ENZYMATIC REMOVAL OF LIGNIN FROM PLANT MATERIALS: POTENTIAL APPLICATIONS

by

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ABSTRACT

Lignin is the major contributor to lignocellulosic recalcitrance to microbial degradation. Lignin acts as a protective matrix making cellulosic and hemicellulosic components inaccessible to microbes, hence slowing down the decomposition process. My research involved application of laccase enzyme for lignin removal from lignocellulosic biomass and its impact on two different applications.

Accumulation of excessive organic matter in the form of thatch layer in turfgrass systems is a major problem and is believed to be due to slow rate of organic matter decomposition. Experiments were conducted in this study to examine the effects of laccase treatment on thatch buildup in turf. Direct application of laccase at 2.06 units cm^{-2} on potted creeping bentgrass in greenhouse every two weeks for nine months demonstrated a 45 and 32% reduction in thatch layer and organic matter relative to control. Field experiments on creeping bentgrass to optimize rate and frequency of laccase application showed that laccase application at rate as low as 0.5 units cm^{-2} applied once every two weeks or at rate 2.0 units cm^{-2} once every twelve weeks was effective to reduce thatch when applied for six months. An 18-22% and 21-30% reduction in

thatch layer was observed with bi-weekly application of laccase at 2.0 units cm^{-2} for a period of six months for bermudagrass and zoysiagrass, respectively.

Lignin removal from lignocellulosic biomass is an essential pretreatment step in bioethanol production to increase accessibility of structural sugars. Experiments were conducted to examine the potential of using laccase in such pretreatment to remove lignin from bioethanol feedstock materials. Sweet sorghum and switchgrass were treated with laccase mediator system consisting of ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), HBT (1-hydroxybenzotriazole), and violuric acid to optimize mediator concentration. A 25.5 and 24% lignin from sweet sorghum was removed at 1.88 and 1.25 mM concentration of HBT and violuric acid, respectively. In switchgrass, reduction of 28% lignin was observed at 0.63 mM concentration of violuric acid.

Application of laccase has the potential to develop as a new method for thatch management. Future research is needed to determine the effectiveness of enzymatic pretreatment for improving the bioethanol production efficiency.

INDEX WORDS: Lignin, Laccase, Lignolytic enzymes, Turfgrass, Thatch management, Biofuels, Laccase mediator system

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2012

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August 2012

DEDICATION

I would like to dedicate this dissertation to my parents Mr. Hardeep Singh and late Ravinder Kaur. Without their unconditional love, support, and sacrifices, I wouldn't have reached this far.

ACKNOWLEDGEMENTS

I would like to thanks my major professors, Dr. Qingguo Huang and Dr. Paul L. Raymer for their tremendous faith in my abilities. Their unwavering support inspired me to work hard. I enjoyed the freedom and encouragement I received from them. I would like to thank Dr. Robert N. Carrow for the time and effort he invested in me. I would also like to thank my committee members, Dr. Miguel L. Cabrera, Dr. Mussie Y. Habteselassie, and Dr. Hong Luo for their suggestions and helping hand.

I owe a lot to Matthew Aderhold, Lewayne White, Brandon Tonnis, Mary G. Flynn, and Rodney Connell for their technical assistance. This research was supported by funding from the Golf Course Superintendents Association of America, Georgia Golf Environmental Foundation, and Georgia Agricultural Experiment Stations.

I would like to express my gratitude to my wife Rashmi for the confidence she has in my abilities and for her selfless support during tough times. I would like to thank my friends Negi-Dolly, Vijendra-Tripti, Dr. Ranjan Singh, Vikas-Neha, Dhananjay, and Harwinder Kang for their invaluable support. Special thanks to my family members Sukhneet, Pooja, Anamika, Rachna, and Seeha for their unconditional love and support. This acknowledgement would not be complete without thanking my Mom & Dad in-law for the faith they always had in me.

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CHAPTER I

INTRODUCTION

Lignin, the second most abundant organic substance next to cellulose in plant cell walls, is a three dimensional amorphous polymer consisting of methoxylated phenyl propane that resists microbial decomposition of organic matter (Ledeboer and Skogley, 1967). The lignin macromolecule structure consists of three monomers derived from three primary hydroxycinnamyl alcohols: *p*-coumaryl, coniferyl and sinapyl alcohols (Wong, 2009). Lignin is extremely recalcitrant to degradation due to its complex heterogeneous structure without a regular pattern. The structure is derived from random oxidative coupling of lignin monomers and cross-linking of polymers via radical mechanisms in a process known as lignification (Chen and Sarkanen, 2003; Davin and Lewis, 2003).

Natural degradation of lignin is carried out in the environment most effectively by certain white-rot fungi which solubilize and mineralize lignin by producing extra-cellular lignolytic enzymes (Kirk et al., 1975; 1976). White-rot fungi preferentially attack lignin more than cellulose or hemicellulose in the wood tissue (Mester et al., 2004). This process of selective delignification exposes cellulosic materials for further bacterial degradation in the environment and produces cellulose- and hemicellulose-enriched wood, which has a wide range of industrial applications (Otjen and Blanchette, 1987).

Four different kinds of lignolytic enzymes are produced by white-rot fungi: lignin peroxidase, manganese peroxidase, versatile peroxidase, and laccase (Baldrian, 2006). The peroxidases catalyze hydrogen-peroxide dependent oxidation of phenolic and non-phenolic

compounds (Farrell, 1987). Laccase is a multi-copper oxidase capable of oxidizing a wide variety of phenols by reducing oxygen to water in a three step process involving electron transfer. However, in the presence of certain chemicals known as mediators, efficacy and substrate range of the laccase enzyme is enhanced (Bourbonnais et al., 1997). For most fungal laccases, the catalytic ability is stable in slightly acidic environment (pH 4-6) and a wide range of temperature (30-55^{o}C) making it suitable for different industrial applications (Baldrian, 2006).

Two potential uses of the laccase enzyme for removal of lignin from biomass were investigated in my dissertation study. First was the application of fungal laccases to facilitate dethatching of turfgrass by removing lignin from thatch biomass and making cellulosic and hemicellulosic sugars available for microbial degradation. The second was the use of laccase-mediator system as a pretreatment for lignocellulosic biomass to enhance lignin removal which may lead to increase in accessibility of structural sugars for hydrolysis and fermentation.

A major problem in modern turfgrass greens is the formation of high organic matter layer known as thatch and mat layer. Thatch is a mixed layer of organic matter containing both living and dead plant tissues intermingled tightly with each other that accumulates between the soil and green turfgrass. It consists of stolons, rhizomes, roots, leaf sheaths and blades (Engel, 1954). The mat layer is generally below the thatch layer where sand or soil is intermingled with thatch due to cultural practices like core aeration and topdressing (McCarty, 2005). The sand or soil content can vary so that the properties of some mat layers may be dominated by organic matter while in others the dominant matrix is sand or soil.

The formation of the thatch-mat layer is due to a greater rate of organic matter accumulation than microbial degradation as presence of lignin in plant cell walls restricts microbial degradation mechanisms (Beard, 1973; Kirk and Farrell, 1987). Lignin limits the

accessibility of microbial degraders to more biodegradable plant materials, such as cellulose and hemicelluloses (Ledeboer and Skogley, 1967).

Several cultural practices like core aeration, topdressing, and vertical mowing are in use today and have been successful in reducing thatch accumulation, however, these practices are energy and cost intensive and often have adverse or disruptive effects on turfgrass surfaces and quality (Landreth et al., 2008; McCarty et al., 2007). We hypothesize that direct application of laccase enzyme on turfgrass could reduce thatch layer buildup. The objectives of this study were to: 1) determine if degradation of soil organic matter can be enhanced by laccase application; and 2) determine if application of laccase enzyme affects turf quality.

The second potential use related to laccase's ability to selectively remove lignin was the application of laccase-mediator system as a pretreatment of lignocellulosic biomass to efficiently remove lignin and enhance the accessibility of cellulosic and hemicellulosic sugars for the subsequent hydrolysis and fermentation processes. Concerns regarding the security and availability of crude oil as well as its negative environmental impacts have increased pressure on our society to find renewable energy resources (Midilli et al., 2006). Bioenergy, in the form of bioethanol, is a viable potential source, but several limitations need to be overcome. Bioethanol can be prepared from sugars and starch, but this would not be practical from socio-economic perspective because it competes for food sources. Lignocellulose is the essential part of cell walls of plants and is one of the most abundant organic sources. Lignocellulose is composed primarily of cellulose (24-54%), hemicellulose (11-38%), and lignin (6-31%) and has been considered as a potential raw material for bioenergy production (Jacques et al., 1999). Cellulose and hemicellulose are embedded in a protective matrix of lignin and requires pretreatment to be accessible for hydrolysis and subsequent conversion into bioethanol.

Several pretreatments such as wet oxidation (Schmidt and Thomsen, 1989) and steam explosion (Galbe and Zacchi, 2002) are used to open up the lignocellulosic material for the subsequent hydrolysis to take place. Loss of cellulose, as well as hemicellulosic sugars, production of microbial inhibitors, and environmentally undesirable chemicals are produced during different pretreatment methods (Petersson et al., 2007). The aim of my study was to optimize the laccase pretreatment conditions including mediator concentrations for lignin removal from different lignocellulosic biomasses. The study was based on the following hypothesis: 1) a laccase-mediator system can effectively remove lignin from lignocellulosic biomass; and 2) enzymatic pretreatment will have minimal impact on structural sugars of the biomass.

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CHAPTER II

LITERATURE REVIEW

The cell walls of seed plants, angiosperms and gymnosperms, are storehouses of fermentable carbohydrates and non-carbohydrate polymers. The major portion of this carbohydrate is in the form of high molecular weight cellulose (24-54%) along with non-cellulosic polysaccharides (11-38%). The carbohydrate and non-carbohydrate polymers are covalently and non-covalently bonded with one another and with non-carbohydrate polymers, particularly lignin (6-31%), and other polymers such as proteins, suberin, and cutin (Garrote et al., 1999). Two types of cell walls, primary and secondary cell walls, are recognized (Basic et al., 1988; Esau, 1953; Harris, 2005). Primary cell walls are typically non-lignified and are formed when the young cells are still developing and enlarging. Secondary walls are typically lignified and are formed after the cells are developed by deposition over the primary cell wall (Basic et al., 1988; Harris, 2005). Cellulose is the structural unit of a plant cell wall (Harris, 2005). The matrix phase polysaccharides include glucans, heteroglucans, heteroxylans, heteromannans, arabino galactans, and pectic polysaccharides (Ridley et al., 2001; Trethewey et al., 2005; Wilkie, 1979).

Lignin is the second most abundant organic substance next to cellulose and the major contributor to lignocellulosic recalcitrance to microbial degradation. Lignin is a three dimensional amorphous polymer consisting of methoxylated phenyl propane that resists microbial decomposition of organic matter (Ledeboer and Skogley, 1967). Lignin macromolecule is composed of three lignin monomers: p-caumaroyl, coniferyl, and sinapyl

alcohols. The corresponding lignin monomers are known as *p*-hydroxy phenyl, guaiacyl and syringyl units, respectively, and often abbreviated as H, G, and S lignin (Wong, 2009). The ratio of G:S:H is generally 70:25:5 in the lignin of grasses and other lignocellulosic materials (Brunow, 2001; Fukushima 2001; Higuchi, 2006). The recalcitrant nature of lignin is attributed to its heterogeneous complex structure, which is derived from random oxidative coupling of lignin monomers and cross-linking of polymers via radical mechanisms, a process known as lignification (Chen and Sarkanen, 2003; Davin and Lewis, 2003). A lignin macromolecule contains monolignols randomly bonded by C-O-C and C-C linkages including β-O-4, β-5, β-β, 5-5, 4-O-5, and β-1 bonds (Alder, 1977; Del Rio et al., 2007; Ralph et al., 2004).

The cellulose and hemicellulose components are covalently bonded with lignin macromolecules in different ways such as ester-ether cross-links (Grabber et al., 2000, 2004; Ralph et al., 1995, 2004), direct ester linkages (Imamura et al., 1994; Joseleau and Gancet, 1981), benzyl ether linkages (Grabber et al., 2004; Lam et al., 1990; Watanabe et al., 1989), and phenyl glycoside linkages (Joseleau and Kesraoui, 1986).

LIGNIN-DEGRADING FUNGI

Lignin is resistant to anaerobic degradation and the aerobic breakdown of lignin is slow (Wong, 2009). Natural degradation of lignin is carried out effectively in the environment only by basidiomyceteous white-rot fungi where water soluble fragments (solubilization) and evolution of ^{14}C labeled CO_2 (mineralization) have demonstrated their efficacy (Kirk et al., 1975, 1976). Some white-rot fungi such as *Ceriporiopsis subvermispora*, *Phellimus pini*, *Phlebia* spp., and *Pleurotus* spp. preferentially attack lignin more than cellulose or hemicellulose in the wood tissue (Blanchette, 1984; Mester et al., 2004; Otjen and Blanchette, 1987). This process of

selective delignification exposes cellulosic materials for further bacterial degradation in the environment (Otjen and Blanchette, 1987).

Many white-rot fungi such as *Trametes versicolor*, *Heterobasidium annosum*, and *Irpex lacteus*, however, exhibit a pattern of simultaneous decay characterized by degradation of all cell wall components with formation of radial cavities (Blanchette, 1991; Eriksson et al., 1990). In contrast to lignin-degrading white-rot fungi, commonly found on angiosperms, brown-rot fungi grow primarily on gymnosperms and degrade wood carbohydrate instead of lignin (Cowling, 1961; Gilbertson, 1980).

Several studies have shown that lignin is degraded and oxidized by white-rot fungi that produce certain enzymes such as lignin peroxidases, manganese peroxidases, laccase and versatile peroxidases (Elisashvili et al., 2008; Rodrigues et al., 2008). Bermudagrass treated with two white-rot fungi Ceriporiopsis *subvermispora* and *Cyathus Stercorius* showed a decrease in the level of hydroxycinnamic alcohols (lignin monomers) by 50 and 65%, respectively (Akin et al., 1996). Similarly, Dinis et al. (2009) observed a 43% decrease in lignin content in wheat straw treated with fungal isolates during 28 days of incubation. The decrease in lignin content was 13% in the first seven days of incubation. Arora et al. (2002) reported a decrease in wheat lignin content by 18.5 and 12.5% when treated with *P. radiate* and *T. vericolor*, respectively.

LIGNOLYTIC ENZYMES

White-rot fungi produce four major types of lignin degrading enzymes: i.e. Lignin peroxidases (LiP; EC1.11.1.14), manganese peroxidases (MnP; EC 1.11.1.13), laccases (Lac; EC 1.10.3.2), and versatile peroxidases (VP; EC 1.11.1.16) (Baldrian, 2006; Farrell, 1987; Gold et al., 2000). Lignin degradation is further enhanced by the cooperative action of several accessory enzymes, which may include glyoxal oxidase (EC1.2.3.5), aryl alcohol oxidase (veratryl alcohol

oxidase; EC 1.1.3.7), pyranose 2-oxidase (glucose 1-oxidase; EC 1.1.3.4), cellobiose/quinone oxidoreductase (EC 1.1.5.1), and cellobiose dehydrogenase (EC 1.1.99.18) (Martinez 2002; Martinez et al., 2005).

Lignin peroxidases catalyze hydrogen-peroxide dependent oxidative de-polymerization of lignin (Hammel et al., 1993; Tien and Kirt, 1983). These enzymes are relatively non-specific and act on a variety of phenolic aromatic as well as non-phenolic substrates and have the unique ability to catalyze oxidative cleavage of C-C bonds and ether (C-O-C) bonds in non-phenolic aromatic substrates of high redox potential (Schoemaker et al., 1994; Valli et al., 1990) as well as lignin (Johjima et al., 1999; Martinez, 2002). Hirai et al. (2005) observed the degradation of lignin model compounds by 84 and 56% by lignin peroxidase from *Phanerochaete sordid* and *Phanerochaete chrysosporium*, respectively.

Manganese peroxidases catalyze manganese-dependent reactions and their extracellular production is dependent on Mn (II) ions where Mn (II) is oxidized to Mn (III), which in turn oxidizes monomeric phenolic compounds (Bonnarme and Jeffries, 1990). Non-phenolic lignin units, the fraction most recalcitrant to degradation, are not oxidized by manganese peroxidase (Gold et al., 2000). Hofrichter et al. (1999) used manganese peroxidase from white-rot fungi *Nematoloma frowardii* on ^{14}C labeled wheat straw and observed the formation of $^{14}CO_2$ (4-10 %) and water soluble ^{14}C-lignin fragments (14-25%). Versatile peroxidases are a group of enzymes which are not only specific for manganese as in manganese peroxidases (Camarero et al., 1996; Kamitsuji et al., 2005), but also are specific for phenol and non-phenolic compounds that are acted upon by lignin peroxidases (Camarero et al., 1999; Rodakiewicz-Nowak et al., 2006).

Laccases, the multi-copper oxidases, are known to act on a wide variety of aromatic compounds by reducing oxygen to water and oxidizing a wide range of diphenols and

monophenols (Baldrian, 2006). Laccases are widely distributed in plants and fungi (Messerschmidt and Huber, 1990) and have also been found in bacteria and insects (Kunamneni et al., 2007). Redox potential of fungal laccase (800 mV) being higher as compared to bacterial and plant laccases is used in several biotechnological applications including degradation of lignin (Thurston, 1994). Redox potential of laccase enzyme from laccase producing fungi is reported as 450 mV (*Myceliophthora thermophila*), 750 mV (*Pycnoporus cinnabarinus*), 780 mV (*Botrytis cinerea*), 790 mV (*Trametes villosa*), and 800 mV (*Trametes versicolor*) (Li et al., 1999; Wong, 2009) along with redox potential of laccase produced from plant *Rhus vernicefera* to be 450 mV (Reinhammer and Vanngard, 1971). Plant laccases due to their low redox potential are involved in the lignin synthesizing system instead of lignin degradation (Bao et al., 1993).

Fungal laccases occur as monomeric or dimeric protein structures. The monomeric protein structures have a molecular mass of 50 to 100 kDa (Thurston, 1994). The process of laccase catalysis occurs in three steps: 1) reduction of type I Cu by substrates; 2) electron transfer from type I Cu to type II and III Cu trinuclear cluster; and 3) reduction of oxygen to water at the trinuclear cluster (Gianfreda et al., 1999). The catalytic activity of most of the fungal laccases varies between 30-55^{o}C and mild acidic conditions in the range of pH 4-6 (Baldrian, 2006; Morozova et al., 2007). Rodriguez Couto (2007) reported that laccase from *Trametes hirsute* was able to decolorize a synthetic non-phenolic dye, indigo carmine, at pH values 8-11.

LACCASE MEDIATOR SYSTEM

Laccase is one of the extra-cellular lignolytic enzymes secreted during oxygen dependent degradation of phenolic compounds by white-rot fungi (Ten-Have and Teunissen, 2001). Low redox potential of fungal laccase restricts its ability to oxidize non-phenolic compounds (Kersten et al., 1990; Ten-Have and Teunissen, 2001). However, addition of low molecular weight

substances, known as mediators, increase the substrate range of laccase enzyme to non-phenolic groups, benzyl and alyl alcohols and ethers (Bourbonnais and Paice, 1992; Bourbonnais et al., 1997; Crestini and Argyropoulos, 1998; Fabbrini et al., 2002; Fabbrini et al., 2001) which contribute the major fraction of the lignin macromolecule (Fritz-Langhals and Kunath, 1998; Johannes and Majcherczyk, 2000; Potthast et al., 1995).

In the laccase mediator system, an oxidized mediator with higher redox potential than laccase, acts on the substrate to carry out its oxidation (Cantarella et al., 2003). Laccase-mediator system catalyzed oxidation of organic substrate could proceed as two different mechanisms. The oxidation of substrate is carried out by mono electron oxidation as in case of mediators like ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), and by abstraction of H atom by a >N-O radical species for N-OH type mediators like HBT (1-hydroxybenzotriazole) and violuric acid (Cantarella et al., 2003). Elegir et al. (2005) reported oxidation of two phenolic model lignin compounds (5-5' phenolic compound, 4,4'-dimethyl-4,4'-dihydroxy, 5,5'dimethoxy-diphenyl; and β-5 phenolic compound, E-methyl-[(2*RS*-3*RS*)-2,3-dihyro-2-(4hydroxy3-methoxyphenyl)-7-methoxy-3-methoxycarbonyl-1-benzofuran-5-yl] proenoate) by application of laccase from *C. subvermispora*, however two non-phenolic model lignin compounds (4,4'-dimethyl-4,4',5,5'tetramethoxy-diphenyl and E-methyl-[(2RS-3RS)-2,3-dihyro-2-(3,4-dimethoxyphenyl)-7-methoxy-3-methoxycarbonyl-1-benzofuran-5-yl] proenoate) were oxidized only in the presence of mediators (ABTS and HBT) and surfactants (Tween 20 and Tween 80).

THATCH/MAT LAYER

Organic matter layer consisting of both living and dead plant tissues intermingled tightly with each other that accumulates between the soil and green turfgrass is known as thatch layer

(Beard 1973). It consists of stolons, rhizomes, roots, leaf sheaths and blades (Engel, 1954). The mat layer consists of thatch layer intermingled with sand or soil due to cultural practices like core aeration and sand topdressing along with earthworm activities (McCarty, 2005). The presence of thatch layer in a limited amount is beneficial to turfgrass as it moderates extreme soil temperatures, increases wear tolerance, and reduces weed invasion (Beard, 1973; Butler, 1965). Excessive thatch accumulation increases disease and insect problems (Musser, 1960; Thompson, 1967), reduces pesticide effectiveness (Latham, 1955; Musser 1960), decreases water infiltration (Carrow, 2003; Murray and Juska, 1977), reduces in hydraulic conductivity (Harris, 1978), increases localized dry spots (Cornman, 1952), reduces tolerance to cold temperatures (Beard, 1973, Thompson 1967), results in poor aesthetic value, and causes shallow rooting (Engel and Alderfer, 1967; Hartwiger, 2004).

Thatch accumulates when the rate of organic matter accumulation is greater than the rate organic matter decomposition. Factors that enhance vegetative growth in turf at a higher rate than its decomposition could result in thatch development. Also, any factor such as acid soil that limits microbial activity can enhance thatch accumulation (Satchell, 1967; Starkley, 1954). Martin and Beard (1975) reported microbial activity, measured by CO_2 evolution, to be maximum at pH 6 while working on red fescue. Lignin content in thatch layer increased whereas microbial activity decreased by seven percent due to addition of clippings in bermudagrass (Meinhold et al., 1973). Thatch is high in lignin, a three-dimensional amorphous polymer consisting of methoxylated phenyl propane that limits microbial degradation of organic matter (Ledeboer and Skogley, 1967). For this reason, turfgrass species high in lignin content are resistant to decomposition (Beard, 1973).

DETHATCHING TECHNIQUES

McWhirter and Ward (1976) observed core aeration done three to six times a year on bermudagrass reduced thatch content by 10 percent. Similar results were reported by Murray and Juska (1977) on a Kentucky bluegrass after five years of core aeration. Weston and Dunn (1985) on the other hand reported 8 and 25% reduction in thatch by core aeration done once and twice a year, respectively. No influence of core aeration was observed in other studies by White and Dickens (1984) and Carrow et al. (1987). McCarty et al. (2005) observed a 58 and 188% increase in water infiltration rates on bentgrass after one and two years of core aeration, respectively. Carrow et al. (1987) reported that saturated hydraulic conductivity increased in bentgrass during the first seven days after cultivation and then decreased 17 to 26 days after treatment.

Several researchers have showed reduction of thatch content on different grasses by vertical mowing. McWhirter and Ward (1976) reported a 12% decrease in thatch content by vertical mowing every 2 to 4 weeks on Tifgreen bermudagrass. Similar results were observed by Danneberger and Turgeon (1986) on Kentucky bluegrass. Vertical mowing once a year reduced thatch content up to 18% on zoysiagrass (Dunn et al., 1981). Similarly, Weston and Dunn (1985) reported an 8 to 10% decrease of thatch in zoysiagrass by vertical mowing once to three times a year. Similar results on Tifway bermudagrass were reported by Carrow et al. (1987). Vertical mowing reduces the thatch build up in different grasses but it reduces turf quality (Dunn et al., 1981; Westen and Dunn, 1985; Carrow et al., 1987). Landreth et al. (2008) observed that verticutting with a blade width of 3 mm removed four times more organic matter than any of the core aeration treatments (various combinations of tine spacing, depth and diameter) on creeping

bentgrass; however, time required to heal from core aeration treatments was half of that required for verticutting treatments. Similar results were also reported by Hanna (2005).

Carrow et al. (1987) reported a 44 to 62% decrease in thatch content by topdressing once or twice a year, respectively on Tifway bermudagrass. Similar results were reported by White and Dickens (1984) on three different bermudagrass cultivars. Barton et al. (2009) observed that topdressing alone was three times more effective in reducing the organic matter content on kikuyu grass than core aeration alone. The reason topdressing is effective in thatch control is that it provides better micro-environment for the microbes. McCarty et al. (2005) observed that combination of various mechanical methods on creeping bentgrass reduced thatch-mat depth and percent organic matter by 18 and 31%, respectively and combinations of different treatments were better than topdressing, vertical mowing and core aeration alone.

Studies of several non-destructive methods in the past using different treatments like sugars, mixtures of sugars and microbial inocula, and some enzymes like cellulase, proved ineffective. Application of commercial microbial inoculum, Biodethatch and Thatch-Away, at rates of 4.9 and 9.8 $g \cdot m^{-2}$ on bermudagrass (*Cynodon dactylon* L.) proved ineffective in thatch layer reduction (Murdoch and Barr, 1976). Similarly, application of two commercial microbial inoculum, Biodethatch and Thatch Away, at a rate of 4.9 $g \cdot m^{-2}$ on creeping bentgrass and annual bluegrass was ineffective in reducing thatch layer depth (Lancaster et al., 1977). Application of three commercial biological dethaching materials Biodethatch, Thatch Away, and Earth Anew on bermudagrass and creeping bentgrass was ineffective in reducing thatch layer thickness (Gilbeault et al., 1976). Ledeboer and Skogley (1967) reported that application of 24.4 kg per m^2 calcium and sucrose at 240 and 490 $kg \cdot ha^{-1}$ on velvet bentgrass (*Agrostis canina* L.) had no

significant effect on thatch decomposition but sucrose significantly increased dollar spot incidence.

Thatch-mat depth on creeping bentgrass increased by 12-15% over control when treated with a biological granular supplement Thach-X (McCarty et al., 2007). Application of the wetting agent, Aqua-Gro®, at 24.6 $kg \cdot ha^{-1}$ and Milogranite, activated sewage sludge, at 275 $kg \cdot ha^{-1}$ on Kentucky bluegrass was ineffective in reduction of organic matter in thatch layer (Murray and Juska, 1977). Reduction in cellulose content and total oxidizable organic matter in bermudagrass and centipedegrass (Sartain and Volk, 1984) and weight loss of bermudagrass pellets, St. Augustinegrass and zoysiagrass stolons (Martin and Dale, 1980) were observed when inoculated with different wood-decaying fungi under controlled greenhouse and laboratory conditions. However, field inoculation experiments on bermudagrass showed no thatch degradation (Martin and Dale, 1980).

PRE-TREATMENT FOR BIOETHANOL PRODUCTION

A general procedure to produce bioethanol from lignocellulose involves three steps: i) pretreatment to reduce lignocellulosic recalcitrance, ii) hydrolysis to produce sugars from carbohydrates, and iii) fermentation to produce ethanol. Hydrolysis, the second step, can be achieved by cellulolytic enzymes such as cellulases (Cardona and Sanchez, 2007); but the efficiency is highly dependent upon the pretreatment step, that is intended to disrupt lignocellulosic structures, making the cellulose and hemicellulose readily accessible by cellulolytic enzymes during hydrolysis (Petersson et al., 2007). Pretreatment is critical in lignocellulose-to-ethanol conversion to remove and/or break down lignin contents and thus disrupt the lignocellulosic structures, leading to increased cellulose accessibility for subsequent

hydrolysis (Conte et al., 2009; Fang et al., 2010; Kaparaju et al., 2009; Kerr and Goring, 1975; Matsushita et al., 2009).

Different pretreatment methods such as steam explosion (Galbe and Zachhi, 2002) and wet oxidation (McGinnis, 1983; Schmidt and Thomsen, 1989) are commonly used before enzymatic hydrolysis. Varga et al. (2003) have shown optimal parameters for corn pretreatment by wet oxidation to be 195^{o}C, 2g·L^{-1} Na_2CO_3, and 12 bar O_2 pressure for 15 minutes. Similar parameters were also used for wheat straw (Klinke et al., 2003). Wet oxidation is energy consuming, and low sugar recovery during pretreatment has been reported (Petersson et al., 2007). Wet oxidation processes produce polymers (Ahring et al., 1999) whereas, in dilute acid hydrolysis, sugar monomers are produced (Taherzadeh et al., 1997). Hydrolysate produced from wet oxidation consists of low molecular weight carboxylic acids (6.0 g·L^{-1}), phenols (2.0g·L^{-1}) and 2-furoic acid (0.007g·L^{-1}) that act as inhibitors to the microorganisms used for hydrolysis (Klinke et al., 2003).

In steam explosion pretreatment, biomass is exposed to pressurized steam with a sudden drop of pressure, thus making cellulose more available for enzymatic hydrolysis. A maximum glucose yield of 16.5 g per 100 g of sunflower stalks pre-treated using steam explosion was reported at 220^{o}C, however the highest hemicellulosic sugar recovery was obtained at 210^{o}C (Ruiz et al., 2008). Kaar et al. (1998) reported 216^{o}C as the optimum temperature for steam explosion pre-treatment for glucose conversion in sugarcane bagasse. The major disadvantages of steam explosion pretreatment are the loss of hemicellulosic sugars (Kaar et al., 1998; Ruiz et al., 2008) and production of furural and hydroxymethyl furfural which act as inhibitors to microbial hydrolysis of cellulose (Buchert, 1990).

The rate of glucose formation by enzymatic saccharification in sugarcane bassage, rice straw and silvergrass pre-treated with sulfuric acid decreased with increasing concentrations (Guo et al., 2009). Sulfuric acid has many disadvantages of being toxic and hazardous, requiring corrosion resistant reactors, being able to chemically modify carbohydrates to reduce glucose availability, and production of cinnamic acids in the residue (Mao, J.-D et al., 2010; Wyman, 1996).

Alteration in biomass structure is required for effective and efficient use of structural sugars for bioethanol production. Loss in structural sugars is a major concern in different pretreatment methods. Specificity of laccase-mediator system to act on phenolic and non-phenolic bonds has the potential to effectively remove lignin from biomass and to increase accessibility of structural sugars for hydrolysis and fermentation. Research is needed to evaluate the efficacy of laccase-mediator system, to optimize concentrations of different mediators for optimum lignin removal, and to determine its impact on structural sugars.

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CHAPTER III

USE OF FUNGAL LACCASES TO FACILITATE BIODETHATCHING: A NEW APPROACH[1]

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ABSTRACT

Formation of high organic content as thatch and/or mat layer is a major problem in management of turfgrass golf greens. A greenhouse experiment using potted bentgrass (*Agrostis stolonifera* L.) determined the efficacy of a ligninolytic enzyme, laccase, in reducing organic matter accumulation in the thatch-mat layer. Laccase was added biweekly at 0, 0.206, 2.06, and 20.6 units of activity cm^{-2} with and without guaiacol (2-methoxyphenol), a mediator of laccase, and sampling was performed after two and nine months. Parameters investigated included thickness of organic layer, thatch layer and mat layer, organic matter content, saturated hydraulic conductivity, and lignin content. Organic matter and thatch layer increased between the two sampling dates in all treatments. Laccase was shown to be effective in slowing the rate of accumulation of organic matter and thatch layer. After two months, application of 20.6 units cm^{-2} of laccase reduced organic layer thickness by 8.7% and extractive-free total lignin content by 8.4% when compared to non-treated control. After nine months, laccase application rates of 2.06 units cm^{-2}, reduced organic matter and thatch layer thickness by 15.6, and 45.0%, respectively below levels observed in the non-treated control. Applications using 0.206 units cm^{-2} of laccase were ineffective. Laccase application had only minor influences on turf quality. These positive responses suggest laccase treatments could be a non-disruptive option for thatch and/or mat control in bentgrass.

INTRODUCTION

Formation of thatch and mat layers is one of the major problems in management of modern turfgrass golf greens. Thatch is a layer of organic matter that accumulates between the soil and green turfgrass and contains both living and dead plant tissues intermingled tightly with each other. Thatch consists of stolons, rhizomes, roots, crown tissue, leaf sheaths, and blades

(Engel, 1954; Roberts and Bredakis, 1960). The mat layer is generally below the thatch layer and is distinguished from thatch by the presence of sand or soil intermingled with thatch as a result of cultural practices like core aeration and topdressing (McCarty, 2005). A small amount of organic matter reduces surface hardness, moderates soil temperature extremes, increases the resilience, and improves wear tolerance of the turfgrass surface (Beard 1973); however, excessive thatch and mat layers are undesirable in turfgrass.

High organic matter accumulation in the form of thatch-mat causes problems such as decreased movement of oxygen through the thatch or mat zone, decreased saturated hydraulic conductivity, and excessive water retention (Carrow, 2003; Hartwiger, 2004; McCarty et al., 2007). These primary problems may further lead to secondary problems like wet wilt, soft surface, black layer, limited rooting, and extra- and intra-cellular freezing damage (Beard, 1973; Carrow, 2004; O'Brien and Hartwiger, 2003). Although structured organic matter, present in live underground plant tissues, is thought to have no adverse effect on the soil physical properties, rapid root death that results in dead gelatinous organic matter swells in the presence of water during decomposition and plugs the soil macro-pores (air-filled pores), causing low oxygen levels in the root zones (Carrow, 2004; O'Brien and Hartwiger, 2003). Excessive accumulation of organic matter causes anaerobic conditions, further reducing the rate of organic matter decomposition (McCoy, 1992). Grasses also generally produce more adventitious roots (surface roots) during anaerobic conditions again further increasing organic matter content (Carrow 2004).

Thatch management techniques such as core aeration, vertical mowing, grooming, and topdressing are currently the most effective strategies to manage thatch-mat buildup but have shown contrasting results (Barton et al., 2009; Carrow et al., 1987; Dunn et al., 1981; McCarty et

al., 2005; McWhirter and Ward, 1976; Weston and Dunn, 1985; White and Dickens, 1984). These cultural practices are intensive in terms of cost, energy, and labor and have adverse effects on turfgrass quality (Barton et al., 2009; Landreth et al., 2008; McCarty et al., 2007). Several non-destructive thatch control studies using glucose, cellulase solutions, (Ledeboer and Skogley, 1967) and commercial inocula containing various microorganisms were ineffective in reducing the amount of thatch (McCarty et al., 2005; Murdoch and Barr, 1976). Reduction in cellulose content and total oxidizable organic matter of bermudagrass (*Cynodon dactylon* L.) and centipedegrass (*Eremochloa ophiuroides*) (Sartain and Volk, 1984) and weight loss of bermudagrass pellets, St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) and zoysiagrass (*Zoysia japonica* Stued., 'Meyer') stolons (Martin and Dale, 1980) were observed when inoculated with different wood-decaying fungi under controlled greenhouse and laboratory conditions. However, field inoculation experiments on bermudagrass showed no thatch degradation (Martin and Dale, 1980).

The formation of the thatch-mat layer is due to a greater rate of organic matter accumulation than degradation (Beard, 1973). Most microbial degradation mechanisms are restricted by the presence of lignin, a plant cell wall constituent. The slow decomposition of soil lignin has long been recognized (Kirk and Farrel, 1987). Lignin limits the accessibility of microbial degraders to more biodegradable plant materials, such as cellulose and hemicelluloses (Ledeboer and Skogley, 1967). Lignin is formed in plants by oxidative coupling of mono-lignols of three primary hydroxycinnamyl alcohols: *p*-coumaryl, coniferyl and sinapyl alcohols. The corresponding lignin monomers are known as *p*-hydroxy phenyl, guaiacyl and syringyl units, respectively (Wong, 2009). Lignification is achieved by cross linking of monomers with a growing polymer via polymer-polymer coupling. Based on the random coupling theory, several

models of lignin molecular structure have been proposed but these models do not imply any particular sequence of monomeric units in the lignin macromolecule (Chen and Sarkanen, 2003; Davin and Lewis, 2003).

Natural degradation of lignin occurs in the environment by certain white-rot fungi which solubilize and mineralize lignin with the help of lignolytic enzymes (Kirk et al., 1975; Kirk et al., 1976). White-rot fungi preferentially attack lignin more than cellulose or hemicellulose in the wood tissue (Blanchette, 1984; Mester et al., 2004). This process of selective delignification exposes cellulosic materials for further bacterial degradation in the environment (Otjen and Blanchette, 1987). Presence of naturally-occurring (guaiacol) and synthetic (1-hydroxybenzotriazole, HBT) chemicals, known as mediators, have shown to enhance the activity of the lignolytic enzyme laccase (Kang et al., 2002; Roper et al., 1995). As lignin content in thatch layer is higher than that of live grass tissues, the thatch layer in turfgrass species with high lignin content is more resistant to microbial decomposition (Beard, 1973; Ledeboer and Skogley, 1967).

We hypothesize that the use of lignin-degrading enzymes such as fungal laccases can effectively reduce the rate of thatch layer accumulation in golf greens. The objectives of our study were: 1) to determine if degradation of soil organic matter can be enhanced by laccase application; 2) to determine if addition of guaiacol enhances laccase efficacy in organic matter decomposition; and 3) to determine if application of laccase enzyme and guaiacol adversely affect turf quality.

MATERIALS AND METHODS

A greenhouse experiment was conducted using “Crenshaw” creeping bentgrass *Agrostis stolonifera* L. (Engelke et al., 1995), established in pots (top diam. 15 cm, height 11.5 cm) at The

University of Georgia, Griffin Campus from October 2008 to July 2009. The bentgrass was acquired from East Lake Country Club, Atlanta, Georgia. Pots were partially filled with 85:15 sand and organic matter mix and sod approximately 3 cm in thickness was cut to fit the pots and placed on top of the mix. All pots were established in June 2008 and grown under management conducive to thatch development in a controlled environment greenhouse for approximately four months prior to initiation of treatments. Pots were irrigated daily, fertilized monthly with a 50-mL solution of 0.4% (w/v) Macron water soluble 28-7-14 fertilizer (Lesco. Strongsville, OH), and maintained by hand clipping weekly at a height of 0.6 cm with clippings removed. The refrigerated air conditioned greenhouse was maintained at 25± 2 / 18 ±2^{o}C, day/night temperature by a Wadsworth Step 50 controller (Wadsworth Control System, Arvada, CO) under natural lighting (approximately 85% ambient light).

The treatment design was as a four by two factorial with all combinations of four levels of laccase and two levels of guaiacol (2-methoxyphenol). The four laccase activity levels were 0 (control), 0.206, 2.06 and 20.6 units cm^{-2} and guaiacol levels were 0 and 0.1 M solution. The experimental design was a randomized complete block with five replications and sampling times of two and nine months. Forty milliliter solutions of the different laccase activity levels and 10 mL of guaiacol solutions were applied uniformly every two weeks to each pot using a hand-held sprayer. The control pots were treated with equivalent amounts of distilled water. After the two months of treatment applications the 20.6 units cm^{-2} treatments with and without guaiacol were discontinued due to limited availability of laccase enzyme. Due to unexpected problems in developing protocols for measurement of saturated hydraulic conductivity, one replication was rendered unusable for measurement of other variables. Therefore, only four replications were used for analysis after nine months treatment duration.

Laccase Activity Assay

The laccase enzyme from *Trametes versicolor,* a white-rot fungus, was purchased from Sigma-Aldrich (product 53739, Sigma Aldrich Inc., St. Louis, MO.). Laccase solutions were standardized based on active units which were quantified using a UV/VIS-spectrophotometer by a colorimetric assay. One activity unit of laccase corresponds to the amount of enzyme that causes an absorbance change at 468 nm at a rate of 1.0 unit min^{-1} in 3.4 mL of 1 mM 2,6-dimethoxyphenol, a specific substrate for laccase, in citrate-phosphate buffer at pH 3.8 (Park et al., 1999). Laccase activity treatments of 0 (control), 0.206, 2.06 and 20.6 units cm^{-2} actually correspond to laccase solutions with activity levels of 0, 0.912, 9.12 and 91.2 units mL^{-1}, respectively. The activity level of laccase applied per unit area was calculated by dividing total number of units of laccase in 40-mL laccase solution by the top surface area of the pot.

Measurements

Effectiveness of treatments was determined by measuring organic matter content (OM) for a depth of 0-5.0 cm, organic layer thickness (OL), extractive-free acid-soluble lignin (L_S) and acid-insoluble lignin (L_I) content after two and nine months of treatment application. Total lignin (L_T) was obtained by addition of acid-soluble and-insoluble lignin contents.

After nine months of treatment application, some additional variables were measured. OL was subdivided into thatch layer thickness (OL_T) and mat layer thickness (OL_M), while organic matter content (OM) was subdivided into 0-2.5, and 2.5-5.0 cm depths to more accurately reflect the effectiveness of laccase on the thatch and mat layers. Saturated hydraulic conductivity (SHC) was also measured after nine months.

Organic Matter Content

The measurement of OM was performed as described by Carrow et al. (1987). Soil cores (2.0 cm diam.) were dried in an oven at 100 ± 5^{o}C for 48 h and weighed. Soil cores were ashed in a muffle furnace at 600 ± 25^{o}C for 24 h and weighed again. Organic matter content was determined as the difference in the two readings and percent organic matter was calculated.

Saturated Hydraulic Conductivity

Intact cores (diam. 4.7 cm and length 7.7 cm) were obtained from the center of each pot using a soil corer. The cores were collected in brass cylinders. The bottom of the core was covered with a double layer of cheesecloth held in place with a rubber band. The core was saturated overnight in a 0.05 N $CaCl_2$ solution to minimize dispersion. A clear plastic cylinder of the same diameter as of the brass cylinder was fastened above the brass cylinder with paraffin wax tape. The SHC of the cores was measured by a constant hydraulic head method using a Marriott tube apparatus. A time of 10-minutes was allowed for the establishment of steady state flow through the samples. The volume of water that passed through the core was measured for one minute and repeated three times. Saturated hydraulic conductivity was calculated using Darcy's equation.

Organic Layer Thickness and Thatch-Mat Layer Thickness

After cores were removed for measurement of organic matter content and saturated hydraulic conductivity, plants were removed from pots and distinct separations among the thatch, mat, and soil interface were clearly visible. The organic layer (OL), thatch layer (OL_T), and mat layer (OL_M) were measured from seven different locations around the edges of the plant/root mass and averaged.

Extractive-free Lignin Content

Thatch was collected from each pot from the top 2.5 cm after sampling for OM and SHC. Extractive-free acid-soluble (L_S) and-insoluble (L_I) lignin content in the thatch layer was determined in a two-step hydrolysis procedure according to the laboratory analytical procedure developed by The National Renewable Energy Laboratory (NREL, 2008). In the first step, extractive-free thatch samples were hydrolyzed for 60 min with 72% H_2SO_4 at 30^oC. In the second step, H_2SO_4 was diluted to 4% and the samples were autoclaved at 121^oC for 1 h. Acid-soluble lignin was determined by measuring the absorbance of this hydrolysis liquid at 240 nm in a UV/VIS spectrophotometer. The solids remaining after acid hydrolysis were dried in an oven at 100 ± 5^oC for 24 h, weighed, ashed in a muffle furnace at 600 ± 10^oC for 24 h, and weighed again. Weight difference was used to calculate the acid-insoluble lignin content.

Turf Quality

Turf quality was determined bi-weekly for the first three months and again for the last two months of the experiment to document the potential for initial and long term phytotoxicity associated with laccase application. The turf quality of each treatment was recorded every two weeks by rating both visual turf quality and canopy spectral reflectance. Visual turf quality ratings were rated on the basis of color, shoot density, and uniformity on a numerical scale where 1 equals no live turf and 9 equals ideal dark green, uniform turf (Johnson et al., 1987). Grass index was determined using TCM 500 turf color meter (Spectrum Technologies, Plainfield, IL). Grass index is a numerical score of the color and density of grass based on the spectral reflectance at 660 and 850 nm. Three grass index readings were recorded from each pot and averaged for statistical analysis.

Statistical Analysis

Analysis of variance (ANOVA) was performed to evaluate the main effects of treatment duration, laccase, and guaiacol and interaction effects of these three factors using general linear model (GLM) (SAS Institute, 1994). Strong treatment duration effects ($P \leq 0.001$) were observed in the initial analysis and therefore each treatment duration was analyzed separately using ANOVA as a two factor study consisting of four levels of laccase enzyme and two levels of guaiacol for the two months treatment duration and three levels of laccase enzyme and two levels of guaiacol for the nine months treatment duration. Fisher's protected LSD test with $\alpha = 0.05$ was used for determining statistical differences among treatment means following each ANOVA.

RESULTS

The full statistical model was used to compare common parameters at the two and nine month sampling dates (Table 3.1). The model included the main and interaction effects of treatment duration, three levels of laccase, and two levels of guaiacol for organic matter (OM) (0-5.0 cm), organic layer thickness (OL), acid-soluble (L_S) and-insoluble lignin (L_I). Only three levels of laccase were used as the 20.6 units cm^{-2} laccase treatment was discontinued after two months of application.

