda seedlings and callus were successfully grown and maintained under the conditions described in the Materials and Methods section. Seedlings could be maintained in growth cones for 15 months without visible signs of distress. Individual callus lines were maintained for one year with similar characteristics of growth and appearance. Liquid suspension cell cultures declined in vigor after 20 or more medium transfers, and lost the ability to grow rapidly. In these cases, cell coloration gradually changed from bright yellow to pale yellow or light tan or gray. Cells at this stage were no longer considered viable.

Interactions of *A. areolatum* with *P. taeda* Tissues

After 3 days there were no visible signs of response to 0.5 mg/ml yeast extract challenge in seedlings; however, puncture wounds for the injection seedlings were darker in color than controls. After 3 weeks all of the seedlings exposed to the yeast cell walls exhibited chlorotic bands on needles, six whorls down from the site of decapitation. These bands also occasionally occurred in wounded controls and thus cannot be considered a specific response to this elicitor.

P. taeda callus cells inoculated onto PDA or malt agar plates browned with medium contact, and no growth was evident after seven days. At 12 days pine cells on either media remained hydrated, but were visibly brown and shriveled where they contacted the medium surface. Fungal medium was demonstrated unsuitable for extended maintenance of pine callus and could not be used as support media for fungus/pine challenge experiments.

Amylostereum areolatum cultured onto Eberhardt medium showed some growth away from the inoculating agar plug and onto the pine medium after seven days, but growth was not

profuse. Microscopic examination revealed that under these conditions the hyphae tended to grow upward and away from the medium. At 12 days, a typical radial growth pattern away from center inoculating agar plug was noted, but growth was slow relative to growth on typical fungal media (malt agar or potato dextrose agar).

The seedling inoculation experiment was inconclusive. Pilot experiments indicated *P. taeda* seedlings could be killed by *A. areolatum* infection alone, and in one case (of nine attempts) the fungus was successfully recultured from dying cambial tissue. No mortality, abscission, needle crazing, or needle color change to gray-green was observed during the course of the fully-replicated experiment, a contrast from the previous pilot results. *Amylostereum areolatum* could not be shown to produce *P. taeda* seedling mortality reproducibly under these growth conditions.

In experiments where fungal growth challenged pine callus on solid medium, a color change in the medium was noticed spreading radially outward from the fungus. The coloration was brown to reddish and seemed to be focused on the side of the medium facing away from the pine callus. The coloration suggested that there was some signaling occurring between the fungus and the pine tissues without actual tissue contact (via the medium). This phenomenon could not be reliably reproduced. The callus tissue eventually became light to dark brown as the fungus began to degrade it. *Amylostereum areolatum* successfully colonizes and kills *P. taeda* callus on solid culture medium.

Discussion

A. areolatum Culture Conditions and Induction of Laccase Activity

Fungal biomass production in the defined medium was greatest at pH 6.5, as was production of laccase activity. Consequently this pH condition was chosen as the standard for all subsequent work in the defined medium. It is likely that laccase production is favored over a

wide range of medium pH. The reliability of this data must be judged against the small sample size used for the assay.

Peroxidase activity was stimulated by carbon deficiency in the medium, whether measured on a volume medium basis or a fungal biomass production basis. The reliability of this data must take into account the small sample size for this assay.

Laccase production in *A. areolatum* liquid culture could not be reproducibly stimulated by 2, 5-xylidine under the described test conditions.

Enzyme Activity

The oxidation of ABTS by liquid culture medium was a positive indication that an oxygen-dependent phenoloxidase activity was present in *A. areolatum* medium. This is the first quantitative description of such an activity in this species and is the subject of a more detailed study as described in Chapter 3.

Amylostereum areolatum cultures grown for 14 days on defined solid medium were shown to give purple coloration (the insoluble oxidation product of syringaldazine) when 0.1% syringaldazine in 95% ethanol was applied in drops to the mycelial mat. For relatively new cultures, there would be little expectation of starvation conditions: at 14 days the colony is quite young and has not exhausted its medium's resources. This indicates that the phenoloxidase activity is likely constitutive and may not require nutrient deficiency to induce its production.

Fungal Mutagenesis Target Tissue Production

Production of *A. areolatum* target tissue for mutagenesis was not achieved through any of the three methods described here. The best targets for point mutations are homokaryotic arthrospores, and none could be isolated from the fungus by the methods used here. Laccase mutants have been created in other wood-degrading basidiomycete fungal systems, and it is likely possible in the *A. areolatum* system as well. Evidence for role of laccase in fungal

pathogenesis in *A. areolatum* will be bolstered if a laccase-less mutant is achieved and demonstrates reduced virulence in pine tissue.

P. taeda Plant Tissue Cultivation

Reliable systems for growing *Pinus taeda* seedlings and for maintaining liquid cultures of callus tissue were successful based on previously reported methods.

Interactions of *A. areolatum* with *P. taeda* Tissues

It was concluded that experiments challenging pine callus with fungal cultures should be carried out on Eberhardt (pine callus inductive) medium. The fungus is at a growth rate disadvantage on Eberhardt medium relative to malt agar or PDA, but the medium is not growth prohibitive. *P. taeda* callus did not survive on malt agar or PDA. Eberhardt medium is the one medium tested on which both tissues can be grown simultaneously.

It was expected that a diffusible signal from the spent fungal medium might give a measurable response in pine explant tissue. Explants have been successfully employed by other investigators to model plant defense responses (Coutts 1969; Bowling and Dolezal 1970; Bailey et al. 1990). In solid culture challenge of *P. taeda* callus by *A. areolatum* (discussed below), there was evidence to suggest that a diffusible signal was present, possibly one with a chromophore. For this reason the UV/VIS response was tested. Experiments using spent fungal medium to challenge floating pine needle explants were not conclusive. There was no difference in elicitor-treated needle response from controls as measured by UV/VIS absorbance.

In pilot experiments, trees subjected to *A. areolatum* infection were killed. In one case the living fungus was recovered from one of the trees. This seemed important, as the literature contained no examples of *P. taeda* seedlings being killed by *A. areolatum* fungus alone. Reproducing this experiment would have suggested that seedlings could be used to screen for genetic susceptibility to *A. areolatum* infection. The fully-replicated experiment which followed

does not support this conclusion. Variations in the growth conditions likely played a key role in this result, and further development of infection conditions is indicated. This study seems to indicate that *A. areolatum*, as has been noted previously in *P. radiata*, is a weak pathogen in *P. taeda* on its own and relies heavily on a secondary tree stress factor for establishment (Coutts 1969).

In the pilot experiment, trees with autoclaved fungus died as readily as those with live fungus. The question of whether the sterilized fungus had actually been killed was raised (intermittent autoclave malfunction may explain this). This was the reason for the double-autoclaving control in the full-scale experiment. Since apparently dead material caused seedling death, the possibility was raised that a small molecule associated with the fungus could be eliciting the mortality. For this reason sterile-filtered fungal medium was included as a control in the full experiment.

Alternative hypotheses why the seedlings were not infected should be investigated. An experiment exploring the role of drought or light stress on infective potential of *A. areolatum* on *P. taeda* seedlings is warranted. Such abiotic stress can increase susceptibility to fungal infection (Madden 1977). It is possible that the pilot study trees were somewhat suppressed due to low light conditions over several weeks. Another possible consideration is that the seedlings chosen for the replicated experiment were inherently resistant to fungal infection. The value of the initial observations in the pilot study remains questionable following the full-scale experiment.

Solid culture *A. areolatum* challenge of *P. taeda* callus tissue did not give fully reproducible results. The reddish-brown coloration of the medium around the fungal tissue was not reproducible in subsequent experiments. On the other hand, callus tissue was invariably killed by the fungus when it grew into contact with it.

Summary

In this portion of the study, the objectives were to establish growth media and conditions for production of *P. taeda* tissue capable of modeling grown tree responses to *A. areolatum* challenge. Phenoloxidase production by *A. areolatum* is expected based upon its identity as a white-rot basidiomycete. Demonstrating phenoloxidase production by the fungus would make a case for further characterization of any phenoloxidase present. Additionally we wished to establish a defined medium for maintenance and optimal growth of *A. areolatum* in the laboratory, and for the optimal production of extracellular phenoloxidase enzymes. Studies by other investigators suggested that the phenoloxidase produced by *A. areolatum* could be involved in fungal virulence in pine. Several plating methods were attempted to produce fungal material suitable for use in experiments to induce point mutations in the fungus. If by mutagenesis the loss of phenoloxidase activity could be induced and demonstrated, the resulting fungal strain could be used to monitor virulence of the fungus. A reduced virulence by a phenoloxidase-less mutant could indicate that the phenoloxidase is involved in fungal virulence.

The results from this study were mixed. Conditions to produce and maintain loblolly callus were established, and extant methods for germinating and growing seedlings were used. While fungal infection of *P. taeda* seedlings was effected, the conditions to reproducibly infect seedlings have not yet been firmly established. Similarly, *P. taeda* callus shows a response to *A. areolatum* in solid culture, but the case has not been firmly established using a full complement of controls. Both these pine tissue systems show promise as models for loblolly tree response to fungal challenge. Further development of seedling growth conditions and assessment of general loblolly callus susceptibility to other pine-pathogenic and non-pathogenic fungi is indicated.

A useful defined medium for *A. areolatum* has been established, and initial conditions for optimal production of fungal biomass as indicated by oven dry weight are described. First steps

have been made to optimize the medium for production of phenoloxidase enzyme activity. Enzyme production conditions bear further investigation with expanded replication to firmly establish their reliability. Induction studies particularly are indicated. The known laccase inhibitor 2, 5-xylidine was not an effective inducer of phenoloxidase activity based on the conditions described here. The defined medium as described here will be useful for future *in vitro* studies of the fungus.

An effective means of isolating arthrospores of the fungus was not demonstrated. Plating studies attempting to develop fungal morphology suitable for radiative induction of point mutations were unsuccessful. A system for isolation of the spores will be a valuable addition to future studies of *A. areolatum* virulence.

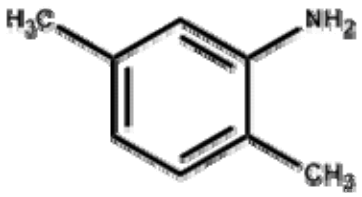


Figure 4: Structure of 2, 5-Xylidine (2, 5-Dimethylaniline). This compound is an arylamine laccase substrate and known fungal laccase inducer.

Table 1: Defined Nutrient Medium for *A. areolatum*. This is the medium used to optimize production of fungal biomass and extracellular phenoloxidases. Modified from Pointing and Levin (Pointing et al. 2000; Levin and Forchiassin 2001).

$AlK(SO4)_2$	(Fluka 60060)	0.4 µM
Ammonium tartrate	(Sigma A-2956)	21.7 mM
$CaCl_2$	(Sigma C-2536)	680 µM
$CoCl_2$	(Fisher C371-100)	4.2 µM
$CuSO_4$	(Baker 1843-01)	40 µM
D-glucose	(Fisher D16-3)	111 mM
2, 2-dimethylsuccinic acid	(Fluka 39660)	10 mM
$FeSO_4$	(Sigma F-2387)	3.6 µM
H_3BO_3	(RPI B32050.1000.0)	1.6 µM
KH_2PO_4	(Baker 3426-05)	15 mM
$MgSO_4$	(Sigma M-7774)	2 mM
$MnSO_4$	(Sigma M-7899)	21 µM
NaCl	(Baker 3624-05)	171 µM
$NaMoO_4$	(Sigma S-6646)	0.4 µM
Nitriloacetic acid	(Sigma N-9877)	785 µM
Phytagar (if solid medium)	(Gibco 10675-023)	8 g/L
Thiamine HCl	(Sigma T-3902)	0.3 µM
$ZnSO_4$	(Sigma Z-0501)	3.5 µM

pH adjusted from 3.5 to 7.5, and autoclaved prior to use.

Table 2: Pine Tissue Culture Medium. This is the medium used to induce and maintain loblolloy pine callus tissue. Modified from Eberhardt (Eberhardt et al. 1993).

$CaCl_2$	(Sigma C-2536)	0.15 mM
$CoCl_2$	(Fisher C371-100)	0.55 μM
$CuSO_4$	(Baker 1843-01)	2 mM
2, 4-dichlorophenoxyacetic acid	(Sigma D-8407)	11 μM
$FeSO_4$	(Sigma F-2387)	0.1 mM
H_3BO_3	(RPI B32050.1000.0)	0.5 mM
KH_2PO_4	(Baker 3426-05)	2.5 mM
KI	(Sigma P-8166)	25 μM
KNO_3	(Sigma P-6030)	18.8 mM
$MgSO_4$	(Sigma M-7774)	7.7 mM
$MnSO_4$	(Sigma M-7899)	0.14 mM
myo-inositol	(Sigma I-3011)	111 μM
Na_2EDTA	(Baker 8993-01)	0.1 mM
$NaMoO_4$	(Sigma S-6646)	5 μM
NH_4NO_3	(Fisher A676)	20.6 mM
nicotinic acid	(Sigma N-0765)	4 μM
phytagar (if solid medium)	(Gibco 10675-023)	8 g/L
pyridoxine HCl	(Sigma P-8666)	0.49 μM
sucrose	(Research Organics 9640S)	3%w/v
thiamine HCl	(Sigma T-3902)	0.3 μM
$ZnSO_4$	(Sigma Z-0501)	0.15 mM

pH adjusted to 5.5 with 1N NaOH, and autoclaved prior to use.

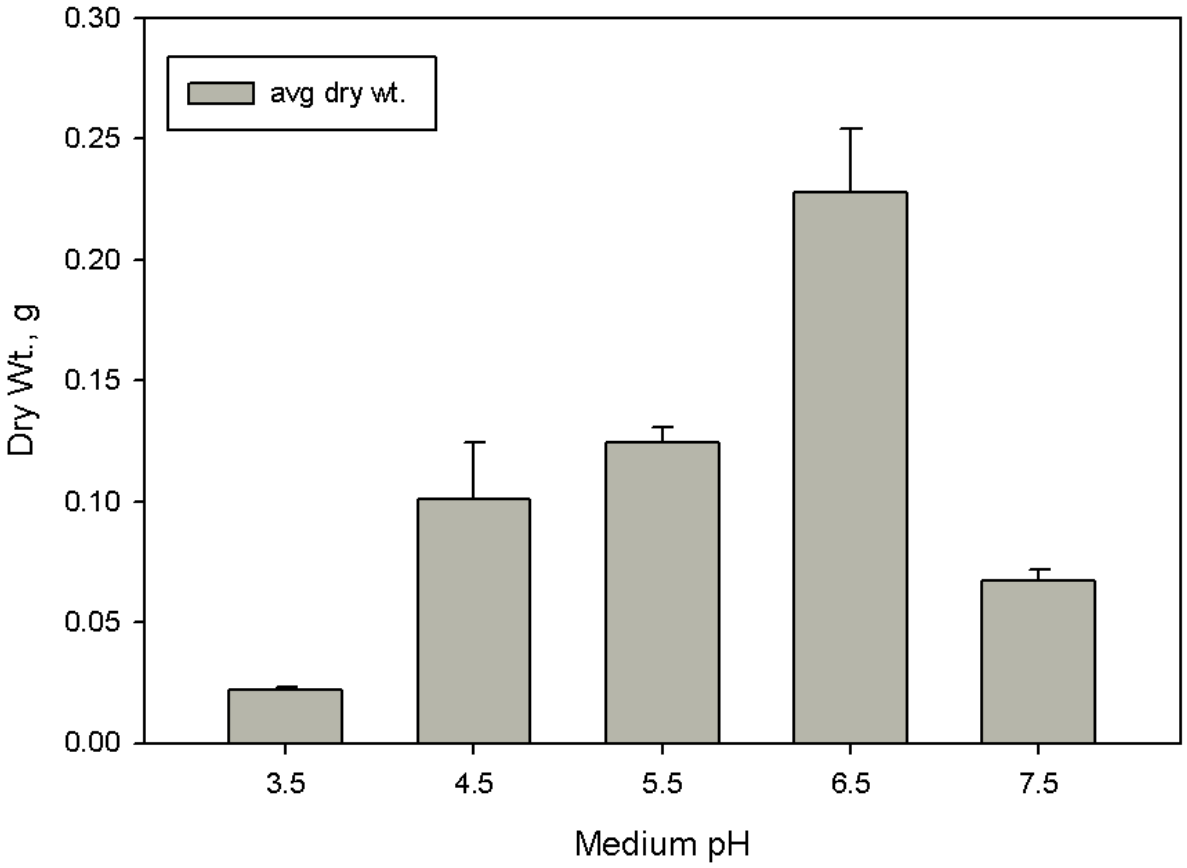


Figure 5: Effect of Medium pH on Fungal Growth as Measured by Oven Dry Weight. Flasks (n=3) containing 50 ml of defined medium previously adjusted for pH were inoculated with a 5 mm agar plug of *Amylostereum areolatum* and grown in darkness at 24°C without shaking for 26 days.

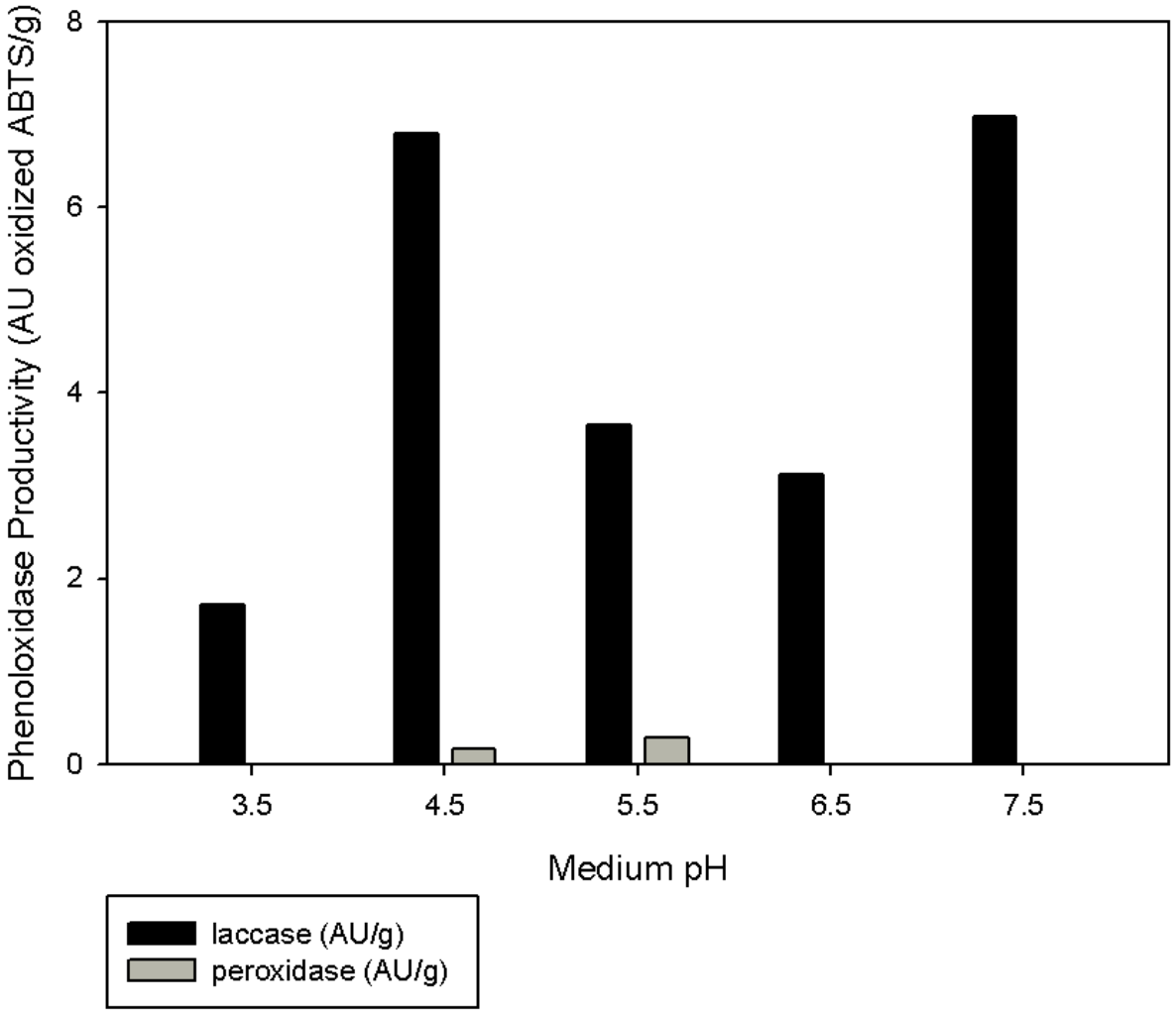


Figure 6: Effect of Medium pH on Phenoloxidase Activity Produced, Mass Basis. Three flasks containing 50 ml of defined medium previously adjusted for pH were inoculated with a 5 mm agar plug of *Amylostereum areolatum* and grown in darkness at 24°C without shaking for 26 days. Three filtrate replicates pooled prior to measurement (n=1).

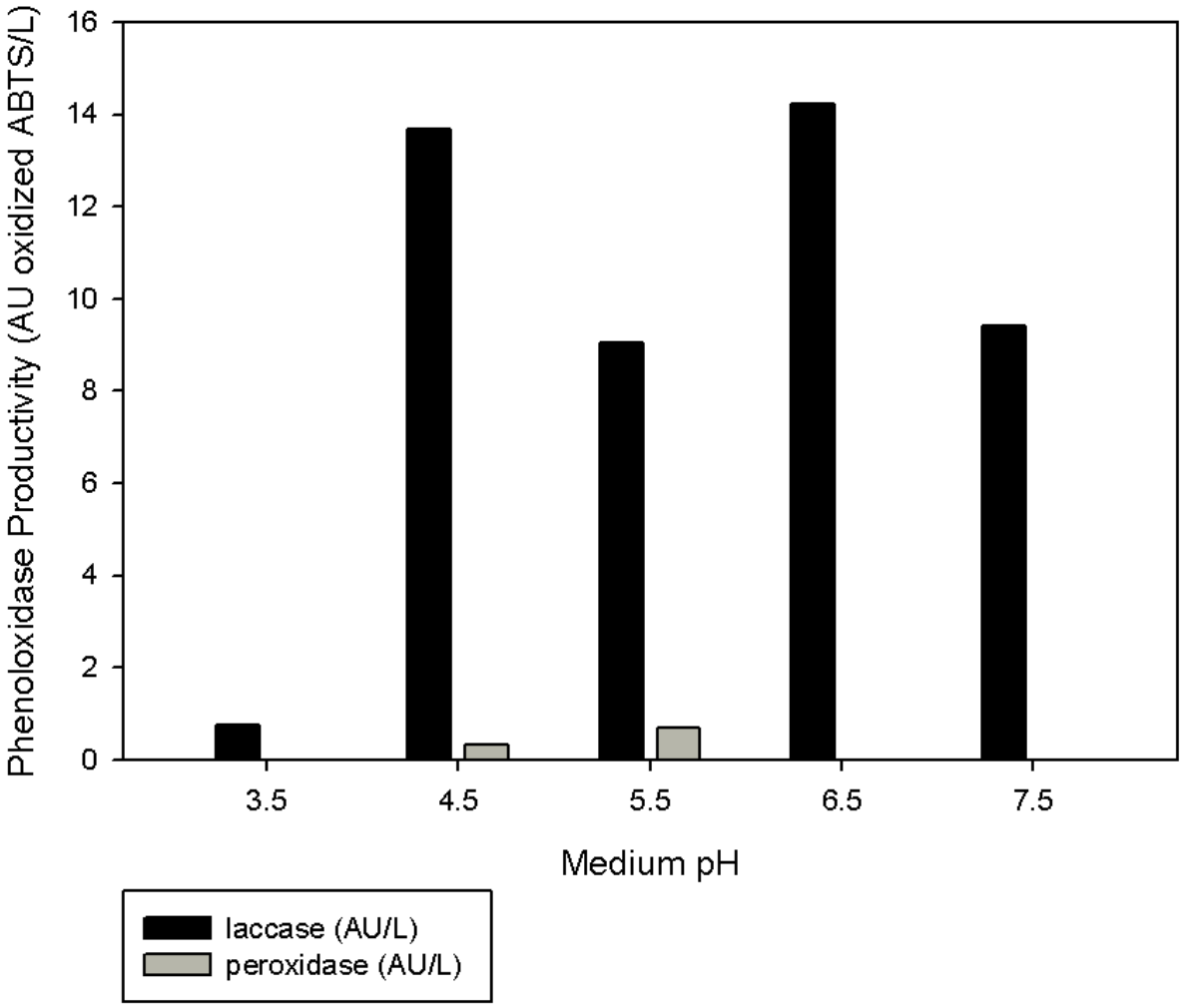


Figure 7: Effect of Medium pH on Phenoloxidase Activity Produced, Volume Basis. Three flasks containing 50 ml of defined medium previously adjusted for pH were inoculated with a 5 mm agar plug of *Amylostereum areolatum* and grown in darkness at 24°C without shaking for 26 days. Three filtrate replicates pooled prior to measurement (n=1).

