A Novel Method to Facilitate Biodethatching Using Fungal Laccases: Optimization in a Field Study

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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 from white-rot fungi, *Trametes versicolor,* 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 applied 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^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^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. 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 ^{o}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·g^{-1} and 4 to 12 mg·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·g^{-1} and 13 to 35 mg·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·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·g^{-1} in year one and by 65 to 105 mg·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.

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.

LITERATURE CITED

Alder, E. 1977. Lignin chemistry - past, present and future. Wood Sci. Technol. 11: 169-218.

Baldrian, P. 2006. Fungal laccases-occurrence and properties. FEMS Microbiol Rev. 30: 215-242.

Barton, L., G.G.Y. Wan, R.P. Buck, and T.D. Colmer. 2009. Effectiveness of cultural thatch-mat controls for young and mature kikuyu turfgrass. Agron. J. 101: 67-74.

Beard, J.B. 1973. Turfgrass: Science and culture. Prentice Hall Inc., Englewood Cliffs, NJ.

Blanchette, R.A. 1984. Screening wood decayed by white rot fungi for preferential lignin degradation. Appl. Environ. Microbiol. 48: 647-653.

Carrow, R.N. 2003. Surface organic matter in bentgrass greens. USGA Turfgrass Environ. Res. Online 2(17): 1-10.

Carrow, R.N. 2004. Surface organic matter in bentgrass greens. Golf Course Mgt. 72(5): 96-101.

Carrow, R.N., B.J. Johnson, and R.E. Burns. 1987. Thatch and quality of Tifway bermudagrass turf in relation to fertility and cultivation. Agron. J. 79: 524-530.

Chen, Y.R., and S. Sarkanen. 2003. Macromolecular lignin replication: A mechanistic working hypothesis. Phytochemistry Rev. 2: 235-255.

Davin, L.B. and N.G. Lewis 2003. A historical perspective on lignin biosynthesis: Monolignol, allylphenol and hydroxycinnamic acid coupling and downstream metabolism. Phytochemistry Rev. 2: 257-288.

Del Rio, J. C., G. Marques, J. Rencoret, A.T. Martinez, and A. Gutierrez. 2007. Occurrence of naturally acetylated lignin units. J. Agric. Food Chem. 55: 5461-5466.

Dunn, J.H., K.M. Sheffer, and P.M. Halisky. 1981. Thatch and quality of Meyer zoysia in relation to management. Agron. J. 73: 949-952.

Engel, R.E. 1954. Thatch on turf and its control. Golf Course Rep. 22 (5): 12-14.

Engelke, M.C., V.G. Lehman, W.R. Kneebone, P.F. Colbaugh, J.A. Reinert, and W.E. Knoop. 1995. Registration of 'Crenshaw' creeping bentgrass. Crop Sci. 35: 589.

Hartwiger, C. 2004. The importance of organic matter dynamics: How research uncovered the primary cause of secondary problems. USGA Green Section Record 42(3): 9-11.

Kirk, T.K., W.J. Connors, R.D. Bleam, and G. Jeikus. 1976. Requirements for a growth substrate during lignin decomposition by two wood-rotting fungi. Appl. Environ. Microbiol. 32: 192-194.

Kirk, T.K., W.J. Connors, R.D. Bleam, W.F. Hackett, and J.G. Jeikus. 1975. Preparation and microbial decomposition of synthetic [^{14}C] lignins. Proc. Natl. Acad. Sci. USA. 72: 2515-2519.

Landreth, J., D. Karcher, and M. Richardson. 2008. Cultivating to manage organic matter in sand based putting greens: University of Arkansas researchers provide important insight for managing organic buildup on putting greens. USGA Turfgrass Environ. Res. Online 46(1): 16-19.

Ledeboer, F.B., and C.R. Skogley. 1967. Investigations into the nature of thatch and methods for its decomposition. Agron. J. 59: 320-323.

Martin, S.B., and J.L. Dale. 1980. Biodegradation of turf thatch with wood-decay fungi. Phytopathology. 70: 297-301.

McCarty, L.B. 2005. Best golf course management practices. 2nd ed. Prentice Hall Inc. Upper Saddle River, NJ.

McCarty, L. B., M.F. Gregg, and J.E. Toler. 2007. Thatch and mat management in an established creeping bentgrass green. Agron. J. 99: 1530-1537.