Treatment duration strongly affected OM (0-0.5 cm; $P \leq 0.001$), OL ($P \leq 0.001$), and L_S ($P \leq 0.001$) (Table 3.1). Laccase application significantly affected OM (0-5.0 cm), OL, L_S and L_I. The very strong interactions for treatment duration and laccase treatment observed in OM (0-0.5 cm; $P \leq 0.001$), OL ($P \leq 0.001$), and L_S ($P \leq 0.01$) were largely due to the lack of response to laccase treatments after two months as opposed to a strong response seen after nine months. Guaiacol treatment as well as the interaction of guaiacol and treatment duration had no effect on any of the parameters (Table 3.1).

We observed an overall increase in OM (0-5.0 cm) and OL in all treatments between the two sampling dates. However, accumulation of OM (0-5.0 cm) and OL were significantly lower in treatments containing laccase when compared to the control. When compared to the control, the rate of accumulation of organic matter (0-5.0 cm) was reduced from 15.8 $mg \cdot g^{-1}$ in control pots to 9.0 $mg \cdot g^{-1}$ in pots treated with 2.06 units cm^{-2} laccase (43%) (Table 3.2). Similarly, application of 2.06 units cm^{-2} laccase reduced OL accumulation from 15.5 mm to 11.7 mm (24%) when compared to the control (Table 3.2). A reduction in lignin content between the two sampling dates was observed at laccase activity levels of 2.06 units cm^{-2} with and without guaiacol (Table 3.3).

Analysis after Two Months Treatment

After two months of treatment application, laccase treatments had no effect on OM (0-5.0 cm) and OL but had significant effects on L_S and L_I (Table 3.1). Neither guaiacol nor the laccase by guaiacol interaction had significant effects on any of the parameters (Table 3.1).

Compared to the control, treatment with 20.6 units cm^{-2} of laccase without guaiacol for two months significantly lowered L_S by 5.2, L_I by 20.5, and L_T content by 25.6 $mg \cdot g^{-1}$ (Table 3.3). Similarly, treatment at the same laccase activity with guaiacol reduced L_S by 4.2, L_I by 18.9, and L_T by 23.4 $mg \cdot g^{-1}$ when compared to control. Treatment with 2.06 units cm^{-2} of laccase with guaiacol also significantly reduced L_S by 1.4 $mg \cdot g^{-1}$ (Table 3.3).

Analysis after Nine Months Treatment

After nine months of treatment application, laccase application impacted OM (0-5.0, and 0-2.5 cm), SHC, OL, OL_T, L_S, and L_I and SHC ($P \leq 0.001$) (Table 3.1). A significant effect of guaiacol was also observed for L_S ($P \leq 0.05$) and SHC ($P \leq 0.01$) (Table 3.1). The interaction of

laccase by guaiacol was significant ($P \leq 0.01$) for SHC. However, none of the treatments affected OM (2.5-5.0 cm) and mat layer thickness after nine months of treatment application.

Treatment with 2.06 units cm^{-2} of laccase with and without guaiacol decreased OL by 10.8 and 9.5 mm, respectively when compared to the control (Table 3.2). These same treatments reduced OL_T by 8.3 (45%) and 6.5 mm (35%) when compared to the control (Fig. 3.1). Laccase applied at same activity was effective in reducing OL and OM while laccase application at 0.206 units cm^{-2} was not different from the control (Table 3.2, Fig. 3.2).

Treatment with 2.06 units cm^{-2} laccase with and without guaiacol reduced OM (0-5.0 cm) by 7.6 and 7.8 $mg \cdot g^{-1}$, and OM (0-2.5) by 25.9 and 30.3 $mg \cdot g^{-1}$, respectively as compared to the control (Table 3.2, Fig. 3.2). Similarly, treatment with 2.06 units cm^{-2} laccase with and without guaiacol increased SHC by 21.6 (322%) and 6.3 cm h^{-1} (94%), respectively over the control (Fig. 3.3). When compared with control, treatment with 2.06 units cm^{-2} laccase without guaiacol reduced L_S by 5.1, L_I by 14.0, and L_T by 19.0 $mg \cdot g^{-1}$, respectively (Table 3.3).

Turf Quality

No significant differences in visual quality ratings were observed among the treatments except for the data collected after thirty eight weeks where 2.06 units cm^{-2} of laccase exhibited a slight but significant reduction in turf quality when compared to the control treatment (Table 3.4). No significant differences from the control were observed for any treatment when means of the visual ratings were compared for the early (2 to 12 weeks), late (32 to 38 weeks), and all periods (Table 3.4). When compared to the control, pots receiving 20.6 units cm^{-2} laccase treatment had a small but significant decrease in grass index at four and six weeks after treatment initiation (Table 3.5). However, no significant differences in grass index values were observed

after six weeks of treatment application. No visual differences among treatments for turf color or growth rate were observed over the duration of the experiment.

DISCUSSION

Laccase Application

This is the first study to report the direct application of laccase enzyme to manage thatch-mat accumulation on creeping bentgrass. Application of laccase, especially at the 2.06 units cm^{-2} activity level, proved to be effective in reducing thatch-mat depth, OM, and significantly increasing SHC. Carley et al. (2011) noted that the nature of temporal dynamics of organic matter accumulation was for small annual changes resulting in long term effects. Our results indicate that application of 2.06 units cm^{-2} laccase alters organic matter dynamics in a positive manner by effectively reducing OM (0-2.5 cm) and OL_T in comparison to the control. However, an increase in OM (0-5.0 cm) and OL was observed for all the treatments over the experiment duration. Application of laccase enzyme was effective in reducing the rate of accumulation of thatch layer thickness and organic matter.

The 2.06 units cm^{-2} laccase treatment, after nine months of application, also resulted in increased SHC where a three-and two-fold increase in SHC was observed with applications of 2.06 units cm^{-2} laccase with and without guaiacol, respectively. This increase can be explained on the basis of thatch layer thickness of the corresponding treatment. Thatch layer thickness more than 1.3 cm was reported to adversely affect water infiltration (McCarty et al., 2005). Thatch layer thickness for the treatment 2.06 units cm^{-2} with and without guaiacol after nine months of treatment was 11.2 and 13.0 mm, respectively.

For both the two and nine months sampling, no effect of guaiacol was observed and no interaction effect with laccase was observed except for saturated hydraulic conductivity after

nine months of application. The 2.06 units cm^{-2} laccase treatment was ineffective when applied with guaiacol after two months of application except for a reduction in extractive-free L_S. The significant replication effect observed for L_S and L_I for two and nine months, respectively may be associated with incomplete acid-hydrolysis for some replications during autoclaving and unavailability of additional sample materials for reanalysis.

The lowest level of laccase application (0.206 units cm^{-2}) proved to be an ineffective treatment even after nine months of application for all the parameters measured. The 20.6 unit cm^{-2} treatment was applied for two months and resulted in no reduction of OM (0-5.0 cm). However, this treatment did result in a significant reduction in OL and extractive-free lignin content (L_S, L_I, and L_T) of the thatch layer.

Laccase application had only minor influences on turfgrass quality. A slight reduction in turf quality was indicated by lower grass index values during the first four to six weeks in response to the 20.6 units cm^{-2} laccase treatment. However, visual quality ratings were not significantly different from controls except for one treatment combination at 38 weeks.

Treatment Duration

If laccase was effective in enhancing organic matter degradation, it would seem reasonable to expect that effects would become more apparent over time. Samples were analyzed after two and nine months of treatment application. Laccase activity levels of 0.206 and 2.06 units cm^{-2} area were continued for nine months. It was observed that treatment duration, and the interaction of treatment duration with laccase treatments had a significant effect on OM (0-5.0 cm), OL, and L_S content.

Why Laccase Application?

Studies in the past using various cultural management practices with different cultivation frequencies have reported contrasting results for reduction in thatch-mat accumulation (Callahan et al., 1998; Carrow et al., 1987; Engel and Alderfer, 1967; McCarty et al., 2005; Rieke, 1994). Degradation of thatch-mat is reported either in terms of thatch-mat depth (Smiley et al., 1985; Soper et al., 1988) or in terms of thatch-mat depth and organic matter content by weight (Barton et al., 2009; McCarty et al., 2007). The organic matter content by weight in different studies is observed for different depths further making it difficult to compare the results (Barton et al., 2009; McCarty et al., 2005; Murray and Juska, 1977). In our study, however, we observed both organic layer thickness (thatch layer and mat layer) and organic matter content in order to provide a better comparison of the effectiveness of laccase on thatch-mat degradation.

Cultural practices like core aeration and vertical mowing are disruptive in nature and have shown to reduce the turf quality both aesthetically and physically, further reducing the playability of the turf (Barton et al., 2009; Landreth et al., 2008; McCarty et al., 2007). However, application of laccase is not disruptive and the effective treatment of 2.06 units cm^{-2} laccase for nine months showed no reduction in turf quality of bentgrass.

Several non-destructive studies in the past using different treatments like sugars, mixtures of sugars and microbial inocula, and some enzymes like cellulase, proved ineffective (Ledeboer and Skogley, 1967; Martin and Dale, 1980; McCarty et al., 2005; Murdoch and Barr, 1976). Most of these studies intended to increase microbial population to degrade organic matter. But it is difficult to maintain higher microbial populations over sustained period of time under field turfgrass management systems due to the inability to maintain proper micro-environment conditions required by particular microbial populations. Another reason that such studies were

ineffective may be that they were focused on degradation of cellulose and hemicellulose by using cellulase enzyme and by increasing bacterial populations. Whereas, our hypothesis is that lignin degradation will open the cell wall structure of thatch biomass, hence making cellulose and hemicellulose more available for further microbial degradation. In our study, we used the end product from the white-rot fungi *Trametes versicolor*, the laccase enzyme, which is stable over a wide pH and temperature (Baldrian, 2006; Munoz et al., 1997; Stoilova et al., 2010; Thurston, 1994) to degrade lignin and to facilitate dethatching.

CONCLUSIONS

This greenhouse research demonstrated that bi-weekly application of laccase enzyme at 2.06 units cm^{-2} can be effective in reducing the rate of accumulation of organic matter in highly maintained turf. However, low activity levels of laccase (0.206 units cm^{-2}) were ineffective in reducing the rate of thatch accumulation. Laccase application had little effect after two months but significantly reduced organic matter after nine months. Implications of these findings point to a novel approach to reduce organic matter in thatch or mat and its associated problems on golf greens. This approach can lead to the development of a new non-disruptive method for thatch management. Future research is needed to observe the effectiveness of laccase under field conditions as well as to optimize the activity level of laccase and the frequency of its application.

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Table 3.1 Analysis of variance (ANOVA) table showing the effects of treatment duration, laccase application, guaiacol application, and their interactions on creeping bentgrass maintained in a greenhouse.

Source of Variation	df	Organic layer thickness (OL)	Organic matter (OM) (0-5.0 cm)	Acid-soluble lignin (L_S)	Acid-insoluble lignin (L_I)	Total lignin (L_T)	Thatch layer thickness (OL_T)	Mat layer thickness (OL_M)	Organic matter (OM) (0-2.5 cm)	Organic matter (OM) (2.5-5.0 cm)	Saturated hydraulic conductivity (SHC)
		mm	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	cm	cm	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	$cm\ h^{-1}$
		mean square values									
Full Model											
Treatment duration	1	3649***	2008***	55.8***	102	309**					
Laccase	2	99.2***	105***	55.5***	476***	852***					
Guaiacol	1	9.69	1.03	9.86	50.7	105					
Duration*laccase	2	109***	60.8***	22.9**	84.4	193*					
Duration*guaiacol	1	13.6	4.46	19.6	76.1	172					
Error	42	7.71	5.83	2.82	30.2	36.1					
2 Months											
Rep	4	1.84	16.9	3.83	10.6	22.3					
Laccase	3	20.8	16.7	50.1***	760***	1195***					
Guaiacol	1	0.70	1.84	0.00028	0.01	0.01					
Laccase*guaiacol	3	3.85	37.5	1.53	3.02	8.58					
Error	28	8.08	7.61	0.96	32.3	33.2					
9 Months											
Rep	3	10.1	4.63	7.85	120	173	7.39	1.68	194	6.14	3.23
Laccase	2	192***	143***	66.6***	430***	835***	96.0***	16.5	1660***	27.4	451***
Guaiacol	1	21.9	4.40	25.7*	113	246*	4.53	6.52	192	4.05	193***
Laccase*guaiacol	2	3.55	2.99	5.80	35.5	71.1	1.35	8.79	153	1.04	140***
Error	15	8.72	8.13	4.07	13.1	13.0	4.03	11.2	100	8.27	3.44

* Significant at the 0.05 probability level
**Significant at the 0.01 probability level
*** Significant at the 0.001 probability level

Table 3.2 Organic layer thickness (OL) and organic matter (OM) content (0-5.0 cm depth) after two and nine months of different treatments applied to creeping bentgrass.

Treatment[z]	Organic layer thickness		Organic matter (0-5.0)	
	2 Months	9 Months	2 Months	9 Months
	--------- mm ---------		--------- $mg \cdot g^{-1}$ ----------	
0L (Control)	48.4a[y] B[x]	69.3a A	33.7a B	49.5a A
0L+G	47.4a B	68.2ab A	34.4a B	47.3a A
0.206L	45.7a B	67.3ab A	36.4a B	50.0a A
0.206L+G	47.6a B	63.8bc A	34.3a B	49.4a A
2.06L	48.1a B	59.8cd A	32.7a B	41.7b A
2.06L+G	47.7a B	58.5d A	35.0a B	41.9b A
20.6L	44.2b	-	36.3a	-
20.6L+G	45.1a	-	33.9a	-

[z]L denotes Laccase level and G the addition of Guaiacol, a mediator.
[y]Means within a column followed by the same lowercase letter are not significantly different according to Fisher's protected LSD at α=0.05.
[x]Means in a row within a parameter followed by the same uppercase letter are not significantly different according to Fisher's protected LSD at α=0.05.

Table 3.3 Extractive-free acid-soluble (L_S), acid-insoluble (L_I), and total lignin (L_T) content after two and nine months of different treatments applied to creeping bentgrass.

	Acid-soluble lignin		Acid-insoluble lignin		Total lignin	
Treatment[z]	2 Months	9 Months	2 Months	9 Months	2 Months	9 Months
	-------- $mg \cdot g^{-1}$ ---------		-------- $mg \cdot g^{-1}$ ---------		-------- $mg \cdot g^{-1}$ ---------	
0L (Control)	43.7ab[y] A[x]	42.2b A	259.9a A	257.4b A	303.5ab A	299.7b A
0L+G	43.8ab A	45.8a A	260.1a A	264.8a A	303.9a A	310.6a A
0.206L	44.1a A	41.4b A	256.9a A	254.5bc A	301.0ab A	295.9b A
0.206L+G	43.3abc A	41.4b A	255.4a A	253.9bc A	298.8ab A	295.5b A
2.06L	42.7bc A	37.1c B	254.0a A	243.4d B	296.7ab A	280.4d B
2.06L+G	42.3c A	39.5bc B	253.9a A	249.6c A	296.2b A	289.0c B
20.6L	38.5d	-	239.4b	-	277.9c	-
20.6L+G	39.5d	-	241.0b	-	280.1c	-

[z]L denotes Laccase level and G the addition of Guaiacol, a mediator.
[y]Means within a column followed by the same lowercase letter are not significantly different according to Fisher's protected LSD at α=0.05.
[x]Means in a row within a parameter followed by the same uppercase letter are not significantly different according to Fisher's protected LSD at α=0.05.

Table 3.4 Mean visual turf quality ratings of creeping bentgrass made over time following continued treatment with different laccase and guaiacol solutions to greenhouse grown plants.

	Weeks												
	Early						Late						
Treatments[z]	2	4	6	8	10	12	32	34	36	38	Early	Late	All
	Visual turfgrass quality[y]												
0L (Control)	7.1ab[x]	6.7a	8.3ab	7.8a	7.2a	8.0ab	7.9a	7.9a	8.3a	8.5a	7.5a	8.0a	7.8a
0L+G	7.0ab	6.9a	8.1ab	7.7a	7.1a	8.1a	7.8a	7.7a	8.1a	8.4a	7.4a	7.9a	7.8a
0.206L	7.2a	6.8a	8.2ab	7.6a	7.1a	7.9ab	8.0a	8.0a	8.1a	8.4a	7.4a	7.8a	7.8a
0.206L+G	7.0a	6.7a	8.0b	7.8a	7.3a	7.8b	7.7a	7.6a	8.3a	8.3ab	7.4a	7.8a	7.5a
2.06L	7.2a	7.0a	8.3ab	7.8a	7.4a	7.9ab	7.9a	7.9a	8.1a	8.1b	7.5a	7.9a	7.6a
2.06L+G	6.9ab	6.7a	8.4ab	7.8a	7.3a	7.7b	7.9a	8.0a	8.2a	8.4a	7.4a	7.9a	7.6a
20.6L	6.7b	6.9a	8.4a	7.8a	-	-	-	-	-	-	7.5a	-	-
20.6L+G	6.9ab	6.7a	8.2ab	7.9a	-	-	-	-	-	-	7.5a	-	-

[z]L denotes Laccase level and G the addition of Guaiacol, a mediator.
[y]Turf quality was visually rated on a 1 to 9 scale with 9=outstanding, 6=acceptable, and 1=dead.
[x]Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD at α=0.05.

Table 3.5 Mean grass index values of creeping bentgrass made over time following continued treatment with different laccase and guaiacol solutions to greenhouse grown plants.

	Weeks												
	Early						Late						
Treatments[z]	2	4	6	8	10	12	32	34	36	38	Early	Late	All
	Grass Index[y]												
0L (Control)	7.03ab[x]	6.47a	7.47a	6.71a	7.70a	8.17ab	8.15a	8.23a	9.14a	9.34a	6.92a	8.46a	7.84a
0L+G	7.11ab	6.45a	6.96ab	6.53a	7.94a	8.31a	8.20a	8.47a	9.04a	9.06a	6.76a	8.50a	7.81a
0.206L	7.21a	6.40ab	7.11ab	6.55a	7.70a	7.96ab	8.22a	7.93a	9.07a	9.10a	6.82a	8.33a	7.73a
0.206L+G	7.15ab	6.33ab	6.90ab	6.53a	7.61a	7.84b	8.36a	8.10a	9.06a	9.34a	6.73a	8.39a	7.72a
2.06L	7.10ab	6.44a	7.27ab	6.53a	7.66a	8.14ab	8.10a	8.29a	9.07a	9.33a	6.84a	8.43a	7.79a
2.06L+G	6.94ab	6.19ab	7.50a	6.49a	7.84a	8.20ab	8.16a	8.10a	9.20a	9.12a	6.78a	8.43a	7.77a
20.6L	6.68b	6.02b	6.70b	6.72a	-	-	-	-	-	-	6.53b	-	-
20.6L+G	6.92ab	6.16ab	6.96ab	6.74a	-	-	-	-	-	-	6.73a	-	-

[z]L denotes Laccase level and G the addition of Guaiacol, a mediator.
[y]Grass index was recorded using TCM 500 based on the spectral reflectance with higher value representing higher quality
[x]Means within a column followed by the same letter are not significantly different according to LSD at α=0.05.

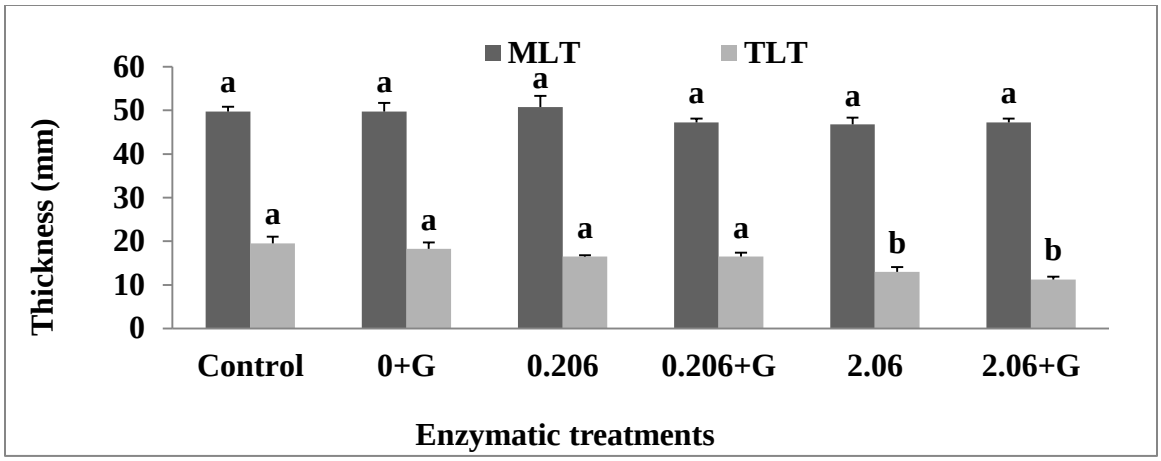

Fig. 3.1 Thatch (OL_T) and mat layer thickness (OL_M) after nine months of treatment on creeping bentgrass with three different levels of laccase (0 (control), 0.206 and 2.06 units cm^{-2}) with and without the mediator, guaiacol (G). Values are means of four replicates and error bars are standard errors. Bars with the same letter (OL_M = bolded and OL_T = standard) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

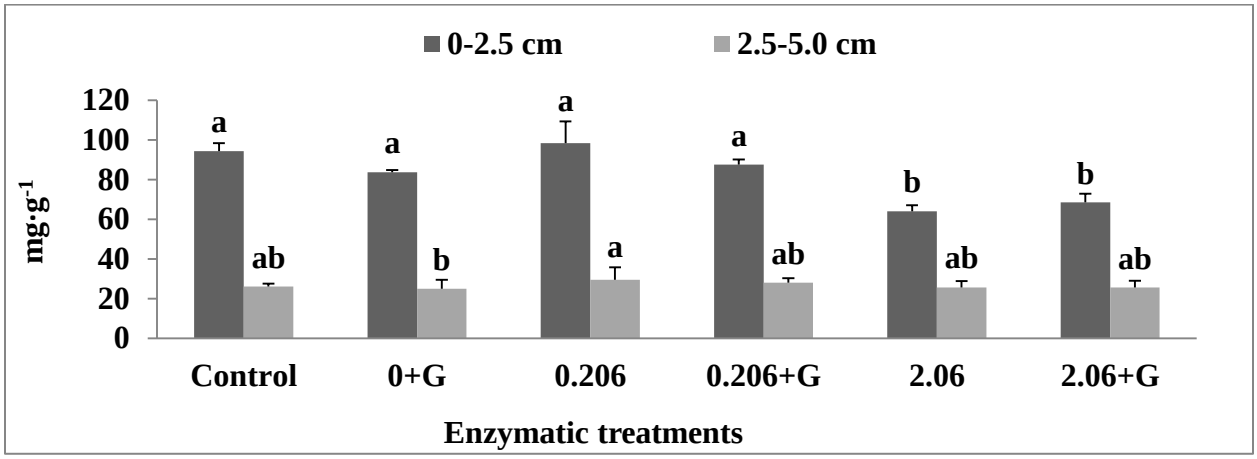

Fig. 3.2 Organic matter (OM) for 0-2.5 cm depth and 2.5 to 5.0 cm depth after nine months of treatment on creeping bentgrass with three different levels of laccase (0 (control), 0.206 and 2.06 units cm^{-2}) with and without the mediator, guaiacol (G). Values are means of four replicates and error bars are standard errors. Bars with the same letter (OM (0-2.5 cm) = bolded and OM (2.5-5.0 cm) = standard) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

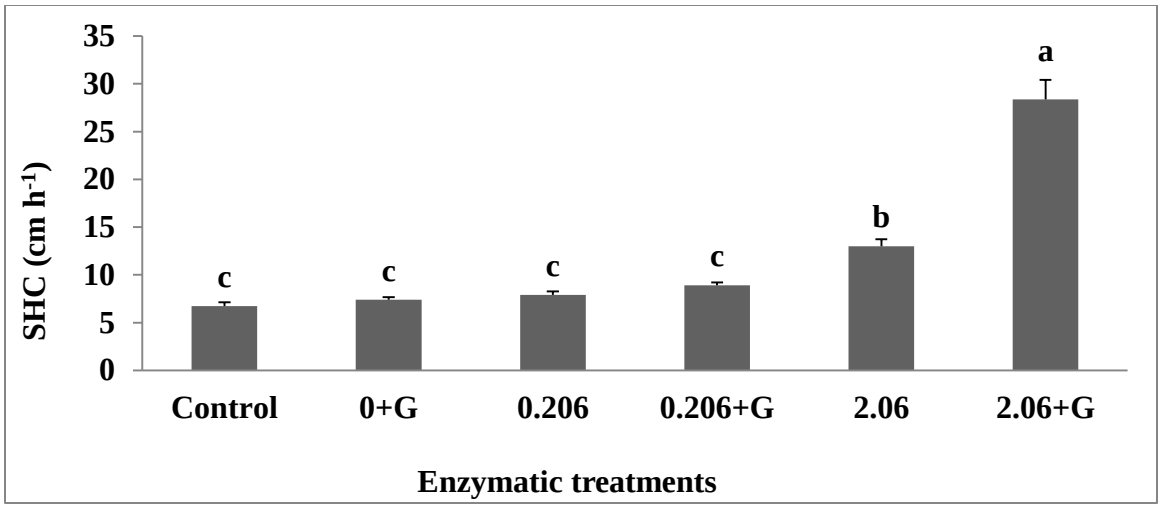

Fig. 3.3 Saturated hydraulic conductivity (SHC) after nine months of treatment on creeping bentgrass with three different levels of laccase (0 (control), 0.206 and 2.06 units cm^{-2}) with and without the mediator, guaiacol (G). Values are means of four replicates and error bars are standard errors. Bars with the same letter are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

CHAPTER IV

LACCASE MEDIATED CHANGES IN PHYSICAL AND CHEMICAL PROPERTIES OF THATCH LAYER IN CREEPING BENTGRASS (*Agrostis stolonifera* L.)[2]

[2] Sidhu, S.S., Q. Huang, R.N. Carrow, and P.L. Raymer. To be submitted to *Journal of Environmental Quality*.

ABSTRACT

Excess thatch, a tightly intermingled layer of dead and living organic matter present below the turf aerial shoots and above the soil, can be a major problem on many turfgrass sites, especially golf greens. A greenhouse experiment conducted on dead potted bentgrass (*Agrostis stolonifera* L.) determined the efficacy of a ligninolytic enzyme, laccase, along with mediator guaiacol in reducing organic matter content in thatch layer biomass. Laccase was added biweekly at 0, 2.06, and 20.6 units of activity cm^{-2} with and without guaiacol (2-methoxyphenol) and sampling was performed after two and six months. Parameters investigated included thickness of thatch layer, organic matter, saturated hydraulic conductivity, lignin content, and structural sugars. After two months of treatment application 22.1 and 12.3% reductions in thatch layer thickness and extractive-free acid-soluble lignin, respectively were observed, while 6.5 and 124.7% increases in extractive-free acid-insoluble lignin content and saturated hydraulic conductivity, respectively were observed. No reduction in organic matter and sugar content was observed after two months of treatment application; but after six months, 62.0, 24.7, and 29.3% reduction in thatch layer thickness, total organic (0-2.5 cm), and total sugar content, respectively were observed. Extractive-free acid-insoluble lignin and saturated hydraulic conductivity increased by 17.1 and 70.8%, respectively in comparison to the control. These positive responses suggest laccase treatments could expedite organic matter degradation in the thatch layer of creeping bentgrass.

INTRODUCTION

A major problem in management of recreational turfgrass sites, especially golf greens is the formation of thatch and/or mat layer that accumulates between the soil and green turfgrass and contains both living and dead plant tissues intermingled tightly with each other. Thatch, a

layer of high organic matter content, consists of stolons, rhizomes, roots, crown tissue, leaf sheaths, and blades (Engel, 1954; Roberts and Bredakis, 1960). The occurrence of mat layer is generally due to presence of sand or soil intermingled with thatch as a result of cultural practices like core aeration and topdressing (McCarty, 2005). A thin layer of thatch aids in reducing surface hardness, moderating soil temperature extremes, and increasing resilience and wear tolerance of the turfgrass surface (Beard, 1973); however, excessive thatch and mat layers are undesirable in turfgrass.

Thatch-mat causes problems such as decreased movement of oxygen through the thatch or mat zone, decreased saturated hydraulic conductivity, low oxygen levels within the thatch/mat layer during wet periods, and increased water retention (Carrow, 2003; Hartwiger, 2004; McCarty et al., 2007). These conditions often lead to secondary problems like wet wilt, soft surface, increased mower scalp, black layer, limited rooting, and extra- and intra-cellular freezing damage (Beard, 1973; Carrow, 2004; O'Brien and Hartwiger, 2003). Adverse effects on the soil physical properties are caused by rapidly decaying dead gelatinous organic matter that swells in the presence of water during decomposition and plugs the soil macro-pores (air-filled pores), causing low oxygen levels in the root zones (Carrow, 2004; O'Brien and Hartwiger, 2003).

Biological and mechanical practices have been used to manage thatch-mat buildup but are not sufficiently effective in most cases in reducing organic matter accumulation and have shown contrasting results (Barton et al., 2009; Carley et al., 2011; Carrow et al., 1987; Dunn et al., 1981; McCarty et al., 2005; McWhirter and Ward, 1976; Weston and Dunn, 1985; White and Dickens, 1984). Biological approaches to enhance organic matter decomposition include microbial inoculation and attempts to enhance microbial activity or limit plant growth.

Mechanical practices like core aeration, vertical mowing, grooming, and topdressing have been the most effective, but adversely impact turf quality and are intensive in terms of cost, energy, and labor (Barton et al., 2009; Landreth et al., 2008; McCarty et al., 2007).

When accumulation of organic matter exceeds the degradation rate, formation of thatch-mat layer is increased (Beard, 1973). The presence of lignin, a plant cell wall constituent, acts as a protective matrix and limits the accessibility of microbial degraders to more biodegradable plant materials, such as cellulose and hemicelluloses (Ledeboer and Skogley, 1967). Lignin is formed in plants by oxidative coupling of mono-lignols of three primary hydroxycinnamyl alcohols: *p*-coumaryl, coniferyl, and sinapyl alcohols (Wong, 2009). Lignification is achieved by random cross linking of monomers through different bonds resulting in a heterogeneous structure resistant to degradation (Ledeboer and Skogley, 1967). Several models of lignin molecular structure have been proposed but these models do not imply any particular sequence of monomeric units in the lignin macromolecule (Chen and Sarkanen, 2003; Davin and Lewis, 2003).

Non-destructive thatch control studies using glucose, cellulase solutions, (Ledeboer and Skogley, 1967) and commercial inocula containing various microorganisms were ineffective in reducing the amount of thatch (McCarty et al., 2005; Murdoch and Barr, 1976). The rate of microbial decomposition is more dependent on the lignin content of the organic matter in the degradation progress (Taylor et al., 1989). A plant litter decomposition study reported a close relationship of mass loss with activity of lignocellulose-degrading enzymes. (Sinsabaugh et al., 1993). Extra-cellular lignolytic enzymes produced by certain white-rot fungi are responsible for natural degradation of lignin (Kirk et al., 1975; Kirk et al., 1976). The preferential degradation of lignin by extra-cellular enzymes produced by white-rot fungi exposes cellulosic materials for

further bacterial degradation in the environment (Blanchette, 1984; Mester et al., 2004; Otjen and Blanchette., 1987). Presence of naturally-occurring (guaiacol) and synthetic (1-hydroxybenzotriazole, HBT) chemicals, known as mediators, have shown to enhance the activity of the lignolytic enzyme laccase (Kang et al., 2002; Roper et al., 1995). Reduction in cellulose content and total oxidizable organic matter of bermudagrass (*Cynodon dactylon* L.) and centipedegrass (*Eremochloa ophiuroides*) (Sartain and Volk, 1984) and weight loss of bermudagrass pellets, St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) and zoysiagrass (*Zoysia japonica* Stued., 'Meyer') stolons (Martin and Dale., 1980) were observed when inoculated with different wood-decaying fungi under controlled greenhouse and laboratory conditions. However, field inoculation experiments on bermudagrass showed no thatch degradation (Martin and Dale., 1980).

A novel approach developed to facilitate thatch-mat degradation using direct application of laccase, an extra-cellular lignolytic enzyme produced from white-rot fungi *Trametes versicolor*, on turfgrass was reported to be effective in reducing organic matter content and thatch layer thickness in creeping bentgrass (Chapter III). However, a net accumulation of organic matter in thatch layer treated with laccase was observed over time (Chapter III). This study was designed to observe the potential of laccase enzyme to facilitate organic matter decomposition in a system where accumulation of organic matter is ceased. The major objectives of this study are: 1) to determine the potential of laccase enzyme to facilitate organic matter degradation in thatch layer; and 2) to determine the changes in physical and chemical composition properties of thatch layer due to application of laccase.

MATERIALS AND METHODS

A greenhouse experiment was conducted using 'Crenshaw' creeping bentgrass (Engelke et al., 1995) *Agrostis stolonifera* L., established in pots (top diam. 15 cm, height 11.5 cm) at The University of Georgia, Griffin Campus from December 2009 to June 2010. The bentgrass was acquired from East Lake Country Club, Atlanta, Georgia. Pots were partially filled with 85:15 sand and organic matter mix and sod approximately 3 cm in thickness was cut to fit the pots and placed on top of the mix. All pots were established in June 2008 and grown in a controlled environment greenhouse for approximately eighteen months prior to initiation of treatments to facilitate development of thatch layer in the pots. The refrigerated air conditioned greenhouse was maintained at 25± 2 / 18 ±2^{o}C, day/night temperature maintained by a Wadsworth Step 50 controller (Wadsworth Control System, Arvada, Co) under natural lighting (approximately 85% ambient light). Pots were irrigated daily, fertilized monthly with a 50-mL solution of 0.4% (w/v) Macron water soluble 28-7-14 fertilizer (Lesco. Strongsville, OH), and maintained by hand clipping weekly at a height of 2.5 cm with clippings removed to develop favorable conditions for thatch development in the pots.

Prior to the treatment initiation in December 2009, creeping bentgrass in the pots was clipped down to the thatch layer and growth was ceased by application of a herbicidal solution containing 1.3% (v/v) of Roundup Pro® (isopropylamine salt of glyphosate, Monsanto, St. Louis, MO) and 1.3% (v/v) Finale® Herbicide (glufosinate ammonium, Bayer Environmental Science, Montvale, NJ). To block any natural or artificial light from reaching the pots and avoid any further growth in the pots, the pots were covered with two 76.2 µm thick sheets of black plastic sheeting cut from Husky Contractor Clean-up bags (item no HK42WC032B, Poly America, Grand Prairie, TX). The treatment design was a three by two factorial with all combinations of

three levels of laccase and two levels of guaiacol (2-methoxyphenol). The three laccase activity levels were 0 (control), 2.06 and 20.6 units cm^{-2} and guaiacol levels were 0 and 0.1 M solution. The experimental design was a randomized complete block with five replications and sampling times of two and six months. Forty milliliter solutions of the different laccase activity levels were applied uniformly every two weeks to each pot using a hand-held sprayer. The control was applied as 40 mL of distilled water. Guaiacol levels were applied as 10 mL of 0.1M solution. Guaiacol is a natural co-substrate and mediator of laccase believed to enhance enzyme performance (Roper et al., 1995). Soil moisture content was maintained in the pots to favor microbial activity during the 6 months by irrigating the pots twice a week.

Measurements

Effectiveness of treatments on chemical and physical properties of thatch-mat layer was determined after two and six months of treatment application. Variables measured included thatch layer thickness (TLT) and saturated hydraulic conductivity (SHC). Similarly, the impact of treatment application on chemical composition properties of thatch layer biomass was determined by measuring organic matter content for a depth of 0-2.5 cm (OM_U), 2.5-5.0 cm (OM_L), and 0-5.0 cm (OM), extractive-free acid-soluble lignin (L_S) and acid-insoluble lignin (L_I), monomeric components of structural polysaccharides including glucose (S_{GLU}), xylose (S_{XYL}), arabinose (S_{ARA}), and galactose (S_{GAL}) content after two and six months of treatment application. Total lignin (L_T) was obtained by addition of acid-soluble and-insoluble lignin contents. Total sugar (S_T) was obtained by addition of values for glucose, xylose, arabinose, galactose, and mannose.

Laccase Activity Assay

The laccase enzyme from *Trametes versicolor,* a white-rot fungus, was purchased from Sigma-Aldrich (product 53739, Sigma Aldrich Inc., St. Louis, MO.). The activity of laccase was quantified using a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA) spectrophotometer by a colorimetric assay where one activity unit of laccase corresponds to the amount of enzyme that causes an absorbance change at 468 nm at a rate of 1.0 unit min^{-1} in 3.4 mL of 1 mM 2, 6-dimethoxyphenol, a specific substrate for laccase, in citrate-phosphate buffer at pH 3.8 (Park et al., 1999). Laccase activity treatments of 0 (control), 2.06 and 20.6 units cm^{-2} actually corresponds to laccase solutions with activity levels of 0, 9.12 and 91.2 units mL^{-1}, respectively. The activity level of laccase applied per unit area was calculated by dividing total number of units of laccase in 40-mL laccase solution by the top surface area of the pot.

Organic matter Content

The measurement of OM was done as described by Carrow et al. (1987). Two soil cores (2.0 cm diam.) were obtained at 0-2.5 cm (OM_U) and 2.5-5.0 cm (OM_L) depth from the pot. The cores were dried in an oven at 100 ± 5^oC for 24 h and weighed. Soil cores were ashed in a muffle furnace at 600 ± 10^oC for 24 h and weighed again. Organic matter content was determined as the difference in the two readings and percent organic matter was calculated.

Saturated Hydraulic Conductivity

An intact core (diam. 4.7 cm and length 7.7 cm) was obtained from the center of each pot in a brass cylinder using a soil corer (Model 0200 soil sampler, Soilmoisture Equip. Corp., Santa Barbara, CA) The bottom of the core was covered with a double layer of cheesecloth held in place with a rubber band and saturated overnight in a 0.05 N $CaCl_2$ solution to minimize dispersion. The SHC of the cores was measured by a constant hydraulic head method using a

Marriott tube apparatus. A steady state flow through the samples was established by flowing 0.05 N $CaCl_2$ through the core for 10 min. After 10 min the volume of water that passed through the core was measured for one minute and repeated three times. Saturated hydraulic conductivity was calculated using Darcy's equation.

Thatch Layer Thickness

After cores were removed for measurement of OM and SHC, contents were removed from pots and distinct separations among the thatch-mat and soil interface were clearly visible. The TLT were measured from seven different locations around the edges of the plant/root mass and averaged.

Extractive-free Lignin Content

Thatch was collected from each pot from the top 2.5 cm after sampling for OM and SHC. Thatch samples were first washed, dried, and ground and then passed through a series of sieves with a 841µm sieve at the top and a 177µm sieve at the bottom. The material left on the top of largest sieve size was reprocessed and the material that passed through the smallest sieve was discarded. The material retained by the 177 µm sieve size was retained and used for analysis. The thatch was extracted for 24 h using the Soxhlet method for water- and alcohol-soluble impurities using de-ionized water and 16.26 M (95 percent USP grade) ethyl alcohol, respectively. Extractive-free L_S and L_I content in the thatch layer was determined in a two-step acid-hydrolysis procedure according to the laboratory analytical procedure developed by The National Renewable Energy Laboratory (NREL, 2008). In the first step, extractive-free thatch samples were hydrolyzed for 60 min with 72% H_2SO_4 at 30^oC. In the second step, H_2SO_4 was diluted to 4% and the samples were autoclaved at 121^oC for 1 h and then vacuum filtered. Acid-soluble lignin was determined using this hydrolysis liquid at 240 nm wavelength in a Beckman

DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The solids remaining after acid hydrolysis were dried in an oven at 100 ± 5^{o}C for 24 h, weighed, ashed in a muffle furnace at 600 ± 10^{o}C for 24 h, and weighed again. Weight difference was used to calculate the acid-insoluble lignin content.

Extractive-free Sugar Content

The sugar content for glucose (S_{GLU}), xylose (S_{XYL}), arabinose (S_{ARA}), and galactose (S_{GAL}) was determined from hydrolysis liquid collected after vacuum filtration in the above step. The hydrolysis liquid was neutralized to a pH range 7.0-8.0 using $NaHCO_3$ (sodium bicarbonate) and monosaccharide sugars were determined using high performance liquid chromatography (HPLC) in an Agilent 1100 HPLC (Aligent Technologies, Waldbronn, Germany) with binary pump and refractive index detector. An AMINEX HPX-87P 7.8 x 300 mm Pb^{2+} carbohydrate analysis column (Bio-Rad, Hercules, CA) was used at 85^{o}C with deionized water as mobile phase at a flow rate of 0.6 mL min^{-1}. These monosaccharide sugars are components of structural polysaccharides, cellulose and hemicellulose.

Statistical Analysis

Analysis of variance (ANOVA) was performed to evaluate the main effects of treatment duration, laccase, and guaiacol and interaction effects of these three factors using general linear model (GLM) (SAS Institute, 1994). Strong treatment duration effects were observed in the initial analysis and therefore each treatment duration was analyzed separately as a two factor study consisting of three levels of laccase enzyme and two levels of guaiacol. However, due to lack of sample availability for extractive-free sugar analysis after two and six months, four and three replications were considered, respectively. Fisher's protected LSD test with $\alpha = 0.05$ was used for determining statistical differences among treatment means following each ANOVA.

RESULTS

The results will be explained on the basis of the ANOVA table showing the effect of treatment application on TLT, OM, L_S, L_I, and SHC (Table 4.1) and on sugar content (Table 4.2). The result section will be divided into the combined analysis and analysis of data collected after two and six months of treatment application.

Combined Analysis

The full statistical model representing the main and interaction effects of treatment duration, three levels of laccase, and two levels of guaiacol for OM_U (0-2.5 cm), OM_L (2.5-5.0 cm), OM (0-5.0 cm), SHC, TLT, L_S, L_I, L_T (Table 4.1) and S_{GLU}, S_{XYL}, S_{GAL}, S_{ARA}, S_T (Table 4.2) was used to compare common parameters at the two and six month sampling dates.

Strong duration effects ($P \leq 0.001$) were observed for OM_U, OM, SHC, TLT, L_S, L_I, L_T (Table 4.1), S_{GLU}, S_{XYL}, S_{GAL}, S_{ARA}, and S_T (Table 4.2). Laccase application significantly effected OM_U ($P \leq 0.001$), OM ($P \leq 0.05$), SHC ($P \leq 0.001$), TLT ($P \leq 0.001$), L_S ($P \leq 0.001$), L_I ($P \leq 0.001$), L_T ($P \leq 0.001$) (Table 4.1), S_{GLU} ($P \leq 0.05$), S_{XYL} ($P \leq 0.001$), S_{ARA} ($P \leq 0.001$), and S_T ($P \leq 0.01$) (Table 4.2). A significant effect of guaiacol was observed for lignin content ($P \leq 0.001$) (Table 4.1) and ($P \leq 0.05$) for S_{GLU}, S_{ARA}, and S_T (Table 4.2). A significant interaction for treatment duration and laccase for OM_U, S_{GLU}, S_{XYL}, S_{ARA}, and S_T (Table 4.1 and Table 4.2) were largely due to the lack of response to laccase treatments after two months as opposed to a strong response seen after six months. Similarly, a significant interaction effect for guaiacol and treatment duration was observed in S_{GLU}, S_{XYL}, S_{ARA}, and S_T (Table 4.2). A very strong interaction effect for treatment duration and laccase ($P \leq 0.001$), and treatment duration and guaiacol ($P \leq 0.001$) was observed for lignin content (Table 4.1).

Analysis after Two Months Treatment

After two months of treatment application, laccase treatments had no effect on OM_U, OM_L, OM (Table 4.1), and sugar content (Table 4.2) but had strong effects ($P \leq 0.001$) on SHC, TLT, L_S, L_I (Table 4.1, Table 4.2). Guaiacol had significant effects on L_I and L_T levels and the laccase by guaiacol interaction was significant for L_I (Table 4.1). The L_I value increased significantly with the application of guaiacol at each laccase activity level.

Saturated hydraulic conductivity increased from 11.6 cm h^{-1} in control pots to 24.4 and 26.1 cm h^{-1} in pots treated with and without 20.6 units cm^{-2} laccase (Fig 4.1). Thatch layer thickness decreased by 2.0 mm (14.5%), 3.0 mm (22.12%), and 2.9 mm (21.9%) when compared to control in pots treated with 2.06 units cm^{-2} with guaiacol, 20.6 units cm^{-2} without guaiacol, and 20.6 units cm^{-2} with guaiacol, respectively (Fig 4.2). Similarly, L_S decreased by 3.1, 7.8, and 7.5 $mg \cdot g^{-1}$ and L_I increased by 10.4, 9.5, and 17.0 $mg \cdot g^{-1}$ over control with the same treatments (Table 4.3). A slight increase of 7.3 and 9.6 $mg \cdot g^{-1}$ over control was observed for L_T with treatments 2.06 and 20.6 units cm^{-2} applied along with guaiacol (Table 4.3). However, no difference was observed in organic matter at different depths (Table 4.1, Fig 4.3), or for sugar content (Fig 4.4, Fig 4.5, Fig 4.6).