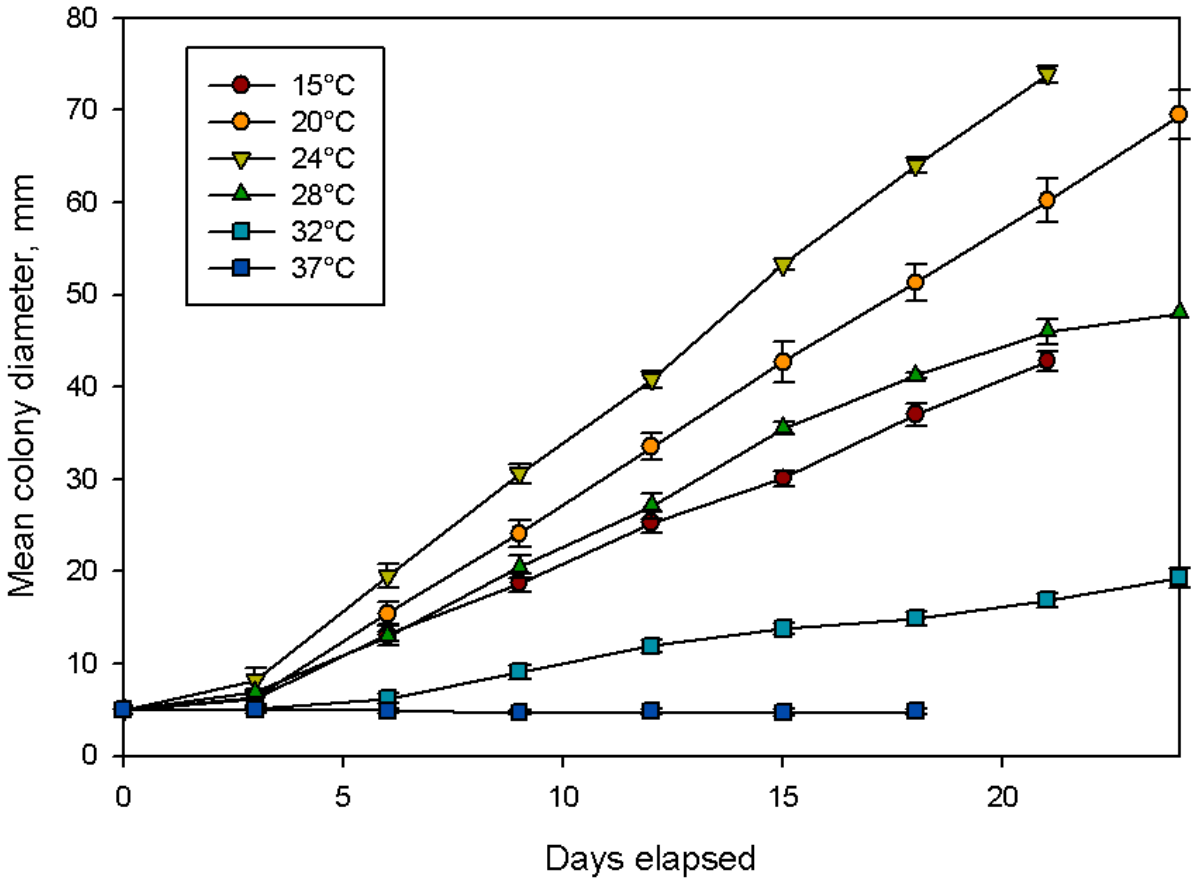


Figure 8: Temperature Dependence of *A. areolatum* Growth. Shown are time-coursed colony diameters on solid medium. Diameter of colonies was measured at three day intervals for up to 24 days (n=5).

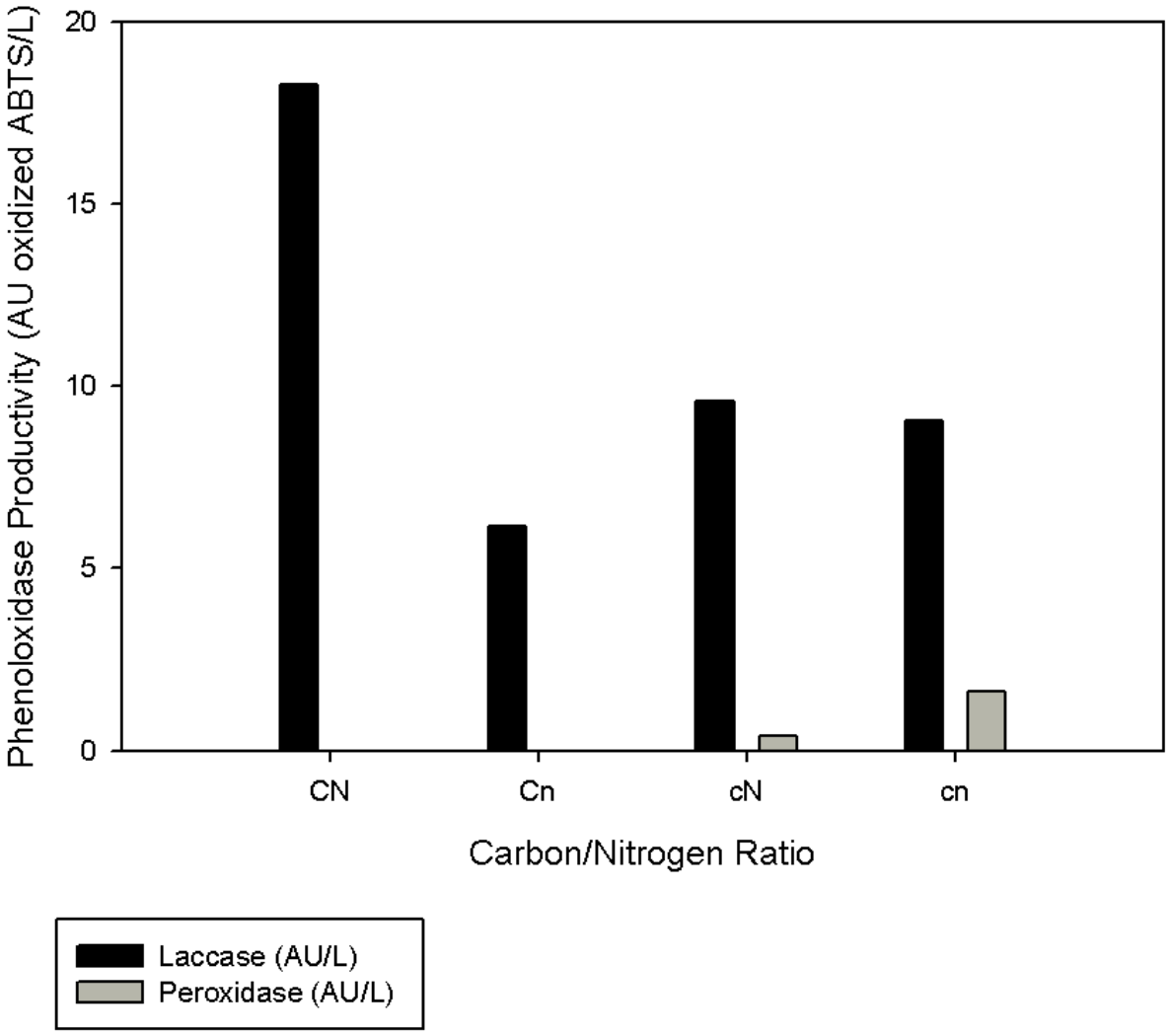


Figure 9: Effect of Carbon/Nitrogen Ratio on Extracellular Phenoloxidase Production, Volume Basis. Three flasks containing 50 ml of defined medium previously adjusted to pH 6.5 were inoculated with a 5 mm agar plug of *Amylostereum areolatum* and grown in darkness at 23°C without shaking for 30 days. Three filtrate replicates pooled prior to measurement (n=1). Legend for X-axis is:

CN: 20 g/L D-glucose, 4 g/L ammonium tartrate
Cn: 20 g/L D-glucose, 0.4 g/L ammonium tartrate
cN: 2 g/L D-glucose, 4 g/L ammonium tartrate
cn: 2 g/L D-glucose, 0.4 g/L ammonium tartrate

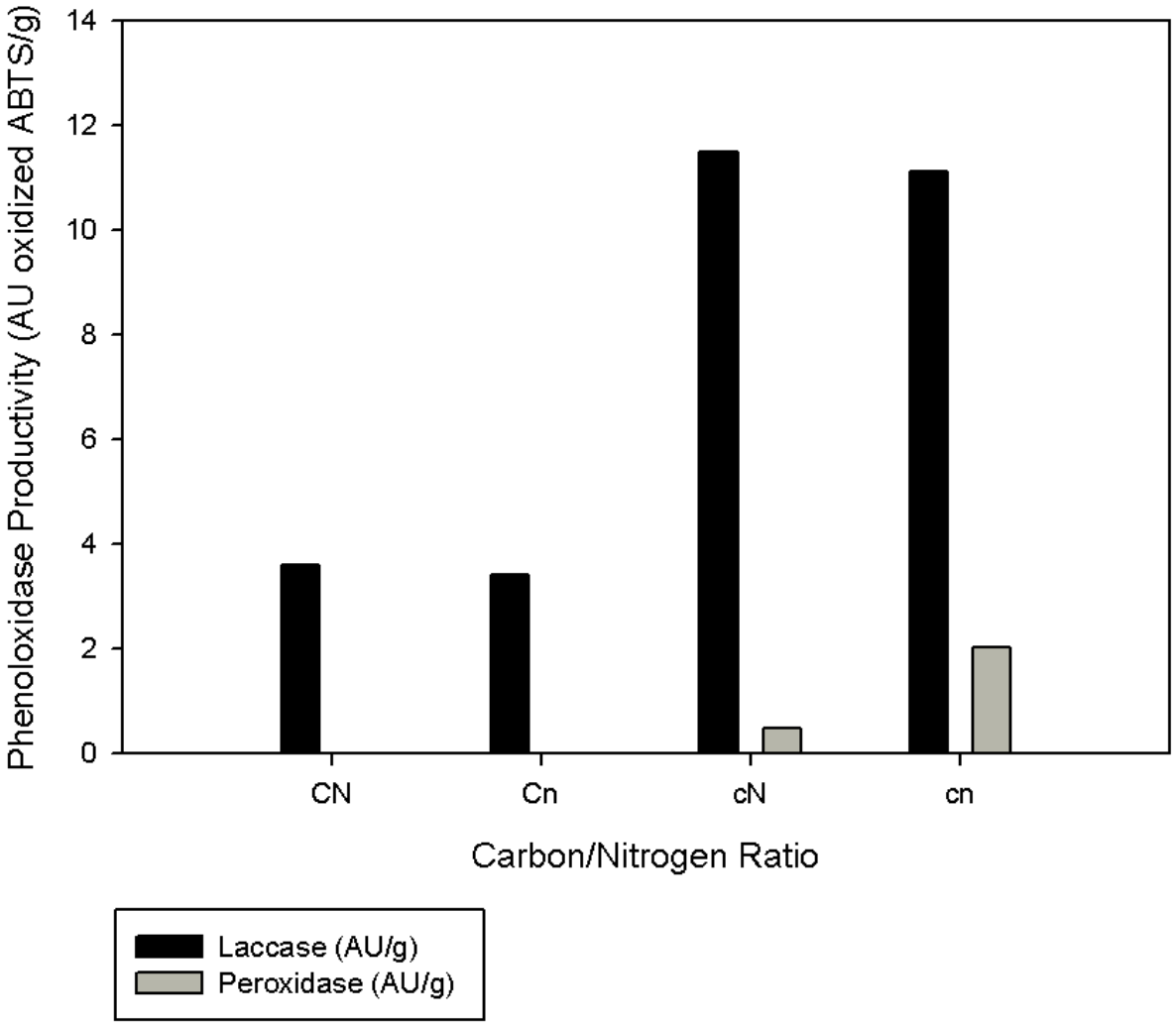


Figure 10: Effect of Carbon/Nitrogen Ratio on Extracellular Phenoloxidase Production, Mass Basis. Three flasks containing 50 ml of defined medium previously adjusted to pH 6.5 were inoculated with a 5 mm agar plug of *Amylostereum areolatum* and grown in darkness at 23°C without shaking for 30 days. Three filtrate replicates pooled prior to measurement (n=1). Legend for X-axis is:

CN: 20 g/L D-glucose, 4 g/L ammonium tartrate
Cn: 20 g/L D-glucose, 0.4 g/L ammonium tartrate
cN: 2 g/L D-glucose, 4 g/L ammonium tartrate
cn: 2 g/L D-glucose, 0.4 g/L ammonium tartrate

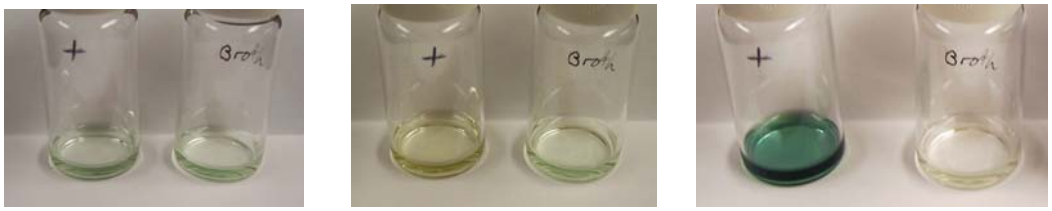


Figure 11: Phenoloxidase Activity in *Amylostereum areolatum* Liquid Culture. Left, 1 ml of 2.3 mM ABTS each. Center, 200 µl 2 week old sterile filtered fungal spent medium added to left, 200 µl PDB unexposed to fungus added to right, 1 minute after addition. Right, same vials 19 hours following addition of 200 µl spent medium or PDB control.

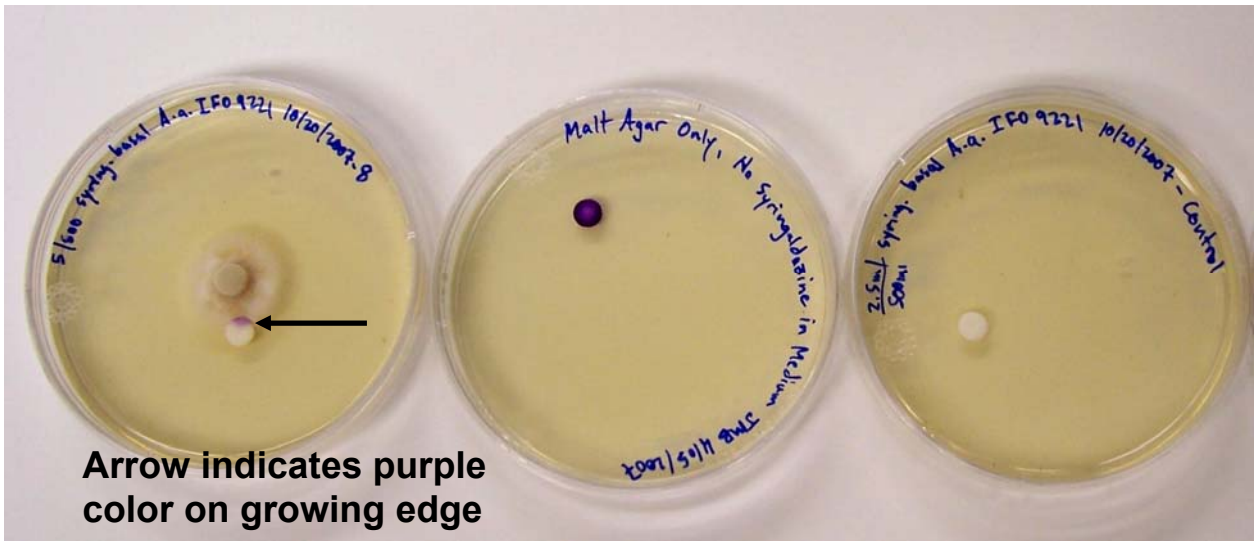


Figure 12: Phenoloxidase Activity in *Amylostereum areolatum* Solid Culture. A filter disk soaked with 10 µl of 0.1% syringaldazine in ethanol was placed on the edge of a 14-day old *A. areolatum* colony (left). Note color change at the top of the disk. A positive control reaction in which horseradish peroxidase and hydrogen peroxide, and then 0.1% syringaldazine were applied to a paper disk on the same medium appears in the center. A negative control with syringaldazine applied in the absence of enzyme or fungal culture appears on the right.

CHAPTER 3: PARTIAL PURIFICATION OF AN EXTRACELLULAR LACCASE-LIKE PHENOLOXIDASE PRODUCED BY *AMYLOSTEREUM AREOLATUM* IN LIQUID CULTURE MEDIUM

Introduction

Amylostereum areolatum is a white-rot fungus of the Basidiomycota. White-rot fungi are those basidiomycetes capable of degrading hemicellulose, cellulose and lignin. The name "white-rot" comes from the characteristic white, bleached appearance of wood attacked by these fungi (Martínez et al. 2005). *Amylostereum areolatum* relies on the degradation of wood as its primary source of nutrition.

Wood is principally composed of the biopolymers cellulose, hemicellulose, and lignin (Martínez et al. 2005). Lignin, an amorphous polymer of guaiacyl, syringyl, and *p*-hydroxyphenyl phenylpropanoid subunits, confers both mechanical strength and chemical resistance to wood (Martínez et al. 2005). White-rot fungi, which are the only fungi capable of degrading lignin to any great extent, begin their degradation of wood carbohydrates (cellulose, hemicellulose, amylose, pectin) and/or lignin with oxidation and hydrolysis reactions. As lignin does not have bonds susceptible to hydrolysis, various oxidase enzymes are required to initiates breakdown of this polymer (Hatakka 2001). Each species of wood-degrading fungus secretes a complex mixture of cellulolytic and ligninolytic enzymes that works in the extracellular environment to produced polymer fragments that can be taken up and catabolized by the fungus (Morozova et al. 2007).

Extracellular phenoloxidase production has been reported in a wide variety of white-rot fungi, and this enzyme activity is critical for lignin degradation (Hatakka 1994; Hatakka 2001;

Morozova et al. 2007). Two phenoloxidases in particular, laccase and peroxidase, are the most important enzymes for the breakdown of lignin (Hatakka 1994; Morozova et al. 2007). Both classes of enzyme require a specific oxygen species as a cosubstrate (Ullrich and Hofrichter 2007).

Laccases (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) play an important role in ligninolytic systems (Morozova et al. 2007). Because their active sites consist of four copper ions, laccases are often called the "multi-copper oxidases" (MCOs) (Morozova et al. 2007). These enzymes oxidize both phenolic and non-phenolic compounds, with concomitant reduction of molecular oxygen to water (Hatakka 1994). While the laccase that acts to degrade lignin is extracellular, intracellular laccases have been described (Rigling and Vanalfen 1993) where they correlate with separate physiological functions. Laccases are characterized by their broad specificity for phenolic and non-phenolic substrates (Leonowicz et al. 2001). Curiously, plant laccases are involved in synthesis of lignin, whereas fungal laccases are involved in its degradation (Dean and Eriksson 1994; Bertrand et al. 2002; Morozova et al. 2007).

Peroxidases (EC 1.11.1.7) are heme-containing enzymes that oxidize lignin using H_2O_2 as a cosubstrate. Wood-degrading fungi produce two main groups of peroxidases, manganese peroxidases (MnPs) and lignin peroxidases (LiPs). In the presence of hydrogen peroxide, MnPs oxidize Mn^{2+} to Mn^{3+}. This oxidized manganese subsequently diffuses into the lignin polymer where it abstracts an electron from an available hydroxyl group. The resulting free radical delocalizes with bond cleavage leading to fragmentation of the lignin polymer. LiPs act similarly in oxidizing the lignin polymer but abstract electrons from non-phenolic portions of polymer to create free radicals and cause the breakup of the lignin (Hatakka 1994).

It has been known for some time that basidiomycetes producing laccase are able to degrade pine defense compounds (Loman 1970). Demonstrating that *A. areolatum* produces a laccase offers a possible mechanism for its pathogenicity in pine. A breakdown of lignin,

coupled with the detoxification of plant defense compounds, surely represents an exploitable breach in the plant cell's defenses.

A cursory mention of "laccase: positive," without further elaboration was made when *A. areolatum*'s presence was first reported in South Africa (Baxter et al. 1995). There are no other reports in the literature describing laccase or peroxidase enzyme production by *A. areolatum*. However, sequence comparisons of LiP and MnP genes were used to define genetic relationships between *Heterobasidion annosum* and other white-rot fungi, including *A. areolatum*. In that study, genes for both lignin peroxidase and manganese peroxidase were identified in *A. areolatum* (Maijala et al. 2003). To date no biochemical or quantitative work on *A. areolatum* laccase enzymes or genes has been reported.

Oxidation of Chromogenic Substrates

The broad substrate specificity of laccase has been exploited in the development of numerous chromogenic assays for laccase activity. Syringaldazine is a useful chromogenic substrate for rapid screening of laccase and peroxidase production on solid media (Harkin and Obst 1973). This substrate does not affect fungal growth, and it does not induce fungal enzyme production on its own (in contrast to substrate 2, 5-xylidine). Syringaldazine can be used to detect laccase activity in the presence of air and absence of peroxide, and it can detect peroxidase activity in the presence of hydrogen peroxide. It is oxidized neither by hydrogen peroxide nor tyrosinase (Harkin and Obst 1973). Moreover, the deep purple oxidation product of syringaldazine has limited solubility in aqueous solution, a valuable property for localizing phenoloxidase activity on solid surfaces (Harkin et al. 1974).

ABTS is a sensitive reagent for detecting phenoloxidase activity, although the ease with which it is oxidized can sometimes lead to confusion due to nonspecific reactions. Mixtures of extracellular enzymes from fungal culture medium containing phenoloxidases will readily oxidize ABTS provided the appropriate cosubstrate is present. Since fungi use extracellular systems for

production of hydrogen peroxide and other oxidants, ABTS oxidation by a fungal culture filtrate can indicate presence of either laccase or peroxidase. Laccase alone may be detected by adding a peroxide scavenger (such as catalase) to the reaction mixture.

The goals of this study were to identify any laccase activity produced by *A. areolatum* and to evaluate some of the fundamental enzymatic characteristics of any laccase discovered. To assure maximum production of copper-containing enzymes, supplementing copper levels in liquid medium seemed prudent. Upregulation of laccase production by copper 2^+ ion has been reported in white-rot fungi and canker-rot fungi, with reports of 4- to 100-fold increased production of laccase above basal levels (Baldrian and Gabriel 2002; Baldrian 2004; Michniewicz et al. 2006; Papinutti and Martinez 2006).

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals used for buffers and gels were reagent grade or higher. Pinosylvin (46619) was supplied by Biofine International (Vancouver, BC Canada). Resveratrol (R5010) was supplied by Sigma-Aldrich (St. Louis, MO). Protein assay solution (500-0006) and protein standard (161-0305) were supplied by Bio-Rad (Hercules, CA). Protein gel standard (LC5925) was supplied by Invitrogen (Carlsbad, CA). Additional reagents were supplied by J. T. Baker, (Phillipsburg, NJ), Fisher Scientific (Pittsburgh, PA), Fluka (St. Louis, MO), Gibco (Carlsbad, CA), Research Organics (Cleveland, OH), Research Products Intl. (Prospect, IL), and Sigma-Aldrich (St. Louis, MO). Potato dextrose broth was supplied by Becton-Dickinson (Franklin Lakes, NJ).

A. areolatum culture conditions and induction of activity

Aliquots (5 ml) of homogenized *A. areolatum* mycelium stored at 4°C were added to Fernbach flasks containing 500ml sterile potato dextrose broth (PDB, 12 g/500 ml DI water,

sterilized 30 minutes at 250°F). Dry weight mass of inoculum was estimated at 0.03 g per flask (n=3). Fungal growth was monitored daily by removing ~0.3 ml aliquots from each flask and measuring laccase activity via oxidation of ABTS. Flasks were shaken under standard laboratory fluorescent lighting at 120 rpm on an orbital shaker. For copper induction studies, each flask was brought to 150 μM $CuSO_4$ two days following fungal inoculation. Mycelial pellets were removed by straining the culture through two layers of cheesecloth. Filtrate was stored frozen at -80°C. Culture filtrate subjected to enzyme purification steps was filtered first (before or after freezing) through Whatman #1 filter paper or Whatman 934-AH glass fiber filters.