Analysis after Six Months Treatment

After six months of treatment application, laccase application impacted OM_U ($P \leq 0.001$), OM ($P \leq 0.01$), SHC ($P \leq 0.001$), TLT ($P \leq 0.001$), L_S ($P \leq 0.01$), L_I ($P \leq 0.001$), L_T ($P \leq 0.001$) (Table 4.1), S_{GLU} ($P \leq 0.05$), S_{ARA} ($P \leq 0.01$), and S_T ($P \leq 0.01$) (Table 4.2). Laccase application had no effect on OM_L, S_{XYL}, and S_{GAL} after six months of application. A significant effect of guaiacol was also observed for L_S, L_I, L_T, S_{GLU}, S_{ARA}, S_T (Table 4.1 and Table 4.2). Strong laccase by guaiacol interaction effect ($P \leq 0.001$) was observed for L_S, L_I and L_T (Table 4.1). An

increase in L_I and L_T and decrease in L_S values were observed with addition of guaiacol along with laccase (Table 4.3).

Compared to control pots, a reduction of 12.0 (22.3%), 10.2 (18.9%), 13.3 (24.7%), and 12.8 $mg \cdot g^{-1}$ (23.9%) for OM_U (Fig 4.3) and 4.5 (43.8%), 3.8 (38.0%), 5.8 (57.2%), and 6.3 mm (62%) for TLT (Fig 4.2) was observed with treatments of 2.06 and 20.6 units cm^{-2} of laccase without and with guaiacol, respectively. However, no effect was observed with any treatment for OM_L and OM after six months of treatment application (data not shown). An increase of 43.9, 41.1, 70.8, 67.1% for SHC (Fig 4.1) and 17.1, 35.0, 27.7, and 46.9% for L_I (Table 4.3) was observed for laccase activity levels of 2.06 and 20.6 units cm^{-2} without and with guaiacol, respectively. However, contrasting results for L_S were obtained after six months of treatment application. Acid-soluble lignin was found to be slightly reduced in laccase treatments with guaiacol and slightly increased over control in laccase treatments without guaiacol (Table 4.3).

After six months of treatment, significant reductions in the monosaccharide components of structural cellulose and hemicellulose sugar were observed. In comparison to the control pots, a reduction of 32.2, 34.5, and 29.3% was observed for S_{GLU}, S_{ARA}, and S_T, respectively in pots treated with 2.06 units cm^{-2} along with guaiacol (Fig 4.4, Fig 4.5, Fig 4.6). Similarly, a reduction of 28.4, 33.2, and 25.7% was observed for S_{GLU}, S_{ARA}, and S_T, respectively in pots treated with 20.6 units cm^{-2} when applied along with guaiacol when compared to control pots (Fig 4.4, Fig 4.5, Fig 4.6). However, when only laccase was applied without guaiacol, the reduction in S_{ARA} and S_T over the control was 25.2 and 15.6% for 2.06 units cm^{-2} and 23.7 and 17% for 20.6 units cm^{-2} treatment (Fig 4.5, Fig 4.6). No significant effect of any treatment was observed for S_{XYL}, and S_{GAL} after six months of treatment application (data not shown).

DISCUSSION

Laccase Application

In our previous greenhouse study on living creeping bentgrass, we reported that the direct application of laccase enzyme at 2.06 units cm^{-2} of activity level applied every two weeks was effective in reducing thatch-mat depth, OM, and significantly increasing SHC (Chapter III). However, an overall increase in OM (0-5.0 cm) and organic layer thickness was observed for all the treatments over the experiment duration. OM (0-5.0 cm) increased by 46.9 and 23.7% in control pots and pots treated with 2.06 units cm^{-2} laccase, respectively over a period of seven months (Chapter III). In our present study, the active growth of creeping bentgrass was ceased by the application of herbicides which created a system where there is no addition of organic matter. Our results indicate that application of 2.06 and 20.6 units cm^{-2} laccase with and without guaiacol could result in altering organic matter dynamics in a positive manner by effectively reducing OM_U (0-2.5 cm) and TLT in comparison to the control.

After two months of treatment application, physical changes in the thatch-mat profile was observed in TLT and SHC with laccase applications at 20.6 units cm^{-2} with and without guaiacol (Fig 4.1, Fig 4.2). However, no significant effect was observed for organic matter at depths of 0-2.5 and 2.5-5.0 cm with any treatment. The structural changes in the thatch biomass after two months were associated with significant reductions in L_S with all laccase treatments and a slight increase in the L_I when laccase was applied without guaiacol (Table 4.3). However, a slight overall reduction in L_T was detected at both laccase treatment levels after two months when applied with guaiacol. The data obtained for monosaccharaide levels showed no significant changes after two months of treatment application (Fig 4.4, Fig 4.5, Fig 4.6).

After six months of treatment application, significant structural changes in TLT and SHC in the thatch-mat profile were observed over control with both activity levels of laccase whether applied with or without guaiacol (Fig 4.1, Fig 4.2). An increase in SHC over control treatments can be explained due to the decrease in the thatch layer depth associated laccase treatments. The presence of higher levels of organic matter in the thatch layer is known to hinder water infiltration through the thatch layer (McCarty et al., 2005). Laccase treatments at 2.06 and 20.6 units cm^{-2} activity were found to be effective in reducing organic matter content at 0-2.5 cm depth when applied with and without guaiacol (Fig 4.3).

Structural changes in the thatch layer biomass after six months of treatment application were recorded by the reduction in S_{GLU}, S_{ARA}, and S_T (Fig 4.4, Fig 4.5, Fig 4.6). This reduction in the sugar content in the biomass can be attributed to the opening up of biomass structure by breaking the protective matrix of lignin, making cellulose and hemicellulosic structural components of plant cell wall more accessible for microbial degradation. The cellulose and hemicellulose components are covalently bonded with lignin macromolecules in different ways such as ester-ether cross-links (Grabber et al., 2000; 2004; Ralph et al., 1995; 2004), direct ester linkages (Imamura et al., 1994; Joseleau and Gancet, 1981), benzyl ether linkages (Grabber et al., 2004; Lam et al., 1990; Watanabe et al., 1989), and phenyl glycoside linkages. These bonds or associations must be broken in order to access the sugars. However, increases in the acid-soluble and -insoluble components were observed after application of both activity levels of laccase with and without guaiacol. This increase in lignin content can be attributed to the overall reduction in the structural carbohydrate (sugar) content where lignin and structural carbohydrates are the major components in the plant cell walls. Lignin percentage is calculated by expressing lignin as a percentage of the total of lignin plus carbohydrates and thereby dependent upon the

carbohydrate content. Therefore, when the carbohydrate content is lowered, lignin percentage increases

Treatment Duration

If laccase application was effective in changing the physical, chemical, and structural properties of thatch layer, it would seem reasonable to expect that effects would become more apparent over time. Samples were analyzed after two and six months of treatment application. After two months, only laccase applications at the highest activity level of 20.6 units cm^{-2} were effective in reducing TLT, L_S, and increasing SHC and application of the lower laccase activity level of 2.06 units cm^{-2} only impacted lignin content. However, after six months of treatment application, laccase treatments of 2.06 and 20.6 units cm^{-2} with and without guaiacol proved equally effective in reducing TLT, OM_U, S_{GLU}, S_{ARA}, and S_T. This result indicates that application of laccase with lower activity levels over a period of time could just be as effective as application laccase solutions with 10 times higher activity . The effect of guaiacol on structural properties of thatch biomass is evident from effects of guaiacol and the interaction of treatment duration and guaiacol on L_S, L_I, L_T, S_{GLU}, S_{ARA}, and S_T (Table 4.1, Table 4.2).

Role of Laccase in Thatch Management

Contrasting results have been reported in the past for reduction in thatch-mat accumulation using various cultural management practices at different cultivation frequencies (Callahan et al., 1998; Carrow et al., 1987; Engel and Alderfer, 1967; McCarty et al., 2005; Rieke, 1994). Cultural practices like core aeration and vertical mowing are disruptive in nature and adversely impact turf quality, further reducing the playability of the turf (Barton et al., 2009; Landreth et al., 2008; McCarty et al., 2007). However, application of laccase was not disruptive,

showed no reduction in turf quality of creeping bentgrass, and proved to be effective when applied at activity levels of 2.06 units cm^{-2} for nine months (Chapter III).

Some efforts in the past to develop non-destructive methods to manage thatch problems using different treatments like sugars, mixtures of sugars and microbial inocula, mixture of amino acids and algae, and some enzymes like cellulase, proved ineffective (Ledeboer and Skogley, 1967; Martin and Dale., 1980; McCarty et al., 2005; Murdoch and Barr, 1976). One of the possible reasons that past studies found these products to be inconsistent in organic matter decomposition is that they focused on degradation of cellulosic and hemicellulosic sugars instead of lignin. Our hypothesis is that the lignin protective matrix has to be at least partially degraded to allow bacterial population to act on the structural sugars. Another possible reason that the above mentioned treatments were ineffective is that most of these treatments intended speed degradation of thatch by simply increasing microbial populations within the thatch layer. Maintaining higher microbial populations over sustained periods of time under field turfgrass management systems is very difficult due to the inability to maintain proper micro-environment conditions, particularly moisture and temperature regimes, required by particular microbial populations.

Laccases are multi-copper oxidases that mediate the oxidation of a wide range of mono- and diphenols using oxygen as the electron acceptor (Baldrian, 2006). Laccase-mediated oxidation leads to decomposition of lignin phenolic components via Cα-Cβ cleavage, alkyl-aryl cleavage, and Cα oxidation (Wong, 2009). This cleavage of different covalent bonds formed within lignin macromolecule and between lignin and structural sugars open up the biomass structure leading to increased availability of easily degradable sugars by microbes. The laccase enzyme we used is a natural product produced by the white-rot fungi *Trametes versicolor*, and is

stable over a wide range of pH and temperature (Baldrian, 2006; Munoz et al., 1997; Stoilova et al., 2010; Thurston, 1994). By using laccase enzyme, we can effectively manage thatch over wide range of environmental conditions and can better utilize the microbial decomposition of organic matter.

CONCLUSIONS

This greenhouse research demonstrated the efficacy of bi-weekly applications of laccase enzyme at 2.06 and 20.6 units cm^{-2} to change physical/structural and chemical composition properties of the thatch layer of creeping bentgrass turf. Laccase application was effective in reducing organic matter content, thatch layer thickness, and sugar content and in increasing saturated hydraulic conductivity. Laccase application at 2.06 units cm^{-2} was not effective after two months of treatment application in reducing thatch layer thickness and sugar content but was effective after six months of application. Duration of treatment application had a significant positive effect on organic matter degradation. Implications of these findings point that laccase application at 2.06 units cm^{-2} can be as effective as laccase activity level of 20.6 units cm^{-2} when applied over a period of time. This approach has the potential as a new non-disruptive method for thatch management. Future research is needed to observe the effectiveness of laccase under field conditions.

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Table 4.1 Analysis of variance (ANOVA) table showing the effects of treatment duration, laccase application, guaiacol application, and their interactions on dead creeping bentgrass thatch maintained in a greenhouse.

Source of Variation	df	Organic matter (OM_U) (0-2.5 cm)	Organic matter (OM_L) (2.5-5.0 cm)	Organic matter (OM) (0-5.0 cm)	Saturated hydraulic conductivity (SHC)	Thatch layer thickness (TLT)	Acid-soluble lignin (L_S)	Acid-insoluble lignin (L_I)	Total lignin (L_T)
		$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	$cm\ h^{-1}$	mm	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$
		mean square values							
Full Model									
Treatment duration	1	641.5***	33.7	895.8***	6340.6***	426.6***	190.5***	65449***	72701***
Laccase	2	414.1***	12.7	94.2*	1290.7***	93.3***	79.7***	18073***	15999***
Guaiacol	1	8.7	27.0	35.0	0.2	1.7	22.6***	5686.6***	4992.1***
Duration*laccase	2	143.6*	41.6	47.5	53.4	7.0	69.7***	11713***	13517***
Duration*guaiacol	1	38.8	2.02	7.5	1.5	0.2	22.8***	2250.4***	1819.7***
Error	50	36.1	36.5	27.6	20.1	2.3	0.7	90.5	090.05
2 Months									
Laccase	2	51.8	7.2	4.0	446.3***	25.8***	146.1***	455.7***	91.24
Guaiacol	1	42.2	7.1	37.5	1.5	0.4	0.03	391.2***	391.90**
Laccase*guaiacol	2	10.8	11.2	10.0	10.4	2.5	0.2	107.8*	109.99
Error	24	50.01	50.1	32.4	29.5	1.4	0.8	25.5	29.09
6 Months									
Laccase	2	505.9***	47.1	137.7**	897.7***	74.53***	3.4**	29331***	29425***
Guaiacol	1	5.3	21.9*	5.0	0.3	1.5	45.4***	7545.8***	6420.0***
Laccase*guaiacol	2	1.9	22.7	9.0	3.9	2.7	4.5***	2719.5***	2510.7***
Error	24	24.5	23.1	23.7	11.9	2.8	0.4	83.9	87.4

* Significant at the 0.05 probability level
**Significant at the 0.01 probability level
*** Significant at the 0.001 probability level

Table 4.2 Analysis of variance (ANOVA) table showing the effects of treatment duration, laccase application, guaiacol application, and their interactions on sugar content on dead creeping bentgrass thatch maintained in a greenhouse.

Source of Variation	df	Glucose (S_{GLU})	Xylose (S_{XYL})	Glactose (S_{GAL})	Arabinose (S_{ARA})	Total sugar (S_T)
		$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$	$mg \cdot g^{-1}$
		----------mean square values----------				
Full Model						
Treatment duration	1	27543.5***	9278.6***	2402.9***	2727.1***	126250***
Laccase	2	2394.7*	417.5***	288.3	497.0***	10525.5**
Guaiacol	1	2964.5*	294.0	32.6	226.4*	8438.9*
Duration*laccase	2	2704.84**	538.9*	67.3	308.8***	9353.0**
Duration*guaiacol	1	4280.7**	759.6*	174.7	139.7*	13815**
Error	32	440.6	152.6	180.8	31.5	1565.9
2 Months						
Laccase	2	78.1	4.6	65.9	13.0	228.2
Guaiacol	1	70.3	63.2	32.8	6.0	384.5
Laccase*guaiacol	2	72.0	8.3	2.1	24.5	317.0
Error	18	18.4	112.4	217.8	26.1	1333.3
6 Months						
Laccase	2	4403.5*	833.4	261.8	695.3***	17222.6**
Guaiacol	1	6286.9**	874.5	156.7	315.8*	19183.9*
Laccase*guaiacol	2	115.7	43.7	23.8	2.5	334.6
Error	12	671.0	231.5	152.1	42.6	2128.1

* Significant at the 0.05 probability level
**Significant at the 0.01 probability level
*** Significant at the 0.001 probability level

Table 4.3 Extractive-free acid-soluble (L_S), acid-insoluble (L_I), and total lignin (L_T) content after two and six months of different treatments applied to thatch layer of dead creeping bentgrass.

	Acid-soluble lignin		**Acid-insoluble lignin**		**Total lignin**	
Treatment†	**2 Months**	**6 Months**	**2 Months**	**6 Months**	**2 Months**	**6 Months**
	-------- mg·g^{-1} ---------		**-------- mg·g^{-1} ---------**		**-------- mg·g^{-1} ---------**	
0L (Control)	63.1a‡	63.2a	259.0c	273.9e	322.1cd	337.2e
0L+G	62.9a	62.3d	259.6c	267.6e	322.5bcd	330.0e
2.06L	60.2b	65.5a	255.7c	320.7d	315.9cd	386.2d
2.06L+G	60.0b	62.0de	269.4b	369.7b	329.4ab	431.7b
20.6L	55.3c	64.2b	268.5b	349.9c	323.8bc	414.2c
206L+G	55.6c	61.2e	276.0a	402.4a	331.7a	463.6a

† L denotes Laccase level and G the addition of Guaiacol, a mediator.
‡ Means within a column followed by the same letter are not significantly different according to LSD at α=0.05.

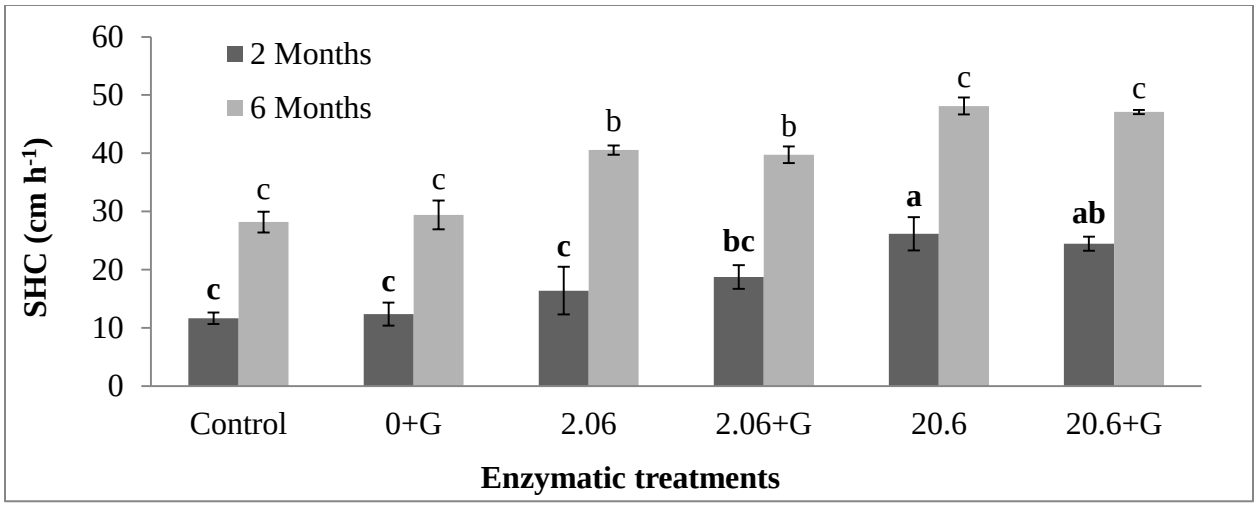

Fig. 4.1 Saturated hydraulic conductivity (SHC) after two and six months of treatment on creeping bentgrass thatch biomass with three different levels of laccase (0 (control), 2.06 and 20.6 units cm^{-2}) with and without the mediator, guaiacol (G). Values are means of five replicates and error bars are standard errors. Bars with the same letter (2 months = bolded and 6 months = standard) are not considered to be statistically different according to LSD at $\alpha = 0.05$.

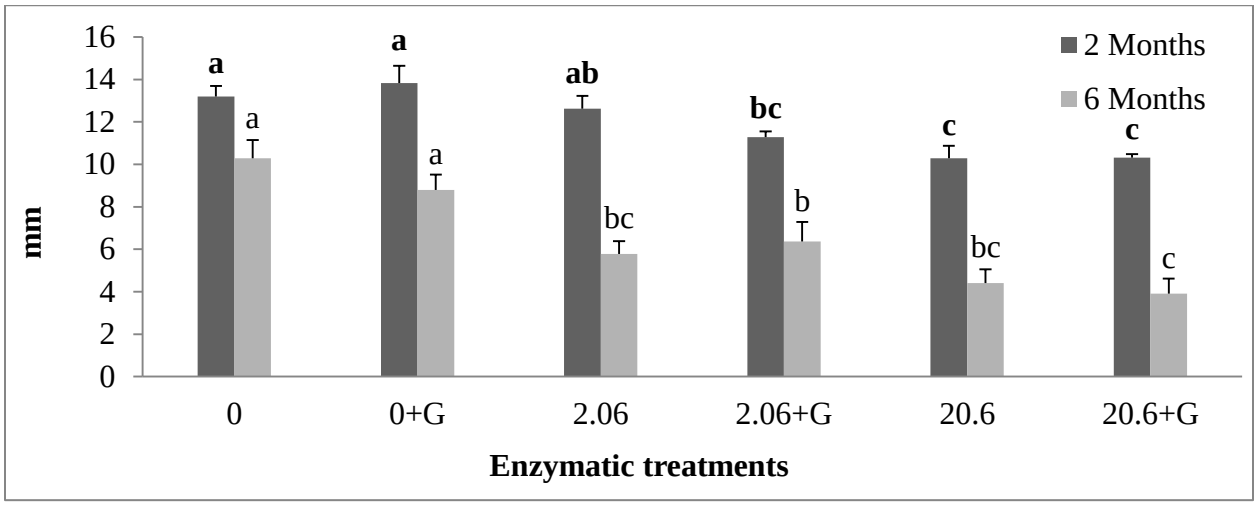

Fig. 4.2 Thatch layer thickness (TLT) after two and six months of treatment on creeping bentgrass thatch biomass with three different levels of laccase (0 (control), 2.06 and 20.6 units cm^{-2}) with and without the mediator, guaiacol (G). Values are means of five replicates and error bars are standard errors. Bars with the same letter (2 months = bolded and 6 months = standard) are not considered to be statistically different according to LSD at $\alpha = 0.05$.

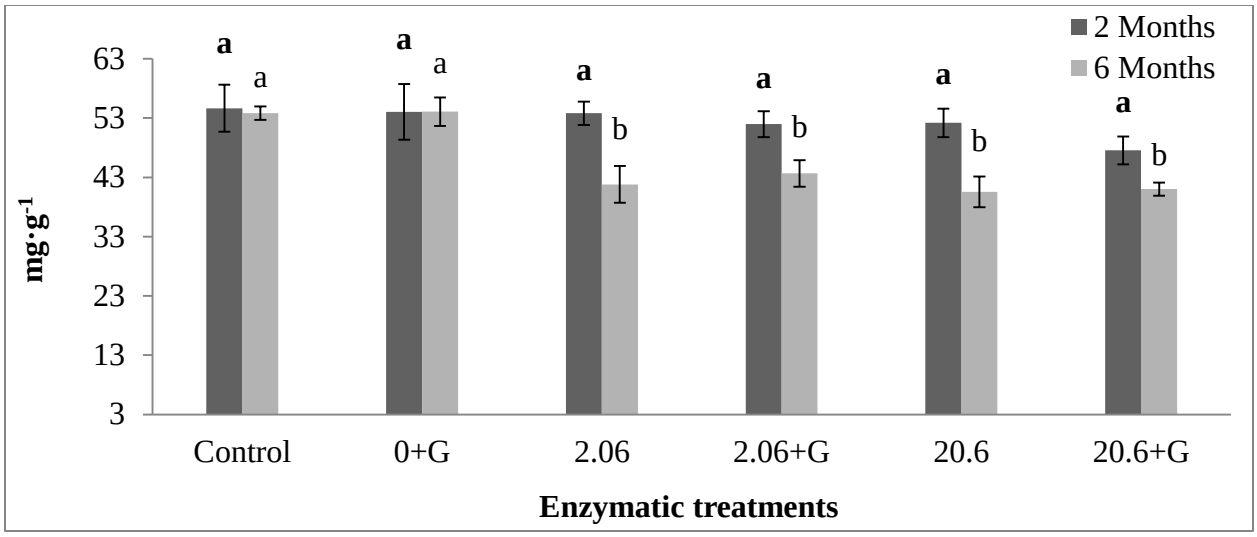

Fig. 4.3 Organic matter content (0-2.5 cm; OM_U) after two and six months of treatment on creeping bentgrass thatch biomass with three different levels of laccase (0 (control), 2.06 and 20.6 units cm^{-2}) with and without the mediator, guaiacol (G). Values are means of five replicates and error bars are standard errors. Bars with the same letter (2 months = bolded and 6 months = standard) are not considered to be statistically different according to LSD at $\alpha = 0.05$.

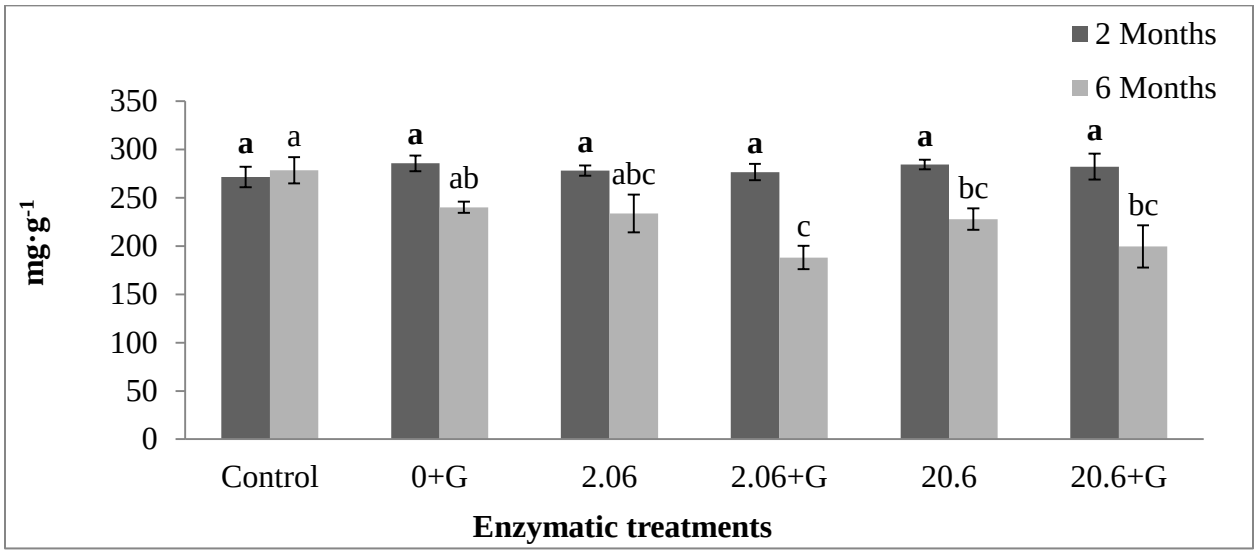

Fig. 4.4 Glucose (S_{GLU}) after two and six months of treatment on creeping bentgrass thatch biomass with three different levels of laccase (0 (control), 2.06 and 20.6 units cm^{-2}) with and without the mediator, guaiacol (G). Values are means of five replicates and error bars are standard errors. Bars with the same letter (2 months = bolded and 6 months = standard) are not considered to be statistically different according to LSD at $\alpha = 0.05$.

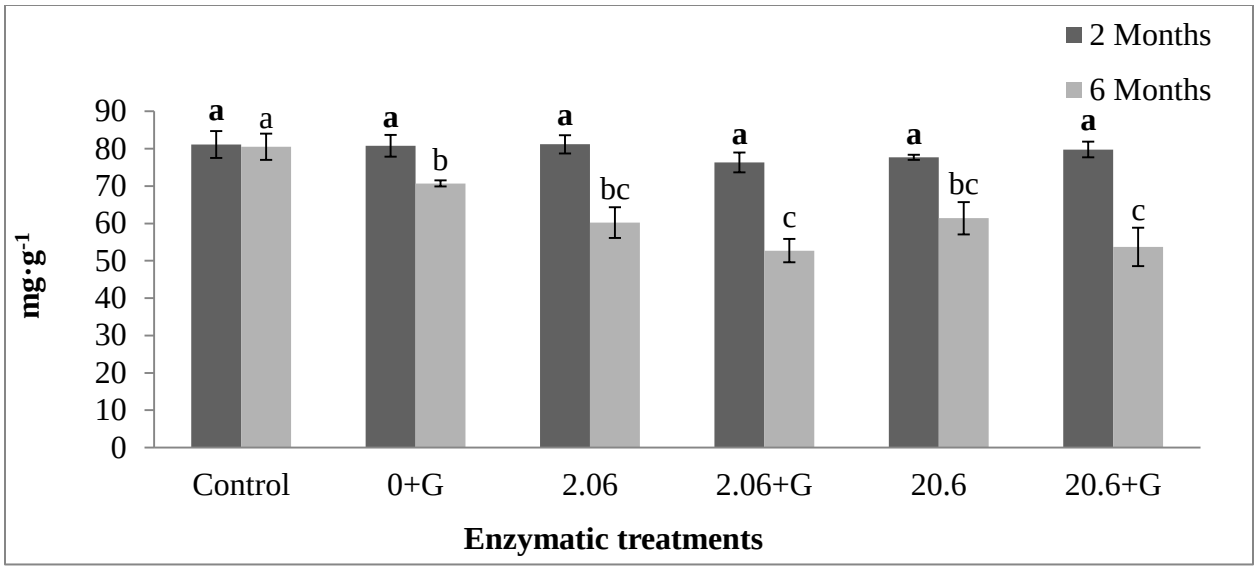

Fig. 4.5 Arabinose (S_{ARA}) after two and six months of treatment on creeping bentgrass thatch biomass with three different levels of laccase (0 (control), 2.06 and 20.6 units cm^{-2}) with and without the mediator, guaiacol (G). Values are means of five replicates and error bars are standard errors. Bars with the same letter (2 months = bolded and 6 months = standard) are not considered to be statistically different according to LSD at $\alpha = 0.05$.

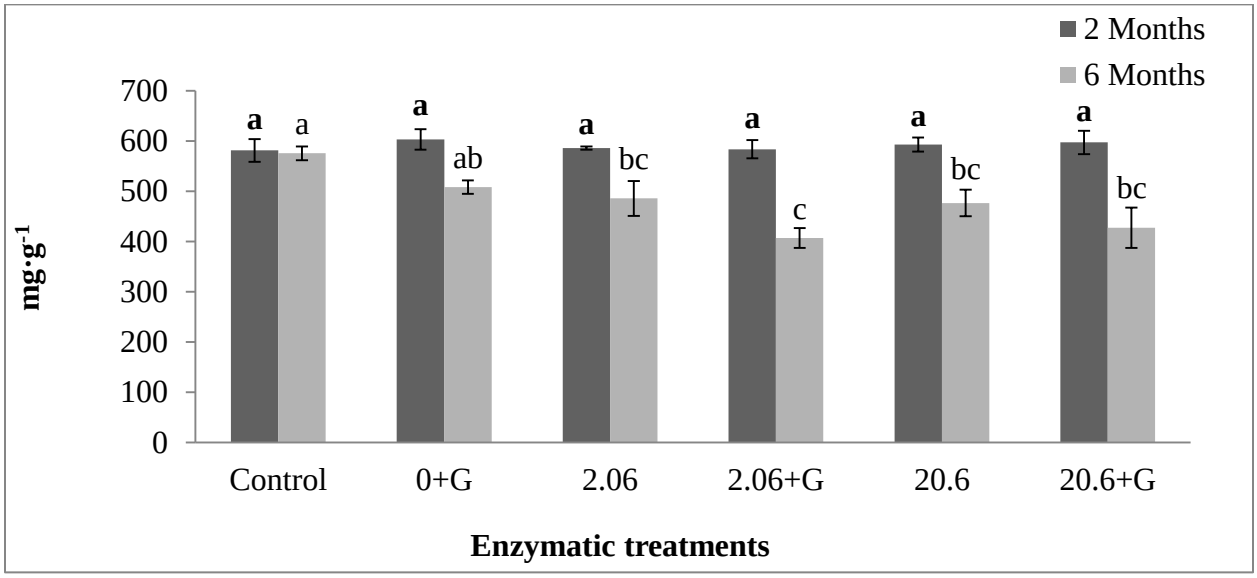

Fig. 4.6 Total sugars (S_T) after two and six months of treatment on creeping bentgrass thatch biomass with three different levels of laccase (0 (control), 2.06 and 20.6 units cm^{-2}) with and without the mediator, guaiacol (G). Values are means of five replicates and error bars are standard errors. Bars with the same letter (2 months = bolded and 6 months = standard) are not considered to be statistically different according to LSD at $\alpha = 0.05$.

CHAPTER V

OPTIMIZING LACCASE APPLICATION ON CREEPING BENTGRASS (*Agrostis stolonifera* L.) TO FACILITATE BIODETHATCHING[3]

[3] Sidhu, S.S., Q. Huang, R.N. Carrow, and P.L. Raymer. To be submitted to *Crop Science*.

ABSTRACT

Organic matter buildup in the form of thatch or mat layers leads to several problems in turfgrass management systems. In a previous study, laccase enzyme solution proved effective in reducing the rate of accumulation of organic matter when applied at an activity level of 2.0 units cm^{-2} every two weeks for nine months on 'Crenshaw' creeping bentgrass (*Agrostis stolonifera* L.). A two year field experiment was conducted on creeping bentgrass to optimize the laccase activity level, frequency of application; to determine potential interactions with core aeration and topdressing cultural practices; and to compare enzyme sources. Laccase enzyme was applied at five activity levels 0, 0.5, 1.0, 2.0 and 4.0 units cm^{-2} applied every two weeks. Frequency of laccase application was tested using a laccase activity level of 2.0 units cm^{-2} applied at frequency of 2, 4, 8, or 12 weeks. The common cultural management practice of core aeration and sand topdressing was compared with and without laccase enzyme at an activity level of 2.0 units cm^{-2} applied once a month. Three sources of laccase enzyme were also compared when applied at a standard activity level of 2.0 units cm^{-2} every two weeks. Results indicated that laccase treatments were effective at rates as low as 0.5 units cm^{-2} applied every two weeks and as infrequent as once a month when applied at rate of 2.0 units cm^{-2}. Monthly applications of laccase further reduced organic matter accumulation when applied in combination with core aeration and sand topdressing.

INTRODUCTION

One of the major problems in management of recreational turfgrass sites, especially golf greens, is accumulation of organic matter in the form of tightly intermingled dead and living plant tissue between the soil and green turfgrass. This high organic matter layer, known as thatch, consists of stolons, rhizomes, roots, crown tissue, leaf sheaths, and blades (Engel, 1954;

Roberts and Bredakis, 1960). Cultural practices like core aeration followed by sand topdressing may cause formation of a mat layer which is a thatch layer mixed with sand or soil with sand most common on golf greens (McCarty, 2005). Mat layers are more common on greens and physical conditions in a mat layer can vary depending upon whether the percent sand or percent organic matter content dominates with best conditions when the organic matter content is < 4.0% by weight (Carrow, 2004).

Soil physical properties are adversely modified due to excessive accumulation of a thatch or mat layer to the point that organic matter, and not sand, is the dominant matrix. Also, within a mat where bentgrass roots are a major component of total organic matter, high temperatures may induce root dieback resulting in rapidly decaying dead gelatinous organic matter that swells in the presence of water during decomposition and plugs the soil macro-pores (air-filled pores), causing low oxygen levels in the root zones (Carrow, 2004; O'Brien and Hartwiger, 2003), decreased movement of oxygen through the thatch or mat zone, decreased water infiltration, low oxygen levels within the thatch/mat layer during wet periods, and increased water retention (Carrow, 2003; Hartwiger, 2004; McCarty et al., 2007). These conditions often lead to secondary problems like wet wilt, soft surface, increased mower scalp, black layer, limited rooting, and extra- and intra-cellular freezing damage (Beard, 1973; Carrow, 2004; O'Brien and Hartwiger, 2003).

The most effective cultural or mechanical techniques used today such as core aeration, vertical mowing, grooming, and topdressing may adversely impact turf quality, hinder playability, and require intensive inputs for labor and energy (Barton et al., 2009; Landreth et al., 2008; McCarty et al., 2007). In addition, these practices have shown contrasting results in their ability to reduce organic matter content in the thatch or mat layer (Barton et al., 2009; Carrow et

al., 1987; Dunn et al., 1981; McCarty et al., 2005; McWhirter and Ward, 1976; Weston and Dunn, 1985; White and Dickens, 1984). Non-destructive thatch management techniques are highly desired and several past studies using different products like glucose, cellulase solutions (Ledeboer and Skogley, 1967), and commercial products containing mixture of amino acids, microbial inoculum, and fertilizers were inconsistent (McCarty et al., 2005; Murdoch and Barr, 1976). The inconsistencies may be because these different products focused on the degradation of cellulosic and hemicellulosic sugars in thatch biomass by attempting to improve microbial populations.

Accumulation of organic matter in the form of thatch or mat layer is due to the presence of lignin, a plant cell wall constituent that acts as a protective matrix and limits the accessibility of microbial degraders to more biodegradable plant materials, such as cellulosic and hemicellulosic sugars (Ledeboer and Skogley, 1967). Lignin is extremely recalcitrant to degradation due to its complex structure formed by random oxidative couplings of mono-lignols of three primary hydroxycinnamyl alcohols: p-coumaryl, coniferyl, and sinapyl alcohols (Ledeboer and Skogley, 1967; Wong, 2009). Mono-lignols randomly bond in the lignin macromolecule by C-O-C and C-C linkages forming β-O-4, β-5, β-β, 5-5, 4-O-5, and β-1 bonds (Alder, 1977; Del Rio et al., 2007; Ralph et al., 2004). Several models of lignin molecular structure have been proposed but these models do not imply any particular sequence of monomeric units in the lignin macromolecule (Chen and Sarkanen, 2003; Davin and Lewis, 2003).

Lignin acts as the rate limiting step in microbial decomposition of the organic matter and the rate of the degradation progress is directly related to the amount to lignin present in organic matter (Taylor et al., 1989). A close relationship of mass loss with activity of lignocellulose-

degrading enzymes has been reported (Sinsabaugh et al., 1993). Extra-cellular lignolytic enzymes produced by certain white-rot fungi are responsible for natural degradation of lignin (Kirk et al., 1975, 1976). Lignin degradation by extra-cellular enzymes produced by white-rot fungi exposes cellulosic sugars for further microbial degradation (Blanchette, 1984; Mester et al., 2004; Otjen and Blanchette., 1987). Several studies have reported weight loss of organic matter from different turfgrass systems when inoculated with white-rot fungi in controlled conditions (Martin and Dale., 1980; Sartain and Volk, 1984). However, field inoculation experiments on bermudagrass showed no thatch degradation (Martin and Dale., 1980).

Direct application of laccase solution to creeping bentgrass was introduced as a novel approach to facilitate the decomposition of organic matter in a greenhouse study with conditions conducive for thatch development (Chapter III). It is very difficult to maintain specific microbial populations for long periods of time under most turfgrass management systems. The direct application of laccase enzyme, the active end product of white-rot fungi that acts on lignin, reduced the limitations associated with maintaining microbial populations. Relative to the control, laccase treatment with an activity level of 2.0 units cm^{-2} at a bi-weekly interval reduced the rate of organic matter and thatch accumulation, but a net accumulation of organic matter in thatch layer was observed overtime including pots treated with laccase (Chapter III). In contrast, a bi-weekly application of laccase enzyme at 2.0 units cm^{-2} on thatch layer of a dead creeping bentgrass for six months verified the effectiveness of laccase in enhancing the rate of organic matter decomposition and the loss in total sugar content of thatch biomass suggesting that laccase application exposed cellulosic and hemicellulosic sugars for microbial degradation by opening up the biomass structure (Chapter IV).

This field study on creeping bentgrass was designed to: a) test the efficacy of using laccase enzyme under field conditions; b) to optimize the rate and frequency of application of laccase enzyme; c) compare laccase with and without core aeration followed by topdressing; and d) to compare the effectiveness of laccase from three different sources.

MATERIALS AND METHODS

A two year field study on 'Crenshaw' creeping bentgrass, *Agrostis stolonifera* L. (Engelke et al., 1995), was conducted at The University of Georgia, Griffin Campus from June 2010 to Jan 2012. The experiment was conducted on a 20-year old bentgrass green established as a sand based putting green with 90:10 sand and organic matter mix (Michigan peat) based on USGA specifications. The bentgrass green was mowed three times a week by Toro Greensmaster 3100 (The Toro Company, Bloomington, MN) and maintained at a height of 0.42 cm.

Bi-weekly fungicide applications on the green were performed from the third week of April to third week of November to control dollar spot (*Sclerotinia homoeocarpa*), brown patch (*Rhizoctonia solani*), anthracnose (*Colletotrichium graminicola*), Pythium blight (*Pythium aphanidermatum*). The fungicide spray routine for both years consisted of applications of Banner MAXX® at 3.2 L ha^{-1} (14.3% propioconazole, Syngenta Crop Protection, Inc., Greensboro, NC) from April to May. From the last week of May to third week of September fungicide treatments every two weeks consisted of a mixture containing Daconil® (40.4% tetrachloroisophthalonitrile, Syngenta Crop Protection, Inc., Greensboro, NC) at the rate of 11.5 L ha^{-1} alternating with Subdue MAXX® (22% Mefenoxam, Syngenta Crop Protection, Inc., Greensboro, NC) at 1.6 L ha^{-1} and with Banner MAXX® at rate of 9.6 L ha^{-1}. Fertilizer application for both years consisted of 50 kg ha^{-1} granular fertilizer 24-4-10 (Lesco. Strongsville, OH) in the third week of March, September, and October and 2 kg ha^{-1} soluble 20-20-20 fertilizer (JR Peters Inc, Allentown, PA)

every two weeks starting the third week of April thru September made in combination with a fungicide application.

The experiment consisted of four replications of thirteen treatments in year one and ten treatments in year two in a completely randomized block design. Treatments were organized to evaluate: laccase rates; frequency of application; influence of management (core aeration and topdressing); and laccase source (Table 5.1). Each block was divided into two halves, one half received only laccase treatments and the other half received laccase and was core-aerated (Ryan Greensaire 24 Aerator, Johnson Creek, WI; tine diam. 1.27 cm; tine depth 6.25 cm; tine spacing 5.0 x 5.0 cm) and sand topdressed (1134 g per plot, Quikrete Premium Play Sand) using Scotts Precision Green Spreader twice a year. Laccase enzyme produced from white-rot fungi *Trametes versicolor* was purchased from Sigma Aldrich ((product 53739, Sigma Aldrich Inc., St. Louis, MO.) and was sprayed as 410-mL solution at five activity levels [0 (control), 0.5, 1.0, 2.0, and 4.0 units cm^{-2}] at every two weeks. Laccase activity level of 2.0 units cm^{-2} was applied at four different frequencies (2, 4, 8, and 12 weeks). Laccase was also applied at 2.0 units cm^{-2} every 4 weeks on plots core-aerated and sand topdressed to observe the effectiveness of laccase in combination with the cultural management practice. During the first year of the study, laccase enzyme from two different sources, Jiangnan University, China (*Picnoporus* genus) and a commercial industrial distributor was also applied at an activity level of 2.0 units cm^{-2} every two weeks to compare the efficacy of laccase from different sources. Based on unavailability of laccase from Sigma Aldrich and similar results from different laccase enzymes observed in year 1 of the study, the second year treatments were applied using laccase enzyme from Jiangnan University, China. For the sake of brevity, laccase treatments hereafter will be mentioned as activity levels (i.e., rate) followed by the frequency of laccase application in parenthesis.

Laccase Activity Assay

The activity of laccase was quantified by a calorimetric assay using a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA) where one activity unit of laccase corresponds to the amount of enzyme that causes an absorbance change at 468 nm at a rate of 1.0 unit min^{-1} in 3.4 mL of 1 mM 2, 6-dimethoxyphenol, a specific substrate for laccase, in citrate-phosphate buffer at pH 3.8 (Park et al., 1999).

Measurements

Organic matter Content

The measurement of OM was conducted by total loss of ignition as described by Carrow et al. (1987). Two soil cores (2.0 cm diam.) were obtained at 0-2.5 cm (OM_U) and 2.5-5.0 cm (OM_L) depth from the plot and used to determine organic matter at 0-5.0 (OM). The cores were dried in an oven at 100 ± 5^oC for 24 h and weighed. Soil cores were ashed in a muffle furnace at 600 ± 10^oC for 24 h and weighed again. Organic matter content was determined as the difference in the two readings and percent organic matter was calculated.

Saturated Hydraulic Conductivity

Saturated hydraulic conductivity of the intact cores was measured by constant hydraulic head method using a Marriot tube apparatus. An intact core (diam. 4.7 cm and length 7.7 cm) was obtained from each plot in a brass cylinder using a soil corer (Model 0200 soil sampler, Soilmoisture Equip. Corp., Santa Barbara, CA) The bottom of the core was covered with a double layer of cheesecloth held in place with a rubber band and saturated overnight in a 0.05 N $CaCl_2$ solution to minimize dispersion. A steady state flow through the samples was established by flowing 0.05 N $CaCl_2$ through the core for 10 min. After 10 min the volume of water that

passed through the core was measured for one minute and repeated three times. Saturated hydraulic conductivity was calculated using Darcy's equation.

Thatch Layer Thickness

Thatch layer thickness was measured by two replaceable wedge-shaped turf profiles (8.9 cm wide and 2.5 cm thick) using AMS Turf Profiler (AMS Inc., American Falls, ID**).** Thatch layer thickness was measured from four points across the width of each profile and averaged. The clear visible distinction between thatch layer and the sand layer below was considered as the boundary for the measurement.

Extractive-free Lignin Content

The top 2.5 cm thatch samples were collected from each intact core after sampling for SHC. Thatch samples were first washed, dried, and ground and then passed through a series of sieves with a 841µm sieve at the top and a 177µm sieve at the bottom. The material left on the top of largest sieve size was reprocessed and the material that passed through the smallest sieve was discarded. The material retained by the 177 µm sieve size was used for analysis. The thatch was extracted for 24 h using the Soxhlet method for water- and alcohol-soluble impurities using de-ionized water and 16.26 M (95 percent USP grade) ethyl alcohol, respectively. Lignin content after removal of water- and alcohol-soluble extractives from biomass was considered to be extractive-free lignin.