Partial purification of extracellular laccase from *A. areolatum*

Fungal filtrate was concentrated 7X by ultrafiltration against Amicon PM30 filters (30 kDa MWCO) (Millipore, Billerica, MA). Solid ammonium sulfate was added to the PM30 filtrate to bring the resulting solution to 70% saturation. The solution was allowed to equilibrate with mixing in darkness at 4°C for 22 hours. Precipitate was removed by centrifugation for one hour at 13,000 x g (11,000 rpm in a Sorvall SLA-3000 rotor). Buffer exchange of the resulting supernatant was carried out against an Amicon PM30 ultrafiltration membrane using 100 mM sodium acetate (pH 5.0). For this step, aliquots (100 ml) of the 70% saturated ammonium sulfate supernatant were diluted with equal volumes of buffer and concentrated back to their original volume (100 ml). This was repeated three times for each aliquot. Following buffer exchange, solutions were concentrated 100X against a PM30 filter and the resulting concentrate was further reduced in volume over 1200-fold using centrifugal concentrators (10 kDa MWCO) (Pall Filtron, East Hills, NY). The concentrate was stored at -80°C until further use.

Protein content and enzyme activity assays

Protein content and enzyme activity were routinely monitored using microplate assays. For protein assays, 40 μl of Bradford protein assay reagent (BioRad, Hercules, CA) were added to 140 μl of test solution, and after mixing were incubated 10 minutes at ambient temperature

prior to measuring absorbance. Bovine serum albumin (BSA) protein standards from 4 to 200 µg/ml were scanned simultaneously with each protein assay. Plates were scanned using a microplate reader (BioTek FL600) using a 590 nm filter in static mode, 3 scans per well, 2 scans per second. Standard curves were plotted using SigmaPlot software (Systat Software, San Jose, CA), and the unknowns were determined against the BSA standard curve.

Enzyme activity was measured by following the oxidation of chromogenic substrates, either ABTS or guaiacol in the presence of catalase. Typical reaction mixtures were as follows:

Laccase (ABTS)

70 µl 50 mM sodium acetate, pH 4.0

10 µl 0.01 mg/ml catalase (0.001 mg/ml final concentration)

10 µl test solution

10 µl 3 mM ABTS (0.3 mM final concentration)

Laccase (Guaiacol)

160 µl 50 mM sodium acetate, pH 4.0

20 µl 0.01 mg/ml catalase (0.001 mg/ml final concentration)

10 µl test solution

10 µl 0.1 M guaiacol (5 mM final concentration)

Peroxidase

36 µl 100 mM sodium acetate, pH 5.0

4 µl 25 mM H_2O_2

10 µl test solution

50 µl 0.03% ABTS

Microplate assays were performed using an FL600 UV/fluorescence plate reader (BioTek Instruments, Winooski, VT) with a 405 nm pass-through filter for ABTS oxidation measurements and a 450 nm pass-through filter for guaiacol oxidation measurements. ABTS assays were allowed to run for 8-20 hours where samples such as raw culture filtrate contained low levels of activity, but 20-30 minute assays were typical for assessing concentrated enzyme solutions. Guaiacol assays typically ran for 60 minutes. For broad spectrum analysis of reaction products, reaction mixtures were sampled at various time points and scanned using a Nanodrop UV/VIS spectrophotometer (Thermo/Fisher, Pittsburgh, PA).

Gel electrophoresis and staining

Tris-tricine SDS-PAGE was used to resolve protein mixtures and compare different stages of enzyme purification using the method of Schägger and von Jagow (Schägger and von Jagow 1987). Stacking gels were 4% total acrylamide (3% crosslinker relative to total acrylamide). Separating gels were 10% total acrylamide (3% crosslinker relative to total acrylamide). Gels were prepared at 1 mm thickness and run at 30 V constant voltage until samples had fully entered the separating gel. Voltage was then increased to 150 V constant voltage and run until loading dye just ran off the end of the gel. Protein samples were boiled prior to loading for gels to determine molecular mass, but not gels that were stained for enzyme activity (zymograms). Typically zymograms were prepared with a pair of gels, one stained for protein and the other for activity. Phenoloxidase activity was detected in zymograms using 1,8-diaminonaphthalene (DAN) (Hoopes and Dean 2001), as follows. Following electrophoresis, gels were incubated in a solution 50 mM sodium acetate pH 5.0 /1% DMSO/2 mM DAN at 40°C for one hour. Following DAN staining, the reaction halted by soaking in 50% methanol, 10% acetic acid for 10 minutes. This was followed by a 10 minute soak in 20% tetrachloroacetic acid (TCA) to darken the DAN oxidation product.

To stain for total protein, gels were first fixed by immersion in 50% methanol/10% acetic acid for 1 hour followed by shaking in 0.3 mM Coomassie Blue G/10% acetic acid overnight at ambient temperature. Destaining was accomplished by soaking gels in 10% acetic acid for 2 hours, changing the solution at least twice. Gels were imaged using a gel imaging system (Alpha Innotech, San Leandro, CA).

Substrate Specificity and Inhibition Studies

The reactions of several potential laccase substrates were tested with the partially purified *A. areolatum* enzyme preparation. Where spectra were collected, reaction mixtures were typically prepared as 200 µl reaction volumes in microtiter plates, which were then sampled over a time-course. Spectral scans were generated using a Nanodrop UV/VIS spectrophotometer, sampling 4 µl aliquots. The pH 3 acetate buffer was required for the resveratrol assay to prevent substrate precipitation. Substrate structures are presented in Figure 13. Reaction mixture compositions were as follows:

ABTS

160 µl 50 mM sodium acetate buffer, pH 4.0

20 µl laccase (1:5000 dilution)

20 µl 3 mM ABTS

Catechol

150 µl 100 mM sodium acetate buffer, pH 5.0

10 µl laccase (1:50 dilution)

40 µl 100 mM catechol

Guaiacol

160 µl 100 mM sodium acetate buffer, pH 5.0

20 µl 0.01 mg/ml catalase

10 µl laccase (1:50 dilution)

10 µl 0.1 M guaiacol

Hydroquinone

179 µl 100 mM sodium acetate buffer, pH 5.0

10 µl laccase (1:50 dilution)

11 µl 100 mM hydroquinone

Phloroglucinol

180 µl 50 mM sodium acetate buffer, pH 4.0

10 µl laccase (1:50 dilution)

10 µl 100 mM phloroglucinol

Pinosylvin

180 µl 50 mM sodium acetate buffer, pH 4.0

10 µl laccase (1:50 dilution)

10 µl 100 mM pinosylvin

P-Phenylenediamine

180 µl 50 mM sodium acetate buffer, pH 4.0

10 µl laccase (1:50 dilution)

10 µl 100 mM *p*-phenylenediamine

Resveratrol

155 µl 50 mM sodium acetate buffer, pH 3.0

10 µl laccase (1:50 dilution)

35 µl 29 mM resveratrol

Putative laccase inhibitors were tested in duplicate in microplate assay using guaiacol as substrate and boiled enzyme as a control. The five putative inhibitors were sodium azide (NaN_3), sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), *L*-cysteine, and sodium dodecyl sulfate (SDS), and each was tested at 1, 5, and 10 mM final concentrations. Two inhibitors (NaN_3 and *L*-cysteine) were also tested at 100 nM, 1 µM, 10 µM, and 100 µM concentrations to obtain a more detailed loss-of-activity profile. (Preliminary experiments with NaN_3 and *L*-cysteine resulted in 100% loss of activity at 1 mM concentrations). After mixing reaction components, allowed to incubate at ambient temperature for 10 minutes. Absorbance readings were then taken at 450 nm for one hour. The measurement at 36 minutes was used. Reaction mixtures were formulated as follows:

160 µl 50 mM sodium acetate pH 4.0

20 µl inhibitor (5 inhibitors x 2 replicates x 3 concentrations)

10 µl laccase (active or boiled)

10 µl 0.1 M guaiacol

To more accurately determine the effects of inhibitors, *A. areolatum* laccase and chromogenic substrate (guaiacol and ABTS) were mixed and monitored at 450 nm for 10 minutes, after which the putative inhibitors were introduced and reactions monitored for at least 30 minutes more. Reaction mixtures were formulated as follows:

Guaiacol

160 µl 50 mM sodium acetate pH 4.0

10 µl laccase (1:50 dilution)

10 µl 0.1 M guaiacol

20 µl inhibitor (10 mM, introduced after 10 minutes)

ABTS

140 µl 50 mM sodium acetate pH 4.0

20 µl laccase (1:5000 dilution)

20 µl 3 mM ABTS

20 µl inhibitor (5 mM and 10mM, introduced after 10 minutes)

This experiment was begun using 10 mM guaiacol as substrate. When repeated using ABTS as substrate, inhibitor concentrations were reduced to 5 mM for NaN_3 and *L*-cysteine (it was already determined that they were extremely effective at higher concentrations). For the apparently less-effective inhibitors (EDTA, SDS, NaCl), trials began with 5 mM concentrations of inhibitor. In attempt to see a more pronounced inhibitory effect, experiments for EDTA, SDS, and NaCl were repeated, increasing inhibitor concentration to 10 mM.

Temperature Stability and pH Optimization

Chromogenic activity assay reaction mixtures were prepared as follows:

640 µl 50 mM sodium acetate pH 4.0

80 µl 0.01 mg/ml catalase

40 µl laccase (1:250 dilution)

(Brought to 5mM guaiacol after 1 hour incubation at specified temperatures)

Five replicates of three reaction mixtures (active laccase/active catalase, boiled laccase/active catalase, or active laccase/boiled catalase) were prepared. Each of the three types of reaction mixtures were incubated at each of 5 temperature conditions for one hour: ambient, 30°, 37°, 50°, and 70°C. Following incubation, each mixture was aliquoted in triplicate (190 µl) to a microtiter plate and guaiacol added to final 5 mM concentration, mixed, and measured for absorbance (450 nm) at 35 minutes. For pH optimization, the activity of a 1:250 dilution of laccase was measured using 5 mM guaiacol as substrate with the following buffers: 50 mM glycine (pH 2-3), 50 mM sodium acetate (pH 3-6), 50 mM sodium citrate (pH 6-7) and 50 mM sodium phosphate (pH 7-8). Reaction mixtures were prepared at indicated pHs, omitting substrate. After 10 minutes incubation, added substrate. Oxidation of 5 mM guaiacol was monitored over one hour via 450 nm absorbance. For pH stability, the lowest and highest pH buffers were eliminated, and the remaining buffers were used (n=8) at exposure times 0, 8, 16, and 24 hours to monitor for enzyme activity. The substrate for this experiment was 5 mM guaiacol, monitored at 450 nm absorbance. For pH experiments, the actual reaction mixture was being monitored, so each assay is a measurement of activity at the exposure pH.

RESULTS

Amylostereum areolatum reached the peak of its laccase activity at 8-10 days post inoculation. The addition of Cu 2^+ ions at two days post-inoculation did not alter the production of laccase activity noticeably but altered the profile (Figure 14). With or without added copper, there seemed to be no peroxidase activity in this system (Figure 15). The fungal cells in this shaken flask system do not take the form of a mycelial mat. Rather, they appear as small, nearly round pellets, with little adherence to one another (Figure 16).

Given the non-specific nature of the laccase active site, it is difficult to identify inhibitors specific for laccase (to the exclusion of any other phenoloxidase present). Consequently assays for laccase will also assay for peroxidase activity in the presence of the peroxidase

cosubstrate H_2O_2. For laccase-specific assays, catalase was included as a peroxide scavenger. Peroxide assays were performed by omitting catalase and including an excess of hydrogen peroxide. Thus the activity measure in such an assay is necessarily laccase + peroxidase. In all the peroxidase (peroxidase + laccase) assays for this fungal liquid culture system, there was no difference in laccase and peroxidase activity, indicating that laccase is the sole measurable phenoloxidase activity produced in this system.

Pilot studies for protein purification indicated a precipitation with 70% saturation ammonium sulfate would result in a 94% reduction of protein but only a 48% loss of laccase activity (Figure 17, Table 3). This was an acceptable loss given the scale of the enzyme production (~4L of liquid culture). A simple three-step procedure resulted in a 1260-fold purification and an activity yield of 445% (based on initial activity of culture filtrate) (Table 4). Activity staining was not responsible for the dark coloration in the lower portion of the culture filtrate and pellet lane; this is evident in the appearance of the unstained gel (Figure 18). Following DAN activity staining, the final, partially-purified laccase gave an apparent molecular weight of ca. 75 kDa (Figure 19). The positive control (horseradish peroxidase, Figure 19, lane 1) as well as the partially-purified laccase (Figure 19, lane 6) suggest that phenoloxidase is capable of surviving a concentration of 1% SDS present in these gels. A non-denaturing gel run exactly as per activity gel, but stained for total protein is shown in Figure 20. Comparing the activity gel with the denaturing protein-stained gel (Figure 21), it appears that the laccase activity may be present in isoforms, although this is less evident in the non-denaturing protein gel. As there is no activity banding below the main activity band in the zymogram, the enzyme appears likely monomeric with ca. 75 kDa molecular weight.

The resulting partially-purified laccase shows oxidation activity against phenoloxidase substrates ABTS, catechol, guaiacol, hydroquinone, phloroglucinol, *p*-phenylenediamine, and resveratrol (Figures 22-28). The oxidation of pinosylvin by the fungal laccase could not be demonstrated spectrophotometrically over 45 minutes (Figure 29). The time-course spectra of

the substrates during oxidation by *A. areolatum* laccase suggest possible wavelengths at which the oxidation may be monitored. Note that no spectrophotometric data was collected for syringaldazine (used here only in solid cultures) or for 1, 8-diaminonaphthalene (used here only for activity staining of gels), but they are also oxidizable substrates and bring the total number of *A. areolatum* laccase substrates demonstrated in this study to nine.

Concentrations of 1, 5, and 10 mM sodium azide and *L*-cysteine completely inhibited laccase activity (Figure 30). Activity dropped sharply between 0.01 and 0.1 mM concentrations for both these inhibitors, and essentially complete inhibition was demonstrated at 0.1 mM sodium azide. *L*-cysteine at 1 mM concentration resulted in complete loss of activity (Figure 31). Sodium chloride showed loss of activity at 1 mM and gave complete inhibition at 5 mM concentration. Both EDTA and SDS demonstrated apparent enzyme activation at 1 mM concentration, but EDTA inhibits most activity at 5 and 10 mM. SDS gave activation at 1 mM, a very slight inhibition at 5 mM, and showed no inhibition at 10 mM.

A true inhibitor should not react with substrate or with oxidation products of the enzyme (Johannes and Majcherczyk 2000). Experiments using guaiacol as substrate appeared at first to suggest that *L*-cysteine and sodium azide were not true inhibitors. The observed reaction profiles indicated that the introduction of the inhibitors resulted in reductive reversal of the enzyme activity (Figure 32). The experiment was repeated with ABTS as substrate; in this case sodium azide gave the expected flattening of the activity curve upon introduction, while *L*-cysteine continued the pattern of reversing the reaction (Figures 33). EDTA and SDS appeared to enhance rather than reverse the activity of the enzyme. *L*-cysteine is thus not a true inhibitor, but rather acts as a reducing agent to inhibit production of the chromogenic product. It is likely that sodium azide is an inhibitor despite the variance in results between ABTS and guaiacol as substrates. The ABTS cationic radical reasonably stable in solution, though the azide ion itself is somewhat reactive with it (Johannes and Majcherczyk 2000). Azide is probably more reactive with the less-stable guaiacol oxidation radical. The structure of the

ABTS monocationic radical (Solis-Oba et al. 2005; Marjasvaara et al. 2008) suggests it is less prone to attack by azide, and this may explain the differences in our results with ABTS vs. guaiacol as substrates.

The laccase enzyme gave highest activity at 30°C (Figure 34). Omitting catalase as a hydrogen peroxide scavenger from the reaction mixture did not alter the results. Activity of the enzyme decreases rapidly between 40° and 70°C with one-hour exposure. Optimum pH selection was based both upon highest activity (Figures 35, 36) and stability over time (Figure 37). Glycine pH 3 and sodium acetate pH 3.5 - 4.5 gave high activity, but only sodium acetate pH 4 and 4.5 maintained activity over the full 24-hour exposure period. The best overall performing buffer was 50 mM sodium acetate pH 4.

DISCUSSION

Production of laccase from *A. areolatum* liquid culture

Potato dextrose broth, a reasonably rich medium, was a suitable growth medium for production of *A. areolatum* laccase activity. To maximize the efficiency of production, it may be useful to test a number of laccase inducers in the medium (Leonowicz et al. 2001). There seems to be sufficient copper present for production of a multicopper enzyme in PDB. It may be that another limiting nutrient prevents the uptake and use of the added copper. On the other hand, the present system shows little if any peroxidase activity, making purification of the final product simpler.

Purification of the enzyme

Judging from the total protein gels, it is clear that we have a partially purified enzyme containing 10 or more constituents. The oxidase activity, however, seems confined to the 75-100 kDa range, with little or no activity outside this range. Given the absence of activity in the lane below 50 kDa, it is likely that the active enzyme is monomeric. Laccases are glycoproteins

(Dean and Eriksson 1994; Baldrian 2006; Morozova et al. 2007), and determination of the level of glycosylation of the purified enzyme would be useful. N-terminal amino acid sequencing of the enzyme could build a stronger case for its identity as a laccase by showing sequence homology with other fungal laccases. Further purification of the enzyme will necessarily precede any further characterization. The laccase we have partially purified may have isoforms, and isoelectric focusing is indicated to confirm this. Isoelectric focusing will allow separation and subsequent characterization of any isoenzymes present. A molecular weight of ca. 75 kDa is consistent with that of many other fungal laccases (Baldrian 2006).

Substrate specificity (*o*-, *m*-, *p*-sites): clues to laccase active site geometry

The broad specificity of the laccase active site is evident in the structures of the many substrates tested (Figure 43). *Amylostereum areolatum*'s extracellular laccase oxidizes hydroquinone, a defining characteristic of a laccase. Perhaps of interest, hydroquinone, the definitive substrate for laccase, is a substance present in trace amounts in unattacked sapwood of two *A. areolatum* host species, *P. radiata* and *P. resinosa* (Hillis and Inoue 1968).

The substrates used in this study fall into the general categories of *o*-substituted (guaiacol, syringaldazine), *m*-substituted (phloroglucinol), and *p*-substituted (hydroquinone, *p*-phenylenediamine, resveratrol) phenols, and mid-molecule nitrogen oxidation target (ABTS, syringaldazine). Oxidation of *p*-phenylenediamine and 1, 8-diaminonaphthalene fit into none of these categories. Of all substrates tested, only pinosylvin could not be shown to be oxidized by the partially-purified *A. areolatum* laccase. It is likely that *A. areolatum* laccase attacks the *p*-hydroxy group on resveratrol and not the two *m*-substituents, as oxidation of pinosylvin (almost exactly like resveratrol, having the two *m*-substituted –OH groups but lacking the *p*-substituted –OH group) could not be demonstrated spectrophotometrically.

There have been no direct reports of pine pathogenic fungal laccases oxidizing resveratrol. There is at least the suggestion that this is so in the literature. The basidiomycete

white-rot pine pathogen *Heterobasidion annosum* P-type produces more laccase than the S-type. Attack by P-type *H. annosum* induces production of higher levels of pinosylvin than in similar attack by S-type (Johansson et al. 1998). This circumstance suggests that pine pathogenic fungal laccase may be capable of degrading pinosylvin, and makes oxidation of pinosylvin by *A. areolatum* – produced laccase a reasonable hypothesis.

The small shifting peaks in the spectral data without definite one-way travel up or down the y-axis were not carefully inspected. These may be the result of unstable, short-lived products (oxidation products, or enzyme-bound reaction intermediates). Since peaks of this type are most noticeable in the pinosylvin spectral data, it could be that pinosylvin is being oxidized but that its products are not stable. An orthogonal means of measuring oxidation for pinosylvin is indicated here. Measurement of oxygen uptake would also serve to strengthen the spectral case for oxidation of the other substrates by *A. areolatum* laccase.

Implications of activity against resveratrol and pinosylvin

Amylostereum areolatum laccase oxidizes resveratrol (3, 5, 4'-trihydroxystilbene), a known plant defense compound against bacterial or fungal challenge. Resveratrol is known to occur in grape in response to *Botrytis cinerea* fungal attack, where it is detoxified by *B. cinerea* fungal laccase (Sbaghi et al. 1996; Schouten et al. 2002). Resveratrol has also been tentatively identified accumulating in response to *Ceratocystis minor* (blue stain fungus) challenge in *P. taeda* (Hemingway et al. 1976). Oxidation of resveratrol indicates a possible role for extracellular laccase in detoxifying plant defense compounds. In order not to overstate the significance of resveratrol, note that *in vitro* assays for wood decay resistance show pinosylvin has a much greater fungistatic effect than resveratrol (Seppänen et al. 2004).

Pinosylvin is a fungicidal or fungistatic compound produced constitutively in pine heartwood, and its production is induced in pine sapwood in response to fungal attack (Hart 1981; Madden 1988; Celimene et al. 1999). Spectrophotometric investigation could not

demonstrate *A. areolatum* laccase oxidation of pinosylvin, a compound nearly identical to resveratrol. Pinosylvin may in fact be oxidized by *A. areolatum* laccase, but oxygen uptake was not monitored during this experiment. If oxygen uptake occurs during the reaction of laccase with pinosylvin, it would serve to demonstrate a positive link between *A. areolatum* laccase and fungal pathogenicity.

Inhibition

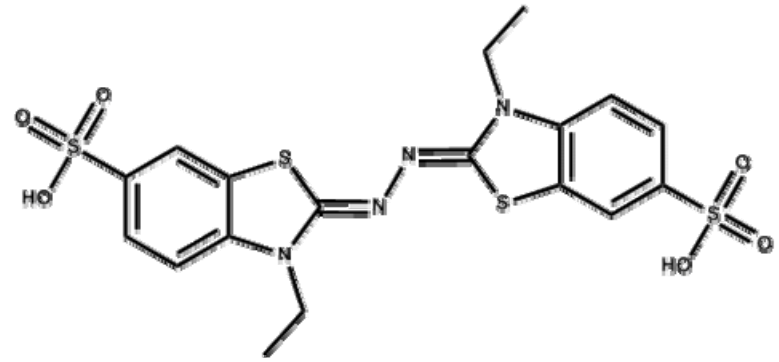
At least two inhibitors of the fungal laccase have been identified. Application of 0.1 mM sodium azide and 5 mM NaCl resulted in essentially complete loss of enzyme activity. Given the reductive nature of *L*-cysteine, it could not be confirmed as an inhibitor by the oxidation of guaiacol used here. Multicopper enzyme activation by SDS has been reported (Diamantidis et al. 2000; Castro-Sowinski et al. 2002; Laufer et al. 2006), but explaining this phenomenon requires further study. Activation by EDTA could be the result of EDTA chelation of metal-containing compounds inhibitory to laccase, but this can only be confirmed with a purified preparation of *A. areolatum* laccase.

Sensitivity to NaCl in the inhibition study was unexpected but reasonable. Halide inhibition of fungal laccase enzymes is variable, but well-documented even in wood-degrading basidiomycetes (Naki and Varfolomeev 1981; Koudelka and Ettinger 1988; Xu 1996). This variation may reflect differences in the active site of the individual laccase (Xu 1996).

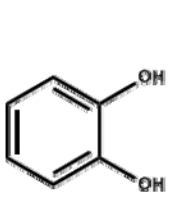
Future studies

Future work with this enzyme will begin with complete purification. Once enzyme purity has been demonstrated, kinetic parameters can be assessed. Further characterization of the enzyme should include N-terminal amino acid sequencing, determination of level of glycosylation, separation and characterization of any isoforms present, and further exploration

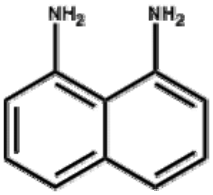
of inhibitors. It would also be useful to measure substrate specificity by an orthogonal procedure (uptake of oxygen) to supplement the spectrophotometric measurements.



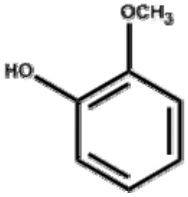
ABTS



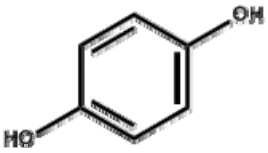
Catechol



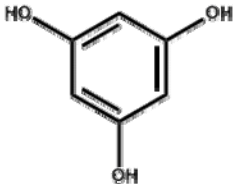
1, 8-Diaminonaphthalene



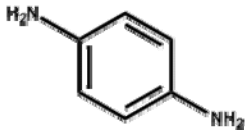
Guaiacol



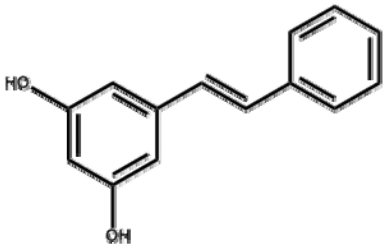
Hydroquinone



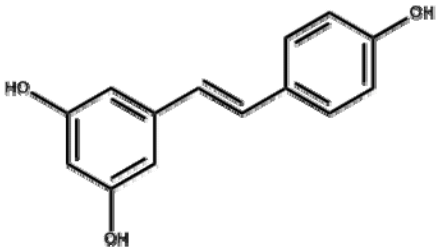
Phloroglucinol



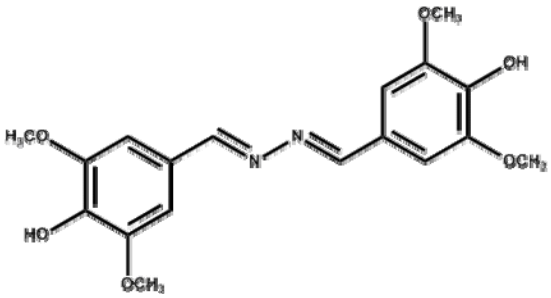
***P*-Phenylenediamine**



Pinosylvin



Resveratrol



Syringaldazine

Figure 13: Structures of Potential *A. areolatum* Laccase Substrates.

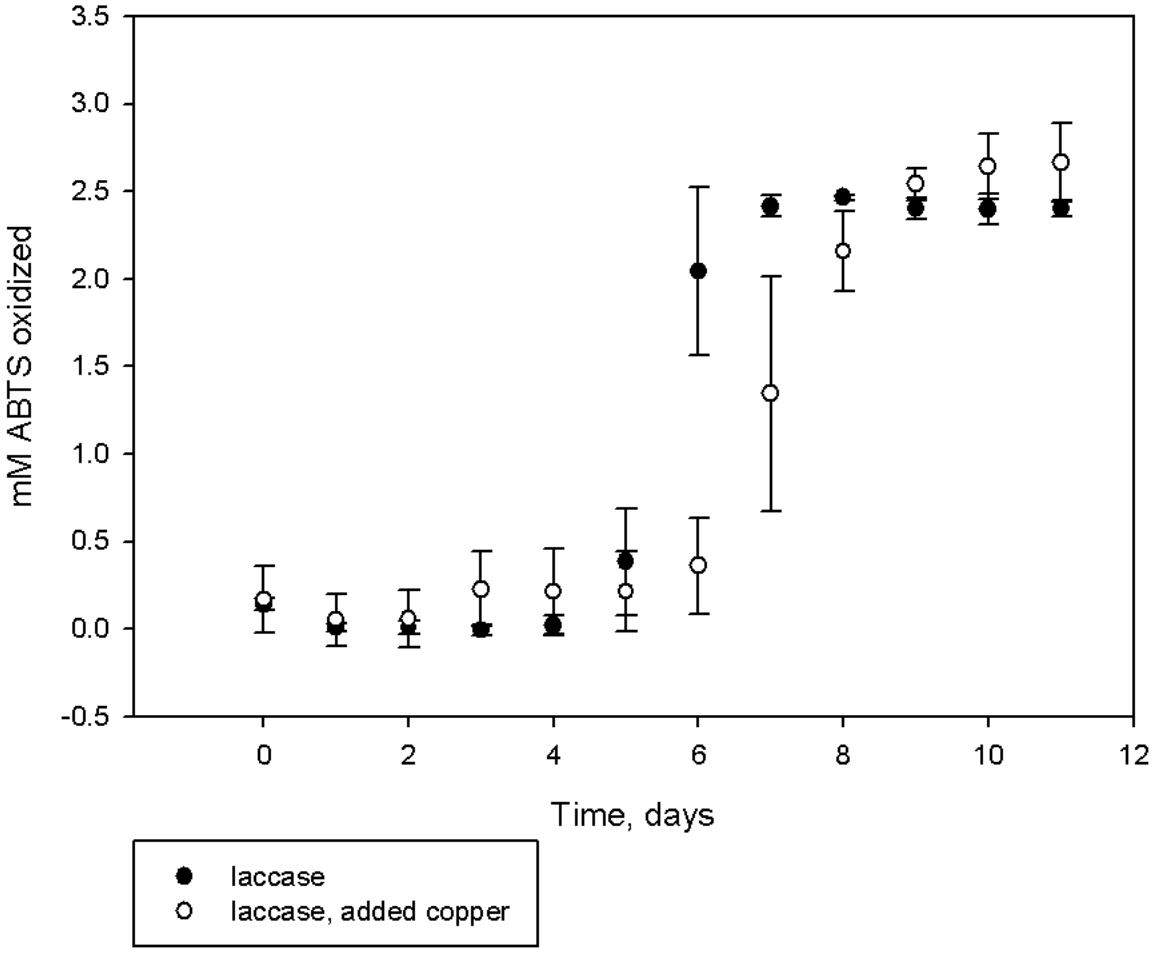


Figure 14: ***A. areolatum*** **Production of Laccase-Like Activity in Potato Dextrose Broth. Comparison of the effect of added copper (150 µM at 2 days) vs. no supplementation (n = 3). For each flask, inoculated with 5 ml (0.03 g dry weight equivalent) of** ***A. areolatum*** **fungal homogenate to 500 ml sterile PDB. Flasks grown under standard laboratory fluorescent lighting at 25°C with shaking (120 rpm) for 11 days. Removed approximately 300 µl every day and stored at -80°C. Measured laccase activity via oxidation of ABTS (UV/VIS) at 405 nm in a microplate reader.**

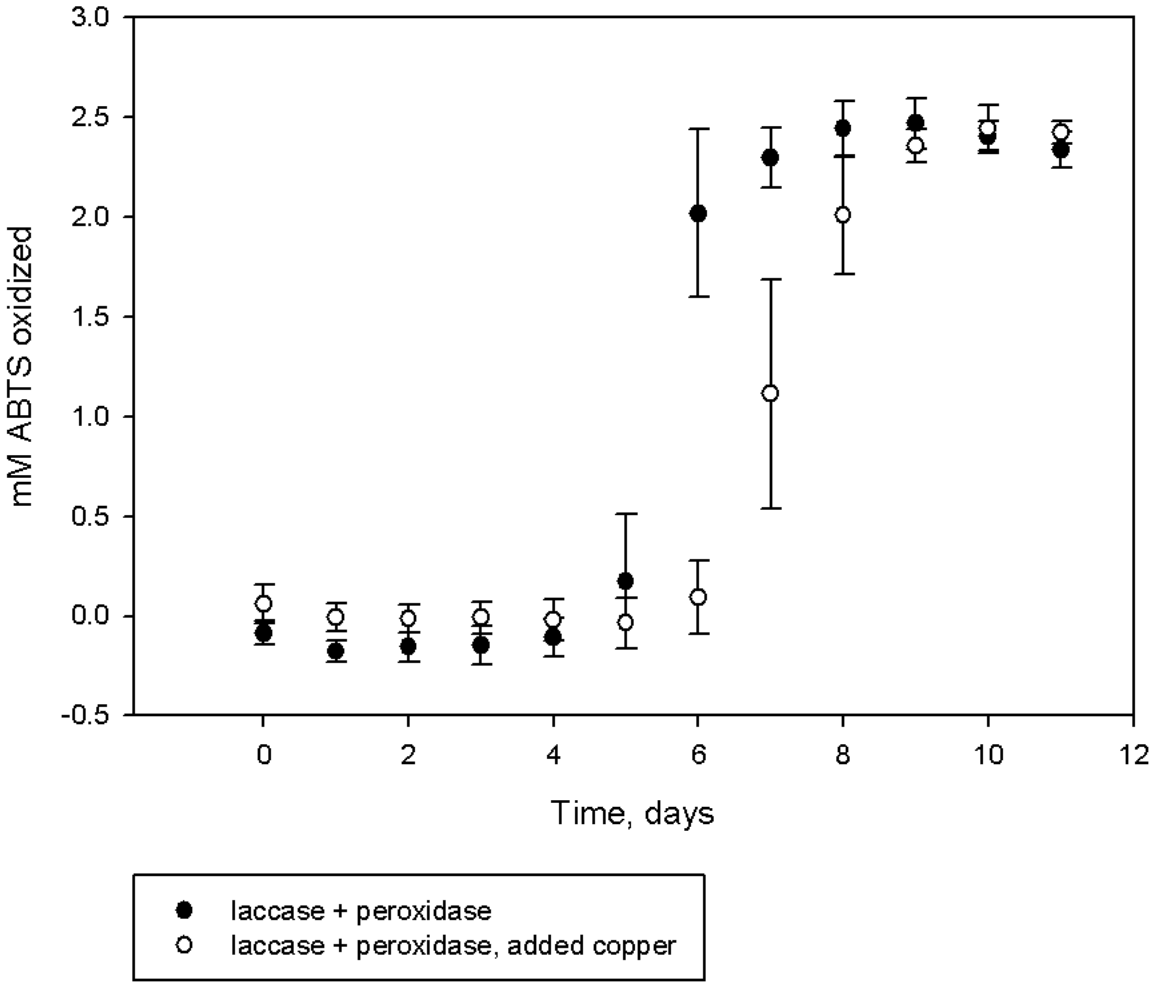


Figure 15: *A. areolatum* production of Total Phenoloxidase in Potato Dextrose Broth. Comparison of the effect of added copper (150 µM at 2 days) vs. no supplementation (n=3). For each flask, inoculated with 5 ml (0.03 g dry weight equivalent) of *A. areolatum* fungal homogenate to 500 ml sterile PDB. Flasks grown under standard laboratory fluorescent lighting at 25°C with shaking (120 rpm) for 11 days. Removed approximately 300 µl every day and stored at -80°C. Measured combined activity of laccase and peroxidase via oxidation of ABTS (UV/VIS) at 405 nm in a microplate reader.

Figure 16: Morphology of *A. areolatum* Fungus Grown in Shaken Flask Culture. Forceps are approximately 11 cm long x 1 cm wide. For each flask, inoculated with 5 ml (0.03 g dry weight equivalent) of *A. areolatum* fungal homogenate to 500 ml sterile PDB. Flasks grown under standard laboratory fluorescent lighting at 25°C with shaking (120 rpm) for 11 days.

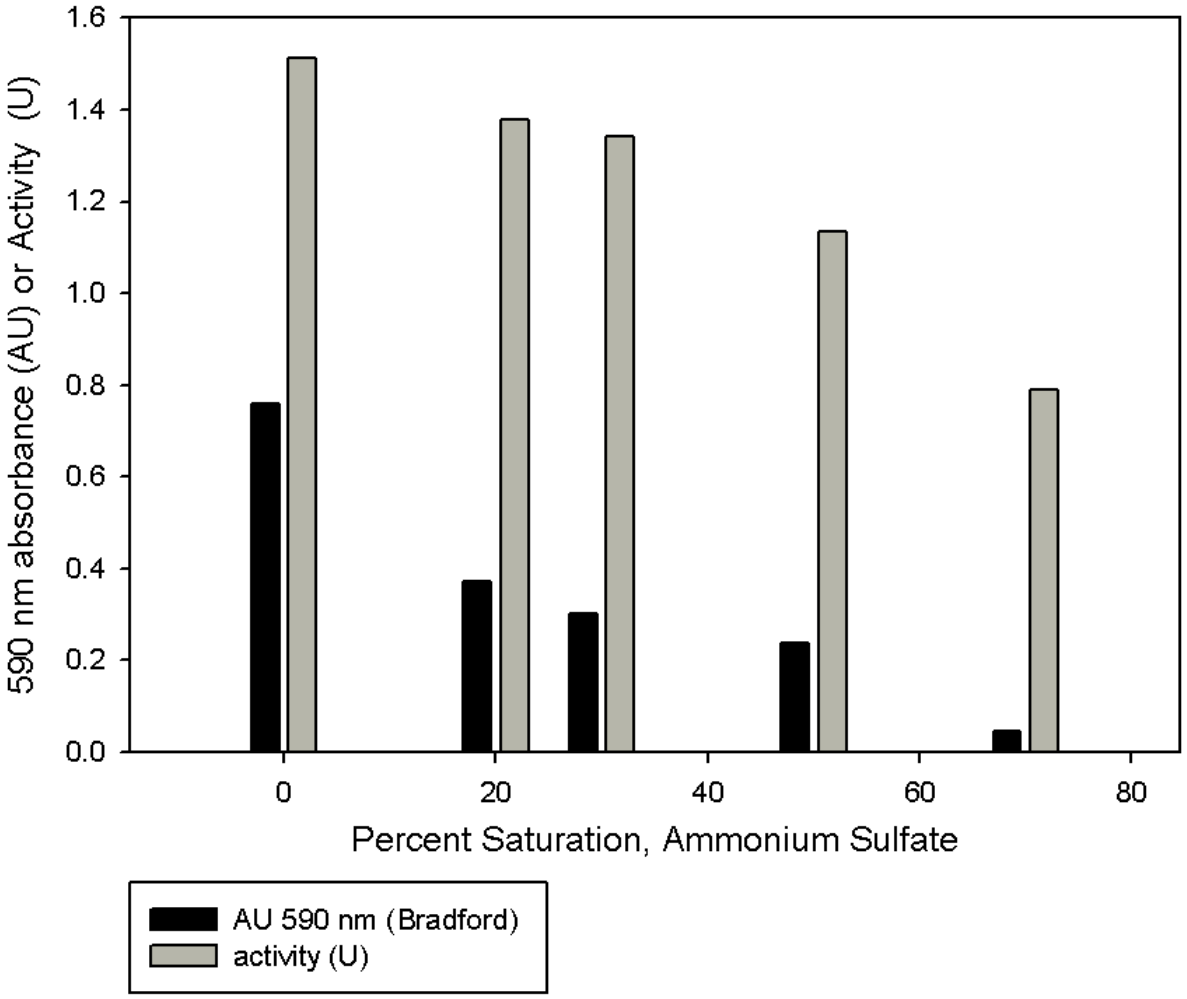


Figure 17: Comparison of Protein Content and Laccase-Like Activity During Pilot Ammonium Sulfate Precipitation. Culture filtrate from 11 day old shaken culture of *A. areolatum* was brought to the specified percentage saturation with solid ammonium sulfate. Following centrifugation, supernatant was decanted. Protein content measured via Bradford assay at 590 nm, and quantitated against BSA standards. Laccase activity measured via ABTS oxidation, monitored at 405 nm.

Table 3: Protein/Activity Comparison with Ammonium Sulfate Precipitation. Prepared from a pilot study. Culture filtrate from 11 day old shaken culture of *A. areolatum* was brought to the specified percentage saturation with solid ammonium sulfate. Following centrifugation, supernatant was decanted. Protein content measured via Bradford assay at 590 nm, and quantitated against BSA standards. Laccase activity measured via ABTS oxidation, monitored at 405 nm.

% Saturation, Ammonium Sulfate	Protein Content (AU, 590 nm)	Activity (U)	Specific Activity (U/AU)	% Loss of Protein	% Loss of Activity
0	0.760	1.5117	1.989	0.0%	0.0%
20	0.373	1.3781	3.695	50.9%	8.8%
30	0.301	1.3410	4.455	60.4%	11.3%
50	0.239	1.1343	4.746	68.6%	25.0%
70	0.046	0.7891	17.153	93.9%	47.8%

Table 4: Summary of Purification of Extracellular Laccase from *Amylostereum areolatum*. At each step of the purification, a quantity was reserved for measuring protein and activity. Protein content measured via Bradford assay at 590 nm, and quantitated against BSA standards. Laccase activity measured via ABTS oxidation, monitored at 405 nm.

Starting Fraction	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification Fold	Activity Yield
Culture Filtrate	3722	170.0	2.1	0.012	---	100%
Post PM-30 Supernatant	566	206.5	3.4	0.016	1.3	161%
70% Ammonium Sulfate Supernatant	677	11.0	4.2	0.388	31.1	201%
Concentration (Amicon and Centrifugal Concentrator)	0.5	0.6	9.4	15.692	1260	445%

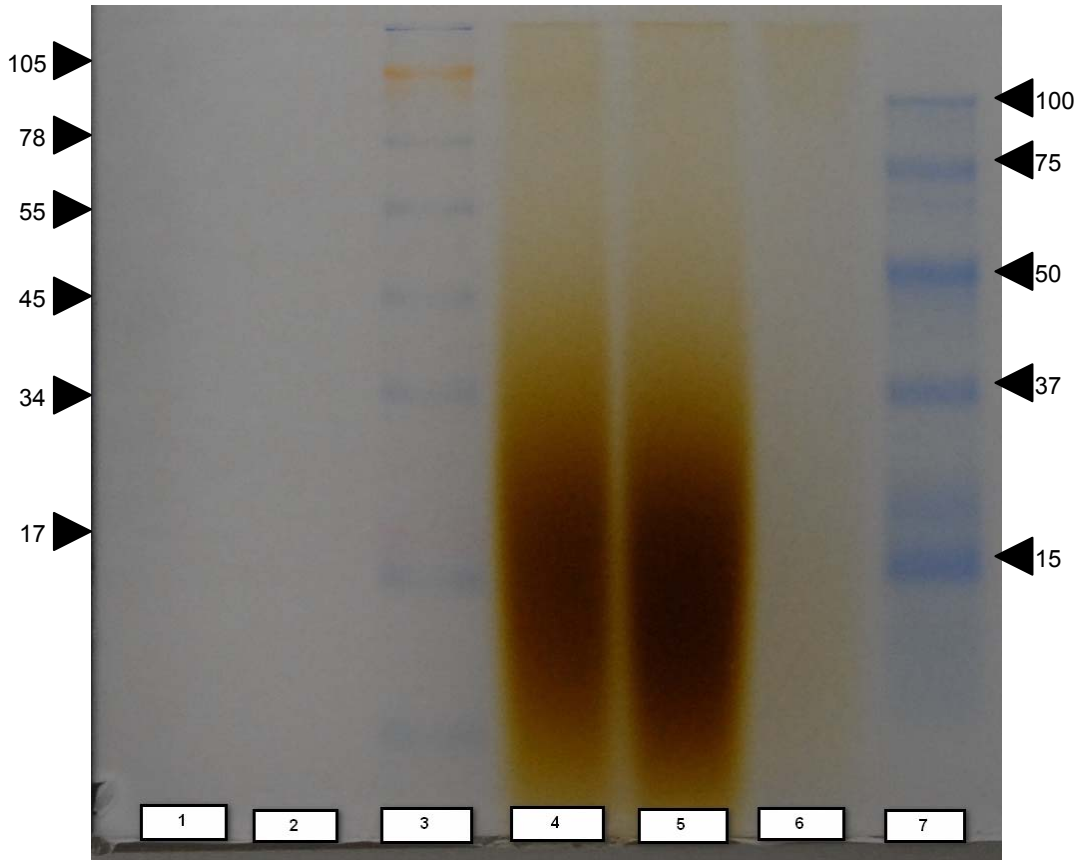


Figure 18: Zymogram Gel Prior to Staining. Arrows indicate molecular weight in kDa. SDS-PAGE (Tris-tricine system), run at constant voltage (30V) until sample entered the separating gel, then 150V until dye electrophoresed off the end of the gel.

1: horseradish peroxidase, 4 µg
2: bovine serum albumin, 8 µg
3: Invitrogen protein standard, 5 µl
4: *A. areolatum* culture filtrate in PDB, post-PM10, 120 µg
5: pellet from 70% ammonium sulfate precipitation, 92 µg
6: partially-purified *A. areolatum* laccase, 40 µg
7: Bio-Rad protein standard, 10 µl

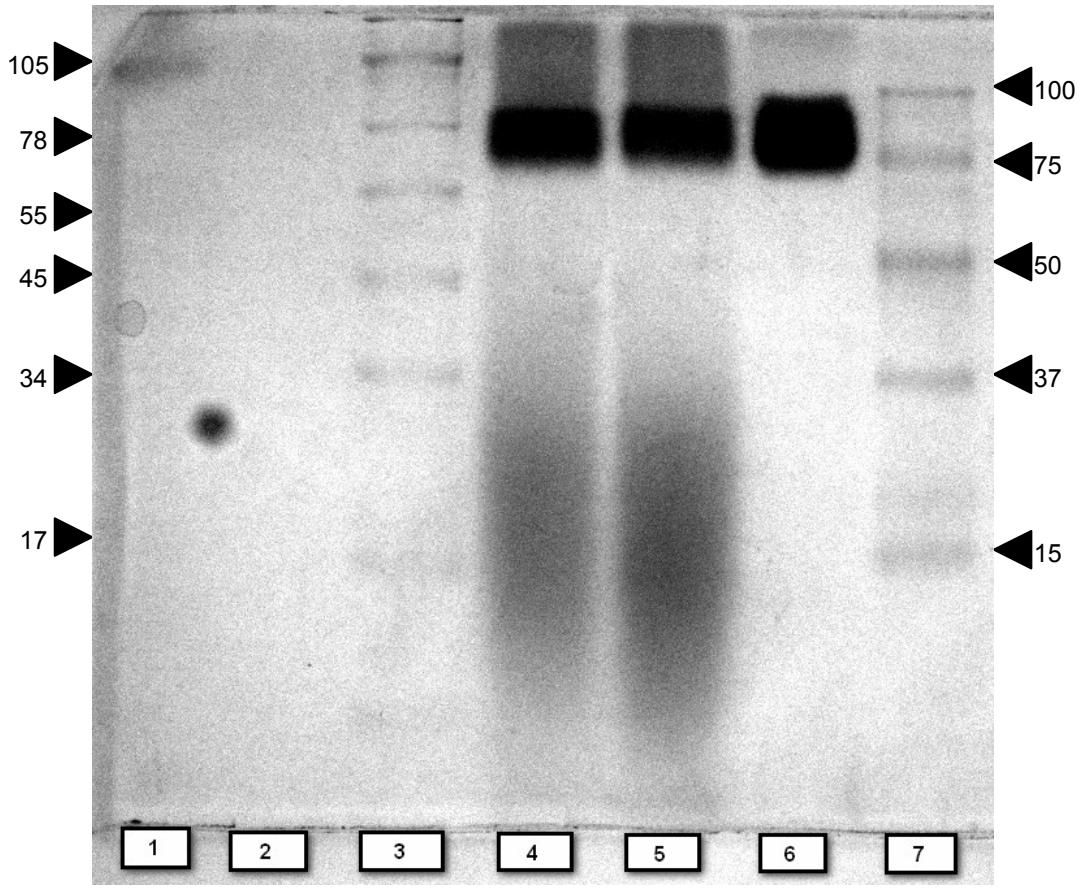


Figure 19: Phenoloxidase Zymogram of the Partially Purified *A. areolatum* Laccase. Arrows indicate molecular weight in kDa. SDS-PAGE (Tris-tricine system), run at constant voltage (30V) until sample entered the separating gel, then 150V until dye electrophoresed off the end of the gel. Phenoloxidase activity detected with 1,8-diaminonaphthalene staining (2 mM DAN in 1% DMSO/50 mM sodium acetate, pH 5).