Extractive-free acid-soluble (L_S) and-insoluble lignin (L_I) content in the thatch layer was determined in a two-step acid-hydrolysis procedure according to the laboratory analytical procedure developed by The National Renewable Energy Laboratory (NREL, 2008). Acid-soluble lignin consists of low molecular weight phenolic components of lignin macromolecule. In the first step, extractive-free thatch samples were hydrolyzed for 60 min with 72% H_2SO_4 at

30°C. In the second step, H_2SO_4 was diluted to 4% and the samples were autoclaved at 121°C for 1 h and then vacuum filtered. Acid-soluble lignin was determined using this hydrolysis liquid at 240 nm wavelength in a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The solids remaining after acid hydrolysis were dried in an oven at 100 ± 5°C for 24 h, weighed, ashed in a muffle furnace at 600 ± 10°C for 24 h, and weighed again. Weight difference was used to calculate the acid-insoluble lignin content. Total lignin (L_T) was calculated by adding acid-soluble and-insoluble lignin content.

Extractive-free Sugar Content

Sugar content determined from biomass after removal of water- and alcohol-soluble extractives is known as extractive-free sugar content. Monosaccharide sugars that are components of structural polysaccharides, cellulose and hemicellulose were measured. The total sugar content (T_S) was determined by addition of sugar content for glucose (S_{GLU}), xylose (S_{XYL}), arabinose (S_{ARA}), galactose (S_{GAL}), and mannose (S_{MAN}). The sugar content is measured using hydrolysis liquid collected after vacuum filtration in the above step. The hydrolysis liquid was neutralized to a pH range 7.0-8.0 using $NaHCO_3$ (sodium bicarbonate) and monosaccharide sugars were determined using high performance liquid chromatography (HPLC) in an Agilent 1100 HPLC (Aligent Technologies, Waldbronn, Germany) with binary pump and refractive index detector. An AMINEX HPX-87P 7.8 x 300 mm Pb^{2+} carbohydrate analysis column (Bio-Rad, Hercules, CA) was used at 85 °C with deionized water as mobile phase at a flow rate of 0.6 mL min^{-1}.

Statistical Analysis

Analysis of variance (ANOVA) was performed to evaluate the main effects of treatments using general linear model (GLM) (SAS Institute, 1994). Treatments were grouped together for

analysis related to rate of laccase application, frequency of laccase application, laccase along with cultural management control, and sources of laccase. Analysis of variance (ANOVA) was performed using general linear model on each group of treatments to evaluate effects of treatments in that particular group. Fisher's protected LSD test with $\alpha = 0.05$ was used for determining statistical differences among durations and treatment means following each ANOVA.

RESULTS

Combined Analysis

Significant treatment effects ($P \leq 0.001$) were observed on TLT, L_S, L_I, L_T, and S_T during the first year of treatment application (Table 5.1). During the second year, significant treatment effects for OM_U ($P \leq 0.01$), TLT ($P \leq 0.001$), SHC ($P \leq 0.001$), L_S ($P \leq 0.001$), L_I ($P \leq 0.001$), L_T ($P \leq 0.001$), and S_T ($P \leq 0.05$) were observed (Table 5.2).

Rate of Laccase Application

Laccase treatments 0 (2), 0.5 (2), 1.0 (2), 2.0 (2), and 4.0 (2), rate of laccase activity is followed by frequency of application in parenthesis, are grouped together to observe the effect of rate of laccase application. A significant effect was observed in year 1 and 2 for TLT ($P \leq 0.001$), L_S ($P \leq 0.001$), L_I ($P \leq 0.001$), L_T ($P \leq 0.001$), and S_T ($P \leq 0.05$) (Table 5.1, 5.2). No differences were observed for organic matter and saturated hydraulic conductivity. All laccase treatments decreased TLT by 3.8 to 4.8 mm (20 to 26%) and 4.9 to 5.5 mm (24 to 28%) in comparison to control during year one and two, respectively (Fig 5.1A). No differences were observed for TLT at different rates of laccase application (Fig 5.1A).

Laccase treatments decreased L_S by 6 to 12 $mg \cdot g^{-1}$ and 4 to 12 $mg \cdot g^{-1}$ when compared to control during year one and two, respectively (Fig 5.2A). In year one, laccase treatment 4.0 (2)

reduced L_S from treatments 0.5 (2) and 1.0 (2). In the second year, L_S decreased significantly with increasing laccase activity rate (Fig 5.2A). A 5 to 29 $mg \cdot g^{-1}$ and 13 to 35 $mg \cdot g^{-1}$ reduction in L_I, which makes up the bulk of L_T, was observed over the control for first and second year, respectively with rate of laccase application up to 2.0 units cm^{-2} (Fig 5.3A). A similar reduction of 13 to 38 and 17 to 43 $mg \cdot g^{-1}$ for L_T with laccase application up to 2.0 units cm^{-2} was obtained for year one and two, respectively when compared to control (Fig 5.4A). However, acid-insoluble lignin content was similar to the control at laccase activity level of 4.0 units cm^{-2} (Fig 5.3A).

Total sugar content (S_T) in the thatch biomass decreased by 27 to 69 $mg \cdot g^{-1}$ in year one and by 65 to 105 $mg \cdot g^{-1}$ in year two relative to control with application of laccase (Fig 5.5A). A reduction in S_{GLU}, S_{XYL}, and S_{GAL} was observed when laccase was applied above 1.0 units cm^{-2} during year one (Fig 5.6A, 5.7A, 5.8A). A reduction in S_{GLU} and S_{XYL} content was observed in comparison to control for all the rates of laccase application during year two (Fig 5.6A, 5.7A). No reduction in S_{GAL} was observed for any laccase treatment during year two (Fig 5.8A).

Frequency of Laccase Application

The frequency group consists of control, 2.0 (2), 2.0 (4), 2.0 (8), and 2.0 (12) to observe effect of laccase application frequency on thatch layer properties. A significant effect was observed both years for TLT ($P \leq 0.001$), L_S ($P \leq 0.001$), L_I ($P \leq 0.001$), L_T ($P \leq 0.001$), and S_T when compared to control; and for SHC in year 1 (Table 5.1, 5.2). Thatch layer thickness was reduced in comparison to control when laccase was applied at all the frequencies in both years (Fig 5.1B). Laccase application at eight and twelve weeks in year two showed a slight increase in TLT in comparison to plots receiving laccase application at two and four weeks frequency (Fig 5.1B). Laccase application at all frequencies reduced L_S content in comparison to control in both

years (Fig 5.2B). The decrease in laccase application frequency showed slight increase in L_S contents as is evident from higher L_S content at 8 and 12 week frequency when compared to laccase treatment applied every two weeks in both years (Fig 5.2B). Laccase treatments applied every two and four weeks were effective in reducing L_I and L_T content in comparison to control in both years (Fig 5.3B, 5.4B). As laccase application frequency decreased, L_I and L_T content increased in both years (Fig 5.3B, 5.4B).

Total sugar content (S_T) in the thatch biomass tended to decrease with application of laccase in both years (Fig 5.5B). No change in S_{GLU} content in comparison to control was observed for different laccase treatments during the first year (Fig 5.6B). During second year, a reduction in glucose content of thatch biomass was evident in all plots treated with laccase regardless of frequency compared to control plots (Fig 5.6B). In both years, the S_{XYL} and S_{GAL} contents in thatch biomass tended to be lower than in the control, especially at the 2 week frequency interval (Fig 5.7B, 5.8B).

Cultural Management

Four treatments in the cultural management group were control, CMP, 2.0 (4), and CMP+2.0 (4). Significant treatments effects were obtained for TLT ($P \leq 0.01$), L_S ($P \leq 0.001$), L_I ($P \leq 0.001$), L_T ($P \leq 0.001$), and ST ($P \leq 0.05$) during first year (Table 5.1); and in the second year for OM_U ($P \leq 0.05$), TLT ($P \leq 0.01$), SHC ($P \leq 0.001$), L_S ($P \leq 0.001$), L_I ($P \leq 0.001$), LT ($P \leq 0.001$), and S_T ($P \leq 0.05$) (Table 5.2). In plots treated with CMP, OM content (0-2.5 cm) decreased by 30.3 and 65.7 $mg \cdot g^{-1}$ during year one and two, respectively when compared to control plots (Fig 5.9). Similarly, OM_U decreased from 132 to 113 $mg \cdot g^{-1}$ during first year and from 193 to 108 $mg \cdot g^{-1}$ during second year in plots treated with CMP+2.0 (4) as compared to control plots (Fig 5.9). Thatch layer thickness was lower in plots receiving cultural management

treatments and laccase treatments when compared to control plots (Fig 5.1C). Plots treated with core aeration followed by sand topdressing along with application of laccase once in four weeks showed a significant reduction in thatch layer when compared to laccase application and cultural management treatment (Fig 5.1C). A significant increase in SHC was observed during second year in plots treated with CMP+2.0 (4) as compared to control plots (Fig 5.10).

Compared to control, other treatments in the cultural management group reduced acid-soluble lignin (Fig 5.2C). Plots receiving laccase application had lower levels of L_S as compared to plots treated with CMP in the both years (Fig 5.2C). Relative to control, acid-insoluble lignin content increased in plots receiving CMP and CMP+2.0 (4) treatments; but decreased in plots receiving only laccase enzyme (Fig 5.3C). Plots receiving CMP+2.0 (4) treatment had increased levels of L_I compared to plots receiving only cultural management practice (Fig 5.3C). Total lignin content in plots receiving CMP+2.0 (4) treatment increased when compared to control; but decreased in plots receiving only laccase treatments (Fig 5.4C). Total sugar content in thatch layer biomass from plots treated with CMP+2.0 (4) treatment was significantly lower than the control plots in both years (Fig 5.5C). Similarly, for the CMP+2.0 (4) treatment S_{GLU} (Fig 5.6C), S_{XYL} (Fig 5.7 C), and S_{GAL} (Fig 5.8C) contents were lower when compared to control treatment for both years. Plots treated with CMP showed reduced content of S_{GAL} when compared to control for year one and two (Fig 5.8C). A reduction in xylose content was observed in plots treated with CMP as compared to control in first year but not in the second year (Fig 5.7C). No effect of CMP was observed on the glucose content (Fig 5.6C). Saturated hydraulic conductivity increased in plots treated with CMP by 12.6 to 13.6 cm h^{-1} when compared to control plots and by 6.4 cm h^{-1} in the second year for CMP+2.0 (4) (Fig 5.10).

Source of Laccase Enzyme

Three laccase source treatments are 2.0 (2), CHI, and CHU. Significant treatment effects were observed for L_S ($P \leq 0.05$), L_I ($P \leq 0.001$), and L_T ($P \leq 0.01$). Laccase enzyme from different sources had no effect on TLT, SHC, OM and sugar content. Acid-soluble lignin content was higher in plots treated with CHU and CHI as compared to laccase enzyme from Sigma Aldrich (Fig 5.2D). Plots treated with CHI showed a slightly higher value for L_I and L_T as compared to CHU and 2.0 (2) treatments (Fig 5.3D, 5.4D).

DISCUSSION

Use of Laccase Application to Manage Thatch

Non-destructive methods to manage thatch are highly desirable. Several efforts in the past using different treatments and commercial products like sugars, mixtures of sugars and microbial inocula, mixture of amino acids and algae, and some enzymes like cellulase have showed contrasting results and mostly proved ineffective (Ledeboer and Skogley, 1967; Martin and Dale., 1980; McCarty et al., 2005; Murdoch and Barr, 1976). The inconsistent results of these studies may be attributed to the fact that they were focused to increase microbial population for organic matter decomposition. Maintaining higher microbial populations over sustained periods of time under field turfgrass management systems is very difficult due to the inability to maintain proper micro-environment conditions, particularly moisture and temperature regimes, required by particular microbial species. Another possible reason for contrasting results of the above mentioned studies was that they focused on degradation of cellulosic and hemicellulosic sugars instead of lignin. Our hypothesis is that the lignin protective matrix has to be at least partially degraded to allow bacterial population to act on the structural sugars.

Laccase is an extra-cellular enzyme, a multi copper oxidase, known to oxidize a wide range of phenolic compounds using oxygen as an electron acceptor (Baldrian, 2006). Lignin phenolic components are oxidized due to laccase-mediated cleavage of different covalent bonds such as Cα-Cβ, alkyl-aryl, and Cα oxidation (Wong, 2009). This cleavage of different covalent bonds formed within lignin macromolecule and between lignin and structural sugars open up the biomass structure leading to increased availability of easily degradable sugars by microbes. The laccase enzyme is stable over a wide range of pH and temperature (Baldrian, 2006; Munoz et al., 1997; Stoilova et al., 2010; Thurston, 1994). By using laccase enzyme, we can enhance thatch management over wide range of environmental conditions and can better utilize the microbial decomposition of organic matter.

Direct application of laccase on potted creeping bentgrass in a greenhouse study, where conditions for thatch development were conducive, was found to be effective relative to control in reducing thatch-mat depth, OM, and significantly increasing SHC (Chapter III). However, an overall increase in OM (0-5.0 cm) and organic layer thickness was observed for all the treatments over the experiment duration; but less increase with laccase treatments. Application of laccase at 2.06 units cm^{-2} was effective in reducing the rate of accumulation of organic matter and organic layer thickness (Chapter III). In our present study, the efficacy of laccase enzyme was verified on the field conditions along with optimization of laccase in terms of rate and frequency of laccase application. Application of laccase in combination with core aeration and topdressing was effective in changing thatch characteristics.

Rate of Laccase Application

In our study, laccase enzyme was applied at five different rates (activity levels) as control, 0.5, 1.0, 2.0, and 4.0 units cm^{-2} applied every two weeks and thatch layer thickness

decreased with all laccase applications (Fig 5.1A). Since there was no difference in the thatch layer thickness from plots treated with different levels of laccase activity. This indicates that when laccase is applied biweekly, we can reduce the rate of application to 0.5 units cm^{-2}. There was no effect of different laccase applications on the organic matter content (Table 5.1, 5.2). This may be attributed to a couple of possible reasons, with one being that the bentgrass green on which the study is conducted is a 20 year old green with high organic matter content within and below the thatch layer. So, a long term application of laccase may be needed to observe any significant differences. The second is the method in which we sample and measure organic matter. The sample is collected for 0-2.5 and 2.5-5.0 cm depth. So, even if there is slight change in the organic matter content of the thatch layer due to application of laccase, it may be masked by very high organic matter content below the thatch layer. In our previous greenhouse studies, a significant decrease in organic matter content was observed with application of laccase (Chapter III, Chapter IV). This was because as the thatch layer thickness decreased with application of laccase, the top 2.5 cm core that was used for organic matter content contacted the increased portion of sand with low organic matter content when using a standard depth of sample. Thus an overall reduction in organic matter content was observed. Whereas, in our field study although we observed that thatch layer thickness was decreased with laccase application, the sample depth never contacted the underlying sand due to deeper thatch/mat layer with high organic matter content and therefore, no differences in organic matter content were observed within the sample depth.

Acid-soluble lignin content decreased relative to the control at all laccase activity levels and L_S tended to decrease as laccase activity increased, which indicates the effectiveness of laccase in oxidizing the bonds of lignin macromolecule (Fig 5.2A). On the other hand,

application of laccase up to the 2.0 units cm^{-2} level decreased L_I but at 4.0 units cm^{-2} L_I was similar to control (Fig 5.3A). This could be explained in term of the decreased total sugar content (i.e. cellulose and hemicellulose) due to application of laccase at 4.0 units cm^{-2} as illustrated in Fig 5.5A. Three major components of plant biomass are cellulosic sugars, hemicellulosic sugars, and lignin. So, with application of laccase, lignin bonds are broken which leads to opening up of the biomass structure making sugars more available for microbial decomposition. As the sugar content is decreased, it tends to increase the lignin content when determined on a dry weight basis. Since structural carbohydrate content decreased with increased application of laccase, this suggests there was greater availability of sugars for microbial degradation.

Frequency of Laccase Application

Laccase at 2.0 units cm^{-2} was applied once every 2, 4, 8, and 12 weeks to optimize the frequency of application. Thatch layer thickness decreased in comparison to control when laccase was applied regardless of frequency (Fig 5.1B). All laccase frequencies of application were similar in year one, but the 2.0 (2) and 2.0 (4) frequencies exhibited the lowest TLT in year two, indicating that 2.0 units cm^{-2} of laccase as infrequent as one application in four weeks would be an effective frequency. A reduction in L_S was observed in plots when laccase was applied at the different frequencies in comparison to control (Fig 5.2B), while L_I decreased only at the 2 and 4 week frequency (Fig 5.3B). The contents of L_S and L_I tend to be higher at 4 and 8 week frequency relative to two week frequency. In year 1, there was trend for total and individual sugar contents to decrease in the plots treated with laccase in comparison to the control plots, but this trend was especially apparent year 2, indicating that laccase application modified the thatch biomass structure leading to increased decomposition by microbes (Fig 5.5B, 5.6B, 5.7B, 5.8B).

Laccase with Core aeration and Topdressing

Organic matter content in the upper 2.5 cm (OM_U) was lower in plots treated with core aeration followed by topdressing as well as plots receiving laccase along with these cultural management practices (Fig 5.9). Application of laccase once in four weeks along with cultural management practice was equally effective in reducing OM content as cultural practice alone. The reduction in OM content may be attributed to the dilution of organic matter caused by application of sand as topdress into this upper zone. Application of only laccase at 2.0 units cm^{-2} once in 4 weeks was not as effective in reducing OM content (Fig 5.9). When laccase was applied along with core aeration and topdressing, it tends to be more effective in reducing TLT and T_S content as well as increasing L_I content as compared to only cultural management practice and only laccase application (Fig 5.3C). This may be attributed to the structural changes in thatch biomass caused by application of laccase making it more favorable for microbial decomposition and core aeration creating favorable environment for microbial population (Carrow et al., 1987; Ledeboer and Skogley, 1967). Increased microbial population in the plots treated with cultural management led to increase in loss of sugars and eventually led to increase in lignin content in the remaining organic matter. Application of laccase in combination with core aeration and sand topdressing may lead to reduced number of cultivations in the long run.

CONCLUSIONS

This field research demonstrated the efficacy of applications of laccase enzyme on physical and chemical composition properties of the thatch layer of creeping bentgrass turf over a wide range of activity levels and frequencies of application. Laccase application rate can be reduced to 0.5 units cm^{-2} when applied as biweekly applications and remain effective in reducing thatch layer accumulation. When laccase at 2.0 units cm^{-2} is applied, the application frequency

can be reduced to once a month. Laccase application at 2.0 units cm^{-2} once in four weeks along with core aeration and topdressing cultural management practices was effective in lowering TLT, OM_U, L_S, S_T, and individual sugar contents and increasing L_I content.

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Table 5.1 Analysis of variance (ANOVA) table for year 1 showing the effects of laccase treatments, rate of application, frequency of application, laccase with cultural management, and laccase sources on creeping bentgrass.

Source of variation	df	Total organic matter OM_U (0-2.5 cm)	Total organic matter OM_L (2.5-5.0 cm)	Total organic matter OM (0-5.0 cm)	Thatch layer thickness TLT	Saturated hydraulic conductivity SHC	Acid-soluble lignin L_S	Acid-insoluble lignin L_I	Total lignin L_T	Total sugars S_T
		----------mean square value----------								
Year 1										
Rep	3	726	368	379*	12	95.8	1	7	13	86
Treatment	12	488	42	77	13***	38.2	46***	918***	1029***	2073***
Error	36	278	86	83	1	24.4	2	8	12	399
Rate of Appl.										
Rep	3	1217	428	534	5	11.9	1	7	6	284
Treatment	4	59	28	030	15***	6.15	81***	1112***	1196***	1879*
Error	12	179	103	077	1	3.18	4	8	18	446
Freq. of Appl.										
Rep	3	417	431	279	3	6.5	1	5	6	8
Treatment	4	61	16	10	21***	4.6*	47***	862***	1184***	1499*
Error	12	218	75	83	1	1.25	4	8	18	228
Cultural Mgt.										
Rep	3	22	93	46	5	123	1	5	11	235
Treatment	3	753	34	72	17**	105	40***	698***	907***	3136*
Error	9	276	100	71	2	71.1	2	6	8	326
Lacc. Sources										
Rep	3	316	181	181	6	7.90	6	33	58	235
Treatment	2	736	011	123	10	1.59	35*	944***	1123***	1620
Error	6	230	208	174	1	1.03	4	16	20	434

* Significant at the 0.05 probability level
**Significant at the 0.01 probability level
*** Significant at the 0.001 probability level

Table 5.2 Analysis of variance (ANOVA) table for year 2 showing the effects of laccase treatments, rate of application, frequency of application, and laccase with cultural management on creeping bentgrass.

Source of variation	df	Total organic matter OM_U (0-2.5 cm)	Total organic matter OM_L (2.5-5.0 cm)	Total organic matter OM (0-5.0 cm)	Thatch layer thickness TLT	Saturated hydraulic conductivity SHC	Acid-soluble lignin L_S	Acid-insoluble lignin L_I	Total lignin L_T	Total sugars S_T
		----------mean square value----------								
Year 2										
Rep	3	4682	1329	1781	1	35.9	1	41	47	12234
Treatment	9	2024**	97	232	14***	66.1***	41***	1334***	1582***	4364*
Error	27	589	124	159	1	6.63	1	27	26	1781
Rate of Appl.										
Rep	3	4651	676	1096	0.3	9.47	3	44	50	6625
Treatment	4	942	140	256	21***	2.23	77***	745***	1026***	6092*
Error	12	684	149	185	0.4	1.39	1	27	23	1550
Freq. of Appl.										
Rep	3	1785	568	683	2	11.2	1	31	38	4087
Treatment	4	1253	164	302	18***	4.4	39***	1260***	1616***	8331**
Error	12	605	163	187	1	3.03	1	37	37	1373
Cultural Mgt.										
Rep	3	968	330	399	1	35.1	1	14	17	8281
Treatment	3	5372*	179	659	38**	125**	28***	1678***	1808***	11808*
Error	9	850	167	205	1	13.8	1	21	19	2187

* Significant at the 0.05 probability level
**Significant at the 0.01 probability level
*** Significant at the 0.001 probability level

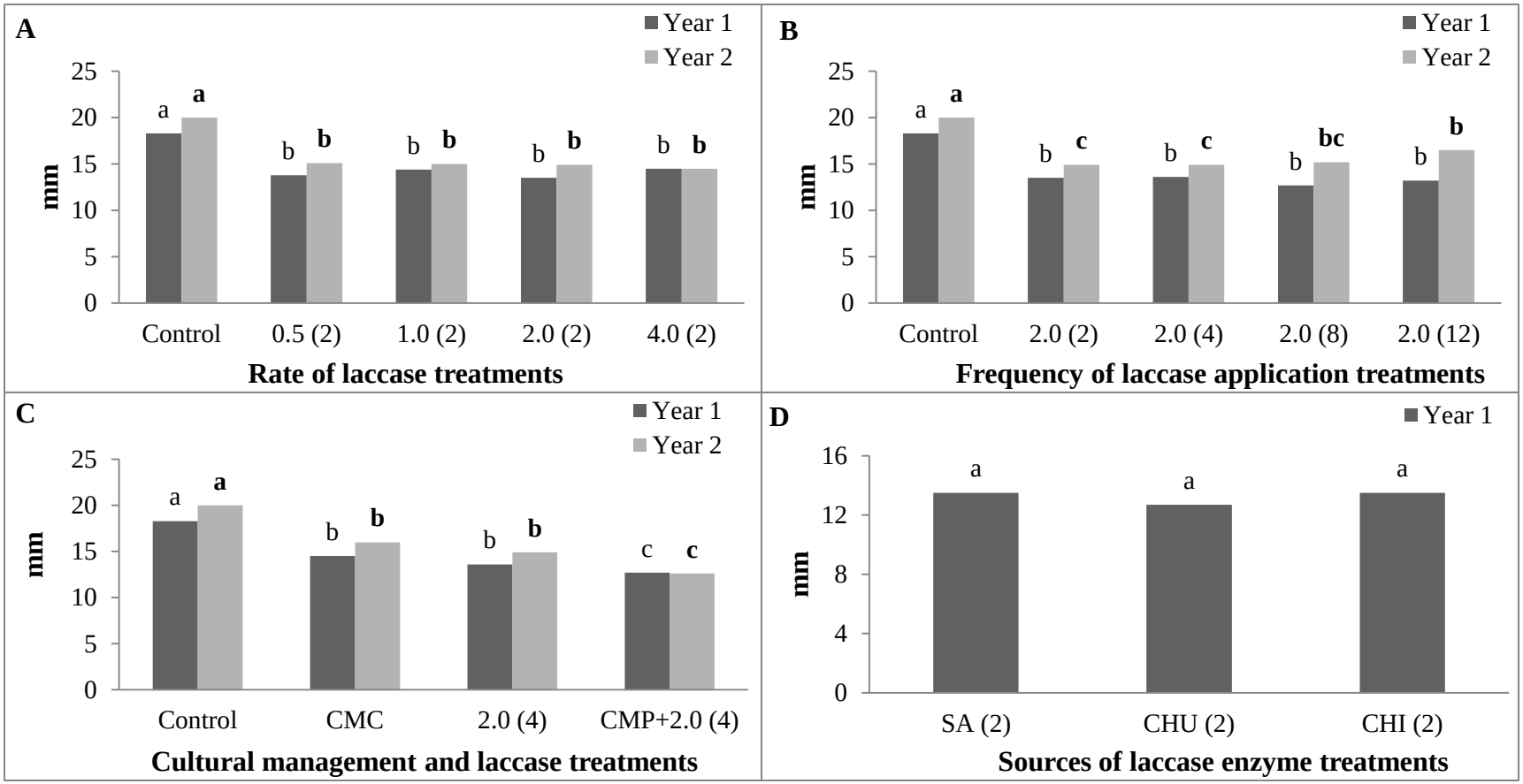

Fig. 5.1 Thatch layer thickness (TLT) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.1A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.1B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice (Fig 5.1C); and laccase enzyme from different sources (Fig 5.1D). Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

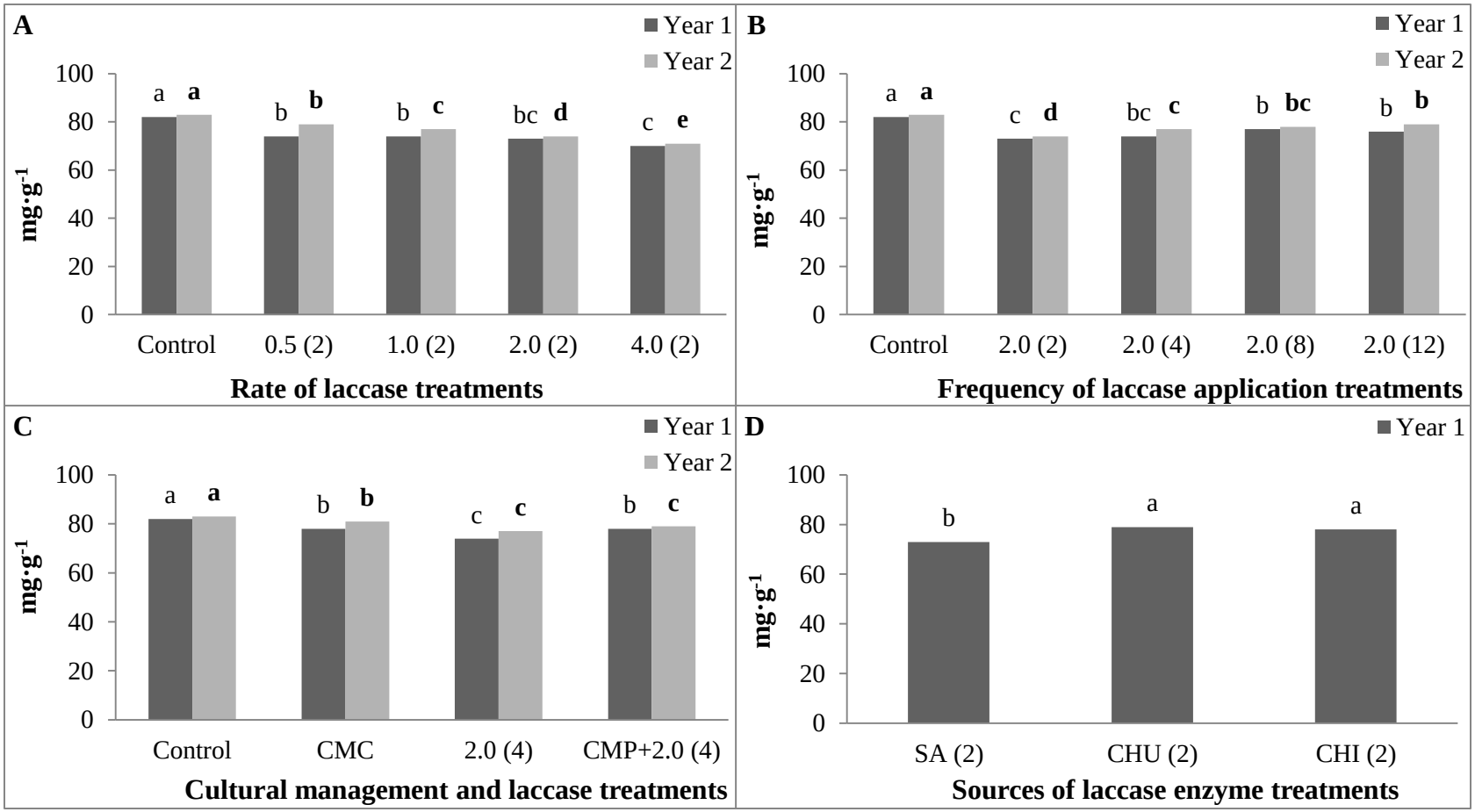

Fig. 5.2 Extractive-free acid-soluble lignin content (L_S) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.2A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.2B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice (Fig 5.2C); and laccase enzyme from different sources (Fig 5.2D). Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

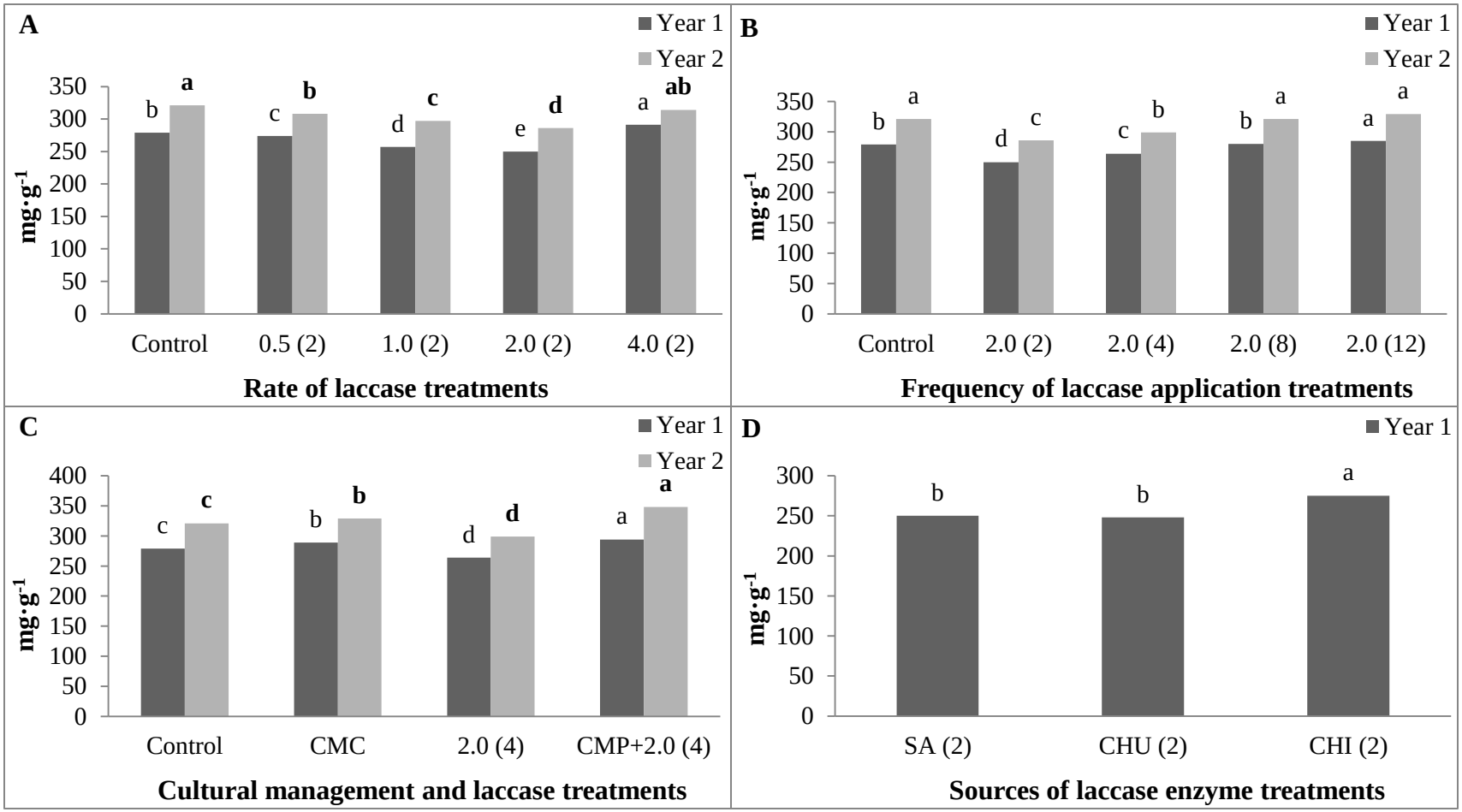

Fig. 5.3 Extractive-free acid-insoluble lignin content (L_I) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.3A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.3B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice (Fig 5.3C); and laccase enzyme from different sources (Fig 5.3D). Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

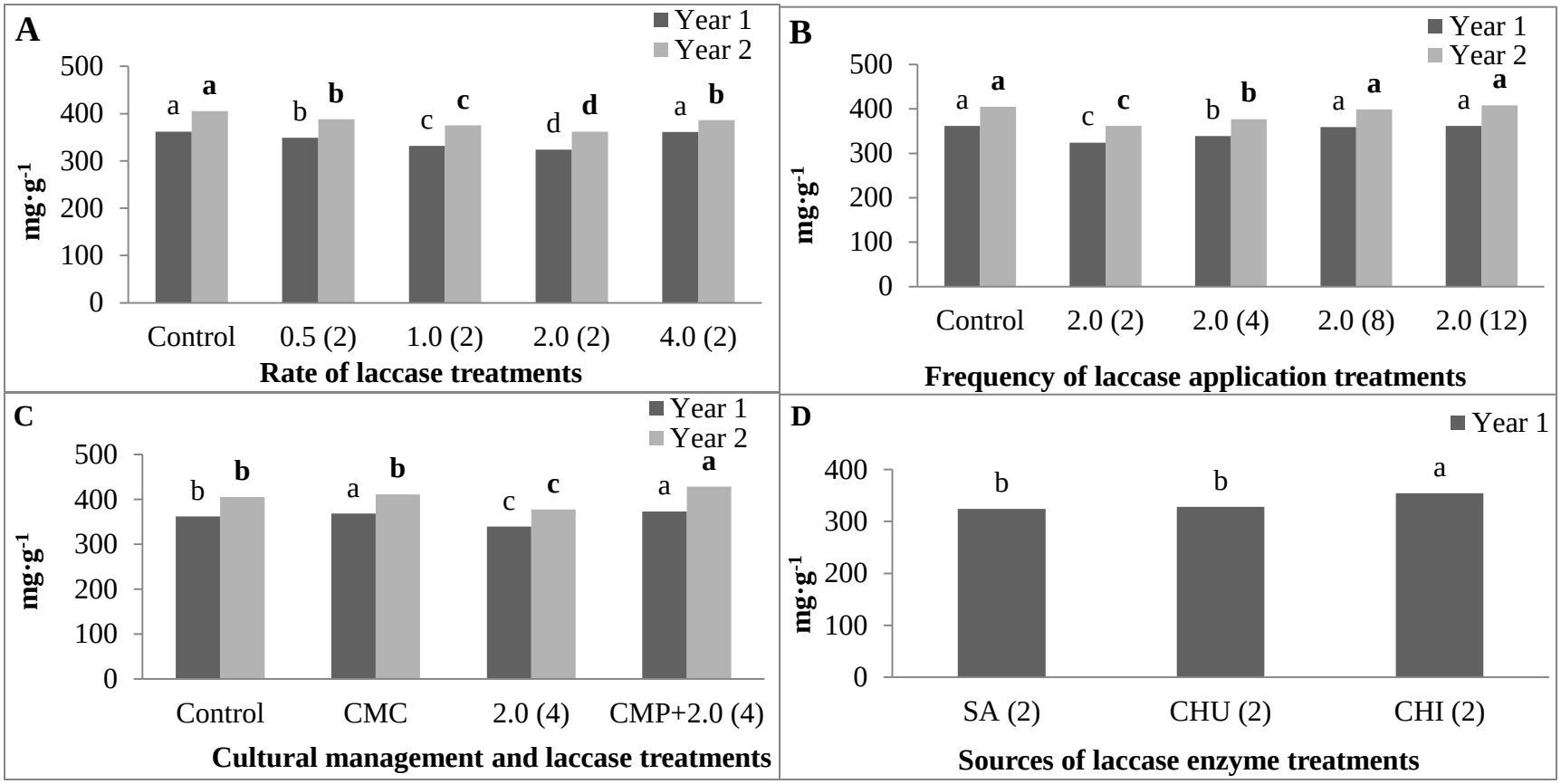

Fig. 5.4 Extractive-free total lignin content (L_I) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.4A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.4B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice (Fig 5.4C); and laccase enzyme from different sources (Fig 5.4D). Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

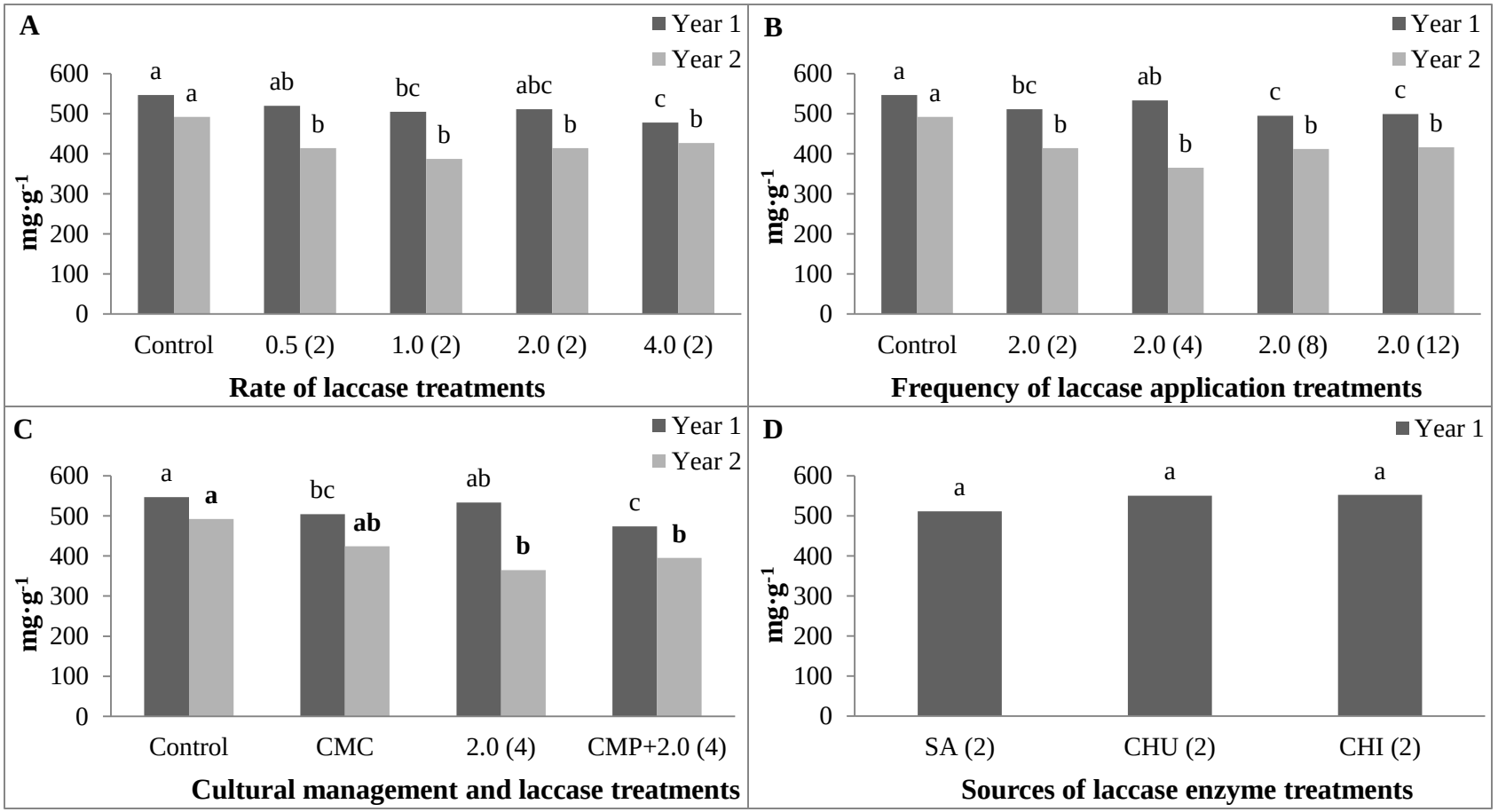

Fig. 5.5 Extractive-free total sugar content (S_T) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.5A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.5B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice (Fig 5.5C); and laccase enzyme from different sources (Fig 5.5D). Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

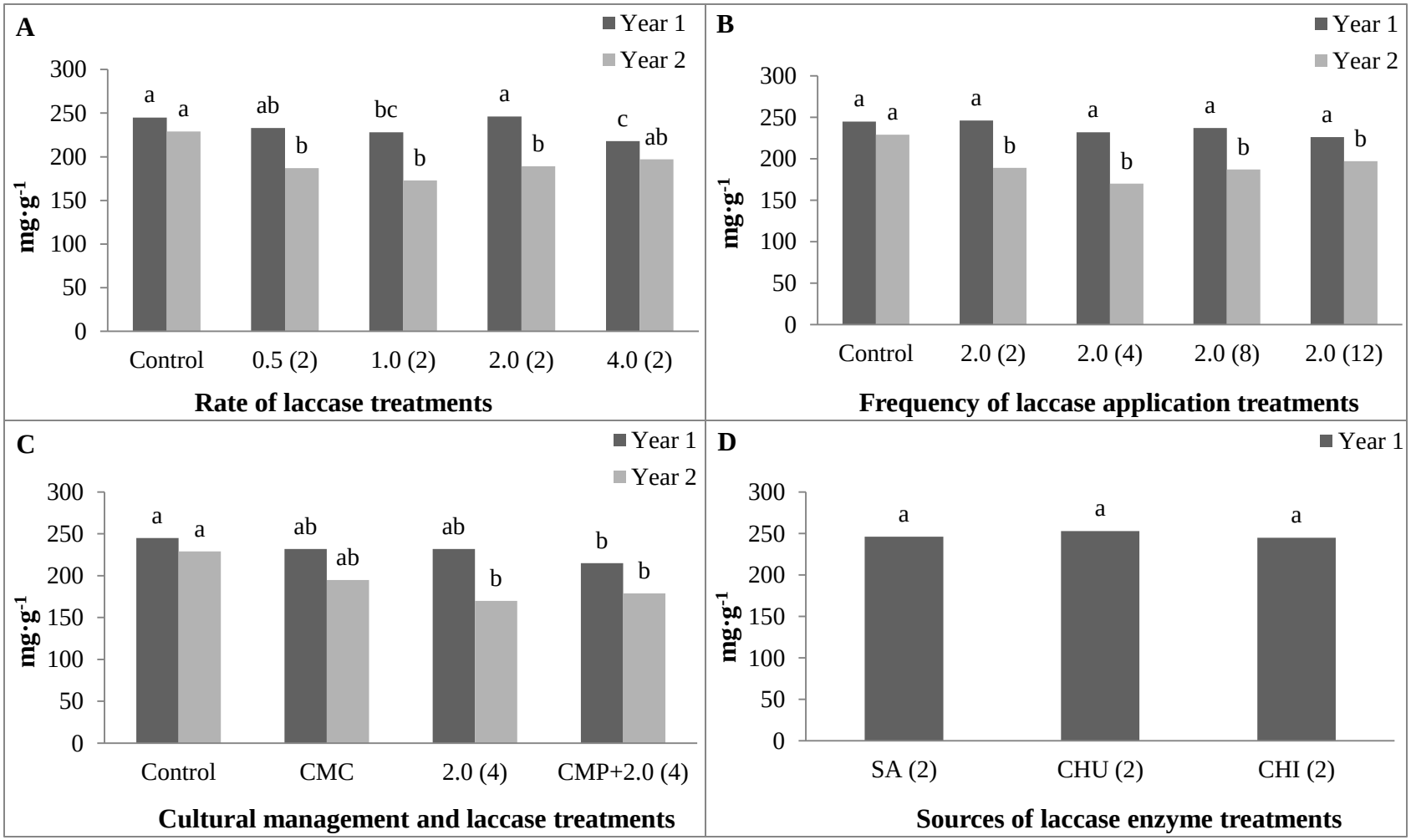

Fig. 5.6 Extractive-free glucose content (S_{GLU}) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.6A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.6B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice (Fig 5.6C); and laccase enzyme from different sources (Fig 5.6D). Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