1: horseradish peroxidase (positive control), 4 µg
2: bovine serum albumin (negative control), 8 µg
3: Invitrogen protein standard, 5 µl
4: *A. areolatum* culture filtrate in PDB, post-PM10, 120 µg
5: pellet from 70% ammonium sulfate precipitation, 92 µg
6: partially-purified *A. areolatum* laccase, 40 µg
7: Bio-Rad protein standard, 10 µl

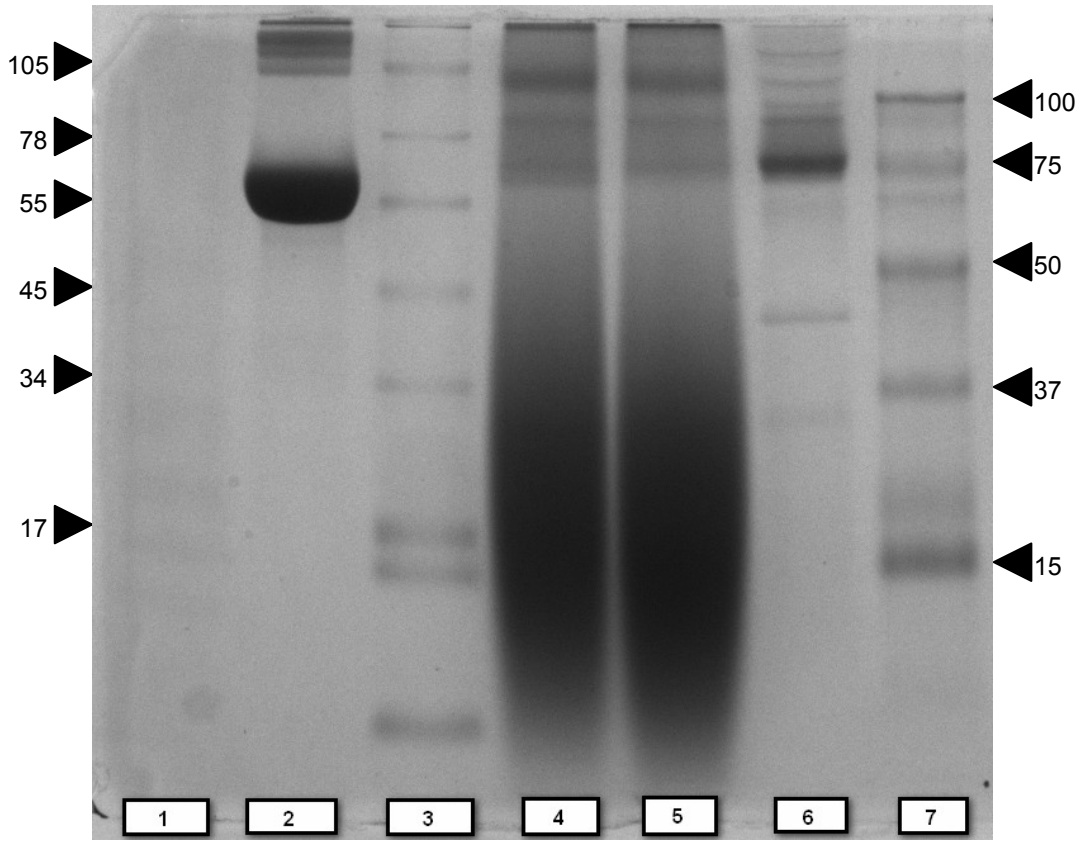


Figure 20: Coomassie-Stained Zymogram Gel Containing the Partially Purified *A. areolatum* Laccase. Samples not boiled prior to loading gel. Arrows indicate molecular weight in kDa. SDS-PAGE (Tris-tricine system), run at constant voltage (30V) until sample entered the separating gel, then 150V until dye electrophoresed off the end of the gel. Stained for phenoloxidase activity with 1, 8-diaminonaphthalene.

1: horseradish peroxidase, 4 µg
2: bovine serum albumin (positive control), 8 µg
3: Invitrogen protein standard, 5 µl
4: *A. areolatum* culture filtrate in PDB, post-PM10, 120 µg
5: pellet from 70% ammonium sulfate precipitation, 92 µg
6: partially-purified *A. areolatum* laccase, 40 µg
7: Bio-Rad protein standard, 10 µl

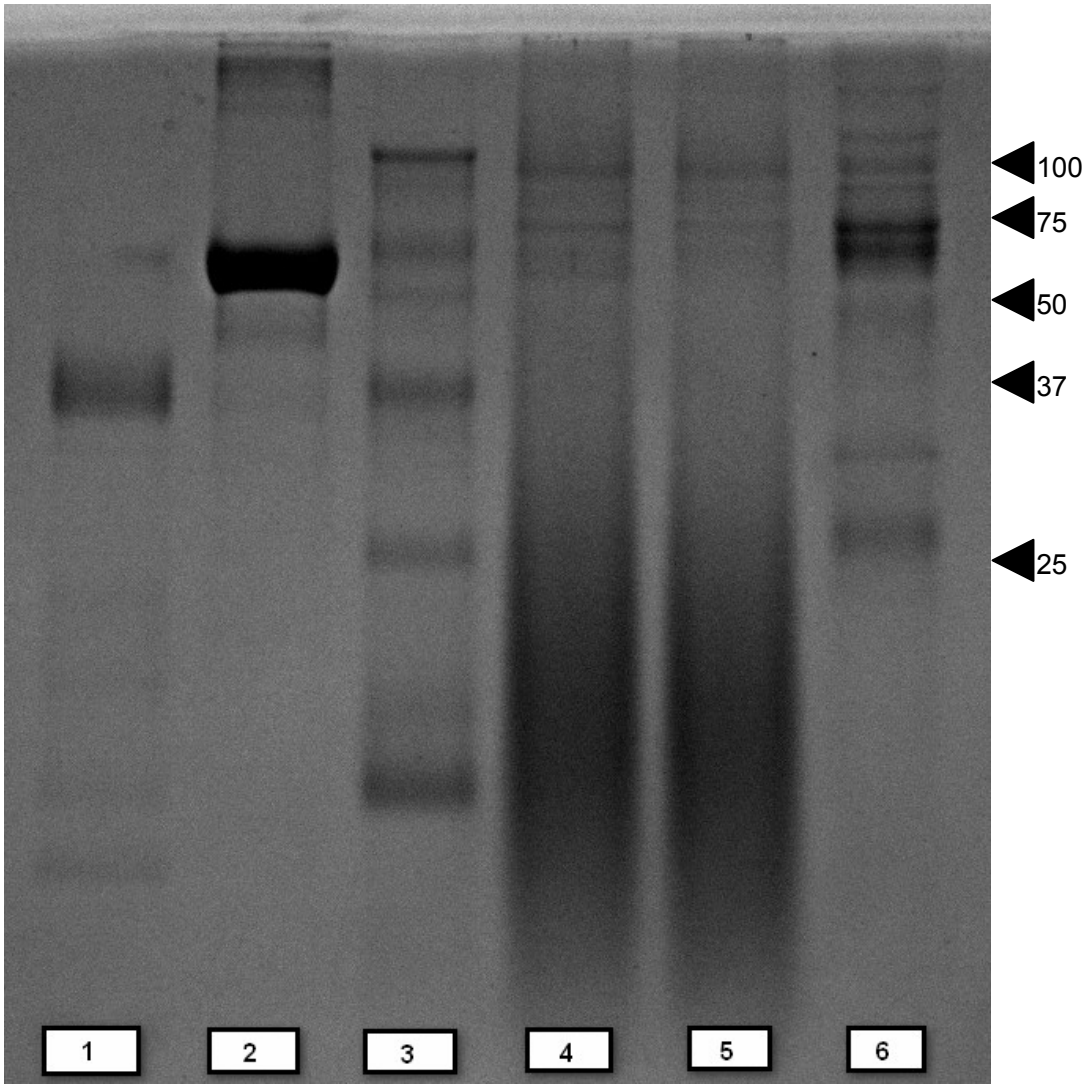


Figure 21: Denaturing SDS-PAGE Analysis of the Partially Purified *A. areolatum* Laccase. Staining is for total protein using Coomassie. Samples boiled in loading buffer prior to loading gel. Arrows indicate molecular weight in kDa. SDS-PAGE (Tris-tricine system), run at constant voltage (30V) until sample entered the separating gel, then 150V until dye electrophoresed off the end of the gel.

1: horseradish peroxidase, 4 µg
2: bovine serum albumin, 2.4 µg
3: Bio-Rad protein markers, 10 µl
4: *A. areolatum* culture filtrate in PDB, post-PM10, 24 µg
5: pellet from 70% ammonium sulfate precipitation, 18.5 µg
6: partially-purified *A. areolatum* laccase, 24 µg

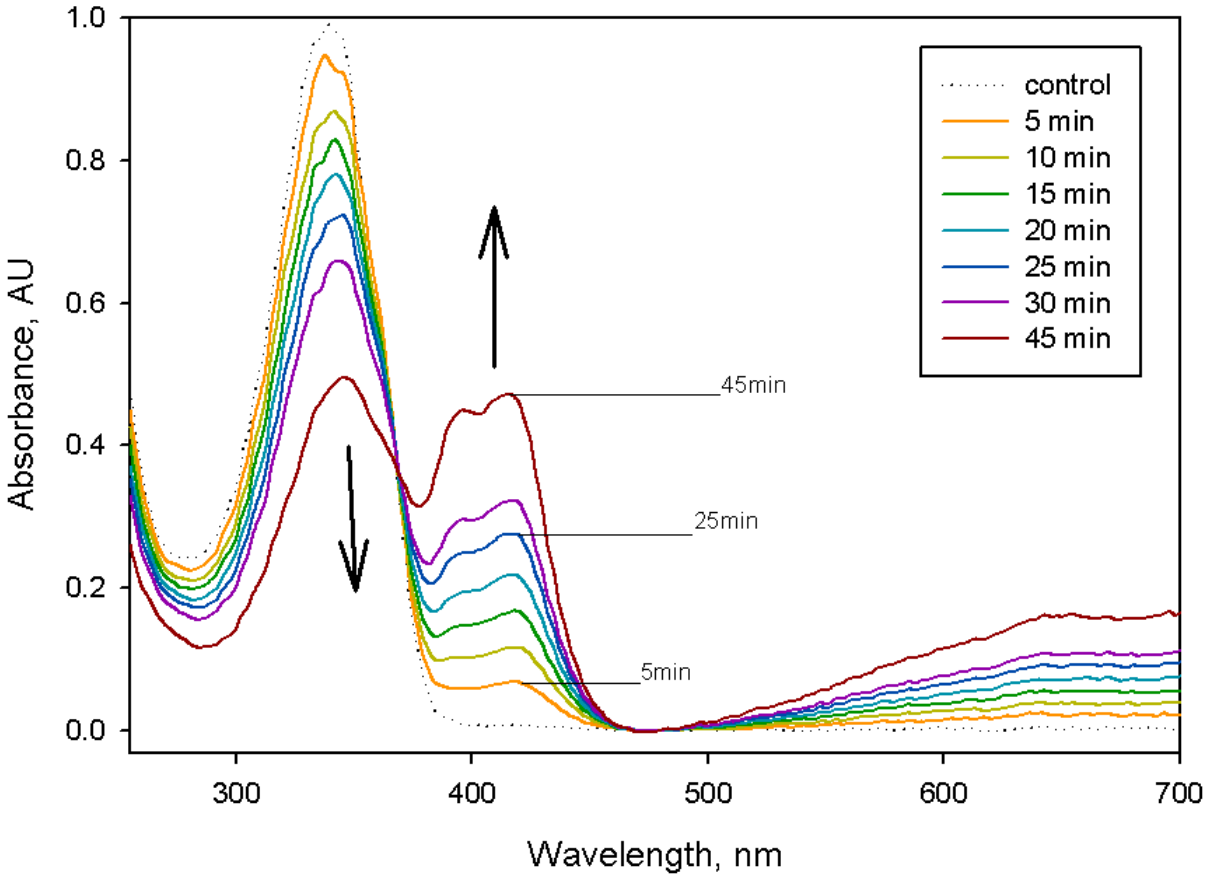


Figure 22: Time Course Spectral Scan of ABTS Oxidation via Partially Purified *A. areolatum* Laccase. Reported wavelength of maximum absorbance: 420 nm (Sterjiades et al. 1992). Autoxidation control contained no enzyme and was measured at 45 minutes. Measured absorption spectrum at specified timepoints via UV/VIS by 4µl aliquots of reaction mixture below:

160 µl 50 mM sodium acetate buffer, pH 4.0

20 µl laccase (1:5000 dilution)

20 µl 3 mM ABTS

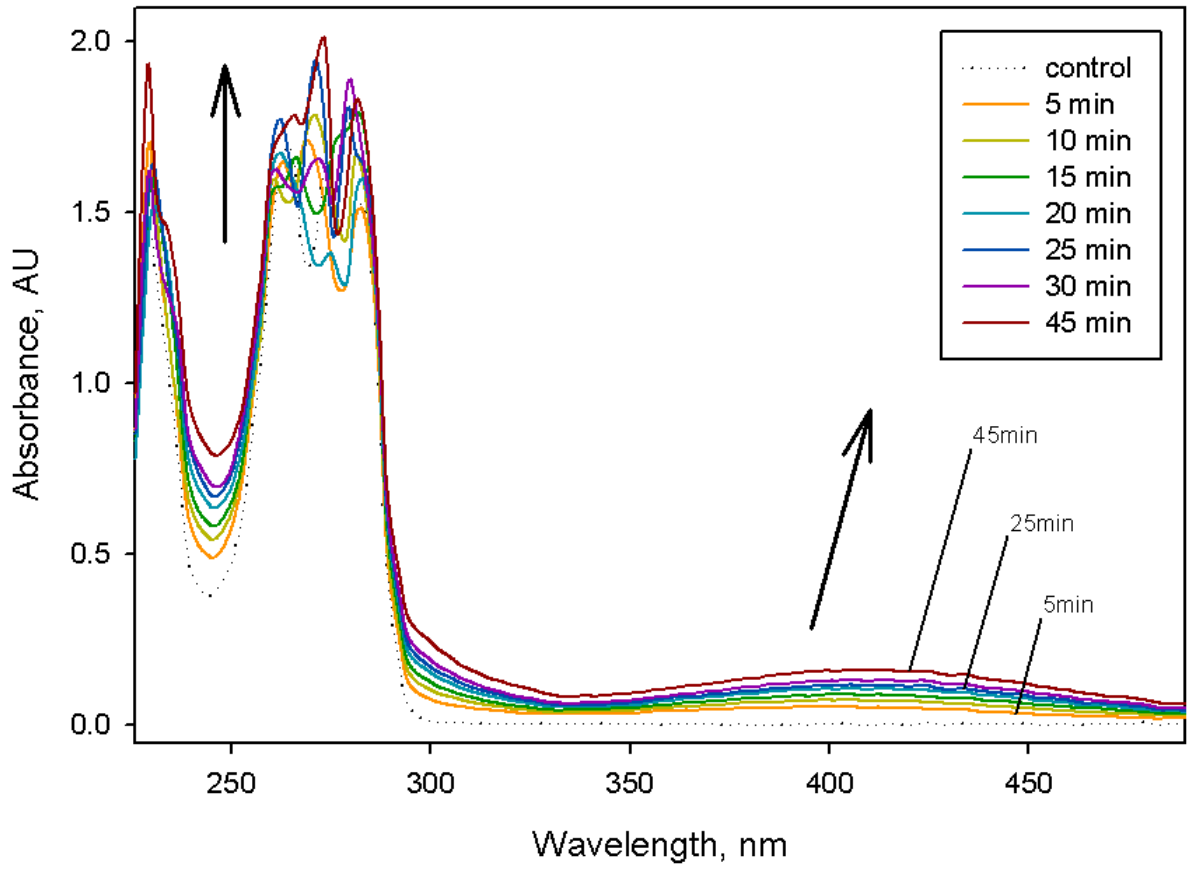


Figure 23: Time Course Spectral Scan of Catechol Oxidation via Partially Purified *A. areolatum* Laccase. Reported wavelength of maximum absorbance: 450 nm (Sterjiades et al. 1992). Control contained no enzyme and was measured at 45 minutes. Autoxidation control contained no enzyme and was measured at 45 minutes. Measured absorption spectrum at specified timepoints via UV/VIS by 4µl aliquots of reaction mixture below:

150 µl 100 mM sodium acetate buffer, pH 5.0

10 µl laccase (1:50 dilution)

40 µl 100 mM catechol

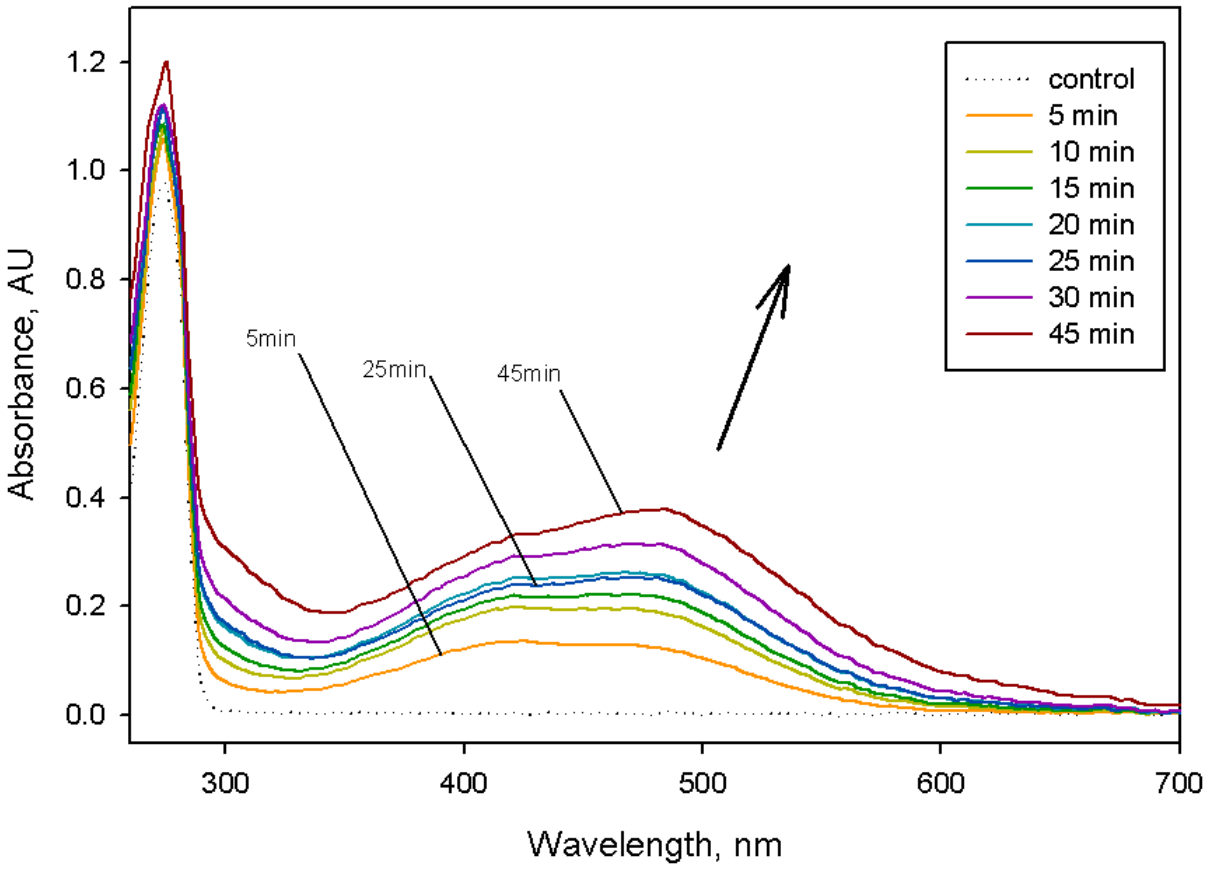


Figure 24: Time Course Spectral Scan of Guaiacol Oxidation via Partially Purified *A. areolatum* Laccase. Reported wavelength of maximum absorbance: 436 nm (Sterjiades et al. 1992). Control contained no enzyme and was measured at 45 minutes. Autoxidation control contained no enzyme and was measured at 45 minutes. Measured absorption spectrum at specified timepoints via UV/VIS by 4µl aliquots of reaction mixture below:

160 µl 100 mM sodium acetate buffer, pH 5.0

20 µl 0.01 mg/ml catalase

10 µl laccase (1:50 dilution)

10 µl 0.1 M guaiacol

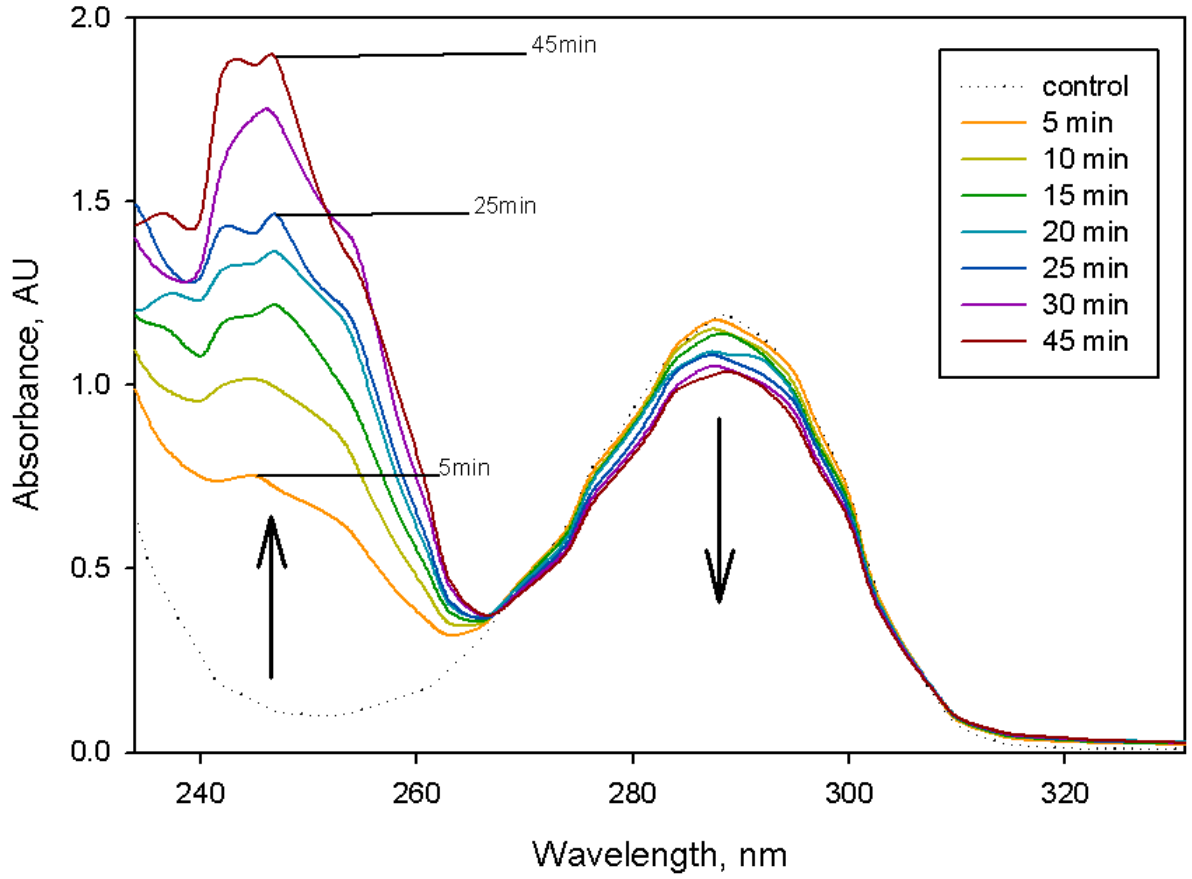


Figure 25: Time Course Spectral Scan of Hydroquinone Oxidation via Partially Purified *A. areolatum* Laccase. Reported wavelength of maximum absorbance: 250 nm (Sterjiades et al. 1992). Control contained no enzyme and was measured at 45 minutes. Autoxidation control contained no enzyme and was measured at 45 minutes. Measured absorption spectrum at specified timepoints via UV/VIS by 4µl aliquots of reaction mixture below:

179 µl 100 mM sodium acetate buffer, pH 5.0

10 µl laccase (1:50 dilution)