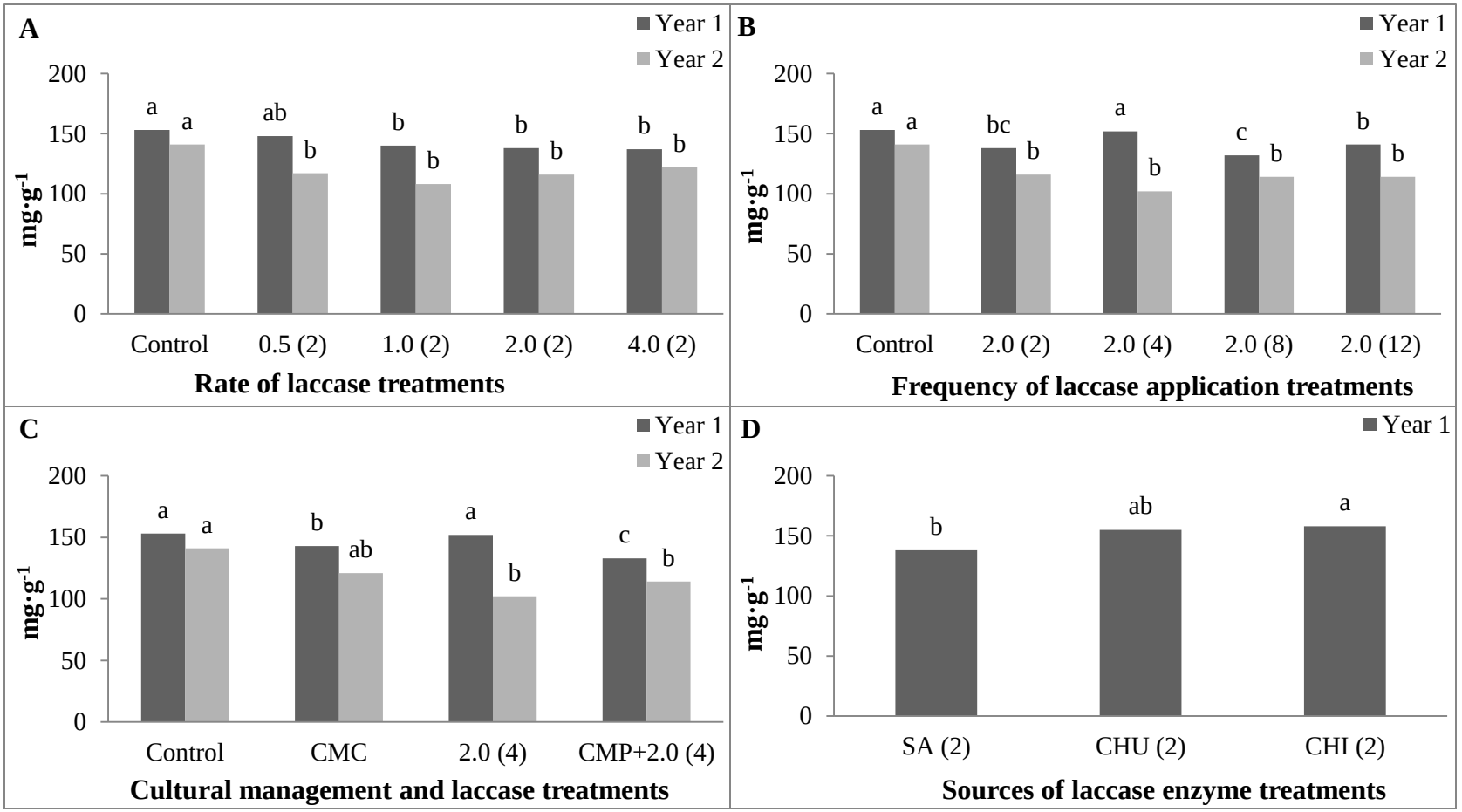

Fig. 5.7 Extractive-free xylose content (S_{XYL}) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.7A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.7B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice (Fig 5.7C); and laccase enzyme from different sources (Fig 5.7D). Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

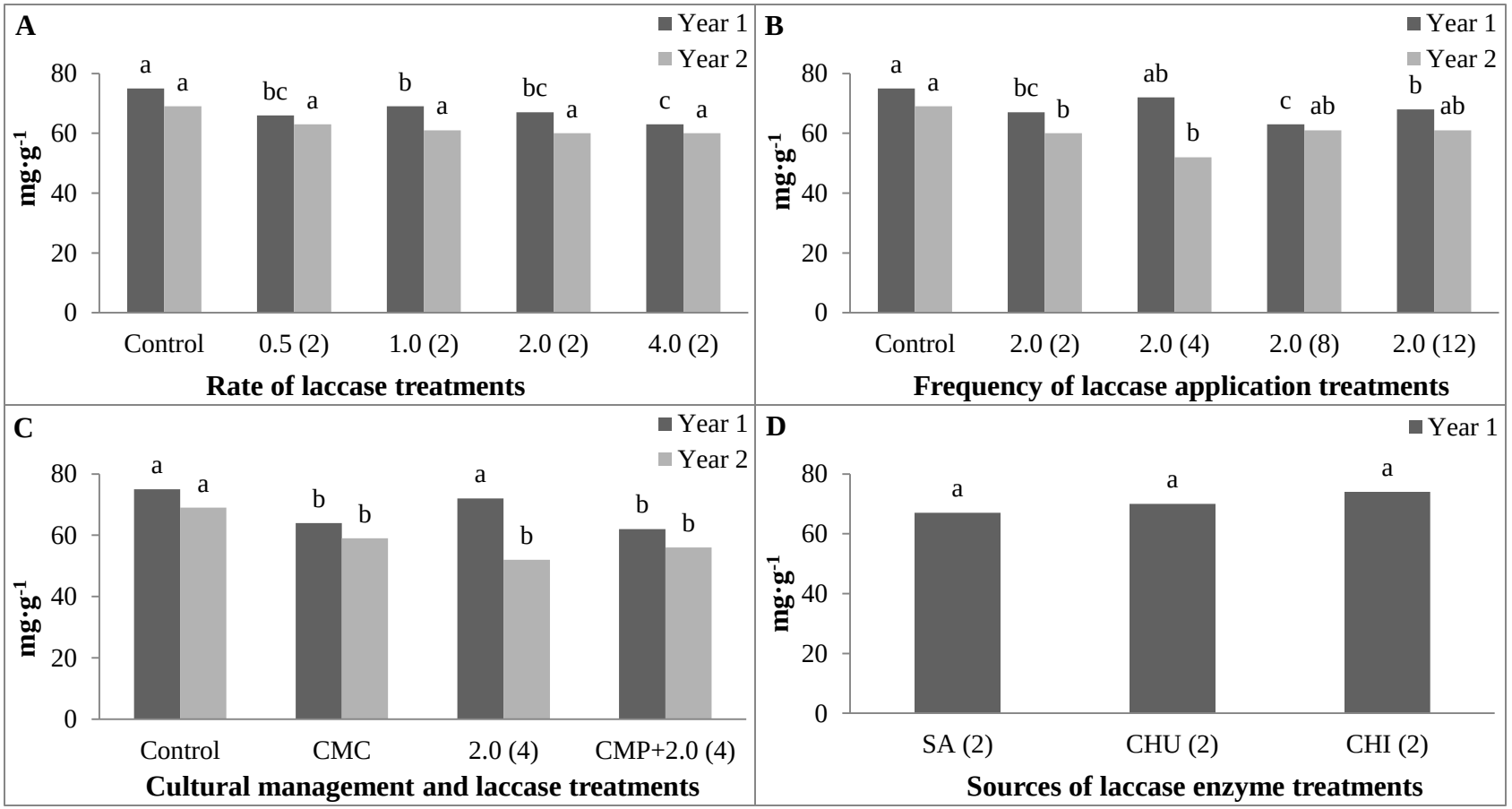

Fig. 5.8 Extractive-free galactose content (S_{GAL}) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.8A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.8B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice (Fig 5.8C); and laccase enzyme from different sources (Fig 5.8D). Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

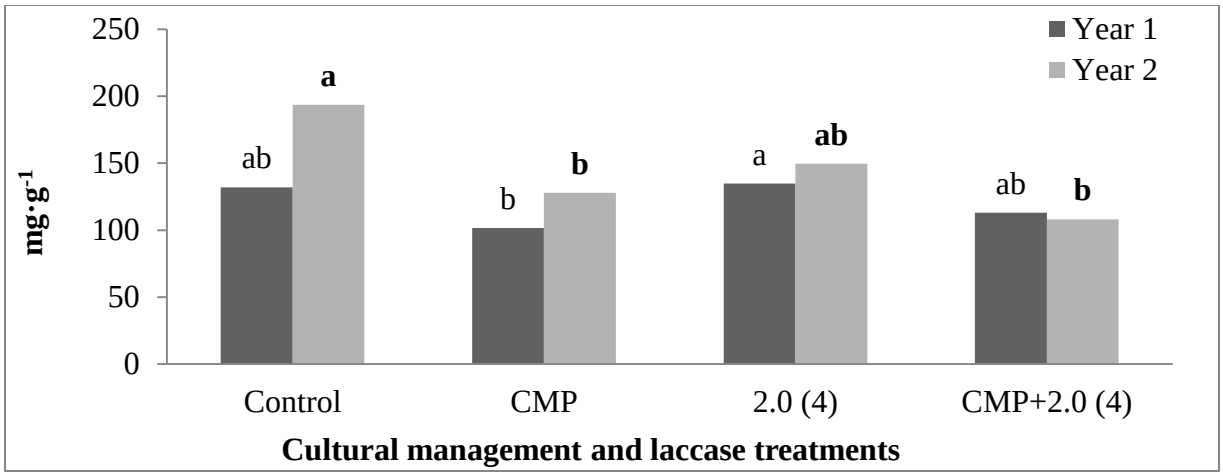

Fig. 5.9 Organic matter content in the 0-2.5 cm surface layer (OM_U) after treatment application on creeping bentgrass with laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice. Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

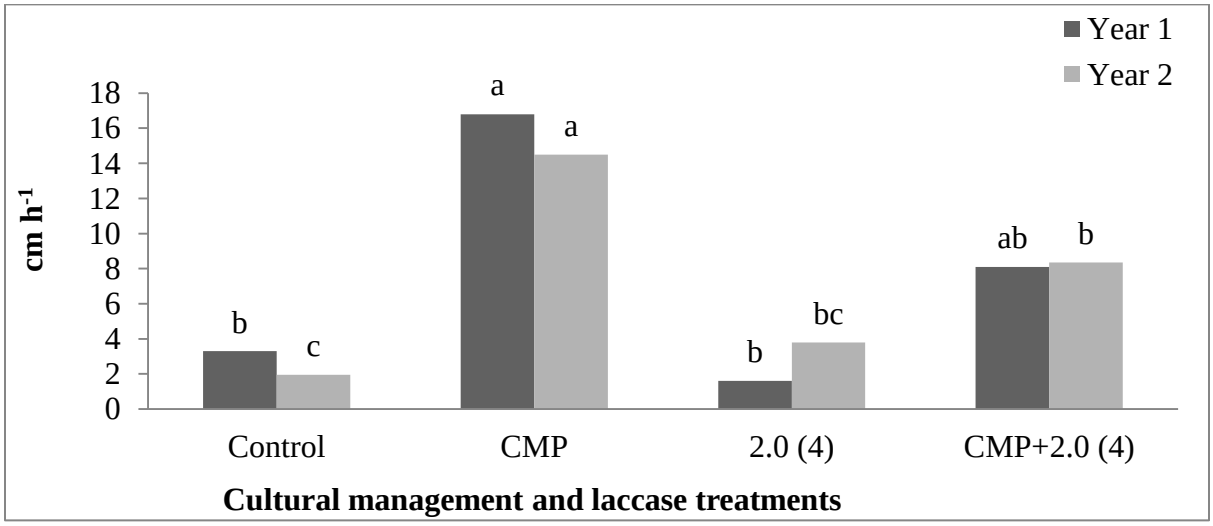

Fig. 5.10 Saturated hydraulic conductivity (SHC) after treatment application on creeping bentgrass with laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice. Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

CHAPTER VI

EFFICACY OF FUNGAL LACCASE TO FACILITATE BIODETHATCHING IN BERMUDAGRASS AND ZOYSIAGRASS[4]

[4] Sidhu, S.S., Q. Huang, R.N. Carrow, and P.L. Raymer. To be submitted to *Agronomy Journal*.

ABSTRACT

Accumulation of excessive organic matter in the form of a thatch and/or mat layer can cause several problems in turfgrass management systems. A previous greenhouse study on creeping bentgrass (*Agrostis stolonifera* L.) demonstrated that direct application of laccase solution every two weeks reduced the rate of accumulation of organic matter and hence buildup of thatch layer. A two year field study was conducted on an ultra-dwarf bermudagrass (*Cynodon dactylon* L., 'TifEagle') green, and zoysiagrass (*Zoysia japonica* Stued., 'Meyer') maintained as a home lawn to observe the influence of laccase enzyme applications on thatch development. Laccase solution was applied bi-weekly at the activity levels of 0 (control) and 2.0 units cm^{-2}. Response to laccase enzyme applications by both the turfgrass species was recorded by measuring thatch layer physical and chemical properties after six months of treatment applications within each year. A significant 18-22% and 21-30% reduction in thatch layer thickness was observed for bermudagrass and zoysiagrass, respectively. Organic matter content (0-2.5 cm) decreased by 23-24% while saturated hydraulic conductivity increased by 19-30% for bermudagrass in both years. Acid-soluble and-insoluble lignin reduced in both the grass species after laccase treatments. The results indicate that bi-weekly application of laccase on bermudagrass and zoysiagrass has positive impact on thatch management.

INTRODUCTION

Accumulation of organic matter in the form of dead and live tissue between soil and turfgrass is known as thatch layer. Thatch layer consists of stolons, rhizomes, roots, crown tissue, leaf sheaths, and blades (Engel, 1954; Roberts and Bredakis, 1960) and is one of the major problems in management of recreational turfgrass sites, especially golf greens. Mat is defined as a thatch layer with sand or soil integrated into the layer. A thin layer of thatch is desirable as it

provides protection from temperature extremes and provides wear tolerance (Beard, 1973). However, excessive thatch layer is undesirable in turfgrass as it leads to decreased saturated hydraulic conductivity, decreased movement of oxygen through the thatch or mat zone, and low oxygen levels within the thatch/mat layer during wet periods, and increased water retention (Carrow, 2003; Hartwiger, 2004; McCarty et al., 2007).

The most effective mechanical techniques to manage thatch consist of core aeration, vertical mowing, sand topdressing, and grooming. However, these techniques have shown contrasting effects on changes in organic matter decomposition (Barton et al., 2009; Carrow et al., 1987; Dunn et al., 1981; McCarty et al., 2005; McWhirter and Ward, 1976; Weston and Dunn, 1985; White and Dickens, 1984). These practices may adversely impact turf quality, playability and be costly (Barton et al., 2009; Landreth et al., 2008; McCarty et al., 2007). Non-destructive techniques are highly desirable for uninterrupted availability of the turfgrass areas for play.

The formation of thatch-mat layer on recreational turfgrass sites, especially golf greens, is accelerated when accumulation of organic matter exceeds the degradation rate. Accumulation of organic matter in the thatch layer is known to be strongly influenced by lignin, a plant cell wall constituent, which is very resistant to microbial degradation and serves as the rate limiting step for organic matter decomposition (Taylor et al., 1989). Lignin acts as a protective matrix restricting the availability of cellulosic and hemicellulosic sugars to microbial degraders thus leading to the accumulation of organic matter (Ledeboer and Skogley, 1967). The recalcitrant nature of lignin may be attributed to its heterogeneous structure as it is composed of random oxidative couplings of lignin monomers and cross-linking of polymers (Ledeboer and Skogley,

1967). After decades of research, the lignin macromolecule structure is known to exhibit no particular sequence of monomeric units (Chen and Sarkanen, 2003; Davin and Lewis, 2003).

In nature, the most rapid natural degradation of lignin is carried out by extracellular enzymes produced by certain white-rot fungi exposing cellulosic sugars to bacterial degradation (Blanchette, 1984; Kirk et al., 1975; 1976; Mester et al., 2004; Otjen and Blanchette., 1987). It is very difficult to maintain viable, specific microbial populations in turf management systems. However, the direct application of laccase enzyme, an extracellular enzyme produced by white-rot fungi, to turfgrass could be used to remove lignin bonds, hence opening up of the structure of thatch biomass for microbes to decompose cellulosic and hemicellulosic sugars. This approach could be an effective means of controlling thatch. In Chapter III, we demonstrated that bi-weekly applications of laccase enzyme solution (2.0 units cm^{-2} activity) reduced the rate of thatch buildup and organic matter accumulation in a greenhouse study on creeping bentgrass. In another greenhouse study, a bi-weekly application of laccase solution (2.0 units cm^{-2} activity) on thatch layer of a dead creeping bentgrass for six months verified the effectiveness laccase in facilitating the organic matter decomposition, loss in total structural sugar content of thatch biomass (Chapter IV). This suggested that laccase application exposed cellulosic and hemicellulosic sugars for microbial degradation by opening up the biomass structure (Chapter IV). Previous studies were conducted on creeping bentgrass; therefore, this study was designed to observe the effectiveness of laccase enzyme on organic matter decomposition of an ultra-dwarf bermudagrass green and zoysiagrass maintained under home lawn conditions. The objective of this study is to determine the influence of laccase application on thatch layer physical and chemical properties of bermudagrass and zoysiagrass under field conditions.

MATERIALS AND METHODS

Two year field studies were conducted on ultra-dwarf bermudagrass and zoysiagrass at The University of Georgia, Griffin Campus as part of a two year experiment from June 2010 to Jan 2012. Bermudagrass plots (60 cm x 30 cm) were established on a sand-based putting green (90:10 sand and Dakota peat moss) based on USGA specifications. The bermudagrass green is maintained under low fertility management and was fertilized three times each year during the growing season at the rate of 50 kg ha^{-1} granular fertilizer 24-4-10 (Lesco., Strongsville, OH). The bermudagrass green was mowed three times a week by Toro Greensmaster 3100 (The Toro Company, Bloomington, MN) and maintained at a height of 0.42 cm. Zoysiagrass was established on a sandy clay loam soil (Table 6.1) and was maintained under home lawn conditions, mowed once a week at 4 cm height with clippings returned. Zoysiagrass was fertilized as 50 kg ha^{-1} granular 24-4-10 (Lesco. Strongsville, OH) once each May, June, July and August each year.

The field experiments on bermudagrass and zoysiagrass were a completely randomized design with two levels of laccase activity (0 and 2.0 units cm^{-2}) replicated four times. During the first year, laccase from white-rot fungi *Trametes versicolor* (Sigma-Aldrich product 53739) was applied as 410 mL solution at 0 and 2.0 units cm^{-2} activity level every two weeks on bermudagrass and zoysiagrass. During the second year laccase from *Pycnoporus* genus was used at the same activity level due to the unavailability of the Sigma Aldrich product.

Laccase Activity Assay

Laccase activity was quantified by a colorimetric assay using a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA) spectrophotometer. One unit of laccase activity is the amount of enzyme that causes an absorbance change at 468 nm at a rate of

1.0 unit min^{-1} in 3.4 mL of 1 mM 2, 6-dimethoxyphenol, a specific substrate for laccase, in citrate-phosphate buffer at pH 3.8 (Park et al., 1999).

Measurements

Effectiveness of laccase application and its impact on physical and chemical properties of thatch layer were determined after six months of treatment application each year. In year one, bi-weekly treatment applications began in June 2010 and continued until Dec 2010. In year two, bi-weekly treatment applications began in July 2011 and continued until Jan 2012. Parameters measured included thatch layer thickness (TLT), organic matter content (OM) for a depth of 0-2.5 cm (OM_U), 2.5-5.0 cm (OM_L), and 0-5.0 cm (OM), saturated hydraulic conductivity (SHC), extractive-free acid-soluble lignin (L_S), and acid-insoluble lignin (L_I). Total lignin (L_T) was obtained by addition of acid-soluble and-insoluble lignin contents.

Thatch Layer Thickness

Thatch layer thickness was measured from two subsamples of the soil profile from each plot. Replaceable wedge-shaped turf profiles (8.9 cm wide and 2.5 cm thick) were pulled using AMS Turf Profiler (AMS Inc., American Falls, ID**).** Thatch layer thickness was measured from four points across the width of each profile and averaged. The clearly visible distinction between thatch layer and the sand layer below was considered as the bottom of the thatch layer for all measurement locations.

Organic Matter Content

Organic matter (OM) was measured by total ignition as described by Carrow et al. (1987). Two soil cores (2.0 cm diam.) were obtained from each plot and divided into 0-2.5 cm (OM_U) and 2.5-5.0 cm (OM_L) depths. The cores were dried in an oven at 100 ± 5^oC for 24 h and weighed. Soil cores were ashed in a muffle furnace at 600 ± 10^oC for 24 h and weighed again.

Total organic carbon content was determined as the difference in the two readings and percent organic matter was calculated.

Saturated Hydraulic Conductivity

The saturated hydraulic conductivity (SHC) from each plot was measured by a constant hydraulic head method using a Marriott tube apparatus and saturated hydraulic conductivity was calculated using Darcy's equation. An intact core (diam. 4.7 cm and length 7.7 cm) was obtained from each plot in a brass cylinder using an undisturbed soil core sampler (Model 0200 soil sampler, Soilmoisture Equip. Corp., Santa Barbara, CA). The bottom of the core was covered with a double layer of cheesecloth held in place with a rubber band and saturated overnight in a 0.05 N $CaCl_2$ solution to minimize dispersion. A steady state flow through the samples was established by flowing 0.05 N $CaCl_2$ through the core for 10 min. After 10 min the volume of water that passed through the core was measured for one minute and the measurement repeated three times.

Extractive-free Lignin Content

Thatch biomass was collected from the top 2.5 cm of each core after measurement of saturated hydraulic conductivity. Thatch samples were first air-dried, ground, washed with water in a glass jar, on a rotary shaker at 200 rpm, and then passed through a series of sieves with a 841µm sieve at the top and a 177µm sieve at the bottom. The biomass retained by the 177 µm sieve size was used for analysis. The thatch biomass was extracted for 24 h using the Soxhlet method for water- and alcohol-soluble extractives using de-ionized water and 16.26 M (95 percent USP grade) ethyl alcohol, respectively. Lignin content determined from an extracted biomass is known as extractive-free lignin content.

Extractive-free acid-soluble lignin (L_S) and acid-insoluble lignin (L_I) content in the thatch layer biomass was determined on weight basis in a two-step acid-hydrolysis procedure (NREL, 2008). In the first step, extractive-free thatch samples were hydrolyzed for 60 min with 72% H_2SO_4 at 30^{o}C in a water bath. In the second step, H_2SO_4 was diluted to 4% and the samples were autoclaved at 121^{o}C for 1 h and then vacuum filtered. Acid-soluble lignin, which consists of low molecular weight phenolic groups, was determined using this hydrolysis liquid at 240 nm wavelength in a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The solids remaining after acid hydrolysis were dried in an oven at 100 ± 5^{o}C for 24 h, weighed, ashed in a muffle furnace at 600 ± 10^{o}C for 24 h, and weighed again to calculate the acid-insoluble lignin content using weight difference. Total lignin content (L_T) of each sample was calculated as the sum of L_S and L_I.

Statistical Analysis

Data from a completely randomized design with treatments consisting of two levels of laccase were analyzed separately for each species by year using one was analysis of variance (ANOVA) using general linear model (GLM) (SAS Institute, 1994). Fisher's LSD test with α = 0.05 was used for determining statistical differences among treatment means following each ANOVA.

RESULTS

Organic Matter Content

For bermudagrass, a significant treatment effect was observed for OM_U ($P \leq 0.05$) and OM ($P \leq 0.05$) during year one and for OM_U ($P \leq 0.05$) during the second year (Table 6.2). The OM_U, OM_L and OM contents at the start of the experiment were 97, 07, and 41 mg·g^{-1}, respectively (baseline reading). Organic matter content (0-2.5 cm) after six months of treatment

was 122 and 93 $mg \cdot g^{-1}$ for control and plots treated with laccase, respectively (Fig 6.1). The results indicate that in laccase treated bermudagrass plots OM_U rate of accumulation was less than the control and there was no net accumulation of organic matter in bermudagrass at the end of 6 months. A reduction of 23 and 24% of OM_U was observed in plots treated with laccase in comparison to control plots in first and second year, respectively (Fig 6.1). No differences were observed for OM_L after six months of laccase application between control and treated plots (Table 6.3). Organic matter (0-5.0 cm) accumulation was observed in control plots after six months. No accumulation was observed in plots treated with laccase enzyme. Organic matter in plots treated with laccase was reduced by 19 and 24% in comparison to control plots after treatment in year one and two, respectively (Table 6.3).

For zoysiagrass, a significant effect of laccase treatment was observed on OM_U ($P \leq 0.05$) during first year of the experiment (Table 6.2). A reduction of 23% OM_U in plots treated with laccase were observed when compared to control plots in year one (Fig 6.1). However, no significant effect was observed for OM_U, OM_L, and OM during the second year (Table 6.3).

Thatch Layer Thickness

A significant effect of laccase application was observed for TLT ($P \leq 0.05$) in the first and second year of the experiment in bermudagrass (Table 6.2). Thatch layer thickness at the start of the experiment was 16.6 mm (baseline reading). The reduction in TLT in plots treated with laccase was 18 and 22% in comparison to control plots during year one and two, respectively (Fig 6.2). A slight accumulation in TLT was observed in control plots above the starting value of 16.6 mm at the end of first year treatment application. However, application of laccase proved effective in reducing TLT after six months of application.

In zoysiagrass, laccase application was effective ($P \leq 0.001$) in reducing TLT in both years relative to the control (Table 6.2). TLT at the start of the experiment was 18.5 mm (baseline reading) with a slight accumulation of that layer noticed in the control plots by the end of year one and two (Fig 6.2). However, laccase application proved to be effective in reducing thatch layer thickness with a reduction of 30 and 21% in TLT observed in plots treated with laccase when compared to control plots in first and second year, respectively (Fig 6.2).

Saturated Hydraulic Conductivity

A significant laccase application effect ($P \leq 0.05$) was observed in bermudagrass with respect to SHC (Table 6.2). An increase of 30 and 19% was observed for SHC in plots treated with laccase over the control plots during the first and second year, respectively (Fig 6.3). In zoysiagrass, a significant effect of laccase on SHC was observed in the second year ($P \leq 0.01$) (Table 6.2). After six months of treatment in the second year, SHC increased from 2.1 to 12.5 cm h^{-1} (Fig 6.3).

Extracted-free Lignin Content

Application of laccase significantly affected L_S, L_I, and L_T in bermudagrass in both years (Table 6.2). A slight but significant reduction of 5-10 $mg \cdot g^{-1}$ in L_S was observed in plots treated with laccase in comparison to control plots in both years (Table 6.3). Content of L_I and L_T content were reduced for plots treated with laccase in the range of 45-48 and 54-57 $mg \cdot g^{-1}$ over control plots in year one and two (Table 6.3, Fig 6.4).

In zoysiagrass, laccase application had a significant effect on L_S ($P \leq 0.001$), L_I ($P \leq 0.001$), and L_T ($P \leq 0.001$) in both year one and two (Table 6.2). Acid-soluble lignin and L_I content decreased from 0.4-0.7 and 38-49 $mg \cdot g^{-1}$ during first and second year, respectively

(Table 6.3). A significant 44-60 $mg \cdot g^{-1}$ reduction in L_T was observed in plots treated with laccase when compared to control plots (Fig 6.4).

DISCUSSION

In our previous study, we tested the effects of direct application of laccase on creeping bentgrass under greenhouse conditions that were conducive for thatch development. In this study, application of laccase at activity levels of 2.06 units cm^{-2} every two weeks was shown to be effective in reducing the rate of accumulation of thatch-mat, OM, and significantly increased SHC over the controls (Chapter III). Although laccase treatment reduced the rate of accumulation and thatch layer buildup, an overall net accumulation of organic matter and thatch buildup was observed after nine months even in the most effective laccase treatments (Chapter III).

In our present study on bermudagrass and zoysiagrass under field conditions, we saw an actual reduction in organic matter and thatch layer in plots treated with laccase at 2.0 units cm^{-2} activity after six months when compared to initial levels pre-treatment levels (Fig 6.1, 6.2). However, the control plots of both grass species showed a slight accumulation of organic matter and thatch layer. This result indicates that bi-weekly laccase treatment applications for six months were effective in reducing OM content and thatch layer thickness in bermudagrass and zoysiagrass. A significant increase was observed in SHC in plots treated with laccase application (Fig 6.3). This may be attributed to the reduction in thatch layer thickness and organic matter content. The presence of higher levels of organic matter in the thatch layer is known to decrease water infiltration through the thatch layer (McCarty et al., 2005).

A reduction in L_S, L_I, and L_T was observed in both years for both grasses in plots treated with laccase (Table 6.3, Fig 6.4). This may be attributed to the oxidation and eventually

degradation of lignin macromolecule. Laccases are produced as extracellular enzymes by white-rot fungi and facilitate the oxidation of wide range of mono- and diphenols using oxygen as the electron acceptor (Baldrian, 2006). Lignin phenolic compounds are oxidized by laccase via Cα-Cβ cleavage, alkyl-aryl cleavage, and Cα oxidation (Wong, 2009). Laccase enzyme acts on bonds formed between lignin macromolecule and between lignin and structural sugars leading to opening up of the biomass structure leading to increased availability of easily degradable sugars by microbes. The laccase enzyme we used is stable over a wide range of pH and temperature (Baldrian, 2006; Munoz et al., 1997; Stoilova et al., 2010; Thurston, 1994). In these studies, laccase enzyme effectively managed thatch over a range of environmental conditions for the period of the studies apparently by allowing better microbial decomposition of organic matter. This suggests that laccase enzyme treatment may be a valuable non-disruptive means to control thatch in these species.

CONCLUSIONS

This field research was the first study to demonstrate the efficacy of laccase application on ultra-dwarf bermudagrass green and zoysiagrass grown under home lawn conditions. The results from the study revealed the positive impacts of laccase on physical and chemical properties of thatch layer of these two grass species with no net accumulation of thatch or organic matter along with an increase in saturated hydraulic conductivity.

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Table 6.1 Characteristics of soils used in zoysiagrass study.

pH	Ca	K	Mg	Mn	P	Zn	Sand	Silt	Clay	Soil Type
	----------Mehlich 1 mg·g^{-1} (ppm)----------						----------%----------			
6.1	1249	147	196.3	65.38	22.16	7.16	55.70	20.0	24.30	Sandy Clay Loam

Table 6.2 Analysis of variance (ANOVA) table showing the effects of laccase treatments, treatment duration, and duration and treatment interactions on ultra-dwarf bermudagrass and zoysiagrass.

Source of variation	df	Organic matter OM_U (0-2.5 cm)	Organic matter OM_L (2.5-5.0 cm)	Organic matter OM (0-5.0 cm)	Thatch layer thickness TLT	Saturated hydraulic conductivity SHC	Acid-soluble lignin L_S	Acid-insoluble lignin L_I	Total lignin L_T
		----------mean square value----------							
Bermudagrass									
Year 1									
Treatment	1	1728*	30	170*	18*	2169*	166**	3987***	5782***
Error	6	218	8	20	2	303	5	31	31
Year 2									
Treatment	1	2062*	336	372	32*	1445*	202***	4532***	6649***
Error	6	232	101	104	1	157	8	41	34
Zoysiagrass									
Year 1									
Treatment	1	1382***	216	54	63***	908	19***	3351***	3892***
Error	6	26	69	9	4	411	0.01	8	10
Year 2									
Treatment	1	384	3	42	31***	216**	107***	4841***	6391***
Error	6	53	17	26	0.4	14.5	1	68	67

* Significant at the 0.05 probability level
**Significant at the 0.01 probability level
*** Significant at the 0.001 probability level

Table 6.3 Organic matter content ($OM_{L,}$ 2.5-5.0 cm; OM, 0-5.0 cm), acid-soluble lignin (L_S), and acid-insoluble lignin (L_I) content after first and second year of laccase treatments on ultra-dwarf bermudagrass and zoysiagrass.

	Organic matter (2.5-5.0 cm) OM_L		**Organic matter (0-5.0 cm) OM**		**Acid-soluble lignin L_S**		**Acid-insoluble lignin L_I**	
	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
	----------mg·g^{-1}----------							
Bermudagrass								
Control	9.9a†	22.2a	47.5a	58.1a	41.5a	49.5a	317.5a	412.1a
2.0 (2)	6.1a	9.2a	38.2b	44.5a	3.67b	39.5b	272.8b	364.5b
Zoysiagrass								
Control	66.9a	68.0a	87.2a	94.0a	23.7a	29.6a	385.7a	430.4a
2.0 (2)	77.3a	66.6a	82.1a	89.4a	20.6b	22.3b	344.7b	381.2b

† Means within a column for grass species followed by the same letter are not significantly different according to LSD at α=0.05.

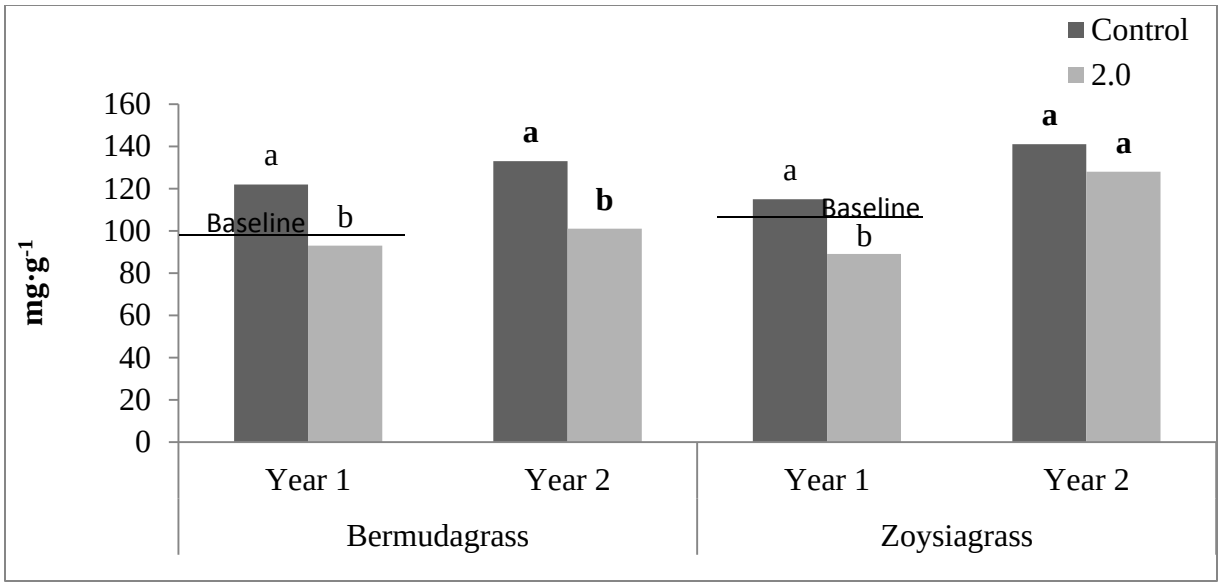

Fig. 6.1 Organic matter content (0-2.5 cm, OM_U) after bi-weekly application of laccase on bermudagrass and zoysiagrass with two levels 0 (control) and 2.0 units cm^{-2}. Values are means of four replicates. Means represented by bars with same letter on top (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's LSD at α = 0.05. The horizontal line represents the baseline reading at the start of experiment.

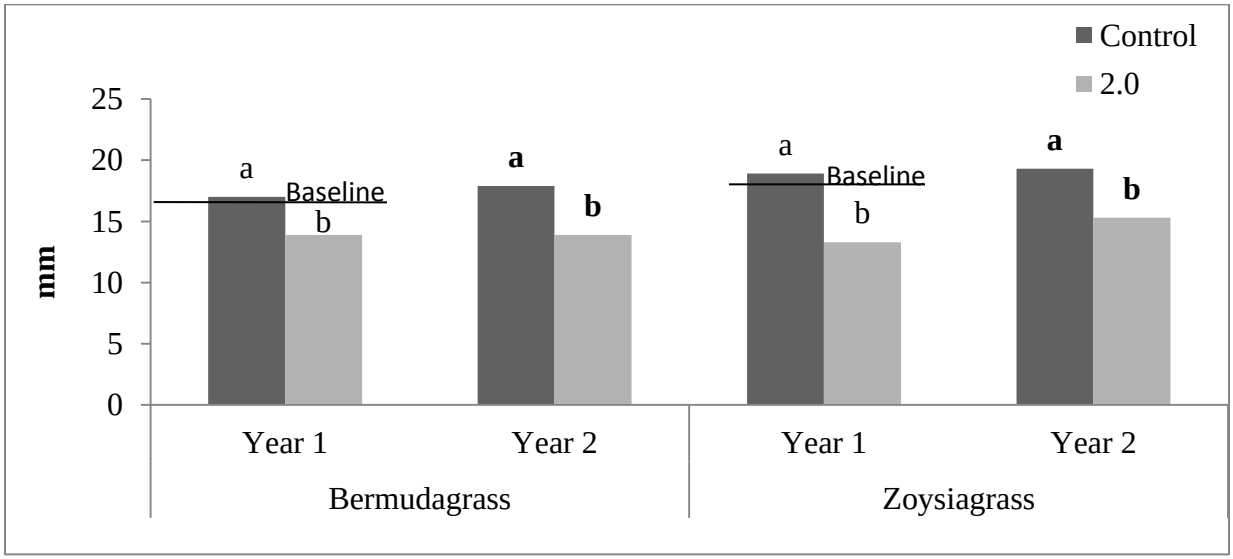

Fig. 6.2 Thatch layer thickness (TLT) after bi-weekly application of laccase on bermudagrass and zoysiagrass with two levels 0 (control) and 2.0 units cm^{-2}. Values are means of four replicates. Means represented by bars with same letter on top (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's LSD at α = 0.05. The horizontal line represents the baseline reading at the start of experiment.

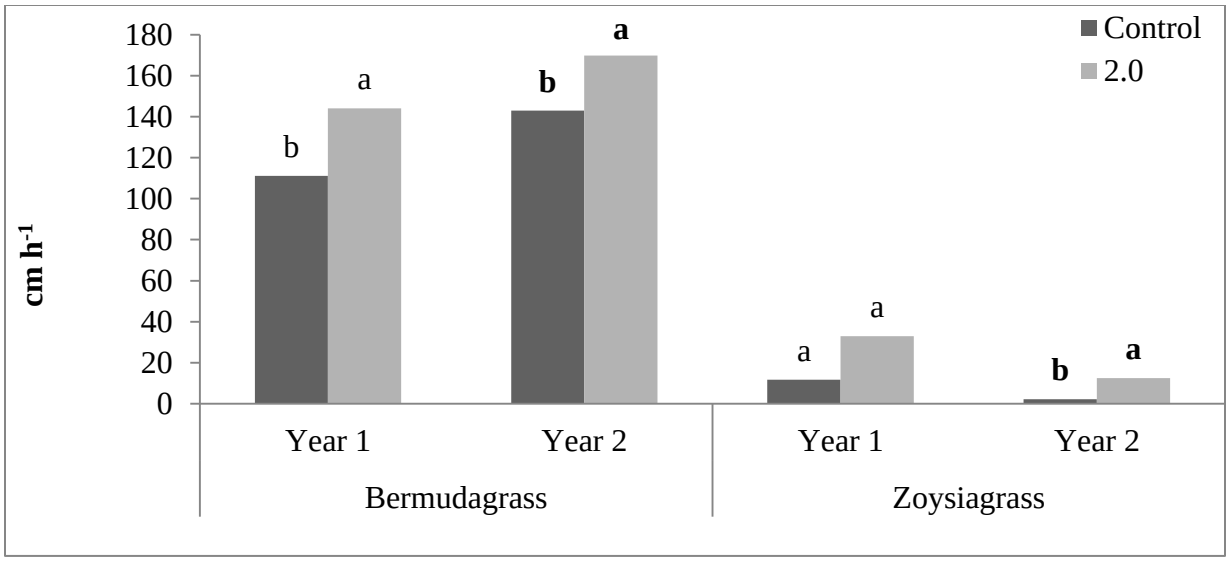

Fig. 6.3 Saturated hydraulic conductivity (SHC) after bi-weekly application of laccase on bermudagrass and zoysiagrass with two levels 0 (control) and 2.0 units cm^{-2}. Values are means of four replicates. Means represented by bars with same letter on top (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's LSD at $\alpha = 0.05$.

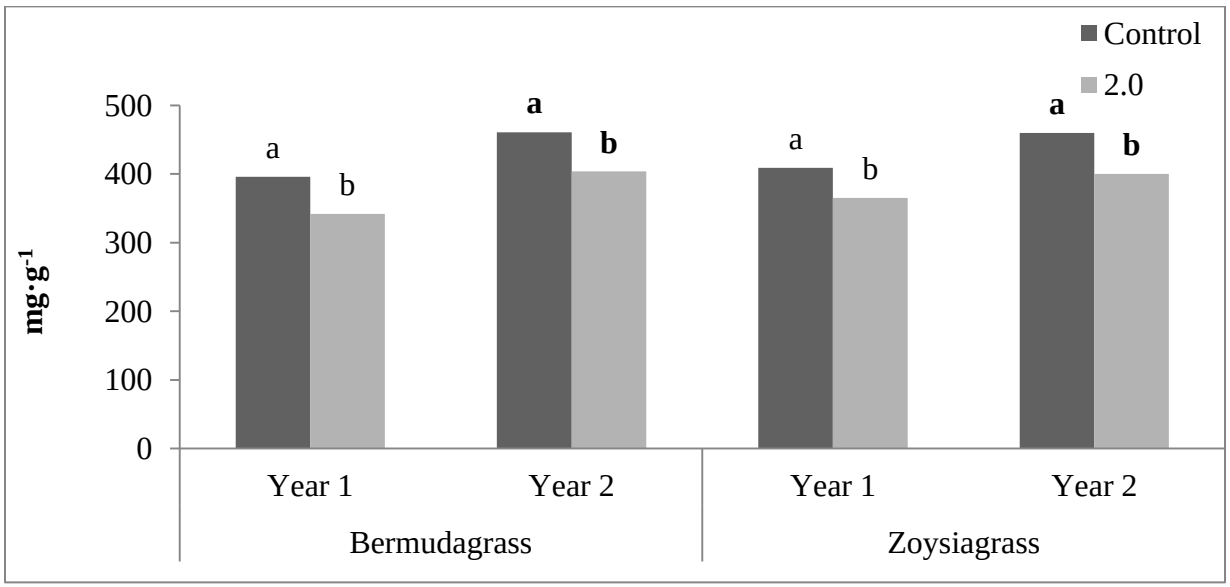

Fig. 6.4 Total lignin content (L_T) after bi-weekly application of laccase on bermudagrass and zoysiagrass with two levels 0 (control) and 2.0 units cm^{-2}. Values are means of four replicates. Means represented by bars with same letter on top (year one = standard, year two = bolded) are not considered to be statistically different according to Fisher's LSD at $\alpha = 0.05$.

CHAPTER VII

RESIDUAL EFFECT OF LACCASE APPLICATION ON THATCH LAYER CHARACTERISTICS OF CREEPING BENTGRASS[5]

[5] Sidhu, S.S., Q.Huang, R.N. Carrow, and P.L. Raymer. To be submitted to *Crop Science*.

ABSTRACT

Organic layer formation in the form of thatch is one of the major problems in turf management systems. Bi-weekly application of laccase enzyme has been well documented to facilitate the degradation of thatch layer and reduce the rate of accumulation of organic matter in "Crenshaw" creeping bentgrass (*Agrostis stolonifera* L.). A field experiment on creeping bentgrass was conducted to observe the residual effect after ceasing laccase application. The experiment consisted of twelve treatments partitioned into sub studies to investigate: different activity levels (rates); frequency; sources of laccase; and its application with cultural management practice i.e. core aeration and sand topdressing. Laccase treatments were applied for six months and response to residual laccase effect was recorded by sampling at six, twelve, and eighteen months after treatment initiation. One of the twelve treatments was applied for six months in year one and for six months in year two to compare the residual effect of laccase with two year laccase application. Parameters investigated were thatch layer thickness, total organic matter (0-2.5 cm, 2.5-5.0 cm, and 0-5.0 cm depth), saturated hydraulic conductivity, and acid-soluble and-insoluble lignin. A significant reduction in thatch layer thickness was observed with laccase at different rates and frequencies at six, twelve, and eighteen months after treatment initiation. Residual effect of laccase was observed in thatch layer depth with no accumulation in after six months of treatment cessation. A significant increase in thatch layer was observed at eighteen months after treatment initiation in plots where application ceased after six months and no accumulation was observed in plots treated with laccase for six months once a year.

INTRODUCTION

Lignin is a plant cell wall constituent that acts as a protective matrix and limits accessibility of microbial degraders to more biodegradable plant materials, such as cellulose and

hemicelluloses leading to the accumulation of organic matter in turfgrass ecosystems (Ledeboer and Skogley, 1967). Lignin is formed in plants by oxidative coupling of mono-lignols of three primary hydroxycinnamyl alcohols: p-coumaryl, coniferyl, and sinapyl alcohols (Wong, 2009). Lignin is extremely recalcitrant to degradation due to its complex structure without a regular pattern, which is derived from random oxidative coupling of lignin monomers and cross-linking of polymers via radical mechanisms, a process known as lignification (Ledeboer and Skogley, 1967). A lignin macromolecule contains monolignols randomly bonded by C-O-C and C-C linkages including β-O-4, β-5, β-β, 5-5, 4-O-5, and β-1 bonds (Alder, 1977; Del Rio et al., 2007; Ralph et al., 2004). Several models of lignin molecular structure have been proposed but these models do not imply any particular sequence of monomeric units in the lignin macromolecule (Chen and Sarkanen, 2003; Davin and Lewis, 2003).