11 µl 100 mM hydroquinone

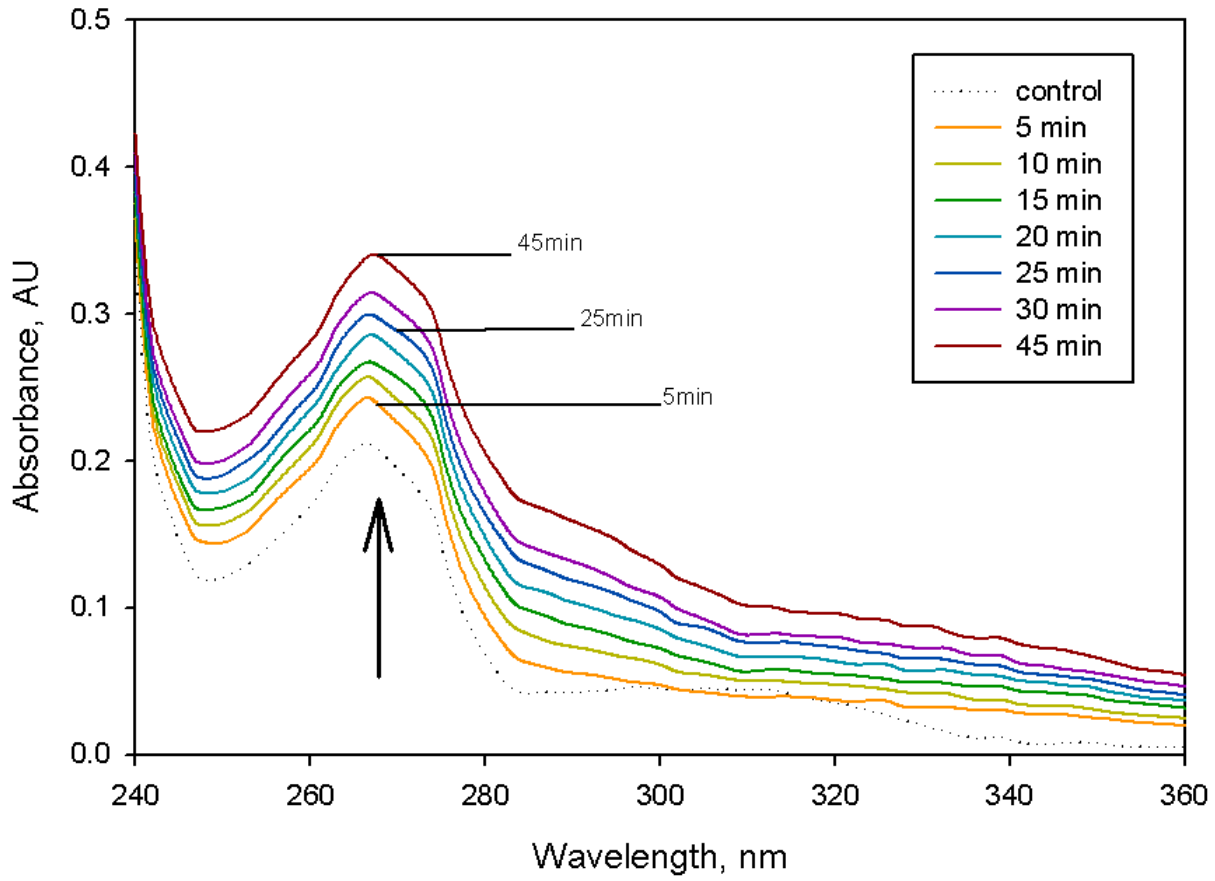


Figure 26: Time Course Spectral Scan of Phloroglucinol Oxidation via Partially Purified *A. areolatum* Laccase. Reported wavelength of maximum absorbance: 268 nm (Sterjiades et al. 1992). Control contained no enzyme and was measured at 45 minutes. Autoxidation control contained no enzyme and was measured at 45 minutes. Measured absorption spectrum at specified timepoints via UV/VIS by 4µl aliquots of reaction mixture below:

180 µl 50 mM sodium acetate buffer, pH 4.0

10 µl laccase (1:50 dilution)

10 µl 100 mM phloroglucinol

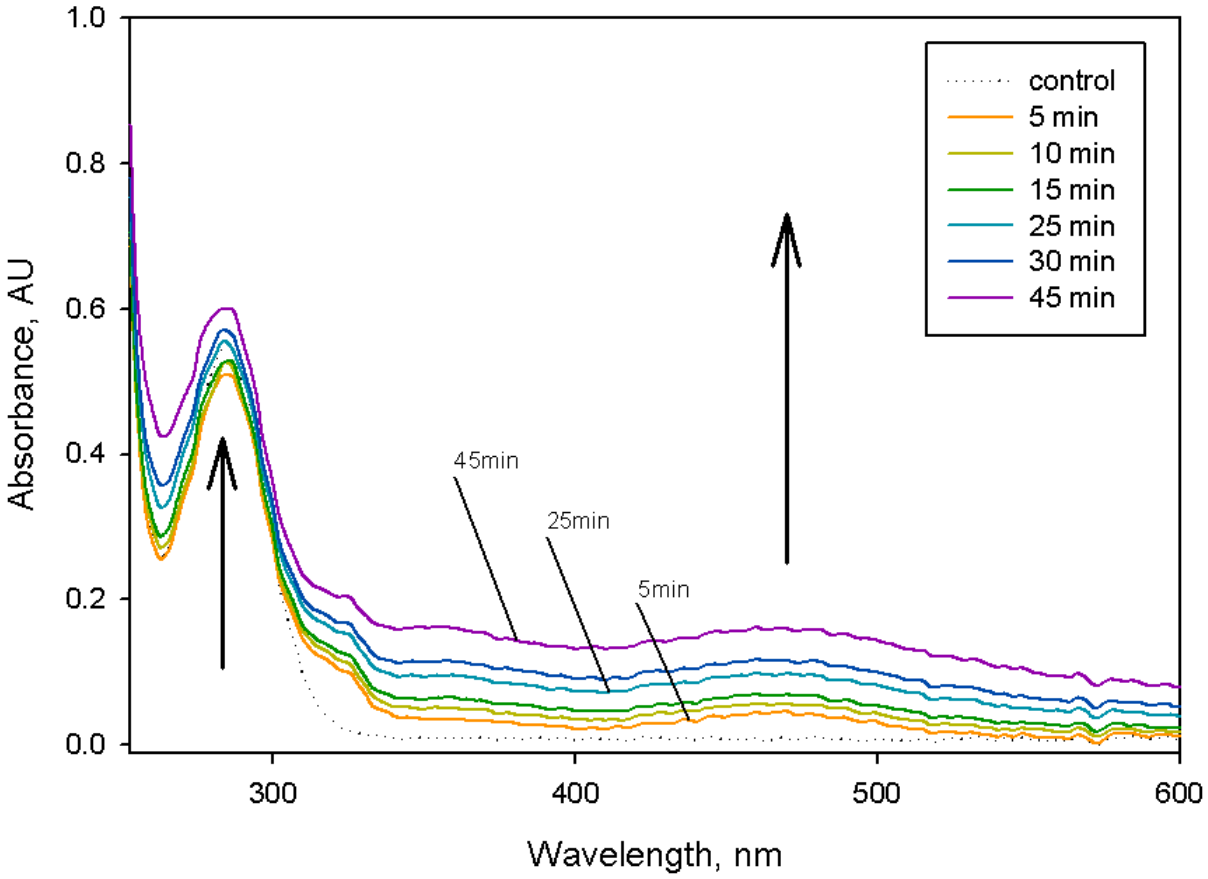


Figure 27: Time Course Spectral Scan of *P*-Phenylenediamine Oxidation via Partially Purified *A. areolatum* Laccase. Recommended wavelengths to monitor: 285, 468 nm. Control contained no enzyme and was measured at 45 minutes. Autoxidation control contained no enzyme and was measured at 45 minutes. Measured absorption spectrum at specified timepoints via UV/VIS by 4µl aliquots of reaction mixture below:

180 µl 50 mM sodium acetate buffer, pH 4.0

10 µl laccase (1:50 dilution)

10 µl 100 mM *p*-phenylenediamine

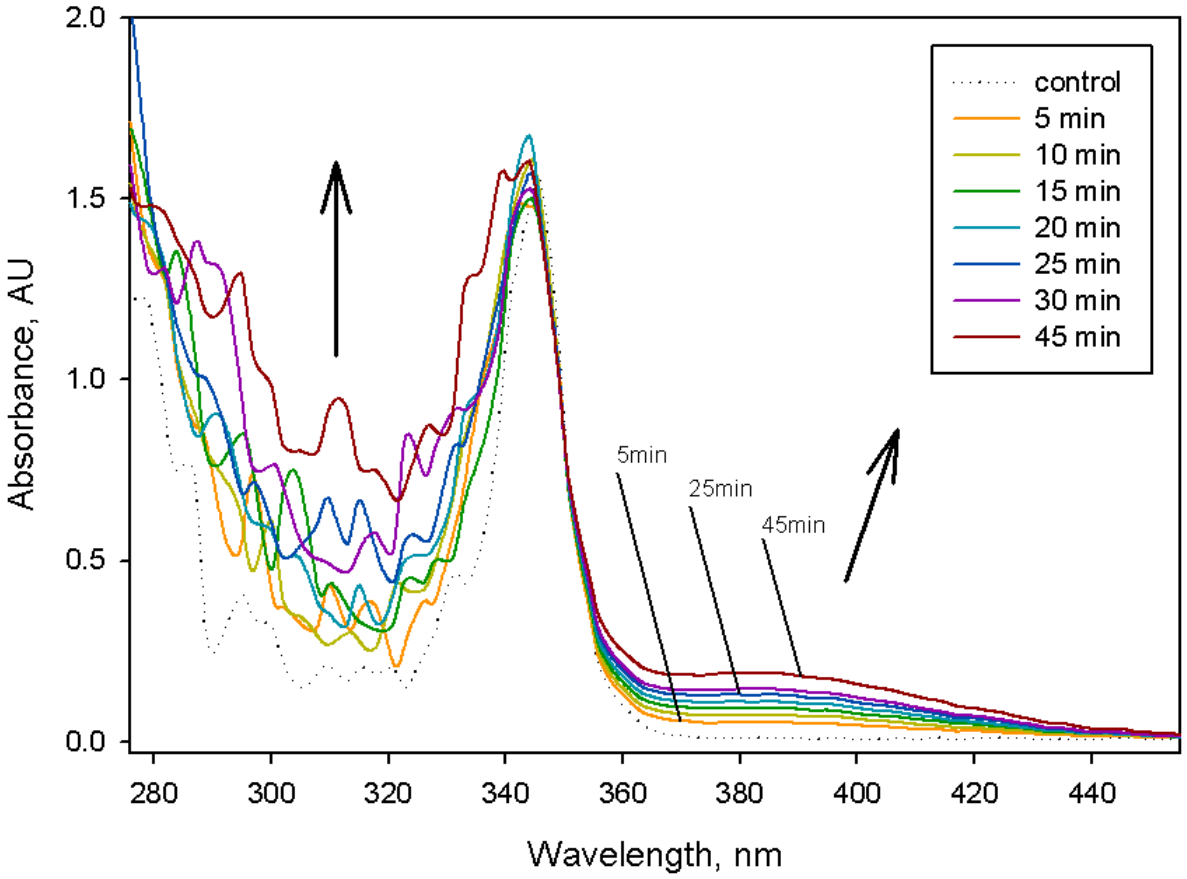


Figure 28: Time Course Spectral Scan of Resveratrol Oxidation via Partially Purified *A. areolatum* Laccase. Recommended wavelengths to monitor: 312, 385 nm. Control contained no enzyme and was measured at 45 minutes. It was necessary to carry out this reaction in 50 mM sodium acetate pH 3.0 to prevent substrate precipitation. Autoxidation control contained no enzyme and was measured at 45 minutes. Measured absorption spectrum at specified timepoints via UV/VIS by 4µl aliquots of reaction mixture below:

155 µl 50 mM sodium acetate buffer, pH 3.0

10 µl laccase (1:50 dilution)

35 µl 29 mM resveratrol

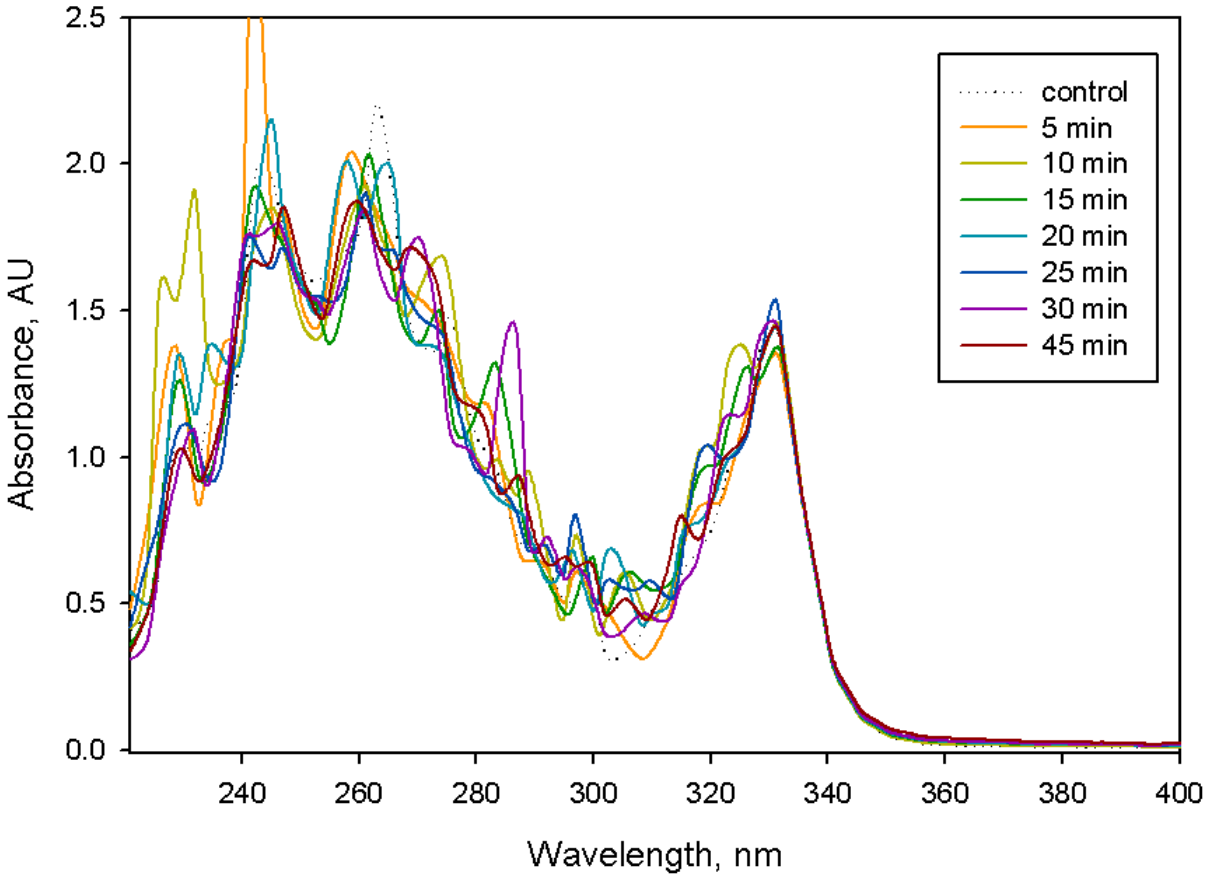


Figure 29: Time Course Spectral Scan of Pinosylvin Oxidation via Partially Purified *A. areolatum* Laccase. Control contained no enzyme and was measured at 45 minutes. Autoxidation control contained no enzyme and was measured at 45 minutes. Measured absorption spectrum at specified timepoints via UV/VIS by 4µl aliquots of reaction mixture below:

180 µl 50 mM sodium acetate buffer, pH 4.0

10 µl laccase (1:50 dilution)

10 µl 100 mM pinosylvin

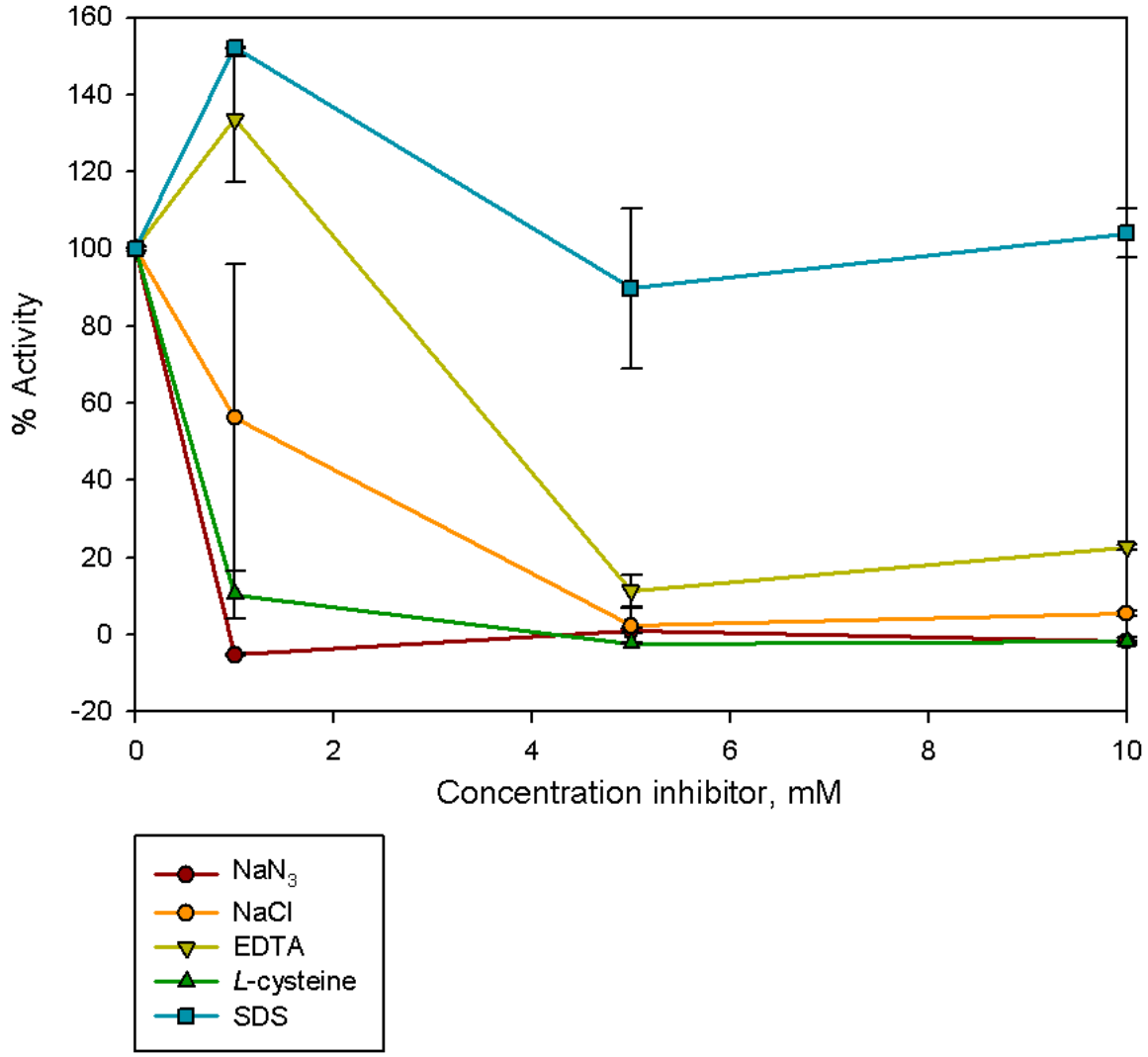


Figure 30: Response of Guaiacol Oxidation by Partially Purified *A. areolatum* Laccase to Putative Enzyme Inhibitors (n = 2). Reaction mixtures incubated at ambient temperature for 10 minutes, then absorbance at 450 nm measured at 36 minutes. Reaction mixtures were formulated as follows:

160 µl 50 mM sodium acetate pH 4.0
20 µl inhibitor (5 inhibitors x 2 replicates x 3 concentrations)
10 µl laccase (active or boiled)
10 µl 0.1 M guaiacol

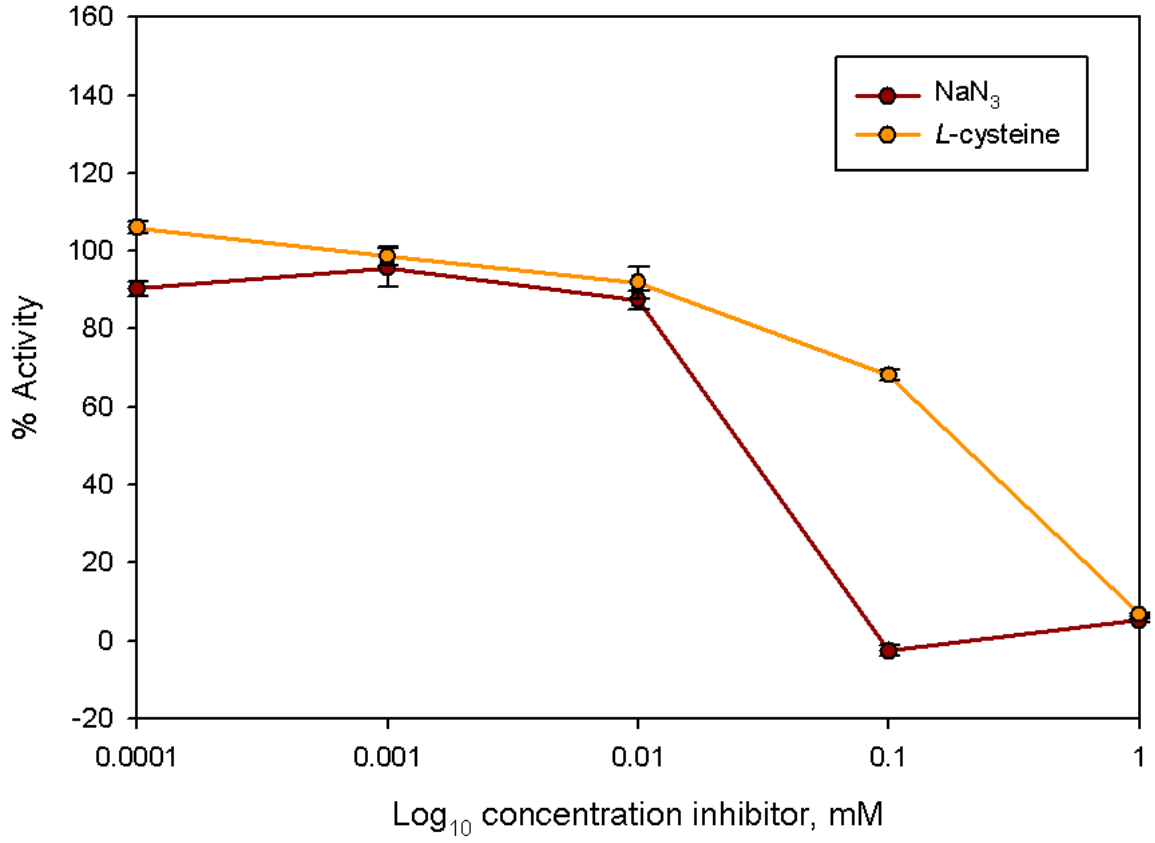


Figure 31: Inhibition of Partially Purified *A. areolatum* Laccase Oxidation of Guaiacol by Low Levels of Sodium Azide and *L*-Cysteine (n = 2). Note log-scale X-axis. Reaction mixtures incubated at ambient temperature for 10 minutes, then absorbance at 450 nm measured at 36 minutes. Reaction mixtures were formulated as follows:

160 µl 50 mM sodium acetate pH 4.0
20 µl inhibitor (2 inhibitors x 2 replicates x 5 concentrations)
10 µl laccase (active or boiled)
10 µl 0.1 M guaiacol

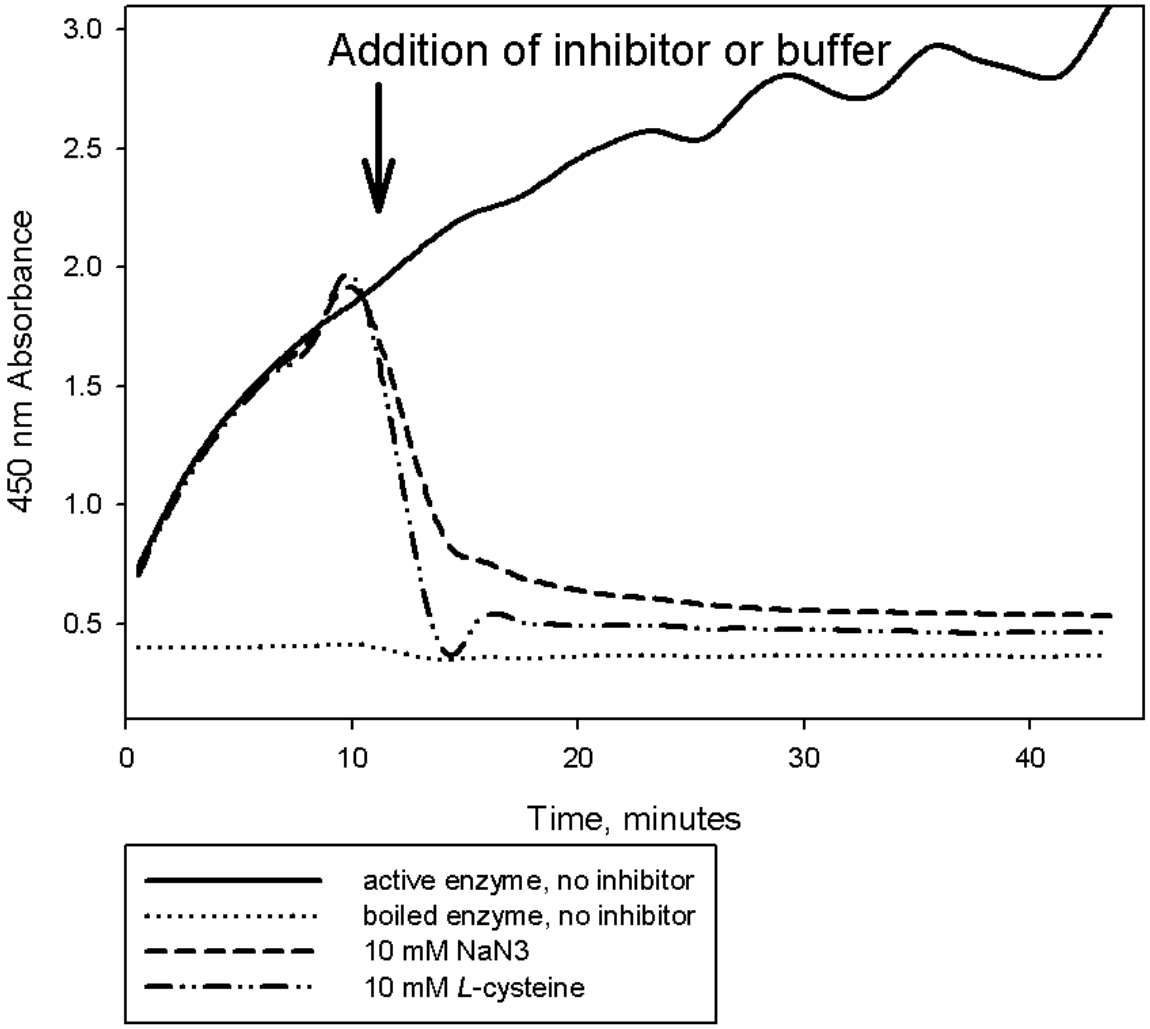


Figure 32: Time Course of NaN_3 vs. *L*-Cysteine Inhibition of Guaiacol Oxidation by Partially Purified *A. areolatum* Laccase. Reaction mixtures containing the fungal phenoloxidase and guaiacol substrate were monitored at 450 nm for 10 minutes. Inhibitor then added at 10 mM concentration, and reaction monitored for at least 30 minutes more.