Formation of thatch-mat layer in home lawn and recreational turfgrass sites, especially golf greens, is accelerated when accumulation of organic matter exceeds the degradation rate. Thatch, a layer of high organic matter content that accumulates between the soil and green turfgrass, consists of dead and living stolon, rhizome, root, crown, leaf sheath, and blade tissues (Engel, 1954; Roberts and Bredakis, 1960). A mat layer is developed by addition of soil and sand in thatch as a result of earthworm activity or cultural practices like core aeration and topdressing (McCarty, 2005). A thin layer of thatch is desirable as it helps to increase resilience and wear tolerance of the turfgrass surface, reduce surface hardness and moderate soil temperature extremes (Beard, 1973). However, excessive thatch or mat layer is undesirable in turfgrass as it leads to decreased saturated hydraulic conductivity, decreased movement of oxygen through the thatch or mat zone, and low oxygen levels within the thatch/mat layer during wet periods, and increased water retention (Carrow, 2003; Hartwiger, 2004; McCarty et al., 2007).

Cultural or mechanical control practices like core aeration, vertical mowing, grooming, and topdressing have been the most effective, but are known to adversely impact turf quality and have intensive requirement for labor, equipment and energy (Barton et al., 2009; Landreth et al., 2008; McCarty et al., 2007) as well as have shown contrasting results in reducing organic matter content in thatch layer (Barton et al., 2009; Carrow et al., 1987; Dunn et al., 1981; McCarty et al., 2005; McWhirter and Ward, 1976; Weston and Dunn, 1985; White and Dickens, 1984). Non-destructive biological and chemical attempts to enhance organic matter degradation in thatch layer have included usage of glucose, cellulase solutions (Ledeboer and Skogley, 1967), and commercial products containing mixture of amino acids, microbial inocula, and fertilizers. But, these have been inconsistent (McCarty et al., 2005; Murdoch and Barr, 1976) as they targeted the degradation of cellulosic and hemicellulosic sugars in thatch biomass by improving conditions for microbial populations.

The rate of microbial decomposition however is dependent on the lignin content of organic matter and lignin degradation acts as the rate limiting step in organic matter decomposition (Taylor et al., 1989). Sinsabaugh et al., (1993) conducted a plant litter decomposition study and reported a close relationship between lignocellulose-degrading enzymes and plant litter mass loss. Certain white-rot fungi are responsible for natural degradation of lignin by producing extra-cellular lignolytic enzymes and hence exposing cellulosic materials for further bacterial degradation in the environment (Blanchette, 1984; Kirk et al., 1975; Kirk et al., 1976; Mester et al., 2004; Otjen and Blanchette., 1987). Weight loss of bermudagrass pellets, St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) and zoysiagrass (*Zoysia japonica* Stued., 'Meyer') stolons were observed when inoculated with different wood-decaying fungi under controlled greenhouse and laboratory conditions (Martin

and Dale., 1980). In similar controlled studies, researchers have reported reduction in cellulose content and total oxidizable organic matter of bermudagrass (*Cynodon dactylon* L.) and centipedegrass (*Eremochloa ophiuroides*) when inoculated with wood-decaying fungi (Sartain and Volk, 1984). However, field inoculation experiments on bermudagrass showed no thatch degradation (Martin and Dale., 1980). Microbial inoculation under field conditions may be ineffective as it is very difficult to maintain suitable microbial environment for longer time periods under turfgrass management systems.

In greenhouse conditions favorable to growth of creeping bentgrass, decrease in the rate of thatch layer build up and accumulation of total organic matter relative to the control was reported in response to direct application of laccase enzyme, an extra-cellular lignolytic enzyme produced from white-rot fungi *Trametes versicolor* (Chapter III). However, a net accumulation of organic matter in thatch layer treated with laccase was observed over time in all treatments (Chapter III). On the other hand, a bi-weekly application of laccase enzyme on thatch layer of a dead creeping bentgrass for six months verified the effectiveness of laccase in facilitating organic matter decomposition and the loss in total sugar content of thatch biomass which suggested that laccase application exposed cellulosic and hemicellulosic sugars for microbial degradation by opening up the biomass structure (Chapter IV). Field studies conducted on creeping bentgrass, ultra-dwarf bermudagrass and zoysiagrass verified the effectiveness of laccase in thatch management on different turfgrass species (Chapter V, VI). However, bi-weekly application of laccase in comparison to core aeration followed by sand topdressing on creeping bentgrass suggested laccase application is as effective in thatch management as cultural practice (Chapter V). In our previous studies, organic matter degradation in response to enzyme treatment was determined during and at the end of the application period. The current project

was designed to expand on the previous studies by investigating the residual effect of laccase application on organic matter degradation. Knowledge of any residual effect would have management and economic implications. The major objectives of this study are: 1) to determine the residual effect of laccase application on thatch layer physical and chemical properties; 2) to compare residual effect of laccase application with continued laccase application on organic matter decomposition.

MATERIALS AND METHODS

A field experiment was conducted on "Crenshaw" creeping bentgrass (*Agrostis stolonifera* L.) (Engelke et al., 1995) at The University of Georgia, Griffin Campus as an 18 month study from July 2010 to Jan 2012. The bentgrass green was established as a sand based putting green on 90:10 sand and organic matter mix (Michigan Peat) as recommended by USGA. Fertilizer application routine on the plot for 2010 and 2011 consisted of 50 kg ha^{-1} granular fertilizer 24-4-10 (Lesco. Strongsville, OH) in the third week of March, September, and October and 2 kg ha^{-1} soluble 20-20-20 fertilizer (JR Peters Inc, Allentown, PA) every two weeks starting third week of April thru September. Bentgrass plots were mowed three times a week by Toro Greensmaster 3100 (The Toro Company, Bloomington, MN) and maintained at a height of 0.42 cm.

The experiment was conducted on plots (30.5 cm x 61.0 cm) in a completely randomized block design with twelve treatments replicated four times. Each block was divided into two halves, one received only laccase treatments and other half was core-aerated (Ryan Greensaire 24 Aerator, Johnson Creek, WI; tine diam. 1.27 cm; tine depth 6.25 cm; tine spacing 5.0 x 5.0 cm) and sand topdressed (1134 g per plot, Quikrete Premium Play Sand) using Scotts Precision Green Spreader twice a year. Laccase was applied for the initial 6 months from July 2010 to Dec

2010 in treatments T2 to T10 listed in Table 7.1. The plots were sampled at the end of treatment application period and again at twelve and eighteen months after treatment initiation to observe the residual effects of laccase application i.e. treatment ceased at six months so the residual effect was after this period. The sample time represents the duration for laccase treatment and residual time period. Laccase treatments were applied as 410 mL solution at different rates and frequencies (Table 7.1). Laccase enzyme from *Trametes versicolor*, a white-rot fungus, was purchased from Sigma-Aldrich (product 53739, Sigma Aldrich Inc., St. Louis, MO.) and was applied at activity levels of 0 (control), 0.5, 1.0, 2.0 and 4.0 units cm^{-2} applied every two weeks and laccase activity level 2.0 units cm^{-2} applied every 2, 4, 8, and 12 weeks to optimize the rate and frequency of laccase application. Laccase was also applied at 2.0 units cm^{-2} every 4 weeks on plots core-aerated and sand top-dressed twice a year to observe the effectiveness of laccase in combination with the cultural management practice. To better understand the treatments, the rate of the laccase activity level will be followed by the frequency of application in parenthesis hereafter – e.g., 2.0 (4) denotes laccase activity of 2.0 units cm^{-2} applied at 4 weeks (Table 7.1).

Laccase from two different sources was procured to compare their effectiveness on thatch management. Laccase from *Pycoporus* genus was procured from Jiangnan University, China (CHU (2)) and from a commercial industrial whole-sale supplier in China (CHI (2) and was applied at activity level of 2.0 units cm^{-2} every two weeks (Table 7.1). The treatment CHU (2) (i.e. T12) was applied from July 2010 to Dec 2010 and from July 2011 to Dec 2011 to compare the effect of application of laccase every year for six months to the residual effect of laccase (Table 7.1).

Laccase Activity Assay

The activity of laccase was quantified by a calorimetric assay using a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA) spectrophotometer where one activity unit of laccase corresponds to the amount of enzyme that causes an absorbance change at 468 nm at a rate of 1.0 unit min-1 in 3.4 mL of 1 mM 2, 6-dimethoxyphenol, a specific substrate for laccase, in citrate-phosphate buffer at pH 3.8 (Park et al., 1999).

Measurements

Residual effect of laccase application on physical and chemical properties of thatch layer was determined at six, twelve, and eighteen months after start of treatment application. Variables measured included total organic matter content for a depth of 0-2.5 cm (OM_U), 2.5-5.0 cm (OM_L), and 0-5.0 cm (OM), thatch layer thickness (TLT), and saturated hydraulic conductivity (SHC). Similarly, extractive-free acid-soluble lignin (L_S) and acid-insoluble lignin (L_I) content was determined to observe the impact of treatment application on chemical composition properties of thatch layer biomass.

Total Organic Matter Content

Total organic content was determined by the method described by Carrow et al. (1987). Two soil cores (2.0 cm diam.) were obtained at two depths; 0-2.5 cm (OM_U) and 2.5-5.0 cm (OM_L) from each plot. The cores were dried in an oven at 100 ± 5^{o}C for 24 h and weighed to determine moisture content and then ashed in a muffle furnace at 600 ± 10^{o}C for 24 h and weighed again. The difference in the two readings was used to calculate total organic matter content.

Thatch Layer Thickness

Thatch layer thickness was measured by two replaceable wedge-shaped turf profiles (8.9 cm wide and 2.5 cm thick) using AMS Turf Profiler (AMS Inc., American Falls, ID**).** Thatch layer thickness was measured from four points across the width of each profile and averaged. Clear visible distinction between thatch layer and the sand layer below was considered for the measurement.

Saturated Hydraulic Conductivity

Saturated hydraulic conductivity (SHC) was measured by a constant hydraulic head method using a Marriott tube apparatus. An intact core (diam. 4.7 cm and length 7.7 cm) was obtained from each plot in a brass cylinder using a soil corer (Model 0200 soil sampler, Soilmoisture Equip. Corp., Santa Barbara, CA). The bottom of the core was covered with a double layer of cheesecloth held in place with a rubber band and saturated overnight in a 0.05 N $CaCl_2$ solution. A steady state flow through the samples was established by flowing 0.05 N $CaCl_2$ through the core for 10 min. After 10 min the volume of water that passed through the core was measured for one minute and repeated three times. Saturated hydraulic conductivity was calculated using Darcy's equation.

Extractive-free Lignin Content

Thatch biomass was collected from the top 2.5 cm of each core after sampling for SHC. Thatch samples were first air-dried, ground, washed by adding water in a mason jar and shaking on a rotary shaker at 200 rpm, and then passed through a series of sieves with a 841µm sieve at the top and a 177µm sieve at the bottom. The material retained by the 177 µm sieve size was used for analysis. The thatch biomass was extracted for 24 h using the Soxhlet method for water- and alcohol-soluble impurities using de-ionized water and 16.26 M (95 percent USP grade) ethyl

alcohol, respectively. Contents of L_S and L_I in the thatch layer were determined in a two-step acid-hydrolysis procedure according to the laboratory analytical procedure developed by The National Renewable Energy Laboratory (NREL, 2008) Acid-soluble lignin is primarily low molecular mass phenolic compounds. In the first step, extractive-free thatch samples were hydrolyzed for 60 min with 72% H_2SO_4 at 30^{o}C. In the second step, H_2SO_4 was diluted to 4% and the samples were autoclaved at 121^{o}C for 1 h and then vacuum filtered. The solids remaining after acid hydrolysis were dried in an oven at 100 ± 5^{o}C for 24 h, weighed, ashed in a muffle furnace at 600 ± 10^{o}C for 24 h, and weighed again to calculate the acid-insoluble lignin content using weight difference. Acid-soluble lignin was determined using this hydrolysis liquid at 240 nm wavelength in a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA).

Statistical Analysis

A repeated measures design was used to analyze the full model for laccase residual effect, consisting of eleven treatments, three levels of treatment duration and four replications. Treatment CHU (i.e. T12) was repeated from July to Dec 2011 and is not considered in the full model. Treatments were combined together to form: a) a cultural management group [control, 2.0 (2), CMC, and CMC+2.0 (4)]; b) a rate of application group [control, 0.5 (2), 1.0 (2), 2.0 (2), and 4.0 (2)]: c) an application frequency group [Control, 2.0 (2), 2.0 (4), 2.0 (8), and 2.0 (12)]; d) a laccase sources group [2.0 (2), CHU (2), and CHI (2)]; and e) and two year application group [2.0 (2) and CHU (2)]. Analysis of variance (ANOVA) was performed to evaluate the main effects of treatment duration, treatments, and interaction effects of duration and treatment using general linear model (GLM) (SAS Institute, 1994). Treatments were grouped together in five groups and analyzed as repeated measures to evaluate the effects of treatment, treatment

duration, interaction effects of treatment and treatment duration. Fisher's protected LSD test with $\alpha = 0.05$ was used for determining statistical differences among durations and treatment means following each ANOVA.

RESULTS

Full Model

Strong treatment effects were observed for OM_U ($P \leq 0.01$), TLT ($P \leq 0.001$), SHC ($P \leq 0.001$), L_I ($P \leq 0.001$), and L_T ($P \leq 0.01$) (Table 7.2). Strong duration (time after treatment applications were initiated) effects ($P \leq 0.001$) were observed for OM_U, TLT and all lignin content measurements (Table 7.2) indicating residual effect of laccase application on these parameters. No duration effects were observed for SHC and OM_L. Interaction effects of duration by treatment ($P \leq 0.001$) were observed for L_I and L_T indicating that different treatments had different effects on extractive-free acid-insoluble and total lignin.

Cultural Management

The cultural management treatment group showed treatment effects for OM_U ($P \leq 0.05$), TLT ($P \leq 0.001$), SHC ($P \leq 0.05$) and L_I ($P \leq 0.001$) and L_T (Table 7.2). Significant duration effects were observed for organic matter content (0-2.5 cm, 2.5-5.0 cm, and 0-5.0 cm), TLT, and L_S, L_I, L_T extractive-free lignin content. Duration by treatment interaction effects ($P \leq 0.001$) were observed for L_I and L_T (Table 7.2).

In comparison to control, no differences were observed for OM_U, OM_L, and OM content at six months after treatment initiation (Table 7.3). When plots were sampled after twelve months of treatment initiation, OM_U content decreased by 50 and 40.7 $mg \cdot g^{-1}$ in plots treated with CMC and CMC+2.0 (4), respectively when compared to control plots. Significant duration effects were observed for OM_L and OM content which is evident from a slight increase of 18.4

and 18.7 $mg \cdot g^{-1}$ in OM_L and OM content in comparison to control when sampled after 18 months of treatment initiation (Table 7.3). No duration effect was observed for OM_U (Table 7.3). After six months, TLT decreased from 18.3 mm in control plots to 13.6 and 15.7 mm in plots treated with 2.0 (4) and CMC+2.0 (4) treatments, respectively (Fig 7.1A). Measurement of thatch layer from plots receiving core aeration and sand topdressing had 3 to 4 mm of sand additions and this was included in the calculations. A TLT reduction of 4.1 mm was observed in plots treated with 2.0 (4) when compared to control plots when sampled at twelve months after start of experiment. At eighteen months sampling, TLT lowered from 21.0 mm in control plots to 16.2 and 17.9 mm in plots receiving 2.0 (4) and CMC+2.0 (4) treatment, respectively (Fig 7.1A). Significant duration effects were observed for control and laccase treatment 2.0 (4) for TLT and no duration effects were observed for plots receiving cultural management practices (Fig 7.1A).

After six months of treatment application, plots receiving core aeration and sand topdressing treatment showed an increase of 13.5 cm h^{-1} in SHC in comparison to control plots (Table 7.4). Plots receiving laccase treatment with or without cultural management had no differences in SHC when compared to control plots (Table 7.4). No change in SHC was observed in comparison to control at other sampling durations. No duration effect was recorded for SHC in this group of treatments (Table 7.4). A reduction of 3.6, 7.8, and 3.8 $mg \cdot g^{-1}$ L_S content was recorded in plots treated with CMC, 2.0 (4), and CMC+2.0 (4), respectively when compared to control plots at six months sampling. However, no differences in L_S content were observed at sampling after twelve and eighteen months of treatment initiation. A slight but significant duration effect was observed for L_S content in 2.0 (4) treatment (Table 7.4). Extractive-free L_I and L_T content after six months of treatment application was lowered in plots treated with laccase treatment alone and increased in plots treated with CMC and CMC+2.0 (4) treatment when

compared to control plots Table 7.4, Fig 7.2A). A similar trend was recorded for L_I at sampling conducted at twelve months after treatment initiation. Acid-insoluble content in all the treatments increased over control at eighteen months after treatment initiation. Significant duration effects were observed for L_I and L_T with increase in lignin content in all the treatments over time (Table 7.4, Fig 7.2A).

Rate of Application

Rate of laccase application significantly effected ($P \leq 0.001$) TLT, L_I, and L_T (Table 7.2). Strong duration effects were observed for OM_U ($P \leq 0.001$), TLT ($P \leq 0.001$), L_S ($P \leq 0.01$), L_I ($P \leq 0.001$), and L_T ($P \leq 0.001$) (Table 7.2). Interaction effects ($P \leq 0.001$) of duration by treatment were observed for L_I and L_T content. After six months of treatment, no differences were observed for OM_U, OM_L, and OM in any of the treatments. Sampling at twelve months after treatment initiation, OM_U at laccase activity level of 4.0 units cm^{-2} decreased by 21.5 $mg \cdot g^{-1}$ when compared to control (Table 7.3). No differences were observed for OM_L and OM for twelve month sampling. At eighteen months sampling after start of experiment a 10.4 $mg \cdot g^{-1}$ increase in OM content was obtained in plots treated with 1.0 (2) over control plots. Organic matter content (0-2.5 cm) increased by 19.5 $mg \cdot g^{-1}$ at 0.5 units cm^{-2} when sampled between six and eighteen months after treatment initiation. A significant reduction of 6.2 and 8.0 $mg \cdot g^{-1}$ in OM_L content from six to eighteen months for treatments 2.0 (2) and 4.0 (2), respectively was observed and reduction of OM (8.5 $mg \cdot g^{-1}$) from six to twelve months was observed for treatment 4.0 (2) suggesting the residual effect of laccase.

Laccase treatments at different activity levels were equally effective and lowered TLT by 3.8 to 4.8 mm in comparison to the control after six months of treatment application (Fig 7.1B). Twelve months after start of the treatment, TLT was lowered by all activity levels of laccase.

However, plots treated with laccase at activity levels of 2.0 and 4.0 units cm^{-2} showed significant reduction in thatch layer thickness in comparison to control plots and plots treated with 0.5 and 1.0 units cm^{-2} laccase activity. A reduction in TLT was observed for all treatments in comparison to control when sampled after eighteen months after start of the experiment. Application of laccase at 0.5, 1.0 and 2.0 units cm^{-2} was effective is maintaining the TLT up to six months after treatment completion whereas in plots treated with laccase at 4.0 units cm^{-2}, TLT was lowered from 14.5 to 13.3 mm from six months after treatment initiation to twelve months after start of treatment. A significant increase in TLT was obtained when laccase was applied at 0.5 and 1.0 units cm^{-2} over the three sampling dates.

No effect of laccase activity levels were observed on SHC on all the sampling dates (Table 7.4). Laccase application up to 2.0 units cm^{-2} lowered L_S by 7.8 to 8.9 $mg \cdot g^{-1}$ when compared to control when sampled after six months of treatment (Table 7.4). Acid-soluble lignin in plots treated with 4.0 cm^{-2} laccase activity was reduced by 12.2 $mg \cdot g^{-1}$ when compared to control plots at the end of treatment (Table 7.4). No differences in L_S were observed at sampling times of twelve and eighteen months after treatment initiation.

Extractive-free L_I content lowered in comparison to control plots when treated with laccase up to 2.0 and 1.0 units cm^{-2} at six and twelve months sampling after start of treatment, respectively (Table 7.4). At the end of the treatment application, L_I was higher in plots treated with 4.0 units cm^{-2} when compared to control. Similarly, L_I content was higher than control plots when treated with 2.0 and 4.0 units cm^{-2} laccase activity, sampled at twelve months after treatment initiation suggesting residual effect of laccase. Plots treated with laccase showed higher L_I content compared to control plots at eighteen months after treatment initiation (Table

7.4). An increase in the L_I content was observed in all the treatments (Table 7.4). Variation in L_T content followed similar trends as for LI content with different laccase activity levels (Fig 7.2B).

Frequency of Application

Laccase application frequency effects ($P \leq 0.001$) were observed for TLT, L_I, and L_T (Table 7.2). Strong duration effects ($P \leq 0.001$) were observed for OM_U, TLT, and L_T content (Table 7.2). Interaction effects of duration by treatment were observed for L_I ($P \leq 0.001$) and L_T ($P \leq 0.001$) (Table 7.2). No differences in OM_U, OM_L, and OM in plots treated with laccase and control plots were observed after six and twelve months of treatment initiation. However, an increase in OM_U and OM content was obtained in plots treated with 2.0 (12) over control plots at eighteen months after start of treatment application. An accumulation of 52.3 $mg \cdot g^{-1}$ in OM_U was obtained in plots treated with 2.0 (12) between the between six and eighteen months of sampling dates (Table 7.3).

Thatch layer thickness was lowered by 4.7 to 5.6 mm, 4.1 to 5.6 mm, and 3 to 4.8 mm in plots treated with laccase at different frequencies in comparison to control plots when sampled at six, twelve, and eighteen months, respectively (Fig 7.1C). No differences among different application frequencies were observed at any sampling date. An increase in thatch layer thickness was observed in all treatments at eighteen months sampling when compared to TLT after six months of treatment. No significant change in TLT was observed at twelve months sampling (Fig 7.1C).

All laccase treatments were effective in lowering the L_S content when compared to control six months after treatment initiation (Table 7.4). However, the decrease in L_S content in comparison to control was more with treatment 2.0 (2) than 2.0 (8) and 2.0 (12) (Table 7.4). It suggests that frequent application of laccase was more effective in lowering L_S content in

comparison to control. No differences in L_S values were observed when sampled at twelve and eighteen months after start of the experiment. After six months of treatment, a reduction in L_I content was observed in plots receiving laccase treatments at every 2 (29.6 $mg \cdot g^{-1}$) and 4 (15.5 $mg \cdot g^{-1}$) weeks when compared to control (Table 7.4). The L_I content increased by 5.8 $mg \cdot g^{-1}$ in plots receiving 2.0 (12) treatment when compared to the control plots. Sampling after twelve months indicated an increase in L_I content when laccase was applied every 2, 8, and 12 weeks. However, with application of laccase every 4 weeks, a slight reduction in L_I content was observed at twelve months sampling in comparison to control. All laccase treated plots showed increase in L_I content in comparison to control plots when sampled at eighteen months (Table 7.4). A significant increase in L_I content was observed in all the treatments when sampled over time (Table 7.4). Similar trends were observed for L_T content with laccase application at different frequencies (Fig 7.2C).

Sources of Laccase Enzyme

Laccase enzymes procured from different sources were similarly effective on organic matter (OM_U, OM_L, and OM; Table 7.3), TLT (Fig 7.1C), and SHC (Table 7.4). Slight differences in L_S and L_I content were observed in plots treated with laccase from different sources (Table 7.4). Six months after treatment initiation, L_S content was lower in plots receiving 2.0 (2) in comparison to other two laccase enzymes. After, 12 and 18 months of sampling, no differences in L_S content were observed in plots treated with different laccase enzymes. When treated with CHI (2) for six months, L_I content was slightly higher than the other two laccase enzymes. However, when sampled after twelve months, L_I content was higher in plots treated with 2.0 (2) followed by CHI (2) and CHU (2). No differences in L_S and L_I content were observed in plots receiving different laccase sources at eighteen months after start of treatment

(Table 7.4). A significant increase in L_I and L_T content was observed from six to eighteen months sampling duration (Table 7.4, Fig 7.2D).

Application Duration

Strong effects of laccase application duration were observed for OM, TLT and L_T at sampling performed at eighteen months after treatment initiation (Table 7.3, Table 7.4, and Fig 7.3). An increase in organic matter was observed at eighteen months sampling after treatment initiation for treatment CHU (2), which was applied for six months in year one and six months in year two, in comparison to application of laccase 2.0 (2) applied for only six months in year one (Table 7.3). The baseline measurement for TLT was 17.2 mm. The TLT value in control plots continue to increase with time whereas in plots treated with 2.0 (2) and CHU (2), after six months, a significant reduction in TLT was observed in comparison to control. When sampled 12 months after treatment initiation, TLT was slightly lower than when sampled after six months in both the treatments. When sampled after eighteen months TLT in plots treated with 2.0 (2) was 3.3 mm higher than plots treated with CHU (2) (Fig 7.3). Lignin content after eighteen months was slightly higher in both the treatments when compared to the control (Fig 7.4).

DISCUSSION

Non-destructive methods to manage thatch are desired but have shown to be ineffective. Use of some commercial microbial inoculum such as Biodethatch, Thatch-Away, and Earth Anew on bermudagrass, creeping bentgrass and annual bluegrass was reported to be ineffective to reduce thatch layer depth (Gilbeault et al., 1976; Lancaster et al., 1977; Murdoch and Barr, 1976). Similarly, application of biological granular supplement Thatch-X on creeping bentgrass (McCarty e al., 2007), wetting agent, Aqua-Gro® and Milogranite, activated sewage sludge, on Kentucky bluegrass (Murray and Juska, 1977) was ineffective in lowering organic matter content

in thatch layer. One of the possible reasons that past studies found these products to be inconsistent in organic matter decomposition is that they focused on degradation of cellulosic and hemicellulosic sugars instead of lignin. We believe that lignin protective matrix has to be removed to open the biomass structure on increase access to readily decomposable structural carbohydrates.

Controlled greenhouse and laboratory studies reported weight loss of bermudagrass pellets, St. Augustinegrass and zoysiagrass stolons (Martin and Dale., 1980) and reduction in cellulose content and total oxidizable organic matter of bermudagrass and centipedegrass (*Eremochloa ophiuroides*) (Sartain and Volk, 1984) when inoculated with different wood-decaying fungi. However, field inoculation experiments on bermudagrass showed no thatch degradation (Martin and Dale., 1980). This may be due to the inability to maintain proper micro-environment conditions, particularly moisture and temperature regimes, required by particular microbial populations under turfgrass management systems which further may lower the possibility of maintaining higher microbial populations over sustained periods of time.

Laccase enzyme is stable over a wide range of pH and temperature (Baldrian, 2006; Munoz et al., 1997; Stoilova et al., 2010; Thurston, 1994). Laccase, a multi copper oxidase, is an extra-cellular enzyme known to oxidize a wide range of phenolic compounds using oxygen as an electron acceptor (Baldrian, 2006). Lignin phenolic components are oxidized due to laccase-mediated cleavage of different covalent bonds formed within lignin macromolecule and between lignin and structural sugars (Wong, 2009). This opens up the biomass structure leading to increased availability of easily degradable sugars by microbes. By using laccase enzyme, turfgrass managers may have a new means to effectively manage thatch over wide range of environmental conditions and can effectively utilize the microbial decomposition of organic

matter. In previous studies, reduction in thatch/mat layer by laccase treatment was demonstrated in greenhouse and field research (Chapter III, IV, V, VI). However, the question of residual effects was not addressed, which is the focus of the current project.

Cultural Management and Laccase

Cultural management using several management techniques has reported contrasting results for reduction in thatch layer thickness and accumulation of organic matter. Carrow et al. (1987) reported a decrease in thatch layer depth of 44-62% with one or two applications of topdressing annually. Sand topdressing four times a year was reported to be effective in reducing thatch layer when compared to single application (White and Dickens, 1984). Barton et al. (2009) reported a significant reduction in organic matter content with sand topdressing twice a year on Kikuyu turfgrass. It was also noted that core aeration along with sand topdressing was equally effective in reducing thatch layer depth and organic matter content. However, Engel and Alderfer (1967), McCarty et al. (2007), and Reiki (1994) observed no reduction in thatch layer by topdressing alone. It has been suggested that application of sand topdressing improves microenvironment for microbial growth (Ledeboer and Skogly, 1967). However, some researchers believe that dilution of organic matter in thatch layer is primary influence of sand topdressing (Couillard et al., 1997; Rieki, 1994). Topdressing alone had no effect on water infiltration rates (McCarty et al., 2007).

A 10% reduction in thatch layer thickness was reported by core aeration four times annually on creeping bentgrass (McCarty et al., 2007) and three to six times a year on Tifgreen bermudagrass (McWhirter and Ward, 1976). Carrow et al. (1987) noted no effect of core aeration applied once or twice a year on Tifway bermudagrass on thatch-mat depth although a reduction in stand density was observed. Several studies have reported an increase in water infiltration in

turfgrass field after core aeration due to formation of water channels and porous profile (Bunnell et al., 2001; Canaway et al., 1986; McCarty et al., 2005).

In our study, organic matter content in the top 2.5 cm was lower in comparison to the control at 12 months with cultural management practice and this may be attributed to the dilution effect created by sand topdressing on the surface layer as sand topdressing showed no effect on organic matter at 2.5-5.0 cm depth (Table 7.3). The increase in OM_L (18 months) and OM (12 and 18 months) for laccase treated thatch may be related to a more dense thatch biomass occurring from laccase activity on cellulose and hemicellulose sugars resulting in a higher content of L_T as seen at 18 months (Fig 7.2A). As raw organic matter decomposes, such as in composting situations, the resulting material increases in lignin content and density.

Lignin dynamics were also apparent in TLT results, where application of laccase along with cultural management effectively decreased TLT at six and eighteen months and increased L_T content at all three sampling times (Fig 7.1A, 7.2A). The increase in L_T may be attributed to the change in thatch biomass structure caused by laccase, making structural sugars more available for decomposition as well as better micro-climate for microbial growth due to core aeration and sand topdressing. Increase in loss of structural sugars from thatch biomass may lead to elevated levels of lignin in the remaining thatch material. A significant loss in structural sugars of creeping bentgrass thatch biomass was observed with application of laccase in greenhouse and field studies (Chapter IV, V).

Saturated hydraulic conductivity was higher at six months in plots with only CMC (Table 7.4). When laccase was applied along with CMC, the SHC was higher at eighteen month sampling when compared to plots receiving only laccase treatments as well as control. The

increase in SHC may be attributed to the core aeration, which creates channels for rapid water movement.

The laccase-CMC data illustrated that the 2.0 (4) treatment of laccase was effective in reducing TLT at 6, 12, and 18 months, when applied alone or with CMC. Laccase alone did not influence SHC, but in combination with CMC, SHC increased relative to the control. These results suggest that laccase has positive effects thatch control and can be used in conjunction with routine CMC.

Laccase Rate and Frequency

Laccase treatments at different rates and frequency of application were ineffective in reducing OM_U, OM_L, and OM content after six months of application (Table 7.3). However, the highest laccase application 4.0 units cm^{-2} applied at every two weeks showed a significant reduction in OM_U content when plots were sampled at twelve months after treatment initiation (Table 7.3). This brings up a very interesting point that laccase application for six months was effective in slowing accumulation of OM_U for over the next six months. As the time progresses the residual effect of laccase declines and OM_U values increase in the treated plots and is similar to the control plots as is evident from the eighteen months data. The reduction in OM_L content was observed over sampling period in plots treated with 2.0 and 4.0 units cm^{-2} (Table 7.3). This may also lead us to believe that a long term application of laccase at a high rate (2.0 and 4.0 units cm^{-2}) may be effective in lowering organic matter content in turf greens that are established for a long time and contain high organic matter content in the thatch/mat layer.

Laccase applied at all frequencies and rates was effective in lowering TLT in comparison to control after six months of treatment (Fig 7.1B, 7.1C). Additionally, TLT readings from plots treated with the various laccase rates and frequencies were lower relative to the control plots

after twelve and eighteen months suggesting strong residual effects of laccase (Fig 7.1B). However, TLT readings were significantly higher at the three highest laccase rates at eighteen months sampling when compared to sampling conducted at twelve months after treatment initiation suggesting that residual effect of laccase in thatch layer is effective for up to six months after treatment cessation (Fig 7.1B). Thatch layer accumulation decreased with application of laccase during second year (Fig 7.3), which suggests that annual application of laccase enzyme for six months in the growing season is effective in reducing or stabilizing thatch accumulation. While laccase treatments did reduce TLT, there were only minor differences in SHC with no apparent trend (Table 7.4).

In the sub-study involving laccase application for six months in year one and again for six months in year two, a significant reduction in thatch layer was observed in comparison to plots receiving laccase for six months in year one when sampled at eighteen months after treatment initiation (Fig 7.3). Residual effect of laccase, after treatment cessation, reduced thatch layer buildup during the next six months. But a significant increase in thatch layer was observed when no laccase enzyme application was performed during second year. However, no thatch buildup was observed when laccase was applied for second year. No further reduction in thatch layer observed during the second year even with application of laccase suggests a threshold level for thatch layer reduction by application of laccase. Increase in OM_U was observed in plots where laccase was applied for six months during second year when compared to laccase applied for six months in year one (Table 7.3). This may be attributed to tight stacking of thatch biomass due to removal of lignin bonds, reduction of structural sugars leading to weak thatch biomass.

Extractive-free L_S content initially decreased with application of laccase as was evident from sampling conducted after conclusion of treatments at 6 months (Table 7.4). The extent of

reduction at 6 months was dependent on the amount of laccase applied to the plots whether by increasing rate or frequency of applications. For L_I, application of laccase up to 2.0 units cm^{-2} decreased L_I in thatch biomass, but application of laccase at 4.0 units cm^{-2} showed an increase in L_I content by dry weight basis in comparison to control (Table 7.4). The increase in L_I content could be attributed to the loss of excessive structural sugars in plots treated with laccase at 4.0 units cm^{-2} (Chapter V). Three major components of plant biomass are cellulosic sugars, hemicellulosic sugars, and lignin. So, with application of laccase, lignin bonds are broken which leads to opening up of the biomass structure making sugars more available for microbial decomposition. As the sugar content is decreased, it tends to increase the lignin content by dry weight basis. A decrease in structural carbohydrate (cellulosic and hemicellulosic sugars) content in thatch biomass after laccase treatment was reported (Chapter IV, V). A decrease in structural sugar content was observed as the rate of laccase activity increased indicating more availability of sugars for microbial degradation (Chapter V). Residual effect of laccase application was observed with an increase in lignin accumulation in thatch biomass from 6 to 18 months samplings suggesting more loss of sugar content from the biomass due to structural changes caused by laccase application (Table 4). As L_I constitutes the major component of L_T, a similar trend in L_T was observed with increasing laccase application rates (Fig 7.2B). For L_S, L_I and L_T, a maximum reduction in comparison to control at 6 months was observed when laccase was applied every two weeks and extent of reduction decreased with decreased frequency of laccase application (Table 7.4, Fig 7.2C) suggesting the extent of lignin reduction was dependent on the amount of laccase applied to the plots.

Laccase Source

Different sources of laccase enzymes proved equally effective in reducing thatch layer thickness (Fig 7.1D). However, slight differences in L_S and L_I were observed with different laccase sources (Table 7.4). Lignin content (L_S and L_I) was significantly lower in plots treated with laccase procured from Sigma Aldrich in comparison to plots treated with CHU and CHI after six months of application but no differences were observed in lignin content when sampled at twelve and eighteen months after treatment initiation (Table 7.4). This suggests that laccase from Sigma Aldrich was initially more effective but other laccase enzymes proved to be equally effective on thatch biomass.

CONCLUSIONS

This field research demonstrated the residual effects of laccase enzyme on creeping bentgrass with TLT reduced by an average across all laccase rates of 23.5, 25.7, and 23.9% of the control for 6, 12, and 18 month sampling times, respectively (Fig 7.1B). In a similar manner, the average TLT reduction across all frequencies of laccase applications were 27.8, 25.5, and 19.7% of the control for 6, 12, and 18 month samplings, respectively (Fig 7.1C). Organic matter contents (OM_U, OM_L, and OM) were not appreciably affected by laccase rates or frequency of the three sample dates. Since total lignin content increased, suggesting decomposition of cellulose and hemicellulose fractions with concentration of lignin, the total OM may not change but the composition did over time. The 2.0 (4) treatment of laccase was effective in reducing TLT at 6, 12, and 18 months, when applied alone or with CMC. Laccase alone did not influence SHC, but in combination with CMC, SHC increased relative to the control. Laccase from different sources were equally effective in organic matter decomposition and thatch layer reduction. Laccase application for six months during second year was effective in ceasing thatch

layer buildup. The results from this study indicate that application of laccase for six months in one year is effective to reduce the organic matter content and thatch layer thickness. Six months application once a year would be effective to maintain thatch layer.

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Table 7.1 Description of laccase treatments applied on creeping bentgrass

Treatment No	Volume of solution	Laccase activity level	Application frequency	Cultural Mgt. practice	Source of laccase	Designation	Application time frame
T1 Control	410 mL	0	2	No	NA	Control	0-6 months 12-18 months
Cultural management							
T2 CMC	0 mL	0	0	Yes	NA	CMC	
T3 CMC+2.0	410 mL	2.0	4	Yes	Sigma Aldrich	CMC+2.0 (4)	0-6 months
Rate of application							
T4 0.5 (2)	410 mL	0.5	2	No	Sigma Aldrich	0.5 (2)	0-6 months
T5 1.0 (2)	410 mL	1.0	2	No	Sigma Aldrich	1.0 (2)	0-6 months
T6 2.0 (2)	410 mL	2.0	2	No	Sigma Aldrich	2.0 (2)	0-6 months
T7 4.0 (2)	410 mL	4.0	2	No	Sigma Aldrich	4.0 (2)	0-6 months
Application frequency							
T8 2.0 (4)	410 mL	2.0	4	No	Sigma Aldrich	2.0 (4)	0-6 months
T9 2.0 (8)	410 mL	2.0	8	No	Sigma Aldrich	2.0 (8)	0-6 months
T10 2.0 (12)	410 mL	2.0	12	No	Sigma Aldrich	2.0 (12)	0-6 months
Laccase sources							
T11 CHI (2)	410 mL	2.0	2	No	Industrial-China	CHI (2)	0-6 months
T12 CHU (2)	410 mL	2.0	2	No	University-China	CHU (2)	0-6 months 12-18 months

Table 7.2 Analysis of variance (ANOVA) table showing the effects of laccase treatments, treatment duration, and duration and treatment interactions on creeping bentgrass.

Source of variation	df	Organic matter (OM_U) (0-2.5 cm)	Organic matter (OM_L) (2.5-5.0 cm)	Organic matter (OM) (0-5.0 cm)	Thatch layer thickness TLT	Saturated hydraulic conductivity SHC	Acid-soluble lignin L_S	Acid-insoluble lignin L_I	Total lignin L_T
		----------mean square value----------							
Full Model									
Rep	3	1429	973	779	5	123	282	60	167
Treatment	10	2583**	316	515	40***	131***	37	2065***	1966***
Error A (rep*treatment)	30	699***	317*	247**	2	24	30	21	56
Duration	2	7934***	15	792**	85***	110	1205***	69963***	79960***
Duration*treatment	20	316	181	106	2	24	36	970***	1127***
Error	66	221	194	105	2	39	66	26	123
Cultural Management									
Rep	3	194	122	58	2	75	110	36	31
Treatment	3	4865*	252	821	49***	272*	28	1668***	1388**
Error A (rep*treatment)	9	906**	195***	217**	2	57	28	22	41
Duration	2	1343**	132*	436***	23**	138	226**	13999***	15053***
Duration*treatment	6	275	65	114	0.7	44	23	206***	317***
Error	24	225	38	47	3	74	32	15	52
Rate of Application									
Rep	3	4105	1368	1747	4	108	182	52	141
Treatment	4	400	139	201	53***	3	55	3029***	2980***
Error A (rep*treatment)	12	313	175***	146**	0.6	4	32	19	42
Duration	2	2900***	112	120	29***	35	507**	37614***	43511***
Duration*treatment	8	225	36	46	2	4	31	1696***	1982***
Error	30	249	37	40	1	23	59	25	101
Appl. Frequency									
Rep	3	1333	1134	522	3	55	73	19	41
Treatment	4	322	488	380	53***	4	59	2170***	1641***
Error A (rep*treatment)	12	663**	464	302	1	5	43	35	78
Duration	2	4108***	173	404	64***	25	690**	35405***	38414***
Duration*treatment	8	496	290	95	1	4	47	2113***	1439***
Error	30	230	346	158	2	17	85	29	168
Laccase Sources									
Rep	3	531	510	418	8	55	92	10	43
Treatment	2	1088*	86	281	1	12*	23	2909***	2703***
Error A (rep*treatment)	6	337	224	207	0.8	12	7	7	5
Duration	1	34	94	87	1	65*	83	46837***	42974***
Duration*treatment	2	37	20	1	0.1	2	13	1360***	1622***
Error	9	193	128	102	2	9	68	32	84
2 Year Application									
Rep	3	862	857	706	4	194	85	113	32
Treatment	1	4150**	780	1520	15	75	57	1447***	926**
Error A (rep*treatment)	3	120	381**	330*	3	56	7	2	12
Duration	2	3337*	105	460*	8*	113	284*	31421***	34666***
Duration*treatment	2	97	174	100	4	41	10	2142***	2362***
Error	12	526	56	74	2	125	60	49	105

* Significant at the 0.05 probability level
**Significant at the 0.01 probability level
*** Significant at the 0.001 probability level

Table 7.3 Total organic matter content at three depths; 0-2.5 cm (OM_U), 2.5-5.0 cm (OM_L), and 0-5.0 cm (OM) at six, twelve and eighteen months after initiation of different laccase treatments applied on creeping bentgrass.

	Total organic matter (0-2.5cm) OM_U			**Total organic matter (2.5-5.0 cm) OM_L**			**Total organic matter (0-5.0 cm) OM**		
Treatment group	6 Months	12 Months	18 Months	6 Months	12 Months	18 Months	6 Months	12 Months	18 Months
	---------- $mg \cdot g^{-1}$ ----------								
Cultural Mgt.									
Control	132.0ab†**A**‡	138.6a **A**	140.1ab **A**	63.9a **A**	62.8a **A**	61.5b **A**	90.4a **A**	89.7ab **A**	91.1b **A**
CMC	107.1b **AB**	88.6b **B**	118.4b **A**	66.7a **A**	60.5a **A**	70.8ab **A**	83.6a **AB**	72.4b **B**	90.51b **A**
2.0 (4)	134.7a **A**	141.7a **A**	160.9a **A**	67.7a **B**	73.2a **AB**	79.9a **A**	93.8a **B**	99.8a **B**	109.8a **A**
CMC+2.0 (4)	113.0ab **AB**	97.9b **B**	118.4b **A**	71.1a **A**	63.9a **A**	71.1ab **A**	89.1a **AB**	77.7b **B**	89.8b **A**
Rate of Appl.									
Control	132.0a **A**	138.6a **A**	140.1a **A**	63.9a **A**	62.8a **A**	61.5ab **A**	90.4a **A**	89.7a **A**	91.1b **A**
0.5 (2)	135.8a **B**	139.6a**AB**	155.3a **A**	69.9a **A**	69.0a **A**	60.6ab **A**	95.8a **A**	95.2a **A**	93.8ab **A**
1.0 (2)	131.5a **A**	133.9ab **A**	165.4a **A**	64.9a **A**	65.5a **A**	67.6a **A**	90.6a **A**	91.6a **A**	101.5a **A**
2.0 (2)	125.2a **A**	127.7ab **A**	156.9a **A**	66.4a **A**	59.0a **B**	60.2ab **B**	90.0a **A**	85.5a **A**	94.4ab **A**
4.0 (2)	129.9a **AB**	117.1b **B**	142.2a **A**	63.1a **A**	56.1a **AB**	55.1b **B**	88.6a **A**	80.1a **B**	85.8b **AB**
Freq. of Appl.									
Control	132.0.a **A**	138.6a **A**	140.1b **A**	63.9a **A**	62.8a **A**	61.5b **A**	90.4a **A**	89.7a **A**	91.1c **A**
2.0 (2)	125.2a **A**	127.7a **A**	156.9ab **A**	66.4a **A**	59.0a **B**	60.2b **B**	90.0a **A**	85.5a **A**	94.4bc **A**
2.0 (4)	134.7a **A**	141.7a **A**	160.9ab **A**	67.7a **A**	73.2a **A**	79.9a **A**	93.8a **A**	99.8a **A**	109.8a **A**
2.0 (8)	134.8a **A**	139.7a **A**	148.8b **A**	64.4a **A**	65.0a **A**	68.6ab **A**	91.7a **A**	93.0a **A**	97.2abc **A**
2.0 (12)	131.6a **B**	129.0a **B**	183.9a **A**	62.6a **A**	94.5a **A**	70.6ab **A**	89.8a **A**	106.7a **A**	107.9ab **A**
Lacc. Sources									
2.0 (2)	125.2b **A**	127.7a **A**	156.9a **A**	66.4a **A**	59.0a **B**	60.2b **B**	90.0a **A**	85.5a **A**	94.4b **A**
CHI (2)	136.8ab **AB**	132.6a **B**	163.9a **A**	69.1a **A**	65.6a **A**	76.6ab **A**	95.5a **AB**	91.5a **B**	108.9a **A**
CHU (2)	152.2b **A**	146.7a **A**		69.5a **A**	68.5a **A**		101.1a**AB**	98.1a **B**	
Cont. appl									
2.0 (2)	125.2a **A**	127.7a **A**	156.9a **A**	66.4a **A**	59.0a **B**	60.2a **B**	90.0a **A**	85.5a **A**	94.4b **A**
CHU (2)	152.2a **A**	146.7a **A**	189.8a **A**	69.5a **A**	68.5a **A**	81.8a **A**	101.1a**AB**	98.1a **B**	118.4a **A**

†Means within a column in a treatment group (treatment effect) followed by the same lowercase letter are not significantly different according to LSD at α=0.05

‡ Means within a row in a treatment group (duration effect) followed by the same uppercase letter are not significantly different according to LSD at α=0.05.