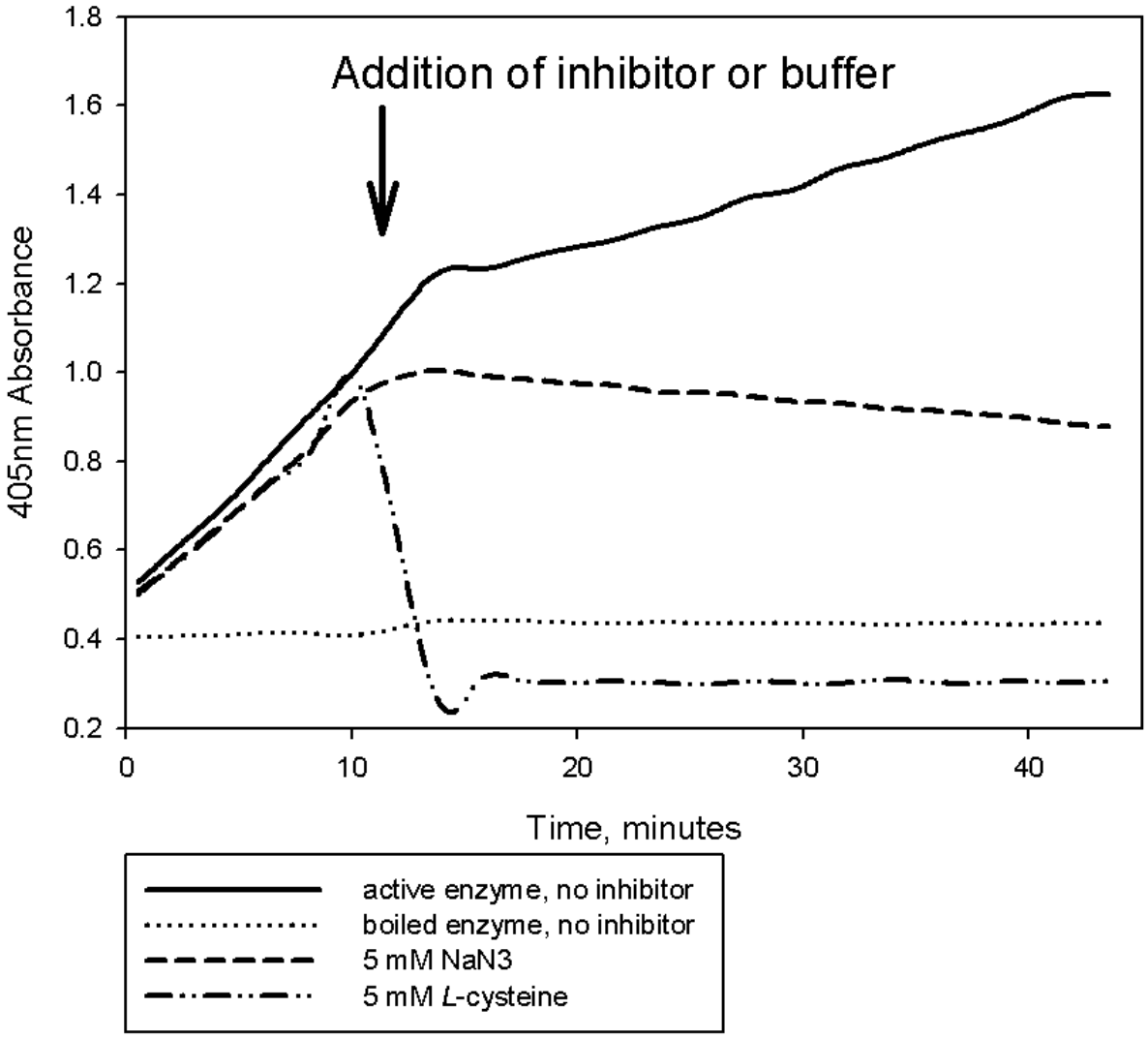


Figure 33: Time Course of NaN_3 vs. *L*-Cysteine Inhibition of ABTS Oxidation by Partially Purified *A. areolatum* Laccase. Reaction mixtures containing the fungal phenoloxidase and ABTS substrate were monitored at 405 nm for 10 minutes. Inhibitor then added at 5 mM concentration, and reaction monitored for at least 30 minutes more.

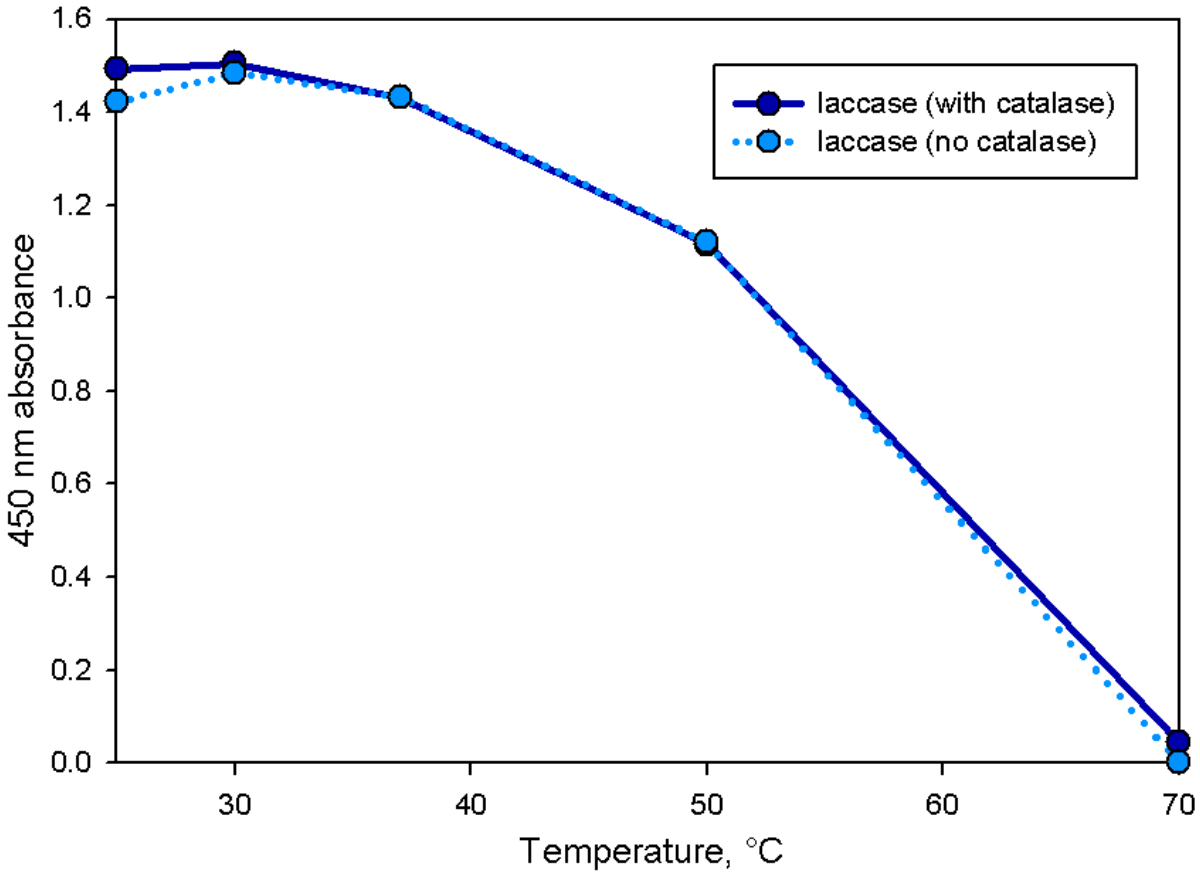


Figure 34: Temperature Stability of Partially Purified *A. areolatum* Laccase. Enzyme was incubated for one hour at various temperatures before residual activity was determined using guaiacol oxidation. Reaction mixtures containing the fungal phenoloxidase and were incubated at specified temperature conditions for one hour. Each mixture was then aliquoted to a microtiter plate and guaiacol added to 5 mM concentration as substrate. The reaction was measured at 35 minutes via UV/VIS absorbance at 450 nm. Inhibitor then added at 5 mM concentration, and reaction monitored for at least 30 minutes more.

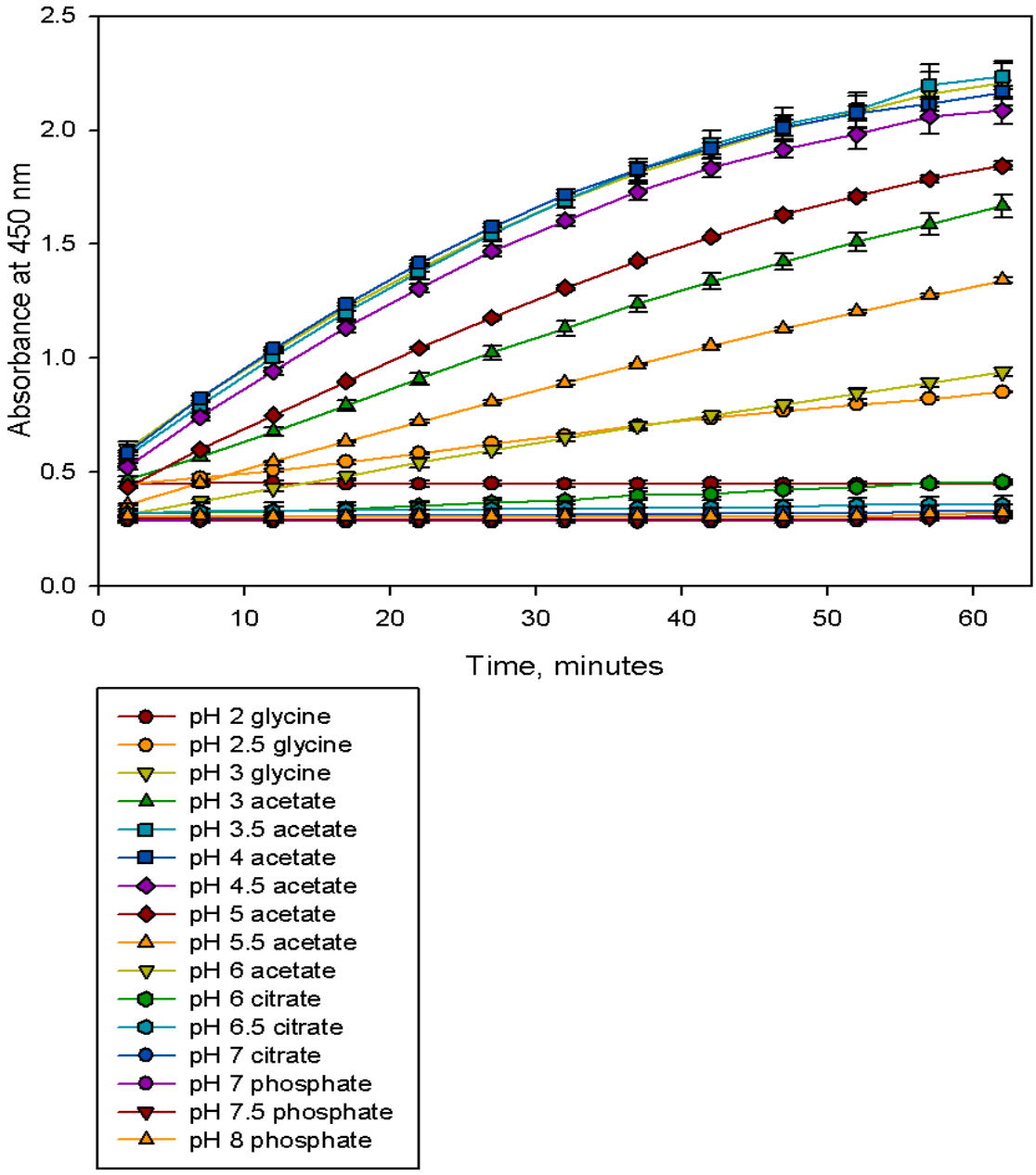


Figure 35: pH Dependence of Guaiacol Oxidation by Partially Purified *A. areolatum* Laccase. Time course data (n = 3). Reaction mixtures prepared in respective pH buffers, omitting substrate. Incubated for 10 minutes, then added substrate. Oxidation of 5 mM guaiacol monitored for one hour via UV/VIS absorption at 450 nm. Partially-purified laccase used was a 1:250 dilution. All buffers used were 50 mM concentration. Activity assayed at indicated pHs.

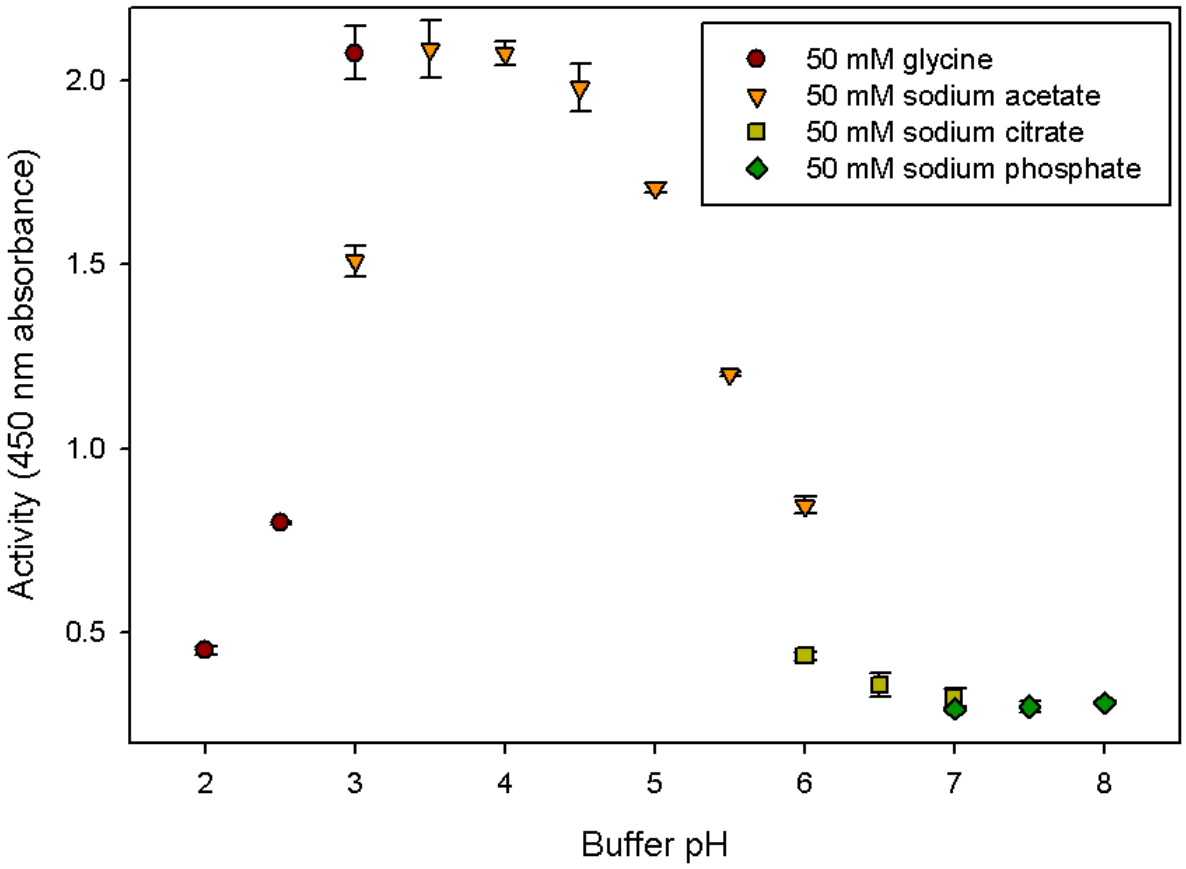


Figure 36: pH Dependence of Guaiacol Oxidation by Partially Purified *A. areolatum* Laccase: Activity vs. pH, 52 minutes (n = 3). Reaction mixtures prepared in respective pH buffers, omitting substrate. Incubated for 10 minutes, then added substrate. Oxidation of 5 mM guaiacol measured at 52 minutes via UV/VIS absorption at 450 nm. Partially-purified laccase used was a 1:250 dilution. Activity assayed at exposure pH.

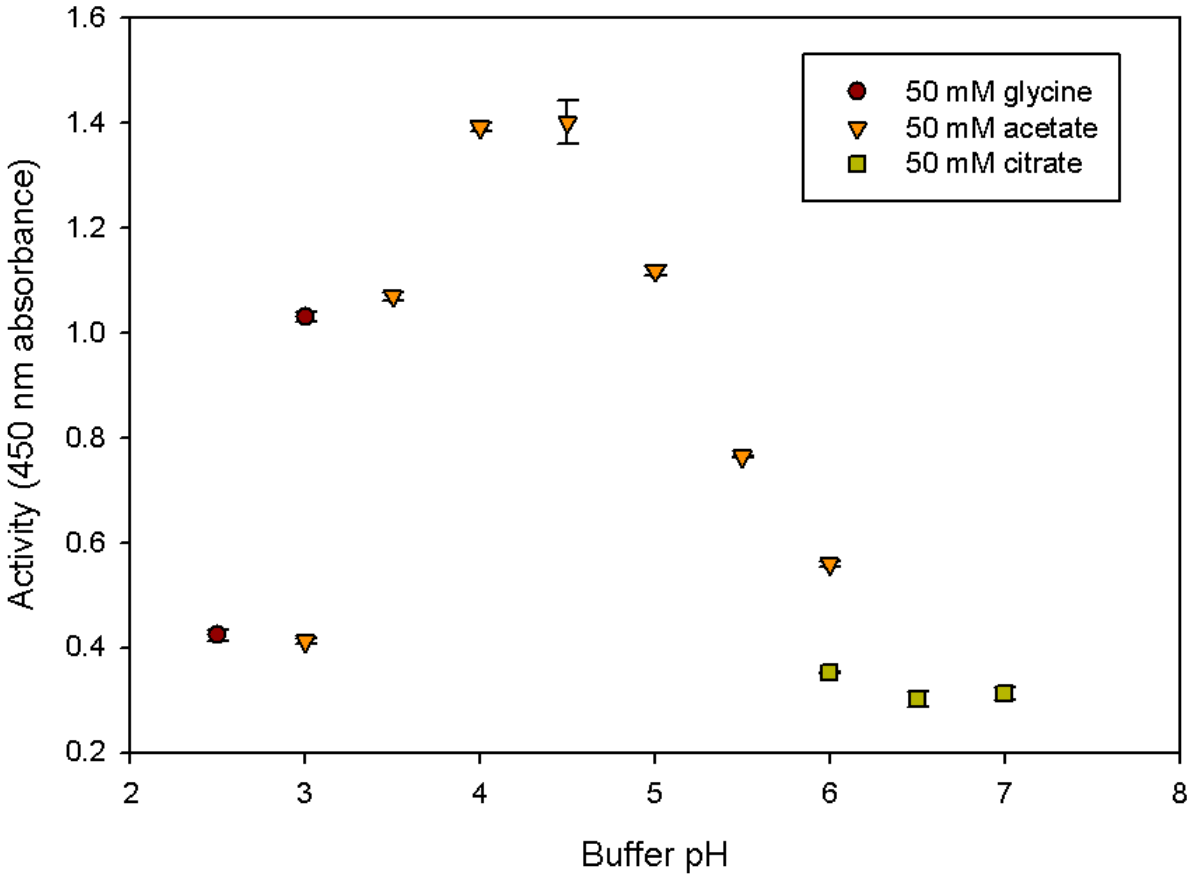


Figure 37: 24-Hour pH Stability of Partially Purified *A. areolatum* Laccase. Enzyme was incubated at indicated pH for 24 hours. Guaiacol added at 5 mM concentration. Oxidation of guaiacol by residual laccase activity following exposure measured at 30 minutes post substrate addition. Activity measured at exposure pH.

CHAPTER 4: SUMMARY

Conclusions from the Present Work

The *S. noctilio* woodwasp-vectored fungus *A. areolatum* is a white-rot basidiomycete and is a fungal pathogen of conifers, especially pine. Since its recent introduction into North America, efforts to understand the pathosystem and search for control targets within the system have gained momentum. This study has undertaken the biology of *A. areolatum* to the exclusion of the *Sirex* wasp, with a focus on fungal growth characteristics and characterization of an extracellular enzyme.

Amylostereum areolatum can be easily propagated in the laboratory on malt agar, potato dextrose agar, and a defined medium described in this study. It grows best at a temperature of 24°C, and dry weight mycelium production is favored by a pH of 6.5. Cultures on wood can be stored for at least 14 months at -20°C or -80°C. Both *Pinus taeda* callus cells on solid medium, and *P. taeda* 10 month old seedlings show response to *A. areolatum* challenge. Both systems require further development to produce highly reproducible systems for measurement of loblolly response to *A. areolatum*.

Amylostereum areolatum produces phenoloxidase activity in both solid and liquid culture, as can be demonstrated with the application of artificial substrates syringaldazine and ABTS. The fungus can be grown in shaken liquid culture to produce laccase. The laccase can be partially purified by a three-step process of ultrafiltration, ammonium sulfate precipitation, and a further ultrafiltration/centrifugal concentration. The resulting partially-purified laccase has an apparent molecular weight of 75 kDa and appears to be monomeric. The laccase can oxidize ABTS, catechol, 1, 8-diaminonaphthalene, guaiacol, hydroquinone, phloroglucinol, *p*-phenylenediamine, resveratrol, and syringaldazine. Oxidation of pinosylvin could not be demonstrated spectrophotometrically. It is inhibited by sodium azide, and appears to be activated by SDS and EDTA.

Further work will serve to more fully characterize the system. It is unknown to what degree the *A. areolatum* laccase is glycosylated, and due to time constraints N-terminal amino acid sequencing has not been performed. The laccase may have isoforms which will be revealed through isoelectric focusing. It has been demonstrated that *A. areolatum* possesses genes for other phenoloxidases, and the full suite of extracellular lignolytic enzymes should be explored. The laccase has been shown to oxidize resveratrol, potentially suggesting a role for the laccase in detoxification of plant defense compounds. Learning to what extent the enzyme is capable of oxidizing pinosylvin, a known defense compound in pine, would be a step further in elucidating the pathogenic mechanism of *A. areolatum*. The generation of a laccase-less mutant of *A. areolatum* will be an important step forward. If such a mutant is incapable of infecting living trees, the *A. areolatum* laccase can be directly linked to pathogenesis. The present work suggests the fungus is a weak pathogen and bolsters previous work suggesting the fungus must be aided in colonizing host trees by the action of the *S. noctilio* wasp mucus secretion.

REFERENCES

(2006). "Brazil: Forest Health/Invasive Species." International Programs: Latin America and the Carribean Retrieved October 6 2006, 2006, from http://www.fs.fed.us/global/globe/l_amer/brazil.htm.

APHIS (2007). Proposed Program for Management of the Woodwasp *Sirex noctilio* Fabricus (Hymenoptera: *Siricidae*), Environmental Assessment. A. a. P. H. I. S. United States Department of Agriculture. Riverdale, Maryland, USDA-APHIS**:** 76.

Bailey, B. A., et al. (1990). "An Ethylene Biosynthesis-Inducing Endoxylanase Elicits Electrolyte Leakage and Necrosis in *Nicotiana tabacum* cv Xanthi Leaves." Plant Physiology **94**(4): 1849-1854.

Baldrian, P. (2004). "Purification and Characterization of Laccase from the White-Rot Fungus *Daedalea quercina* and Decolorization of Synthetic Dyes by the Enzyme." Applied Microbiology and Biotechnology **63**(5): 560-563.

Baldrian, P. (2006). "Fungal Laccases - Occurrence and Properties." Fems Microbiology Reviews **30**(2): 215-242.

Baldrian, P. and J. Gabriel (2002). "Copper and Cadmium Increase Laccase Activity in *Pleurotus ostreatus*." Fems Microbiology Letters **206**(1): 69-74.

Barnett, J. P. and B. F. McLemore (1967). "Germination of Loblolly Pine Seed Hastened by Soakings in Aerated Cold Water." Tree Planter's Notes **18**: 24-25.

Baxter, A. P., et al. (1995). "*Amylostereum areolatum* (Aphyllophorales: Stereaceae) in South Africa." South African Journal of Botany **61**(6): 352-354.

Bertrand, T., et al. (2002). "Crystal Structure of a Four-Copper Laccase Complexed with an Arylamine: Insights into Substrate Recognition and Correlation with Kinetics." Biochemistry **41**(23): 7325-7333.

Bonello, P. and J. T. Blodgett (2003). "*Pinus nigra-Sphaeropsis sapinea* as a Model Pathosystem to Investigate Local and Systemic Effects of Fungal Infection of Pines." Physiological and Molecular Plant Pathology **63**(5): 249-261.

Borchert, D. F., Glenn; Jackson, Lisa (2006). Organism Pest Risk Analysis: Risk to the Conterminous United States Associated with the Woodwasp, *Sirex noctilio* Fabricius, and the Symbiotic Fungus, *Amylostereum areolatum* (Fries: Fries) Boidin. Raleigh, NC USA, USDA-APHIS-PPQ-CPHST-PERAL**:** 1-46.

Boros, C. B. (1968). The Relationship Between the Woodwasp *Sirex noctilio* F. and the Wood-Rot Fungus *Amylostereum* sp. Entomology. Adelaide, University of Adelaide. Master of Agricultural Science

Bowling, J. P. and J. E. Dolezal (1970). "Initial Note on the Development of a "Mucus Test" to Determine Resistance of *Pinus radiata* to Attack by *Sirex noctilio*." Australian Forestry Research **5**(1): 57-62.

Brown, C. L. and R. H. Lawrence (1968). "Notes: Culture of Pine Callus on a Defined Medium." Forest Science **14**: 62-64.

Brown, J. T. (1990). The Initiation and Maintenance of Callus Cultures. Plant Cell and Tissue Culture. J. M. W. Jeffrey W. Pollard. Clifton, N.J., Humana Press. **6:** 57-63.

Brownleader, M. D., et al. (1997). "Elicitor-Induced Extensin Insolubilization in Suspension-Cultured Tomato Cells." Phytochemistry **46**(1): 1-9.

Buchanan, P. K. (2001). "Aphyllophorales in Australasia." Australian Systematic Botany **14**: 417-437.

Buchner, P. (1965). Siricids. Endosymbiosis of Animals with Plant Microorganisms. New York, J. Wiley & Sons**:** 83-92.

Campbell, M. M. and B. E. Ellis (1992). "Fungal Elicitor-Mediated Responses in Pine Cell Cultures: Cell Wall-Bound Phenolics." Phytochemistry **31**(3): 737-742.

Campbell, W. G. (1932). "The Chemistry of the White Rots of Wood
The Effect on Wood Substance of *Ganoderma applanatum* (Pers.) Pat., *Fomes fomentarius* (Linn.) Fr., P*olyporus adustus* (Willd.) Fr., *Pleurotus ostreatus* (Jacq.) Fr., *Armillaria mellea* (Vahl.) Fr., *Trametes pini* (Brot.) Fr., and *Polystictus abietinus* (Dicks.) Fr." Biochemical Journal **26**(6): 1829-1838.

Caponetti, J. D. (1996). Nutrition of Callus Cultures. Plant Tissue Culture Concepts and Laboratory Exercises. R. N. a. D. J. G. Trigiano. Boca Raton, CRC Press**:** 374 p.

Castro-Sowinski, S., et al. (2002). "Laccase Activity in Melanin-Producing Strains of *Sinorhizobium meliloti*." Fems Microbiology Letters **209**(1): 119-125.

Celimene, C. C., et al. (1999). "Efficacy of Pinosylvins Against White-Rot and Brown-Rot Fungi." Holzforschung **53**(5): 491-497.

Choi, G. H., et al. (1992). "Molecular Analysis of the Laccase Gene from the Chestnut Blight Fungus and Selective Suppression of its Expression in an Isogenic Hypovirulent Strain." Molecular Plant-Microbe Interactions **5**(2): 119-128.

Ciesla, W. M. (2003). "European Woodwasp: A Potential Threat to North Americas Conifer Forests." Journal of Forestry **101**: 18-23.

Coutts, M. P. (1968). "Rapid Physiological Change in *Pinus radiata* Following Attack by *Sirex noctilio* and its Associated Fungus, *Amylostereum* sp. ." Australian Journal of Science **30**(7): 275-277.

Coutts, M. P. (1969). "The Formation of Polyphenols in Small Blocks of *Pinus radiata* Sapwood With and Without the Fungal Symbiont of *Sirex*." Australian Forestry Research **4**(1): 29-34.

Coutts, M. P. (1969). "Mechanism of Pathogenicity of *Sirex noctilio* on *Pinus radiata* .2. Effects of *S. noctilio* Mucus." Australian Journal of Biological Sciences **22**(5): 1153-&.

Coutts, M. P. (1969). "Mechanism of Pathogenicity of *Sirex Noctilio* on *Pinus radiata* .I. Effects of Symbiotic Fungus *Amylostereum* sp. (Thelophoraceae)." Australian Journal of Biological Sciences **22**(4): 915-&.

Coutts, M. P. and J. E. Dolezal (1965). *Sirex noctilio* and the Physiology of *Pinus radiata.* 38th A.N.Z.A.A.S. Congress, Hobart, Australia.

Coutts, M. P. and J. E. Dolezal (1966). Polyphenols and Resin in the Resistance Mechanism of *Pinus radiata* Attacked by the Wood Wasp, *Sirex noctilio*, and its Associated Fungus. F. a. T. B. Department of National Development, Commonwealth of Australia**:** 1-19.

Coutts, M. P. and J. E. Dolezal (1966). "Some Effects of Bark Cincturing on the Physiology of *Pinus radiata*, and on *Sirex* Attack." Australian Forestry Research **2**(2): 17-28.

Coutts, M. P. and J. E. Dolezal (1969). "Emplacement of Fungal Spores by Woodwasp, *Sirex noctilio*, During Oviposition." Forest Science **15**(4): 412-&.

Curir, P., et al. (2006). "The Plant Antifungal Isoflavone Genistein is Metabolized by *Armillaria mellea* Vahl to Give Non-Fungitoxic Products." Plant Biosystems **140**(2): 156-162.

Dean, J. F. D. and K. E. L. Eriksson (1994). "Laccase and the Deposition of Lignin in Vascular Plants." Holzforschung **48**: 21-33.

Diamantidis, G., et al. (2000). "Purification and Characterization of the First Bacterial Laccase in the Rhizospheric Bacterium *Azospirillum lipoferum*." Soil Biology & Biochemistry **32**(7): 919-927.

Dowd, P. F. (1992). "Insect Fungal Symbionts: a Promising Source of Detoxifying Enzymes." Journal of Industrial Microbiology **9**(3-4): 149-161.

Dunkle, R. L. (2005). Detection of the European wood wasp, *Sirex noctilio* (Fabricius) in New York, USDA, APHIS**:** 1.

Dunkle, R. L. (2006). Detection of *Sirex noctilio* Fabricius (Hymenoptera: Siricidae) (Sirex woodwasp) in Allegany, Erie, Genesee, Jefferson, Livingston, Monroe, Niagara, Oneida, Ontario, Orleans, Schuyler, Wyoming and Yates Counties in New York, USDA-APHIS**:** 1-2.

Dunkle, R. L. (2006). Detection of *Sirex noctilio* Fabricius (Sirex woodwasp) in Tioga County, Pennsylvania, USDA-APHIS**:** 1.

Eberhardt, T. L., et al. (1993). "Lignification in Cell Suspension Cultures of *Pinus taeda*. *In Situ* Characterization of a Gymnosperm Lignin." J. Biol. Chem. **268**(28): 21088-21096.

Eggert, C., et al. (1997). "Laccase is Essential for Lignin Degradation by the White-Rot Fungus *Pycnoporus cinnabarinus*." Febs Letters **407**(1): 89-92.

Eggert, C., et al. (1996). "The Ligninolytic System of the White Rot Fungus *Pycnoporus cinnabarinus*: Purification and Characterization of the Laccase." Applied and Environmental Microbiology **62**(4): 1151-1158.

Francke-Grosmann, H. (1939). "Über das Zusammenleben von Holzwespen (Siricinae) mit Pilzen." Zeitschrift für Angewandte Entomologie **25**: 647-680.

Gauld, I. D., et al. (1990). The Biological Significance and Conservation of Hymenoptera in Europe. Strasbourg, Council of Europe.

Gaut, I. P. C. (1969). "Identity of the Fungal Symbiont of *Sirex noctilio*." Australian Journal of Biological Sciences **22**: 905-914.

Gilmour, J. W. (1965). "The Life Cycle of the Fungal Symbiont of *Sirex noctilio*." New Zealand Journal of Forestry **10**: 80-89.

Graham, K. (1967). "Fungal-Insect Mutualism in Trees and Timber." Annual Review of Entomology **12**: 105-126.

Harkin, J. M., et al. (1974). "Use of Syringaldazine for Detection of Laccase in Sporophores of Wood Rotting Fungi." Mycologia **66**(3): 469-476.

Harkin, J. M. and J. R. Obst (1973). "Syringaldazine, an Effective Reagent for Detecting Laccase and Peroxidase in Fungi." Experientia **29**(4): 382-387.

Hart, J. H. (1981). "Role of Phytostilbenes in Decay and Disease Resistance." Annual Review of Phytopathology **19**: 437-458.

Hatakka, A. (1994). "Lignin-Modifying Enzymes from Selected White-Rot Fungi: Production and Role in Lignin Degradation." FEMS Microbiol Reviews **13**: 125-135.

Hatakka, A. (2001). Biodegradation of Lignin. Biopolymers. Lignin, Humic Substances and Coal. A multivolume handbook. . M. S. Hofrichter, A. Weinheim, Germany, Wiley-VCH. **1:** 129-180.

Hemingway, R. W., et al. (1976). "Polyphenols in *Ceratocystis minor* infected *Pinus taeda*: Fungal Metabolites, Phloem and Xylem Phenols." Abstracts of Papers of the American Chemical Society **172**(SEP3): 53-53.

Hillis, W. E. and T. Inoue (1968). "Formation of Polyphenols in Trees .4. Polyphenols Formed in Pinus Radiata after Sirex Attack." Phytochemistry **7**(1): 13-&.

Hoopes, J. T. and J. F. D. Dean (2001). "Staining Electrophoretic Gels for Laccase and Peroxidase Activity Using 1,8-Diaminonaphthalene." Analytical Biochemistry **293**(1): 96-101.

Hotter, G. S. (1998). "Elicitor-Induced Oxidative Burst and Phenylpropanoid Metabolism in *Pinus radiata* Cell Suspension Cultures." Functional Plant Biology **24**(6): 797-804.

Hurley, B. P., et al. (2007). "A Comparison of Control Results for the Alien Invasive Woodwasp, *Sirex noctilio*, in the Southern Hemisphere." Agricultural and Forest Entomology **9**: 159-171.

Isaac, S. (1992). Fungal-Plant Interactions. London, Chapman & Hall.

Jamieson, J. P. S. (1957). "Some Observations on the Relation of the Internal Temperature of *P.radiata* to Infestation by *Sirex*." New Zealand Journal of Forestry **VII**(4): 32-34.

Johannes, C. and A. Majcherczyk (2000). "Laccase Activity Tests and Laccase Inhibitors." Journal of Biotechnology **78**(2): 193-199.

Johansson, M., et al. (1998). "Differential Phenol-Induced Laccase Activity and Total Oxidative Capacity of the S and P Intersterility Groups of the Conifer Root Pathogen *Heterobasidion annosum*." Microbiological Research **153**(1): 71-80.

Kile, G. A. and C. R. A. Turnbull (1974). "The Effect of Radiata Pine Resin and Resin Components on the Growth of the *Sirex* Symbiont *Amylostereum areolatum*." Australian Forestry Research **6**(4): 27-34.

King, J. M. (1966). "Some Aspects of Biology of Fungal Symbiont of *Sirex noctilio*." Australian Journal of Botany **14**(1): 25-&.

Koudelka, G. B. and M. J. Ettinger (1988). "Fluoride Effects on the Activity of *Rhus* Laccase and the Catalytic Mechanism Under Steady-State Conditions." Journal of Biological Chemistry **263**(8): 3698-3705.

Lange, B. M., et al. (1994). "Elicitor-Induced Formation of Free and Cell-Wall-Bound Stilbenes in Cell-Suspension Cultures of Scots Pine (*Pinus sylvestris* L.)." Planta **V194**(1): 143-148.

Laufer, Z., et al. (2006). "Co-Occurrence of the Multicopper Oxidases Tyrosinase and Laccase in Lichens in Sub-Order Peltigerineae." Annals of Botany **98**(5): 1035-1042.

Leonowicz, A., et al. (2001). "Fungal Laccase: Properties and Activity on Lignin." Journal of Basic Microbiology **41**(3-4): 185-227.

Levin, L. and F. Forchiassin (2001). "Ligninolytic Enzymes of the White Rot Basidiomycete *Trametes trogii*." Acta Biotechnologica **21**(2): 179-186.

Loman, A. A. (1970). "The Effect of Heartwood Fungi of *Pinus contorta* var. Latifolia on Pinosylvin, Pinosylvin Monomethyl Ether, Pinobanksin, and Pinocembrin." Canadian Journal of Botany **48**(4): 737-747.

Madden, J. L. (1968). "Physiological Aspects of Host Tree Favourability for the Woodwasp, *Sirex noctilio* F." Proceedings of the Ecological Society of Australia **3**: 147-149.

Madden, J. L. (1975). "Analysis of an Outbreak of Woodwasp, *Sirex noctilio* F (Hymenoptera, Siricidae), in *Pinus radiata*." Bulletin of Entomological Research **65**(3): 491-500.

Madden, J. L. (1975). "Bacteria and Yeasts Associated with *Sirex noctilio*." Journal of Invertebrate Pathology **25**: 283-287.

Madden, J. L. (1977). "Physiological Reactions of *Pinus radiata* to Attack by Woodwasp, *Sirex noctilio* F. (Hymenoptera-Siricidae)." Bulletin of Entomological Research **67**(3): 405-426.

Madden, J. L. (1988). *Sirex* in Australasia. Dynamics of Forest Insect Populations. A. A. Berryman. New York, Plenum Publishing Corporation**:** 407-429.

Maijala, P., et al. (2003). "A Peroxidase Gene Family and Gene Trees in *Heterobasidion* and Related Genera." Mycologia **95**(2): 209-221.

Marjasvaara, A., et al. (2008). "Oxidation of a Laccase Mediator ABTS as Studied by ESI-FTICR Mass Spectrometry." Journal of Mass Spectrometry **43**(4): 470-477.

Martínez, Á. T., et al. (2005). "Biodegradation of Lignocellulosics: Microbial, Chemical and Enzymatic Aspects of the Fungal Attack of Lignin." International Microbiology **8**: 195-204.

Michniewicz, A., et al. (2006). "The White-Rot Fungus *Cerrena unicolor* Strain 137 Produces Two Laccase Isoforms with Different Physico-Chemical and Catalytic Properties." Applied Microbiology and Biotechnology **69**(6): 682-688.

Morgan, F. D. (1968). "Bionomics of Siricidae." Annual Review of Entomology **13**(1): 239-256.

Morozova, O., et al. (2007). ""Blue" Laccases." Biochemistry (Moscow) **72**(10): 1136-1150.

Moss, G. P. (2008, June 26 2008). "IUBMB Enzyme Nomenclature: EC 1.10.3.2." Retrieved June 30, 2008, from http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/10/3/2.html.

Moss, G. P. (2008, June 26 2008). "IUBMB Enzyme Nomenclature: EC 1.11.1.13." Retrieved June 30, 2008, from http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/11/1/13.html.

Moss, G. P. (2008). "IUBMB Enzyme Nomenclature: EC 1.11.1.14." Retrieved June 30, 2008, from http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/11/1/14.html.

Müller, W. (1934). "Untersuchungen über die Symbiose von Tieren mit Pilzen und Bakterien." Archives of Microbiology **5**(1): 84-147.

Naki, A. and S. D. Varfolomeev (1981). "Mechanism of the Inhibition of Laccase Activity from *Polyporus versicolor* by Halide Ions." Biochemistry-Moscow **46**(9): 1344-1350.

NYDAM. (2008). "Forest Health Invaders." Retrieved July 17, 2008, 2008.

Papinutti, L. and M. J. Martinez (2006). "Production and Characterization of Laccase and Manganese Peroxidase from the Ligninolytic Fungus *Fomes sclerodermeus*." Journal of Chemical Technology and Biotechnology **81**(6): 1064-1070.

Pointing, S. B., et al. (2000). "Optimization of Laccase Production by P*ycnoporus sanguineus* in Submerged Liquid Culture." Mycologia **92**(1): 139-144.

Popp, M. P., et al. (1996). "Defense Responses Elicited in Pine Cell Suspension Cultures." Plant Cell Tissue and Organ Culture **47**(3): 199-205.

Preisig-Mueller, R., et al. (1999). "Characterization of a Pine Multigene Family Containing Elicitor- Responsive Stilbene Synthase Genes." Plant Molecular Biology **V39**(2): 221-229.

Rawlings, G. B. (1948). "Recent Observations on the *Sirex noctilio* Population in *Pinus radiata* Forests in New Zealand." New Zealand Journal of Forestry **5**: 411-421.

Rawlings, G. B. (1953). "Rearing of *Sirex noctilio* and Its Parasite *Ibalia leucospoides*." New Zealand Forestry Research Notes **1**(8): 20-34.

Rigling, D., et al. (1989). "Reduction of Laccase Activity in dsRNA-containing Hypovirulent Strains of *Cryphonectria* (Endothia) *parasitica*." Phytopathology **79**(2): 219-223.

Rigling, D. and N. K. Vanalfen (1993). "Extracellular and Intracellular Laccases of the Chestnut Blight Fungus, *Cryphonectria parasitica*." Applied and Environmental Microbiology **59**(11): 3634-3639.

Rogalski, J., et al. (1991). "Influence of Aromatic Compounds and Lignin on Production of Ligninolytic Enzymes by *Phlebia radiata*." Phytochemistry **30**(9): 2869-2872.

Saparrat, M. C. N., et al. (2002). "Induction, Isolation, and Characterization of Two Laccases from the White Rot Basidiomycete *Coriolopsis rigida*." Applied and Environmental Microbiology **68**(4): 1534-1540.

Sbaghi, M., et al. (1996). "Degradation of Stilbene-Type Phytoalexins in Relation to the Pathogenicity of *Botrytis cinerea* to Grapevines." Plant Pathology **45**(1): 139-144.

Schägger, H. and G. von Jagow (1987). "Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis for the Separation of Proteins in the Range from 1 to 100 kDa." Analytical Biochemistry **166**(2): 368-379.

Schouten, A., et al. (2002). "Resveratrol Acts as a Natural Profungicide and Induces Self-Intoxication by a Specific Laccase." Molecular Microbiology **43**(4): 883-894.

Seppänen, S. K., et al. (2004). "Antifungal Activity of Stilbenes in *in vitro* Bioassays and in Transgenic *Populus* Expressing a Gene Encoding Pinosylvin Synthase." Plant Cell Reports **22**(8): 584-593.

Shein, I. V., et al. (2003). "Effect of Pine Callus Elicitation by the Fusarium Strains of Various Pathogenicity on the Content of Phenolic Compounds." Russian Journal of Plant Physiology **V50**(5): 634-639.

Slippers, B., et al. (2001). "Population Structure and Possible Origin of *Amylostereum areolatum* in South Africa." Plant Pathology **50**(2): 206-210.

Smith, J. A., et al. (2006). "Proteomic Comparison of Needles from Blister Rust-Resistant and Susceptible *Pinus strobus* Seedlings Reveals Upregulation of Putative Disease Resistance Proteins." Molecular Plant-Microbe Interactions **19**(2): 150-160.

Solis-Oba, M., et al. (2005). "An Electrochemical-Spectrophotometrical Study of the Oxidized Forms of the Mediator 2,2 '-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) Produced by Immobilized Laccase." Journal of Electroanalytical Chemistry **579**(1): 59-66.

Sterjiades, R., et al. (1992). "Laccase from Sycamore Maple (*Acer pseudoplatanus*) Polymerizes Monolignols." Plant Physiol. **99**(3): 1162-1168.

Sterjiades, R., et al. (1993). "Extracellular Laccases and Peroxidases from Sycamore Maple (*Acer pseudoplatanus*) Cell Suspension Cultures: Reactions with Monolignols and Lignin Model Compounds." Planta **190**(1): 75-87.

Talbot, P. H. B. (1964). "Taxonomy of the Fungus Associated with *Sirex noctilio*." Australian Journal of Botany **12**: 46-52.

Talbot, P. H. B. (1977). "*Sirex-Amylostereum-Pinus* Association." Annual Review of Phytopathology **15**: 41-54.

Teasdale, R. D. (1984). "Application of Growth Analysis to Trace Element Nutrition: Study of Copper Uptake with a Loblolly Pine (*Pinus taeda)* Cell Suspension Culture." J. Exp. Bot. **35**(10): 1495-1506.

Thomsen, I. M. and S. Harding (1996). A Technique for Isolation of Fungi from Woodwasps. In: *Amylostereum areolatum* and *Amylostereum chailletii*, Symbiotic Fungi of Woodwasps (*Sirex* sp. and *Urocerus* sp.). Forest and Landscape. Denmark. Ph.D.

Thomsen, I. M. and J. Koch (1999). "Somatic Compatibility in *Amylostereum areolatum* and *A. chailletii* as a Consequence of Symbiosis with Siricid Woodwasps." Mycological Research **103**: 817-823.

Ullrich, R. and M. Hofrichter (2007). "Enzymatic Hydroxylation of Aromatic Compounds." Cellular and Molecular Life Sciences CMLS **64**: 271-293.

Vaartaja, O. and J. King (1964). "Inoculation Experiments with *Amylostereum* sp. from a Woodwasp." Plant Disease Reporter **48**: 438-440.

Vasiliauskas, R. (1999). "Spread of *Amylostereum areolatum* and *A. chailletii* Decay in Living Stems of *Picea abies*." Forestry **72**(2): 95-102.

Velazquez-Cedeno, M., et al. (2007). "Interspecific Interactions with *Trichoderma longibrachiatum* induce *Pleurotus ostreatus* Defense Reactions Based on the Production of Laccase Isozymes." Biotechnology Letters **29**(10): 1583-1590.

Wesenberg, D., et al. (2003). "White-Rot Fungi and Their Enzymes for the Treatment of Industrial Dye Effluents." Biotechnology Advances **22**: 161-187.

Xu, F. (1996). "Oxidation of Phenols, Anilines, and Benzenethiols by Fungal Laccases: Correlation Between Activity and Redox Potentials As Well As Halide Inhibition." Biochemistry **35**(23): 7608-7614.

Zhu, X. and P. R. Williamson (2004). "Role of Laccase in the Biology and Virulence of *Cryptococcus neoformans*." Fems Yeast Research **5**(1): 1-10.

Zhu, X. D., et al. (2001). "Laccase of *Cryptococcus neoformans* is a Cell Wall-Associated Virulence Factor." Infection and Immunity **69**(9): 5589-5596.

Zondag, R. and M. J. Nuttall (1977). *Sirex noctilio* Fabricius (Hymenoptera:Siricidae). N. Z. F. Service, Forest Research Institute**:** 1-8.