Table 7.4 Saturated hydraulic conductivity (SHC), extractive-free acid-soluble (L_S) and-insoluble lignin (L_I) at six, twelve and eighteen months after initiation of different laccase treatments applied on creeping bentgrass.

	Saturated hydraulic conductivity SHC			**Extractive-free Acid-soluble lignin L_S**			**Extractive-free Acid-insoluble lignin L_I**		
Treatment group	6 Months	12 Months	18 Months	6 Months	12 Months	18 Months	6 Months	12 Months	18 Months
	----------cm h^{-1}----------			----------mg·g^{-1}----------					
Cultural Mgt.									
Control	3.3b† **A**‡	4.2a **A**	6.9b **A**	82.5a **A**	76.2a **A**	82.9a **A**	279.6c **C**	316.6c **B**	326.8c **A**
CMC	16.8a **A**	9.9a **A**	14.3ab **A**	78.9b **A**	74.4a **A**	82.2a **A**	289.2b **C**	327.9b **B**	339.9b **A**
2.0 (4)	1.6b **A**	3.6a **A**	4.0b **A**	74.7c **B**	75.7a **B**	85.0a **A**	264.1d **C**	306.2d **B**	338.7b **A**
CMC+2.0 (4)	8.1ab **A**	7.7a **A**	21.3a **A**	78.7b **A**	72.8a **A**	79.0a **A**	294.1a **B**	343.3a **A**	350.4a **A**
Rate of Appl.									
Control	3.3ab **A**	4.2a **A**	6.9a **A**	82.5a **A**	76.2a **A**	82.9a **A**	279.6b **C**	316.6c **B**	326.8d **A**
0.5 (2)	2.3b **A**	3.9a **A**	4.7a **A**	74.1b **AB**	69.5a **B**	80.8a **A**	274.5c **C**	297.6e **B**	346.2c **A**
1.0 (2)	2.5b **A**	3.8a **A**	6.5a **A**	74.7b **A**	72.2a **A**	82.6a **A**	257.6d **C**	306.7d **B**	359.1b **A**
2.0 (2)	2.5b **A**	5.5a **A**	5.9a **A**	73.6bc **A**	72.9a **A**	83.4a **A**	250.0e **B**	366.4a **A**	365.7b **A**
4.0 (2)	5.3a **A**	3.5a **A**	4.9a **A**	70.3c **A**	75.6a **A**	84.0a **A**	291.2a **C**	355.1b **B**	379.3a **A**
Freq. of Appl.									
Control	3.3ab **A**	4.2a **A**	6.9a **A**	82.5a **A**	76.2a **A**	82.9a **A**	279.6b **C**	316.6d **B**	326.8d **A**
2.0 (2)	2.5b **A**	5.5a **A**	5.9a **A**	73.6c **A**	72.9a **A**	83.4a **A**	250.0d **B**	366.4a **A**	365.7a **A**
2.0 (4)	1.6b **A**	3.6a **A**	4.0a **A**	74.7bc **B**	75.7a **B**	85.0a **A**	264.1c **C**	306.2e **B**	338.7c **A**
2.0 (8)	4.5a **A**	4.2a **A**	4.5a **A**	77.7b **AB**	65.4a **B**	85.0a **A**	280.8b **C**	348.9b **B**	371.0a **A**
2.0 (12)	3.1ab **A**	4.3a **A**	5.1a **A**	76.7b **A**	67.7a **A**	80.3a **A**	285.4a **C**	338.4c **B**	356.1b **A**
Lacc. Sources									
2.0 (2)	2.5a **A**	5.5a **A**	5.9a **A**	73.6b **A**	72.9a **A**	83.4a **A**	250.0b **B**	366.4a **A**	365.7ab **A**
CHI (2)	2.5a **A**	7.3a **A**	4.3a **A**	78.5a **AB**	73.1a **B**	84.5a **A**	275.9a **B**	359.6b **A**	355.1b **A**
CHU (2)	3.4a **A**	8.0a **A**		79.0a **AB**	73.9a **B**		248.6b **C**	313.4c **B**	
Cont. Appl									
2.0 (2)	2.5a **A**	5.5a **A**	5.9a **A**	73.6a **A**	72.9a **A**	83.4a **A**	250.0a **B**	366.4a **A**	365.7a **A**
CHU (2)	3.4a **A**	8.0a **A**	14.8a **A**	79.0a **AB**	73.9a **B**	86.3a **A**	248.6a **C**	313.4b **B**	373.4a **A**

†Means within a column in a treatment group (treatment effect) followed by the same lowercase letter are not significantly different according to LSD at α=0.05.

‡ Means within a row in a treatment group (duration effect) followed by the same uppercase letter are not significantly different according to LSD at α=0.05.

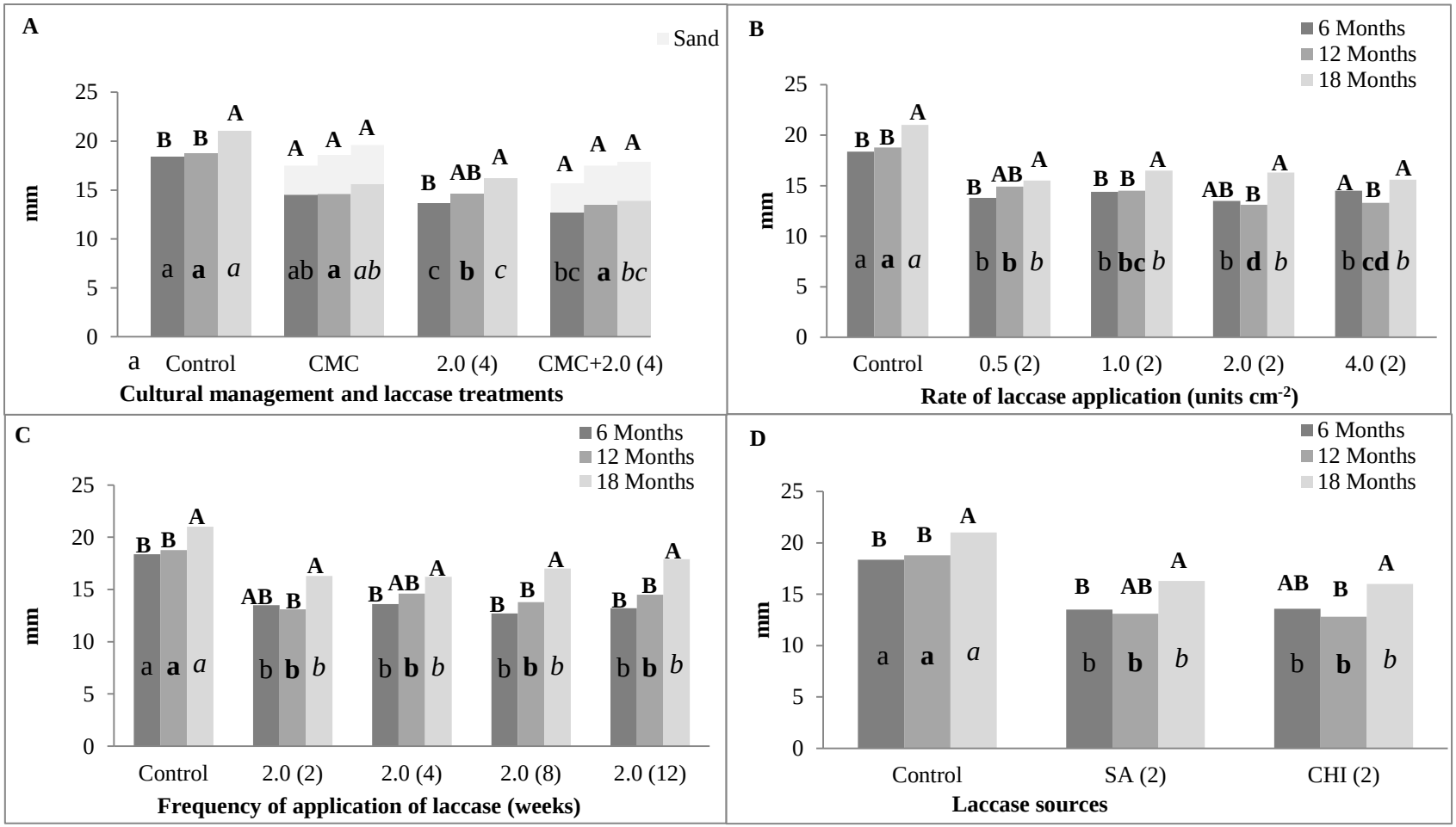

Fig. 7.1 Thatch layer thickness (TLT) in mm at six, twelve, and eighteen months after treatment initiation on creeping bentgrass with cultural management and laccase treatments (Fig 7.1A); rate of laccase application (7.1B); frequency of application of laccase (Fig 7.1C); and laccase sources (Fig 7.1D). Values are means of four replicates. Same letter within the bars (6 months = lowercase standard, 12 months = lowercase bold, and 18 months = lowercase italics) and same letter on top of the bars (duration effect = uppercase bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

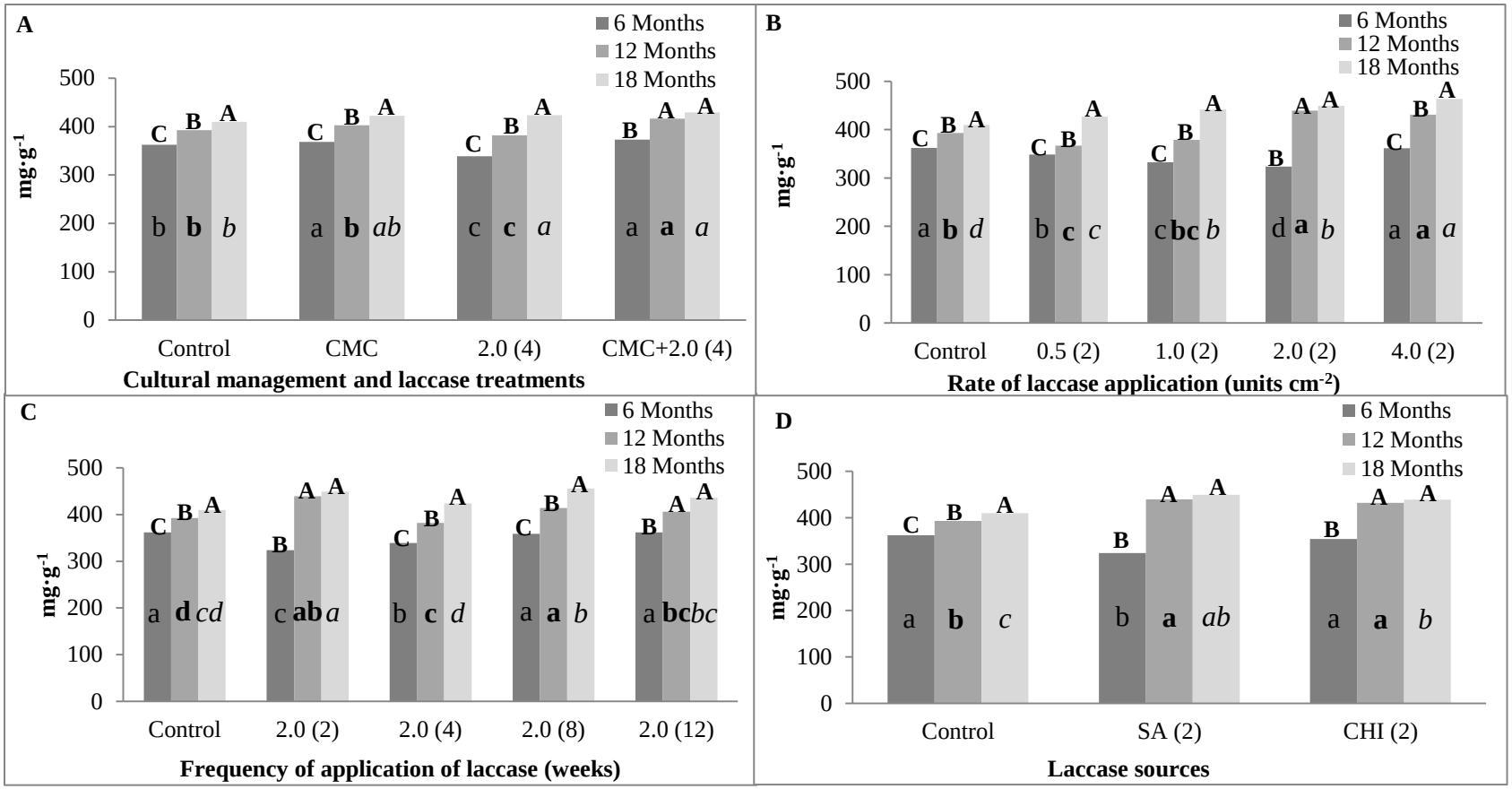

Fig. 7.2 Extractive-free total lignin content (L_T) in $mg \cdot g^{-1}$ at six, twelve, and eighteen months after treatment initiation on creeping bentgrass with cultural management and laccase treatments (Fig 2A); rate of laccase application (Fig 2B); frequency of application of laccase (Fig 2C); and laccase sources (Fig 2D). Values are means of four replicates. Same letter within the bars (6 months = lowercase standard, 12 months = lowercase bold, and 18 months = lowercase italics) and same letter on top of the bars (duration effect = uppercase bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

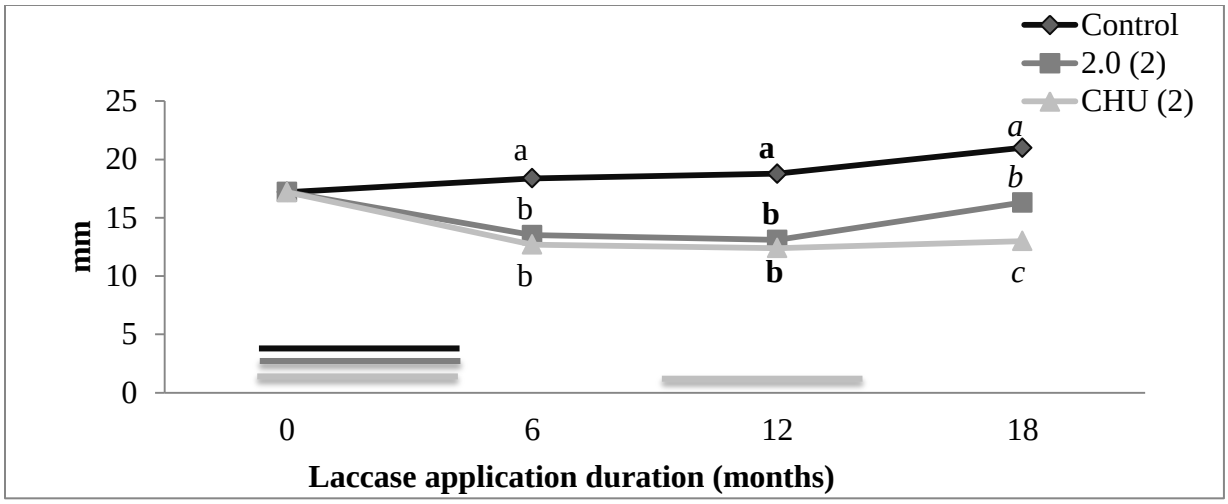

Fig. 7.3 Thatch layer thickness (TLT) in mm at six, twelve, and eighteen months after treatment initiation on creeping bentgrass with laccase treatments 0 (control), and 2.0 (2) for six months in year one, CHU (2) for six months in year one and six months in year two. Values are means of four replicates. Same letter within treatment (6 months = lowercase standard, 12 months = lowercase bold, and 18 months = lowercase italics) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

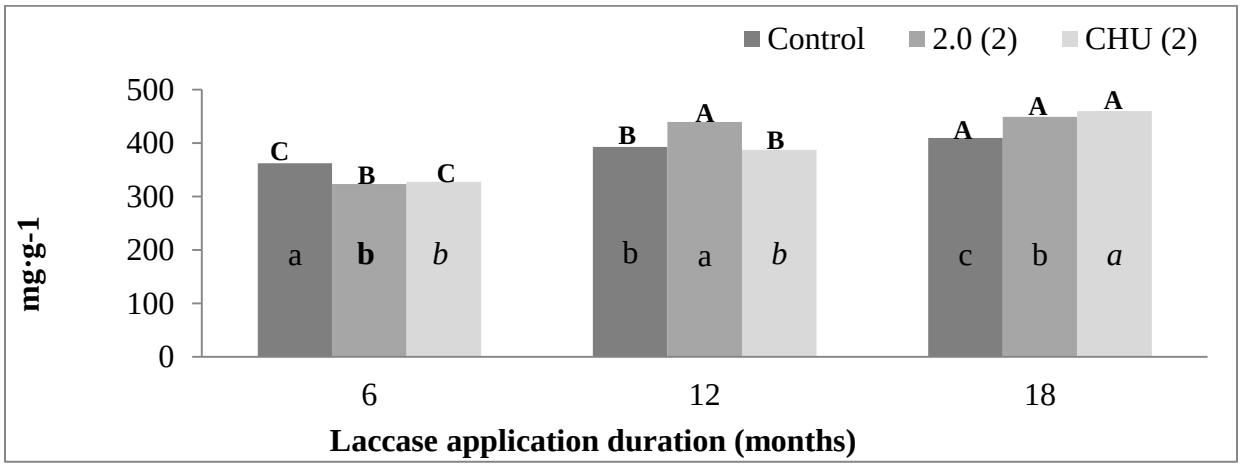

Fig. 7.4 Extractive-free total lignin content (L_T) in $mg \cdot g^{-1}$ at six, twelve, and eighteen months after treatment initiation on creeping bentgrass with laccase treatments 0 (control), and 2.0 (2) for six months in year one, CHU (2) for six months in year one and six months in year two. Values are means of four replicates. Same letter within treatment (control = lowercase standard, 2.0 (2) = lowercase bold, and CHU (2) = lowercase italics) and same letter on top of the bars (duration effect = uppercase bolded) are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

CHAPTER VIII

LIGNIN REMOVAL FROM SWEET SORGHUM AND SWITCHGRASS BIOMASS USING LACCASE MEDIATOR SYSTEM[6]

[6] Sidhu, S.S., P.L. Raymer, and Q. Huang. To be submitted to *Bioresource Technology*.

ABSTRACT

Lignocellulosic materials are renewable resources for bioethanol production from sugars. Pretreatment of lignocellulosic materials is a necessary element in bioconversion of cellulosic and hemicellulosic sugars to ethanol. Removal of lignin from lignocellulosic biomass was optimized by laccase-mediator system. A 300 mg sample of sweet sorghum and switchgrass in a 50 mL Erlenmeyer flask was subjected to 20 mL reaction mixture of laccase or laccase mediator system (LMS). Activity of laccase enzyme in the reaction mixture was 10 units mL^{-1} along with varying concentrations of one of the three mediators; HBT (1-hydroxybenzotriazole), violuric acid (5-isonitrosobarbituric acid)**,** and ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid). The concentration of HBT and ABTS in the reaction mixture was 0, 0.13, 0.25, 0.31, 0.63, 1.25, and 1.88 mM and violuric acid concentration was 0.31, 0.63, 1.25, and 1.88 mM to optimize the mediator concentration for maximum lignin removal. A 5-8% and 5-14% lignin removal was observed sweet sorghum and switchgrass, respectively after 24 h treatment with laccase-ABTS system. A 25.5% lignin removal at 1.88 mM HBT and 24% at 1.25 mM VA after 24 h treatment was observed in sweet sorghum. Similarly, in switchgrass a 28% lignin removal at 0.63 mM VA in combination with laccase was observed after 24 h treatment. A slight loss is structural sugars were observed at treatments with enzymatic treatments. The optimum mediator concentration for maximum removal of lignin changed with mediators and lignocellulosic biomass.

INTRODUCTION

Population growth and industrial development has led to increased energy consumption in the world, which has increased 17-fold over the period of last 100 years (Ayhan, 2007). The conventional energy resources like fossil fuels are limited in quantity and cannot meet the

increasing energy demand, besides having a considerable negative environmental impact such as emitting greenhouse gases like carbon dioxide. Use of alternate and renewable energy options like biofuels have several advantages such as lower CO_2 emissions and lesser dependency of non-oil producing nations for crude oil imports, and has thus drawn widespread attention.

Lignocellulosic materials are heterogeneous complexes of cellulose, hemicellulose and lignin. Removal of lignin from plant cell wall opens up the structure of lignocellulosic biomass and leads to opening of the pore-space in the cell wall structure and thus increase cellulose accessibility for further hydrolysis(Conte et al., 2009; Fang et al., 2010; Kaparaju et al., 2009; Kerr, 1975; Matsushita et al., 2009). Lignocellulosic biomass has to undergo a pretreatment for bioconversion of polymeric sugars to monomers and further fermentation to ethanol (Cheng et al., 2008). Pretreatment recognized as a key step in the bioethanol conversion process must improve the availability of sugars (both cellulosic and hemicellulosic) from enzymatic hydrolysis, prevent loss of sugars or carbohydrates, and avoid formation of chemical inhibitors for subsequent hydrolysis and fermentation processes.

The several methods that have been used as pretreatment till date include dilute acid hydrolysis, wet oxidation, and steam explosion. However, commonly known disadvantages are associated with the existing pretreatment methods including loss of carbohydrates (Abatzoglov et al., 1986; Bouchard et al., 1989; Bouchard et al., 1992; Conner et al., 1985), modification of cellulose polymers or oligomers (Abatzoglov et al., 1986; Bouchard et al., 1989; Mok et al., 1992; Qian et al., 2005), and re-polymerization reaction among carbohydrates by products and lignin intermediates (Li et al., 2007; Xiang et al., 2004).

Natural degradation of lignin is carried out in the environment by certain white-rot fungi which solubilize and mineralize lignin with the help of lignolytic enzymes (Kirk et al., 1975;

1976). White-rot fungi preferentially attack lignin more than cellulose or hemicellulose in the wood tissue (Blanchette, 1984).

Laccase is one of the lignolytic enzymes secreted during oxygen dependent degradation of organic material by white-rot fungi (Ten Have and Teunissen, 2001). Low oxidation potential of laccase restricts its ability to oxidize non-phenolic lignin components (Kersten et al., 1990; Ten Have and Teunissen, 2001). However, addition of low molecular weight substances, mediators, increases the substrate range of laccase enzyme to non-phenolic groups, benzyl and alyl alcohols and ethers (Bourbonnais and Paice, 1992; Bourbonnais et al., 1997; Crestini and Argyropoulos, 1998; Fabbrini et al., 2002; Fabbrini et al., 2001) which comprise the major moieties in lignin macromolecule (Fritz-Langhals and Kunath, 1998; Johannes and Majcherczyk, 2000; Potthast et al., 1995).

In laccase mediator system, the oxidized mediator with a higher redox potential than laccase, acts on the substrate to carry outs its oxidation (Cantarella et al., 2003). Oxidation of organic substrates in laccase-mediator system can proceed by two different mechanisms (Cantarella et al., 2003). In case of mediators like ABTS, the oxidation of substrate is carried out by single electron oxidation, whereas for N-OH type mediators like HBT and violuric acid, the oxidation is carried out by abstraction of H atom by a >N-O radical species.

There are several studies indicating that mediators enhance the oxidation of substrate of laccase (Kang et al., 2002; Kim and Nicell, 2006; Tsutsumi et al., 2001). However, little has been done to optimize the use of mediators in lignin removal from lignocellulosic biomass. We hypothesize that the use of lignin-degrading enzyme such as fungal laccases when applied along with mediators can effectively reduce lignin content from lignocellulosic biomass and our study was designed to test two specific hypothesis: 1) degradation of lignin can be enhanced by

laccase-mediator system; and 2) Different mediators have different optimum effectiveness in reducing lignin.

MATERIALS AND METHODS

Biomass Preparation

The sweet sorghum [*Sorghum bicolor* L., PI 17077) and switchgrass (*Panicum virgatum* L.) biomass were obtained from Dr. M. L. Wang's USDA laboratory at The University of Georgia, Griffin Campus. The biomass sample was ground (177-841 µm) and extracted with water and alcohol to remove water-and alcohol-soluble extractives as specified by the protocol developed by National Renewable Energy Laboratory (NREL, 2008a). The extractive-free biomass samples were air-dried to a moisture level less than 10% before treatment application.

Chemicals

Laccase used in the experiment was obtained from Jiangnan University, China. The enzyme was purified from a laccase producing fungal strain *Pycnoporus* sp. JL-N with accession number GU 182936. Three mediators used in the experiment; HBT (1-hydroxybenzotriazole), violuric acid **(**5-Isonitrosobarbituric acid), and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) along with surfactant, Pluronic F-68 (polyoxyethylene-polyoxypropylene polymer,$C_3H_6O.C_2H_4O$) were purchased from Sigma-Aldrich (Sigma Aldrich Inc., St. Louis, MO.).

Laccase Activity Assay

The activity of laccase was quantified using a UV/VIS-spectrophotometer by a colorimetric assay. One activity unit of laccase corresponds to the amount of enzyme that causes an absorbance change at 468 nm at a rate of 1.0 unit min^{-1} in 3.4 mL of 1 mM 2,6-dimethoxyphenol in citrate-phosphate buffer at a particular pH (Park et al., 1999). Laccase

enzyme activity was assayed over a range and was found to be most active at pH 3.0 in the initial laboratory tests conducted to optimize the pH for this enzyme (Fig 1).

Enzymatic Treatment

The reaction mixture consisted of lignocellulosic biomass, surfactant, laccase, citrate-phosphate buffer at pH 3.0, and mediators. A 300 mg extractive-free biomass sample of sweet sorghum and switchgrass was collected in a 50 mL Erlenmeyer flask and was allowed to stand overnight in a 10 mL solution (3% w/w) of surfactant, Pluronic F-68. Effectiveness of laccase enzyme was observed at activity level of 0, 2, 5, 10 and 20 units mL^{-1} in the reaction mixture. Laccase mediator system consisted of laccase at 10 units mL^{-1} along with one of the three mediators. The concentration of HBT and ABTS in the reaction mixture was 0.13, 0.25, 0.31, 0.63, 1.25, and 1.88 mM and violuric acid concentration was 0.31, 0.63, 1.25, and 1.88 mM to examine the effect of mediator concentration on lignin removal. The reaction mixture was put on rotary shaker at 150 rpm at 25^{o}C. Samples were removed from the reaction mixture after 24, 48, and 72 h and were washed three times with 100 mL water.

Measurements

After enzymatic treatment the samples were dried and weighed to observe dry mass loss. The effectiveness of treatments was determined by measuring acid-soluble lignin (L_S) and acid-insoluble lignin (L_I) and structural sugars. The lignin and sugar content will be designated as extractive-free lignin and extractive-free structural sugar content due to the use of extractive-free biomass for the experiment. Total lignin (L_T) was obtained by addition of acid-soluble and-insoluble lignin contents.

Extractive-free Lignin Content

Lignin content in the sweet sorghum and switchgrass biomass was determined in a two-step hydrolysis procedure according to the laboratory analytical procedure developed by The National Renewable Energy Laboratory (NREL, 2008b). In the first step, 100 mg of air dried treated biomass samples were hydrolyzed for 60 min with 1mL of 72% H_2SO_4 at 30^oC in a water bath. In the second step, H_2SO_4 was diluted to 4% and the samples were autoclaved at 121^oC for 1 h. After autoclave the samples were vacuum filtered and the hydrolysis liquid was used for analyzing acid-soluble lignin content and structural sugars. Acid-soluble lignin was determined using this hydrolysis liquid at 240 nm wavelength in a UV/VIS spectrophotometer. The solids remaining after acid hydrolysis were dried in an oven at 100 ± 5^oC for 24 h, weighed, ashed in a muffle furnace at 600 ± 10^oC for 24 h, and weighed again. Weight difference was used to calculate the extractive-free acid-insoluble lignin content.

Structural Sugars

Structural sugar content for glucose, xylose, arabinose, mannose, and galactose was determined for selected samples from the hydrolysis liquid collected after vacuum filtration in the above step. The hydrolysis liquid was neutralized to a pH range 6.0-8.0 using $NaHCO_3$ (sodium bicarbonate) and structural sugars were determined using high performance liquid chromatography (HPLC) in an Agilent 1100 HPLC (Aligent Technologies, Waldbronn, Germany) with a binary pump and a refractive index detector. An AMINEX HPX-87P 7.8 x 300 mm Pb^{2+} carbohydrate analysis column (Bio-Rad, Hercules, CA) was used at 85^oC with deionized water as mobile phase at a flow rate of 0.6 mL min^{-1}. These monosaccharide sugars are components of structural polysaccharides, cellulose and hemicellulose. Total sugars were calculated by addition of the sugar contents of these monomers.

Statistical Analysis

Analysis of variance (ANOVA) was performed to evaluate the main effects of laccase, mediator, and interaction effects of these two factors using general linear model (GLM) (SAS Institute, 1989). Fisher's protected LSD test with $\alpha = 0.05$ was used for determining statistical differences among treatment means following each ANOVA.

RESULTS

Lignin Content

Laccase Application

Treatment of sweet sorghum and switchgrass with different levels of laccase for 24 h had no effect on extractive-free acid-insoluble and total lignin content (Table 8.1). However, slight but significant differences were observed for L_S content (Table 8.1). A slight increase in L_S content ($P \leq 0.05$) of sweet sorghum was obtained when reaction mixture consisted of laccase enzyme at 20 units mL^{-1} whereas in switchgrass a slight reduction in L_S content ($P \leq 0.05$) was observed in reaction mixture containing 5 units mL^{-1} laccase activity level as compared to the control (Table 8.1).

Laccase-ABTS Mediator System

A significant effect ($P \leq 0.001$) of laccase-ABTS system was observed on L_S, L_I and L_T content of sweet sorghum and switchgrass. After 24 h of laccase-ABTS treatment, L_S content of sweet sorghum increased by 8.9 (23%), 15.8 (41%), and 24.7 (65%) $mg \cdot g^{-1}$ over the control with ABTS concentration of 0.63, 1.25, and 1.88 mM, respectively when applied along with laccase (Fig 8.1A). However, when compared with control, L_I content decreased by 8.0 (5%), 19.4 (11%), 37.7 (21%), and 23.7 (13%) $mg \cdot g^{-1}$ in reaction mixture containing laccase along with ABTS at 0.31, 0.63, 1.25, 1.88 mM, respectively (Fig 8.1B). A slight reduction of 5 and 8% in

total lignin content when compared to control was obtained when ABTS was applied at 0.63 and 1.25 mM along with laccase (Table 8.2).

Switchgrass biomass treated with laccase-ABTS system showed a slight but significant reduction in L_S content up to ABTS concentration of 0.31 mM when applied along with laccase. Extractive-free acid soluble content increased over the control by 2.3, 4.2, and 4.8 $mg \cdot g^{-1}$ when with ABTS concentration of 0.63, 1.25, and 1.88 mM, respectively, in the reaction mixture containing laccase (Fig 8.2A). Extractive-free acid-insoluble lignin content of switchgrass biomass was lowered by 9.4, 13.4, 14.4, 16.7, 22.4, and 41.2 $mg \cdot g^{-1}$ when ABTS concentration was 0.13, 0.25, 0.31, 0.63, 1.25, and 1.88 mM, respectively, in the presence of laccase (Fig 8.2B). Similarly, L_T content in switchgrass biomass decreased in the range of 12.2 (5%) and 36.4 (14%) $mg \cdot g^{-1}$ in comparison with control for the same concentration of ABTS (Table 8.2). Reaction mixture containing ABTS at different concentrations without laccase had no effect on acid-soluble and-insoluble lignin content in sweet sorghum and switchgrass (Fig 8.1A, 8.1B and Fig 8.2A, 8.2B).

Duration of laccase-ABTS system had no effect on L_S content in sweet sorghum for ABTS concentration of 0.25, 0.31, and 1.25 mM (Table 8.3). After 48 and 72 h of treatment at ABTS concentration of 0.63 mM, a reduction in L_S content was observed (Table 8.3). However, a reduction in L_S content was observed with increase in treatment duration switchgrass biomass when ABTS concentration was 0.63mM or higher in laccase-ABTS system (Table 8.4). No duration effect was observed for L_I content in sweet sorghum for ABTS concentrations of 0.25 and 1.25 mM (Table 8.3). When ABTS was applied at 0.31 and 0.63 mM in presence of laccase, L_I content significantly decreased from 168.6 to 157.7 and 157.2 to 138.4 $mg \cdot g^{-1}$ when measured after 24 and 72h (Table 8.3). Similarly, L_T content in sweet sorghum decreased from 210.6 to

201.4, 209.9 to 197.7, and 204.2 to 182.4 mg·g^{-1} when measured after 24 and 72h at ABTS concentration of 0.25, 0.31, and 0.63 mM, respectively (Table 8.3). In switchgrass biomass a significant duration effect was observed for L_I and L_T content at ABTS concentration of 0.63 and 1.25 mM (Table 8.4). A reduction of 14.7 and 16.1 mg·g^{-1} and 17.4 and 18.7 mg·g^{-1} was obtained for L_I and L_T content in switchgrass when measured after 24 and 72h at ABTS concentration of 0.63 and 1.25 mM (Table 8.4).

Laccase-HBT Mediator System

Laccase-HBT system had significant effects ($P \leq 0.001$) on L_S, L_I and L_T content of sweet sorghum and switchgrass. After 24 h of treatment with laccase along with HBT mediator up to concentration of 0.63 mM, a reduction in the range of 1.5-2.2 mg·g^{-1} in L_S content was obtained in sweet sorghum (Fig 8.3A). No reduction in L_S content was observed with higher HBT content (Fig 8.3A). HBT application without laccase had no significant effect on the L_S content when compared to control (Fig 8.3A). Extractive-free acid-insoluble content of sweet sorghum was significantly lowered by 13.3 (8%), 27.4 (16%), 31.8 (18%), 41.7 (24%), 49.2 (28%), and 55.1 (31%) mg·g^{-1} with HBT concentration of 0.13, 0.25, 0.31, 0.63, 1.25, and 1.88 mM, respectively when applied along with laccase (Fig 8.3B). A slight reduction in L_I content was observed when sweet sorghum was treated with 0.63 mM concentration of HBT without laccase. A 25.5% reduction in total lignin content of sweet sorghum was observed when HBT was applied at concentration of 1.88 mM along with laccase enzyme (Table 8.2).

A significant reduction in L_S content in switchgrass was observed in the range of 6.5-6.8 mg·g^{-1} when treated with HBT alone at all the concentrations (Fig 8.4A). When HBT was applied with laccase L_S content was lowered by 7.2, 7.4, and 8.1 mg·g^{-1} at HBT concentration of

0.13, 0.25, and 0.31, respectively. However, at HBT concentration of 1.25 and 1.88mM along with laccase, a significant increase in L_S content was observed (Fig 8.4A). No effect of HBT alone was observed on L_I content of switchgrass. However, L_I content was significantly lowered by 9.7, 11.9, 15.9, 18.5, and 25.1 $mg \cdot g^{-1}$ at HBT concentration of 0.25, 0.31, 0.63, 1.25, and 1.88, respectively when compared to control (Fig 8.4B). Similarly, L_T content was lowered by 9.9 (4%), 16.8 (6%), 20.1 (8%), 19.5 (8%), 15.9 (6%), and 23.9 (9%) $mg \cdot g^{-1}$ when compared to control at HBT concentration of 0.13, 0.25, 0.31, 0.63, 1.25, and 1.88 mM, respectively when applied with laccase enzyme (Table 8.2).

A slight but significant decrease in L_S content was observed for sweet sorghum between 24 and 72 h at HBT concentration of 0.31 mM (Table 8.3). Similarly, L_I content of sweet sorghum decreased by 14.5 and 7.5 $mg \cdot g^{-1}$ and L_T content decreased by 14.1 and 7.8 $mg \cdot g^{-1}$ when measured between 24 and 72 h at HBT concentration of 0.31 and 0.63 mM (Table 8.3). However, for switchgrass not significant duration effect was observed for L_S, L_I, and L_T except a slight reduction in L_S content with duration at HBT concentration of 0.63 mM (Table 8.4).

Laccase-VA Mediator System

A significant treatment effect ($P \leq 0.001$) of laccase-VA system was observed on L_S, L_I and L_T content of sweet sorghum and switchgrass. After 24 h of laccase-VA treatment of sweet sorghum, no effect on L_S content was observed with application of VA without laccase. However, a 1.9-7.5 $mg \cdot g^{-1}$ (5-20%) increase in L_S content was observed over control when VA was applied along with laccase at concentrations 0.63 to 1.88 mM (Fig 8.5A). Extractive-free acid-insoluble lignin (L_I) content of sweet sorghum biomass was not affected when reaction mixture consisted of mediator without laccase enzyme. In the presence of laccase, L_I content of sweet sorghum was lowered by 38.9 (22%), 45.5 (26%), 53.6 (30%), and 52.6 (29%) $mg \cdot g^{-1}$

when compared to control at VA concentration of 0.31, 0.63, 1.25, and 1.88 mM, respectively (Fig 8.5B). At the same VA concentrations with laccase in the reaction mixture, reduction in L_T content were 38.5, 46.7, 50.7, and 45.1 $mg \cdot g^{-1}$ representing 18, 22, 24, and 21% reduction, respectively (Table 8.2).

After 24 h of treatment of switchgrass biomass with VA without laccase, a significant decrease in L_S content in the range of 7.5-8.8 $mg \cdot g^{-1}$ was observed when compared to the control (Fig 8.6A). When applied along with laccase, a slight decrease in L_S content (21%) was observed at VA concentration of 0.31 mM when compared to control (Fig 8.6A). At VA concentration of 0.63-1.88 mM, L_S content increased by 1.3-3.5 $mg \cdot g^{-1}$ over the control (Fig 6A). No effect of VA was observed on switchgrass biomass L_I content when treated without laccase enzyme (Fig 8.6B). When applied along with laccase, a significant reduction in L_I content was observed for all the concentrations of VA (Fig 8.6B). However, the maximum reduction in L_I content was 75 (34%) $mg \cdot g^{-1}$ when compared to control at VA concentration of 0.63 mM (Fig 8.6B). With increasing concentration of VA, the extent of L_I reduction was lowered. Similarly, the maximum reduction in extractive-free total lignin content in switchgrass amounted to 73.2 (28%) $mg \cdot g^{-1}$ obtained when VA at 0.63 mM was applied along with laccase enzyme (Table 8.2). At higher VA concentrations, the impact on lignin reduction decreased significantly (Table 8.2).

A significant reduction in sweet sorghum L_S content was observed when reaction time for laccase-VA system was increased from 24 to 72 h at VA concentration of 0.31 and 0.63 mM (Table 8.3). However, no further reduction in L_I content of sweet sorghum was obtained with increase in reaction time (Table 8.3). An overall decrease of 20 $mg \cdot g^{-1}$ in sweet sorghum L_T content was obtained with the duration of reaction at VA concentration of 0.31 mM (Table 8.3).

No significant effect of laccase-VA system duration effect was observed for switchgrass L_I and L_T content at any VA concentration (Table 8.4). However, a reduction in L_S content of switchgrass was obtained with increased duration for VA concentration of 0.63, 1.25, and 1.88 mM (Table 8.4).

Structural Sugars

Structural sugar content (S_T) in treated sweet sorghum biomass was lowered by 20-40 $mg \cdot g^{-1}$ in comparison to control when ABTS was applied along with laccase enzyme at 0.63, 1.25, 1.88 mM concentration (Table 8.5). At the same ABTS concentrations a slight but non-significant reduction in structural sugar content was obtained (Table 8.5). However no further reduction in sugar content was observed for both species when reaction time increased from 24 h to 72 h (data not shown). A 24-34 and 23-31 $mg \cdot g^{-1}$ reduction in structural sugar content in comparison to control was observed for sweet sorghum and switchgrass, respectively when treated with the laccase-HBT system consisting of HBT at 0.63, 1.25, and 1.88 mM. No reduction in sugar content was observed with increase in treatment duration from 24 h to 72 h at the same HBT concentrations. Laccase-VA system consisting of the same VA concentrations significantly reduced sugar content in sweet sorghum when compared to control at VA concentration of 1.88 mM (Table 8.5). However, S_T content in treated switchgrass was reduced at VA concentrations of 0.63 (62.6 $mg \cdot g^{-1}$) and 1.25 mM (18.6 $mg \cdot g^{-1}$) compared to the control.

Dry Mass Loss

Weight loss in sweet sorghum and switchgrass was measured after the enzymatic treatments. A weight loss of 35-49, 70-78, and 63-69 $mg \cdot g^{-1}$ in comparison to control were observed in sweet sorghum for ABTS, HBT, and VA mediator system, respectively (Fig 8.7A). Similarly, 51-81, 53-56, and 56-63 $mg \cdot g^{-1}$ loss in switchgrass weight was obtained when

compared to control after treatment with ABTS, HBT, and VA mediator system, respectively (Fig 8.7B). Loss in sweet sorghum and switchgrass weight after enzymatic treatment were calculated by addition of total lignin removal and structural sugar loss. Calculated and measured weight loss for both species is similar for HBT and VA mediator systems. However, for ABTS mediator system, calculated and measured weight loss in switchgrass are better related when weight loss due to acid-insoluble lignin is considered instead of the total lignin (Fig 8.7B).

DISCUSSION

Treatment of biomass with laccase without a mediator was not effective in removal of lignin from both biomass sources. Laccase-mediator system was effective in decreasing lignin content from the two biomass sources; however the efficacy of different mediators was different in different biomass species for L_S and L_I. The efficiency of laccase mediator system to remove lignin can be attributed to the high oxidation potential of the oxidized mediator and the small size of the mediators in comparison to laccase which makes it easier for them to reach deep within biomass structure to oxidize lignin bonds (Bourbonnais et al., 1997).

No significant reduction in L_S content was observed in sweet sorghum when ABTS concentration was 0.31 mM or less (Fig 8.1A). However, in switchgrass a significant reduction was observed at the same concentrations (Fig 8.2A). Optimal concentration for L_I removal was 1.25 and 1.88 mM for sweet sorghum and switchgrass, respectively. In sweet sorghum 8% total lignin reduction was obtained at 1.25 mM as compared to 14% in switchgrass at 1.88 mM concentration of ABTS when applied with laccase enzyme (Fig 8.1B, Fig 8.2 B).

Optimum HBT concentration in the presence of laccase for L_S content reduction was up to 0.63 and 0.31 mM in sweet sorghum and switchgrass, respectively (Fig 8.3A, Fig 8.4A). Optimum reduction of L_I in both grass species was observed at 1.88 mM concentration in the

presence of laccase. Laccase-HBT system was more effective in reducing L_I content in sweet sorghum (31%) as compared to switchgrass (12%) (Fig 8.3B, Fig 8.4B). Extractive-free total lignin content was lowered in sweet sorghum at 1.88 mM HBT along with laccase whereas L_T content in switchgrass decreased when HBT concentration in the reaction mixture was in the range of 0.25-1.88 mM along with laccase (Table 8.2).

When VA was applied along with laccase, an increase in L_S content was observed in sweet sorghum at concentration 0.63 mM and above (Fig 8.5A). On the other hand, a reduction in switchgrass L_S content was observed at concentration of 0.31 mM and an increase in L_S content was obtained at 0.63 mM concentration or above (Fig 8.6A). The optimum concentration of VA in laccase-VA system to lower the L_I and L_T content was 1.25 and 0.63 mM for sweet sorghum and switchgrass, respectively (Table 8.2, Fig 8.5B, 8.6B).

The different optimum concentrations of the three mediators on the same lignocellulosic biomass may stem from the difference in the mode of actions of these mediators. ABTS oxidizes lignin bonds by extraction of electrons whereas HBT and VA abstracts hydrogen atom for oxidation (Cantarella et al., 2003). Our results suggest that structure of the lignocellulosic biomass may also influence the extent of lignin removal (Chunxia et al., 2010).

Effect of enzymatic treatment duration on sweet sorghum and switchgrass varied with the choice of mediator. The duration effect of laccase-ABTS system was observed at different ABTS concentrations in the two biomass species. The effect was observed at 0.31 and 0.63 mM and 0.63 and 1.25 mM ABTS in sweet sorghum and switchgrass, respectively (Table 8.3, 8.4). It is seen that with higher ABTS concentration optimum lignin removal reached after 24 h and had no further duration effect, whereas with lower ABTS concentrations duration effect was shown, but the amount of delignification is not increased with increase in the reaction time. This may

suggest that optimum amount of lignin removal in laccase-ABTS system can be achieved after 24 h with optimum mediator concentration.

The duration effect of laccase-HBT system on sweet sorghum was observed with HBT concentrations of 0.31 and 0.63 mM for L_I and L_T contents and with 0.31 mM HBT for L_S content (Table 8.3). However, no significant duration effect for L_I and L_T contents was observed at any HBT concentration in switchgrass biomass except a slight reduction in switchgrass L_S content was obtained at 72 h when compared to 24 h at 0.63 mM concentration (Table 8.4).

Laccase-VA system had no duration effect on total lignin content of switchgrass at different VA concentrations, while a slight reduction of L_T content in sweet sorghum was seen at VA concentration of 0.31 mM VA (Table 8.3, 8.4). This suggests that violuric acid as laccase mediator functions differently in different biomass species. The duration effect of VA on L_S content was observed at 0.31 and 0.63 mM for sweet sorghum and 0.63, 1.25, and 1.88 mM for switchgrass.

Different laccase mediators impacted the S_T content to different extents on sweet sorghum and switchgrass. Laccase-ABTS system reduced a S_T to a significant extent from the sweet sorghum but no significant reduction from switchgrass biomass. Laccase-HBT system impacted S_T on both biomass sources to the extent of 23-31 $mg \cdot g^{-1}$. Laccase-VA system lowered the sugar content from sweet sorghum at 1.88 mM, but in switchgrass no S_T content was impacted at this concentration (Table 8.5).

CONCLUSIONS

The accessibility of structural sugars in lignocellulosic biomass needs to be improved for enhancing bioethanol production efficiency. Structural sugar availability for fermentation can be increased by removing lignin protective matrix. The results from this experiment suggest the

efficacy of laccase-mediator system in delignification of sweet sorghum and switchgrass. The ability of these mediators to remove lignin varied with biomass species. Lignin removal from sweet sorghum was in the range of 24-25.5% at 1.25 mM VA and 1.88 mM HBT in combination with laccase after 24 h treatment. In switchgrass, with application of laccase-ABTS system a 5-14% removal of lignin was observed and 28% lignin removal at 0.63 mM VA in combination with laccase after 24 h treatments. Future research is needed to study the impact of laccase-mediator system on overall bioethanol production efficiency.

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Table 8.1 Extractive-free acid-soluble lignin (L_S), acid-insoluble lignin (L_I), and total lignin (L_T) in sweet sorghum and switchgrass after 24 h of treatment with laccase enzyme at 0, 2, 5, 10, and 20 units mL^{-1} activity in a 20 mL reaction mixture.

Laccase activity levels (units mL^{-1})	Sweet sorghum			Switchgrass		
	Acid-soluble lignin (L_S)	Acid-insoluble lignin (L_I)	Total lignin (L_T)	Acid-soluble lignin (L_S)	Acid-insoluble lignin (L_I)	Total lignin (L_T)
	$mg \cdot g^{-1}$					
0	38.1b†	176.6a	214.7a	43.0a	219.2a	262.2a
2	38.8ab	175.4a	214.2a	42.0ab	218.2a	260.2a
5	38.6ab	175.2a	213.8a	39.1b	221.6a	260.6a
10	38.5ab	175.5a	214.1a	41.3ab	219.0a	260.3a
20	39.2a	174.4a	213.6a	43.5a	221.6a	265.3a

† Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD at α=0.05.

Table 8.2 Total lignin content (extractive-free) in sweet sorghum and switchgrass biomass after 24 h treatment with laccase-mediator system. The 20 mL reaction mixture consisted of laccase at 10 units mL^{-1} activity with one of the three mediators; ABTS, HBT, and VA at different concentrations.

	Sweet sorghum			Switchgrass		
Treatment†	ABTS	HBT	VA	ABTS	HBT	VA
	$mg \cdot g^{-1}$					
Control	214.7ab‡	214.7b	214.7a	262.2a	262.2a	262.2a
0+10L	214.1ab	214.1bc	214.1a	260.3a	260.3ab	260.3a
0.12	214.3ab	213.6bc	-	263.0a	258.8abc	-
0.12+10L	214.4ab	199.0d	-	250.6c	252.3cd	-
0.25	215.7ab	217.2ab	-	260.1ab	258.5abc	-
0.25+10L	210.6abc	185.7e	-	247.3c	245.0ef	-
0.31	215.6ab	216.3ab	209.7a	263.0a	257.3abc	255.8a
0.31+10L	209.9bc	181.4e	176.2b	246.5c	242.1ef	197.7c
0.63	217.7a	209.8c	215.2a	260.1ab	257.2abc	256.5a
0.63+10L	204.2c	170.7f	171.0b	247.8c	242.7ef	189.0d
1.25	216.6ab	220.8a	214.9a	262.5a	257.5abc	259.9a
1.25+10L	196.7d	165.1g	164.0c	244.0c	246.3ed	218.6c
1.88	216.1ab	217.2ab	214.4a	251.9bc	254.9bc	259.0a
1.88+10L	215.7ab	160.4h	169.6bc	225.8d	238.3f	232.8b

† L denotes laccase at 10 units mL^{-1} activity level

‡ Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD at $\alpha=0.05$.

Table 8.3 Extractive-free acid-soluble lignin (L_S), acid-insoluble lignin (L_I), and total lignin (L_T) in sweet sorghum after 24, 48, and 72 h of enzymatic treatment. The 20 mL reaction mixture consisted of laccase at 10 units mL^{-1} activity with one of the three mediators; ABTS, HBT, and VA at different concentrations.

	Duration (hours)								
Treatments	24	48	72	24	48	72	24	48	72
	Acid-soluble lignin (L_S)			Acid-insoluble lignin (L_I)			Total lignin (L_T)		
	$mg \cdot g^{-1}$								
ABTS									
Control	38.1a†	38.1a	38.2a	176.6a	176.7a	175.1a	214.7a	214.8a	213.4a
0.25	40.5a	39.7a	39.0a	170.2a	166.3a	162.3a	210.6a	206.0ab	201.4b
0.31	41.4a	41.2a	39.5a	168.6a	160.0b	157.7b	209.9a	201.2b	197.2b
0.63	47.0a	45.7b	44.0c	157.2a	147.8ab	138.4b	204.2a	193.4ab	182.4b
1.25	53.9a	54.7a	51.6a	138.8a	141.9a	141.5a	196.7a	196.6a	193.2a
HBT									
Control	38.1a	38.1a	38.2a	176.6a	181.7a	182.4a	214.7a	220.2a	220.8a
0.31	36.7a	36.2ab	35.8b	144.7a	140.9a	130.2b	181.4a	177.2a	166.0b
0.63	35.9a	35.6a	35.5a	134.8a	127.1b	127.3b	170.7a	162.7b	162.9b
1.25	37.8a	38.4a	38.1a	127.3a	123.3a	123.8a	165.1a	161.6a	161.8a
1.88	39.0a	38.4a	38.1a	121.4a	121.2a	124.8a	160.4a	159.5a	162.9a
VA									
Control	38.1a	38.1a	38.2a	176.6a	177.4a	178.7a	214.7a	215.5a	216.1a
0.31	38.5a	37.7ab	37.2b	137.7a	133.9a	129.0a	176.2a	171.6ab	166.2b
0.63	40.0a	38.6b	38.2b	131.1a	123.4a	121.0a	171.0a	162.0a	159.2a
1.25	40.9a	40.7a	40.3a	123.0a	128.3a	129.7a	164.0a	169.0a	170.0a
1.88	45.7a	44.1a	44.1a	124.0a	126.6a	127.3a	169.6a	170.7a	171.4a

† Means within a row for a parameter followed by the same letter are not significantly different according to Fisher's protected LSD at α=0.05.

Table 8.4 Extractive-free acid-soluble lignin (L_S), acid-insoluble lignin (L_I), and total lignin (L_T) in switchgrass after 24, 48, and 72 h of enzymatic treatment. The 20 mL reaction mixture consisted of laccase at 10 units mL^{-1} activity with one of the three mediators; ABTS, HBT, and VA at different concentrations.

Treatments	Duration (Hours)								
	24	48	72	24	48	72	24	48	72
	Acid-soluble lignin (L_S)			Acid-insoluble lignin (L_I)			Total lignin (L_T)		
	$mg \cdot g^{-1}$								
ABTS									
Control	43.0a	42.2a	42.1a	219.2a	217.8a	219.6a	262.2a	260.1a	261.8a
0.25	41.6a	40.3a	40.6a	205.7a	208.8a	203.2a	247.3a	249.1a	243.8a
0.31	41.8a	41.2a	40.7a	204.7a	203.2a	198.8a	246.5a	230.6a	239.5a
0.63	45.3a	44.1b	43.4c	202.4a	196.5a	187.0b	247.8a	240.6b	230.4c
1.25	47.2a	46.2a	44.6b	196.8a	191.2a	180.7b	244.0a	237.4b	225.3c
1.88	47.9a	47.1b	46.6b	177.9a	180.7a	179.7a	225.8a	227.8a	226.3a
HBT									
Control	43.0a	42.2a	42.1a	219.2a	217.8a	219.6a	262.2a	260.1a	261.8a
0.31	34.9a	34.5a	34.0a	207.2a	206.4a	204.3a	242.1a	240.9a	238.3a
0.63	39.5a	38.5ab	38.2b	203.2a	204.6a	198.5a	242.7a	243.1a	236.7a
1.25	45.6a	43.5b	43.0b	200.7a	191.9a	193.2a	246.3a	235.4a	236.2a
1.88	44.2a	43.9a	43.2a	194.0a	193.3a	194.7a	238.3a	237.2a	238.0a
VA									
Control	43.0a	42.2a	42.1a	219.2a	217.8a	219.6a	262.2a	260.1a	261.8a
0.31	33.8a	34.9a	34.9a	163.9a	176.2a	176.5a	197.7a	211.1a	211.4a
0.63	44.3a	43.1b	42.4c	144.1a	147.5a	149.6a	189.0a	190.6a	191.9a
1.25	46.5a	45.8a	44.4b	172.1a	172.0a	174.1a	218.6a	217.8a	218.4a
1.88	46.1a	45.6a	44.1b	186.7a	178.6a	179.7a	232.8a	224.2a	223.8a

† Means within a row for a parameter followed by the same letter are not significantly different according to Fisher's protected LSD at α=0.05.

Table 8.5 Extractive-free total structural sugar content (S_T) of sweet sorghum and switchgrass biomass after 24 h of enzymatic treatment. The 20 mL enzymatic treatment mixture consisted of the three mediators; ABTS, HBT, and VA at concentration of 0.63, 1.25, and 1.88 mM along with laccase enzyme at 10 units mL^{-1}.

Treatments†	Sweet sorghum	Switchgrass
	----------mg·g^{-1}----------	
Control	709.3a‡	694.8ab
ABTS		
0.63+10L	690.9bcd	709.1a
1.25+10L	687.1bcde	677.8bc
1.88+10L	670.9e	679.3bc
HBT		
0.63+10L	676.8de	693.8bc
1.25+10L	685.5cde	663.8c
1.88+10L	675.3de	671.3c
VA		
0.63+10L	696.1abc	632.2d
1.25+10L	702.1ab	676.2bc
1.88+10L	683.2cde	694.3ab

† L denotes laccase at 10 units mL^{-1} activity level

‡ Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD at α=0.05.

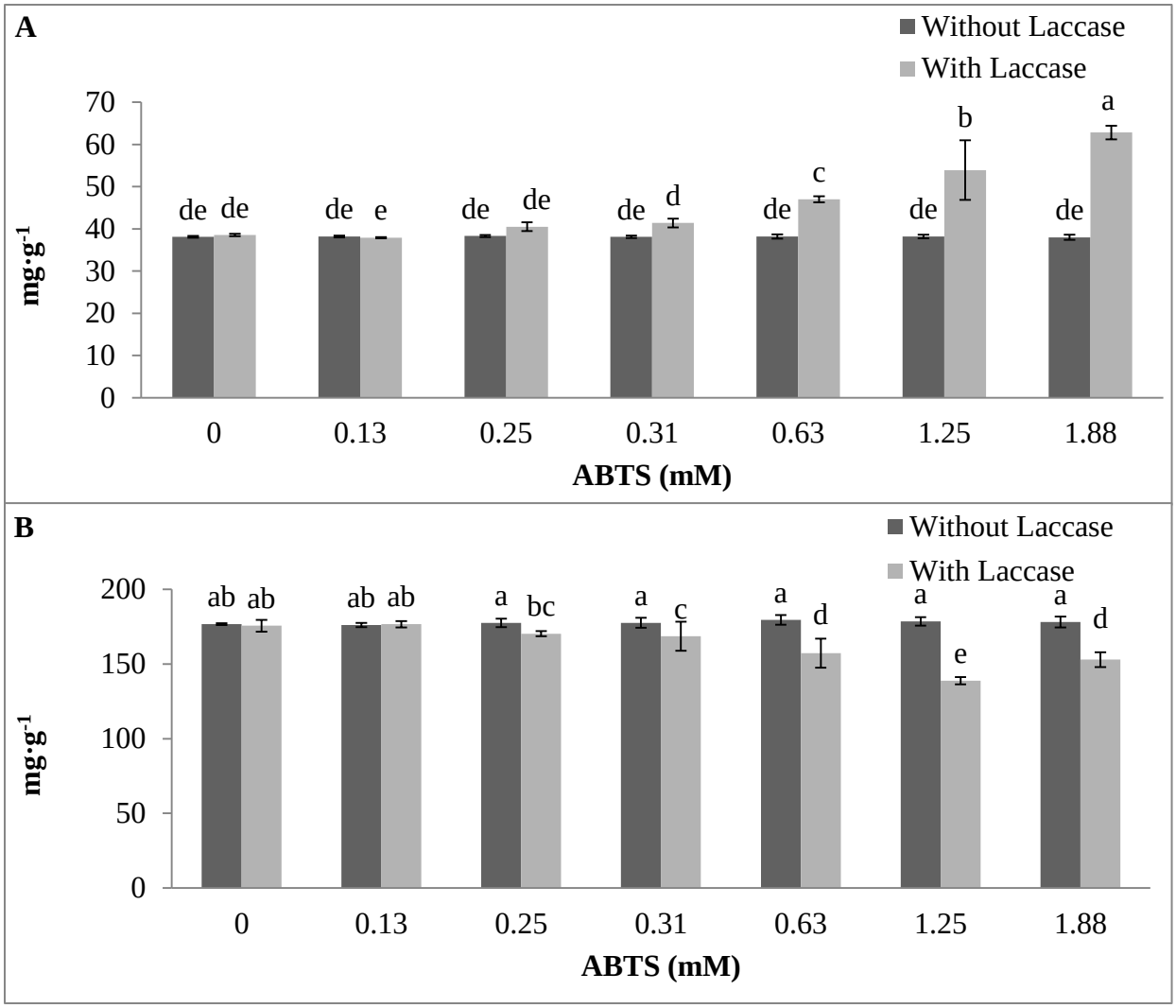

Fig. 8.1 Extractive-free acid-soluble lignin content (L_S, 8.1A) and acid-insoluble lignin content (L_I, 8.1B) of sweet sorghum after 24 h of enzymatic treatment in a 20 mL reaction mixture with seven different levels of mediator ABTS 0, 0.13, 0.25, 0.31, 0.63, 1.25, and 1.88 mM with and without laccase at activity 10 units mL^{-1}. Values are means of three replicates and error bars represent standard deviation. Bars with the same letter are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

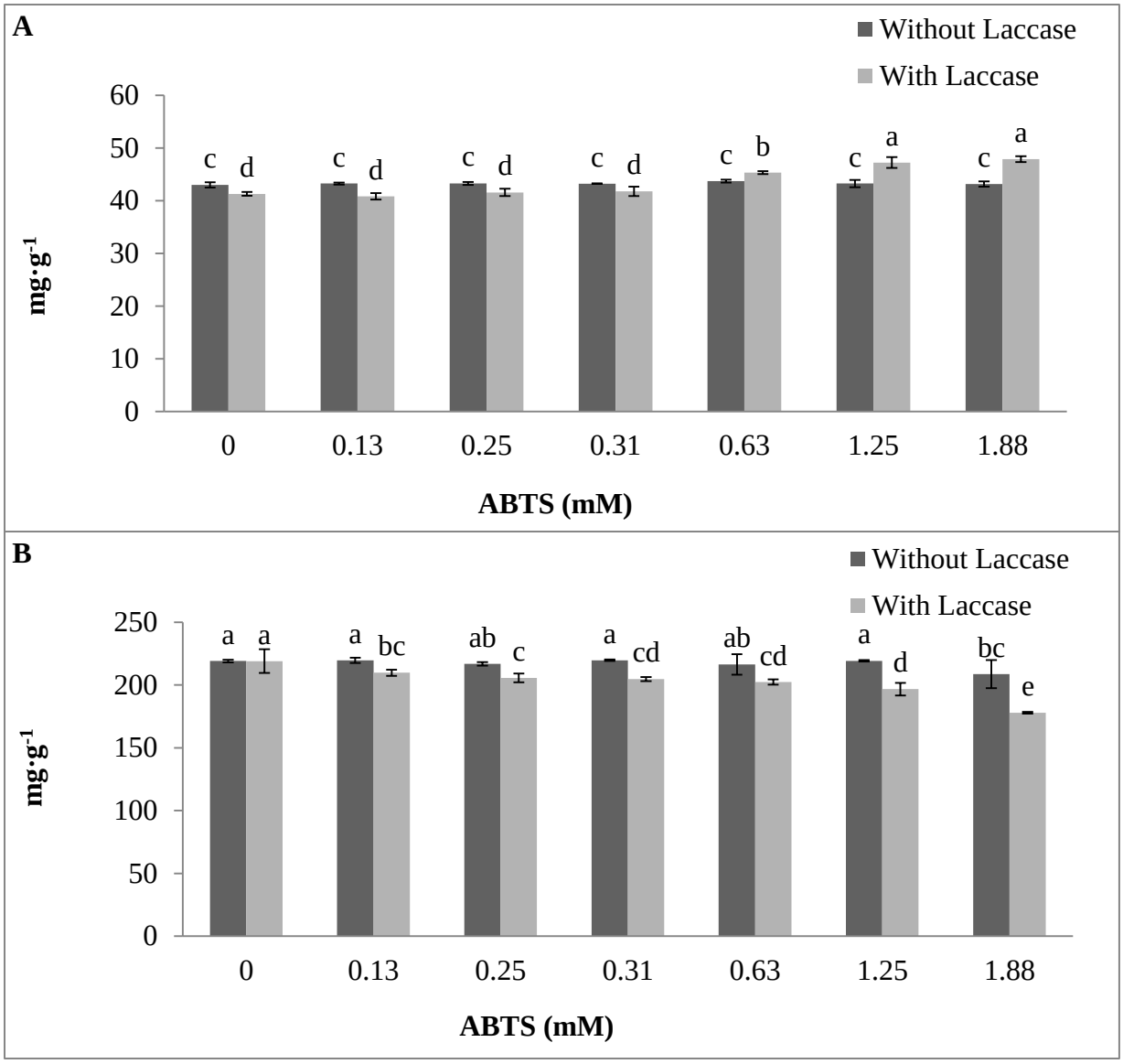

Fig. 8.2 Extractive-free acid-soluble lignin content (L_S, 8.2A) and acid-insoluble lignin content (L_I, 8.2B) of switchgrass after 24 h of enzymatic treatment in a 20 mL reaction mixture with seven different levels of mediator ABTS 0, 0.13, 0.25, 0.31, 0.63, 1.25, and 1.88 mM with and without laccase at activity 10 units mL^{-1}. Values are means of three replicates and error bars represent standard deviation. Bars with the same letter are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

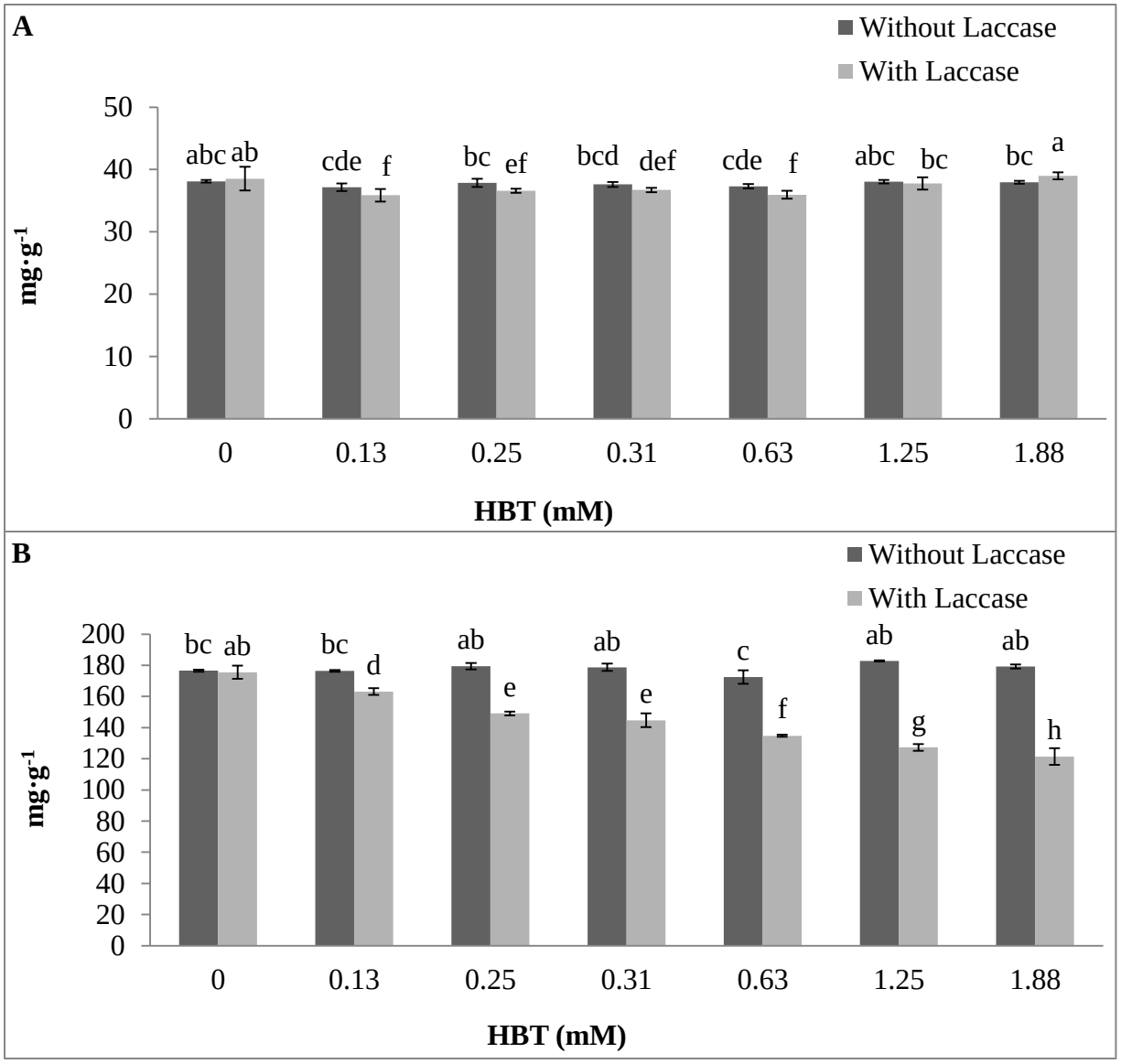

Fig. 8.3 Extractive-free acid-soluble lignin content (L_S, 8.3A) and acid-insoluble lignin content (L_I, 8.3B) of sweet sorghum after 24 h of enzymatic treatment in a 20 mL reaction mixture with seven different levels of mediator HBT 0, 0.13, 0.25, 0.31, 0.63, 1.25, and 1.88 mM with and without laccase at activity 10 units mL^{-1}. Values are means of three replicates and error bars represent standard deviation. Bars with the same letter are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

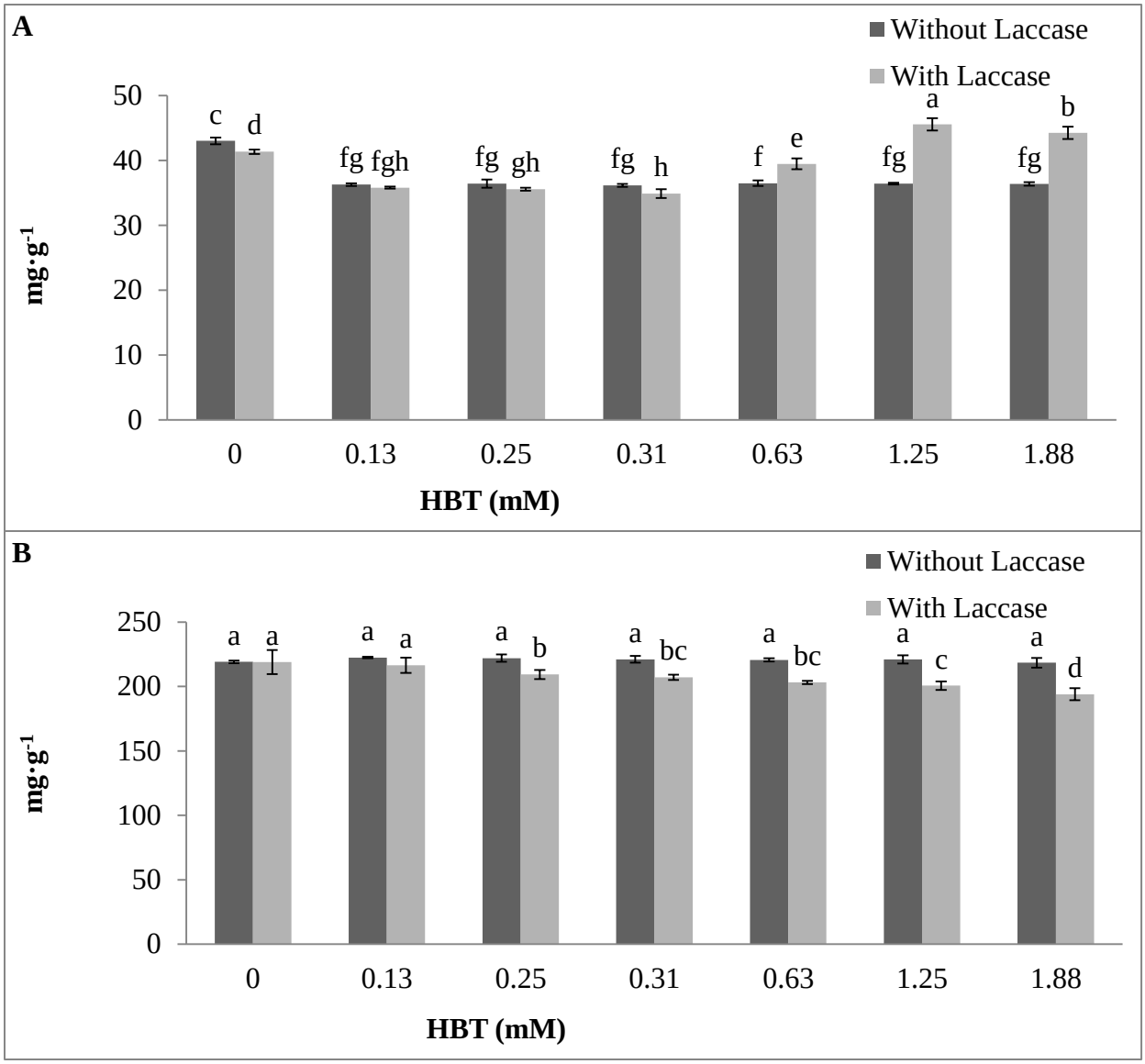

Fig. 8.4 Extractive-free acid-soluble lignin content (L_S, 8.4A) and acid-insoluble lignin content (L_I, 8.4B) of switchgrass after 24 h of enzymatic treatment in a 20 mL reaction mixture with seven different levels of mediator HBT 0, 0.13, 0.25, 0.31, 0.63, 1.25, and 1.88 mM with and without laccase at activity 10 units mL^{-1}. Values are means of three replicates and error bars represent standard deviation. Bars with the same letter are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

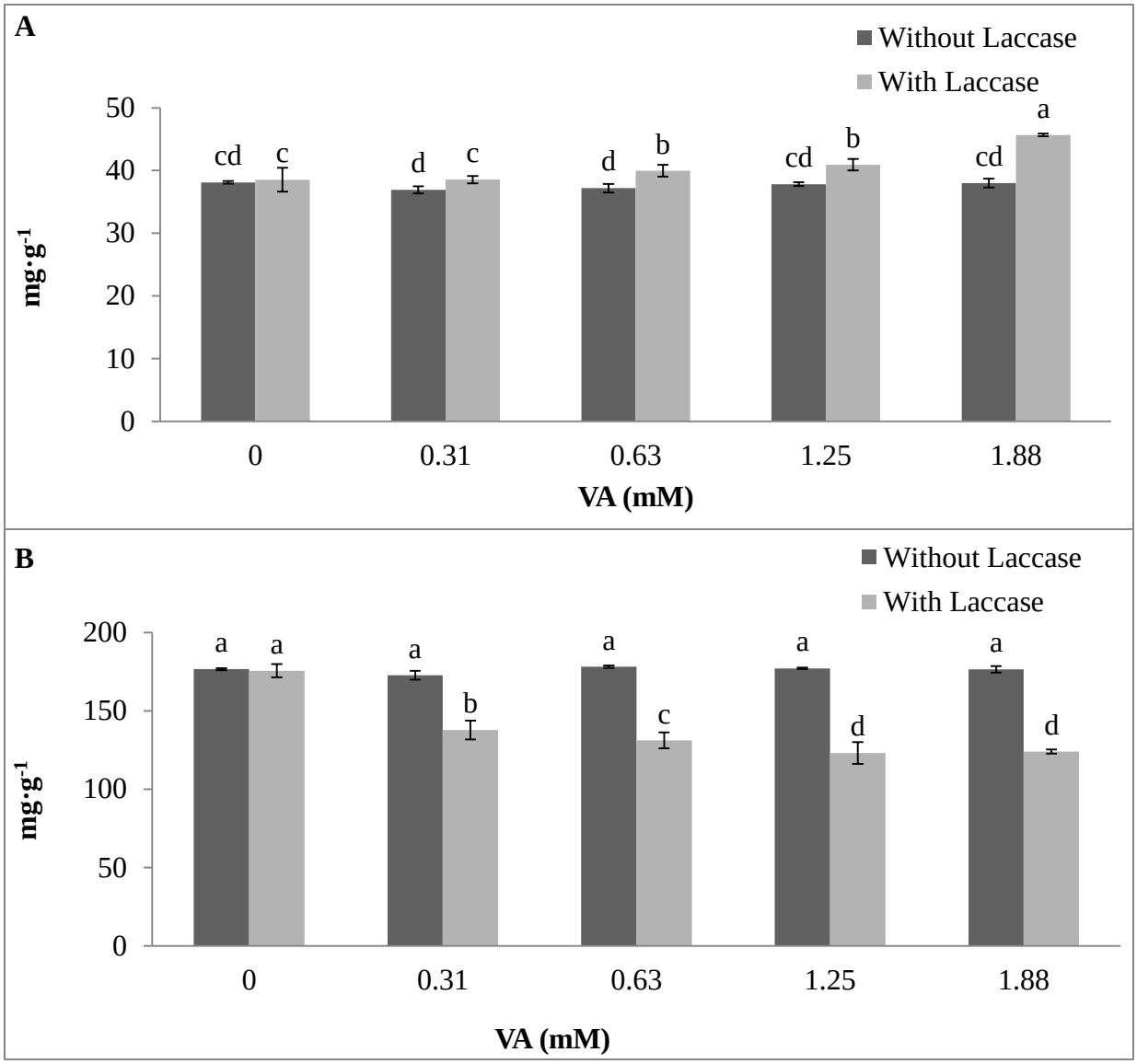

Fig. 8.5 Extractive-free acid-soluble lignin content (L_S, 8.5A) and acid-insoluble lignin content (L_I, 8.5B) of sweet sorghum after 24 h of enzymatic treatment in a 20 mL reaction mixture with five different levels of mediator VA 0, 0.31, 0.63, 1.25, and 1.88 mM with and without laccase at activity 10 units mL^{-1}. Values are means of three replicates and error bars represent standard deviation. Bars with the same letter are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

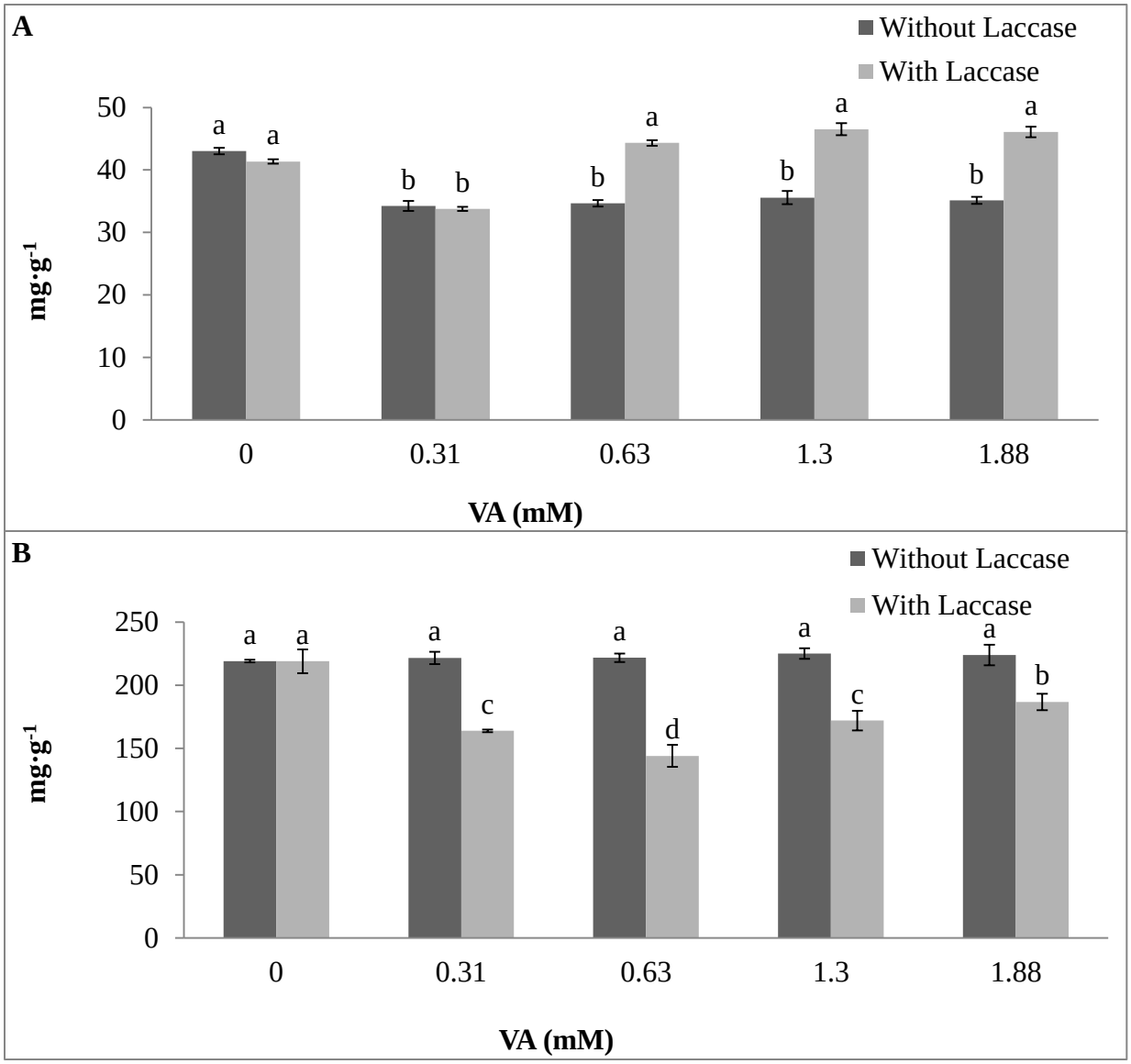

Fig. 8.6 Extractive-free acid-soluble lignin content (L_S, 8.6A) and acid-insoluble lignin content (L_I, 8.6B) of switchgrass after 24 h of enzymatic treatment in a 20 mL reaction mixture with five different levels of mediator VA 0, 0.31, 0.63, 1.25, and 1.88 mM with and without laccase at activity 10 units mL^{-1}. Values are means of three replicates and error bars represent standard deviation. Bars with the same letter are not considered to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

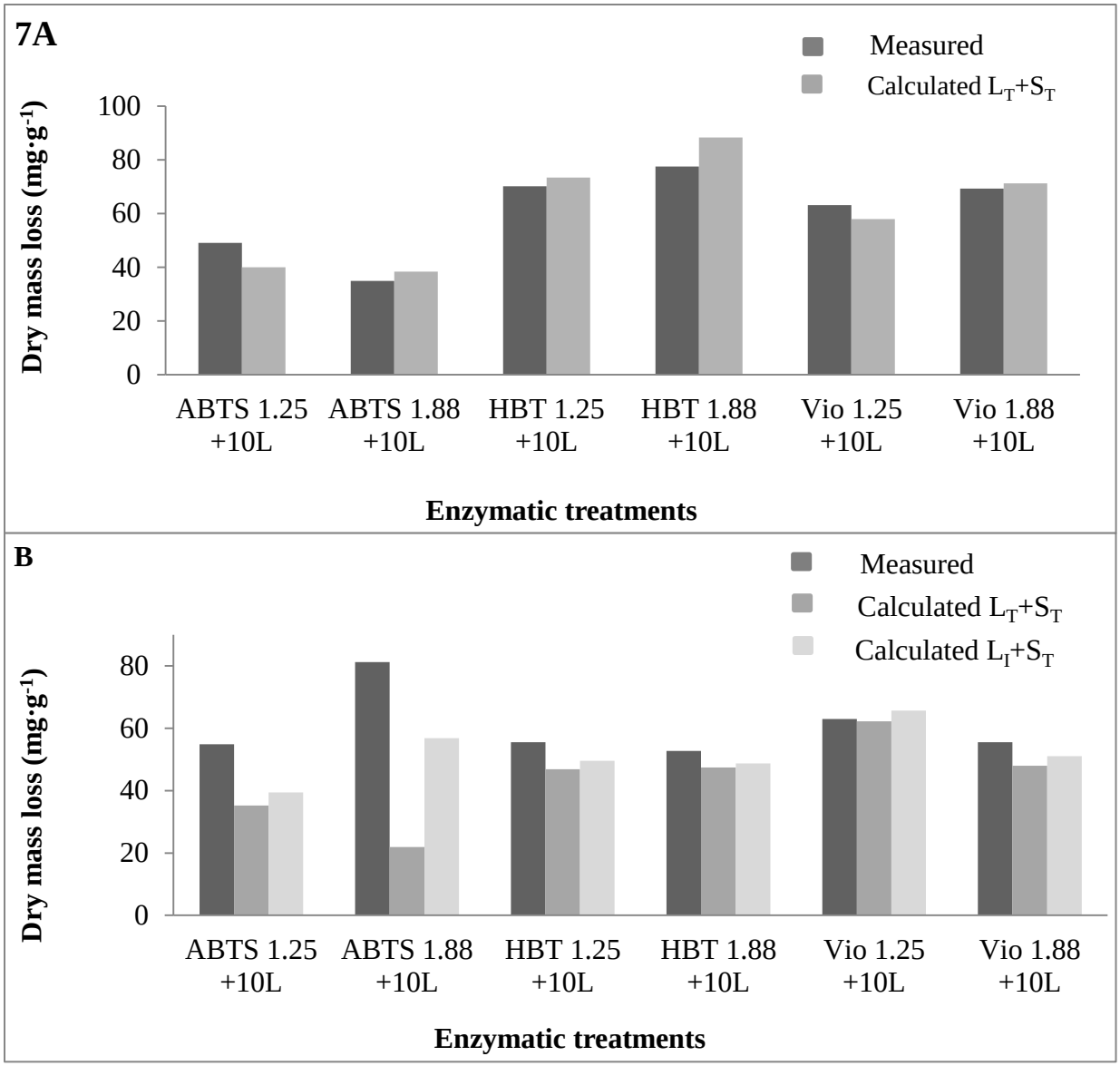

Fig. 8.7 Measured and calculated dry mass loss from sweet sorghum (8.7A) and switchgrass (8.7B) biomass after 24 h of laccase treatment (10 units mL^{-1}) in a 20 mL reaction mixture with two different levels of mediators ABTS, HBT, and VA at 1.25, and 1.88 mM concentration.

CHAPTER IX

OVERALL CONCLUSIONS

The greenhouse research demonstrated that direct application of laccase enzyme at 2.06 units cm^{-2} for nine months on creeping bentgrass every two weeks was effective in slowing the rate of accumulation of organic matter and thatch layer in highly maintained turf. However, low activity level of laccase (0.206 units cm^{-2}) was ineffective in reducing the rate of thatch accumulation. Laccase application at 2.06 units cm^{-2} had little effect after two months, but after nine months significantly reduced organic matter (30.3%), thatch layer (45%), lignin content (19%) while increasing saturated hydraulic conductivity (322%) relative to control.

Bi-weekly applications of laccase enzyme on dead creeping bentgrass, with a distinct thatch layer but where new thatch development would not occur, at 2.06 and 20.6 units cm^{-2} was effective to cause changes in physical and chemical properties and compositions of the thatch layer. No reduction in organic matter and sugar content was observed after two months of treatment; but after six months, 62.0, 24.7, and 29.3% reduction in thatch layer thickness, total organic matter (0-2.5 cm), and total sugar content, respectively were observed. Application of laccase for six months increased saturated hydraulic conductivity and acid-insoluble lignin content by 70.8 and 17.1%, respectively.

The field research to optimize laccase application on creeping bentgrass demonstrated that a bi-weekly application of laccase for six months at 0.5 units cm^{-2} is equally effective in managing thatch as 1.0, 2.0 and 4.0 units cm^{-2} applied every two weeks. Laccase application frequency can be reduced to once a month when applied at 2.0 units cm^{-2} to effectively manage

thatch layer accumulation. Organic matter content at 0-2.5 and 2.5-5.0 cm depths was not affected with application of laccase across different rates and frequencies. Laccase alone did not influence saturated hydraulic conductivity, but in combination with core aeration and sand topdressing, saturated hydraulic conductivity increased relative to the control. Laccase application at 2.0 units cm^{-2} once in four weeks along with core aeration and topdressing was effective in lowering thatch layer thickness, organic matter content (0-2.5 cm), acid-soluble lignin and sugar contents while increasing acid-insoluble lignin content. Laccase enzyme from different sources was equally effective in thatch layer reduction.

After six months of laccase application on creeping bentgrass, effects of residual laccase were observed over the next six and twelve months after cessation of laccase treatment across all rates and frequencies. An average reduction in thatch layer thickness across all laccase rates was 23.5, 25.7, and 23.9% of the control when sampled at 6, 12, and 18 months after start of treatment. Similarly, thatch layer reduced on an average across all laccase application frequencies, by 27.8, 25.5, and 19.7% of the control for 6, 12, and 18 month samplings. The field study demonstrated that application of laccase enzyme for six months once a year would be effective to reduce the organic matter content and thatch layer thickness.

Laccase enzyme when applied on ultra-dwarf bermudagrass and zoysiagrass once every two weeks at 2.0 units cm^{-2} for six months effectively changed properties of the thatch layer of both grass species. In bermudagrass, a reduction of 24%, 18.2-22.3%, and 12.3 to 13.6% was observed for organic matter content at depth 0-2.5 cm, thatch layer thickness, and total lignin content, respectively while increasing saturated hydraulic conductivity (18.8-29.5%) or both years during two year experiment. In zoysiagrass, a significant reduction in organic matter (0-2.5 cm) content in plots receiving laccase was observed in the first year but not during the second

year. Thatch layer thickness and total lignin content decreased by 20.7-29.6% and 10.7-13%, respectively in both years.

Lignin removal from two lignocellulosic biomasses of sweet sorghum and switchgrass was optimized using laccase mediator system. A total lignin reduction of 8% with 1.25 mM ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) and 14% with 1.88 mM ABTS was observed in sweet sorghum and switchgrass, respectively when used with laccase enzyme after 24 h treatment. Optimum reduction in total lignin after 24 h treatment of laccase-HBT (1-hydroxybenzotriazole) mediator system was 25 and 9% for sweet sorghum and switchgrass at HBT concentration of 1.88 mM in the reaction mixture. Violuric acid as a mediator with laccase was effective in removing total lignin to the extent of 24.6% at 1.25 mM and 28.0% at 0.63 mM concentration in sweet sorghum and switchgrass, respectively. All three mediators without laccase enzyme in reaction mixture had no effect on acid-soluble lignin in sweet sorghum while application of HBT and VA significantly reduced L_S content in switchgrass. Enzymatic treatment was effective in lignin removal from biomass while negligibly effecting sugar content of both biomass species. The results of this study suggest that laccase mediator system has the potential to be used as pre-treatment for lignocellulosic biomass for bioethanol production while future research is needed to observe the downstream process of hydrolysis to observe its impact on bioethanol production efficiency.