McCarty, L.B., M.F. Gregg, J.E. Toler, J.J. Camberato, and H.S. Hill. 2005. Minimizing thatch and mat development in a newly seeded creeping bentgrass golf green. Crop Sci. 45: 1529-1535.

McWhirter, E.L., and C.Y. Ward. 1976. Effect of vertical mowing and aerification on golf green quality. Mississippi Agric. For. Exp. Stn. Res. Rep. 2 (12): 1-2.

Mester, T., E. Varela, and M. Tien. 2004. Wood degradation by brown-rot and white-rot fungi. The Mycota II: Genetics and biotechnology. 2nd edition. Springer-Verlag, Berlin, Heidelberg.

Munoz, C., F. Guillen, A.T. Martinez, and M.J. Martinez. 1997. Laccase isozymes of *Pleurotus eryngii*: Characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. App. Environ. Microbiol. 63: 2166-2174.

Murdoch, C.L., and J.P. Barr. 1976. Ineffectiveness of commercial microorganism inoculums in breaking down thatch in common bermudagrass in Hawaii. HortScience. 11: 488-489.

NREL- National Renewable Energy Laboratory. 2008. Determination of structural carbohydrates and lignin in biomass. Available at: http://www.nrel.gov/biomass/pdfs/42618.pdf (Verified 01 July, 2012). NREL, Golden, CO, USA.

O'Brien, P., and C. Hartwiger. 2003. Aeration and topdressing for the 21st century: Two old concepts are linked together to offer up-to-date recommendations. USGA Green Section Record 41(2): 1-7.

Otjen, L., and R. Blanchette. 1987. Assessment of 30 white rot basidiomycetes for selective lignin degradation. Holzforschung. 41: 343-349.

Park, J. W., J. Dec, J. E. Kim, and J. M. Bollag. 1999. Effect of humic constituents on the transformation of chlorinated phenols and anilines in the presence of oxidoreductive enzymes or birnessite. Environ. Sci. Tech. 33: 2028-2034.

Ralph, J., M. Bunzel, J.M. Marita, R.D. Hatfield, F. Lu, H. Kim, P.F. Schatz, J.H. Grabber, and H. Steinhart. 2004. Peroxidase dependent cross linking reactions of p-hydroxicinnamates in lant cellwalls. Phytochemistry Rev. 3: 79-96.

Roberts, E.C., and E.J. Bredakis. 1960. What, why and how of turfgrass root development. Golf Course Rep. 28: 13-24.

Sartain, J.B., and B.G. Volk. 1984. Influence of selected white-rot fungi and topdressings on the composition of thatch components of four turfgrasses. Agron. J. 76: 359-362.

SAS Institute Inc. 1994. The SAS system for windows. Release 9.2. SAS Inst., Cary, NC.

Sinsabaugh R.L., R.K. Antibus, A.E. Linkins, C.A. Mcclaugherty, L. Rayburn, D. Repert, T. Weiland. 1993. Wood decomposition - nitrogen and phosphorus dynamics in relation to extracellular enzyme-activity. Ecology. 74: 1586-1593.

Stoilova, I., A. Krastanov, and V. Stanchev. 2010. Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. Adv. Bioscience Biotech. 1: 208-215.

Taylor B.R., D. Parkinson, W.F.J. Parsons. 1989. Nitrogen and lignin content as predictors of litter decay rates: A microcosm test. Ecology. 70: 97-104.

Thurston, C.F. 1994. The structure and function of fungal laccases. Microbiol. 140: 19-16.

Weston, J.B., and J.H. Dunn. 1985. Thatch and quality of Meyer zoysia in response to mechanical cultivation and nitrogen fertilization. P. 449-458. *In* F. Lemaire (ed.) Proc. 5th Int. Turfgrass Res. Conf., Avignon, France. 1-5 July 1985. Institut National de la Recherche Agronomique, Paris, France.

White, R.H., and R. Dickens. 1984. Thatch accumulation in bermudagrass as influenced by cultural practices. Agron. J. 76: 19-22.

Wong, D.W.S. 2009. Structure and action mechanism of ligninolytic enzymes. Appl. Biochem Biotechnol. 157: 174-209.

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

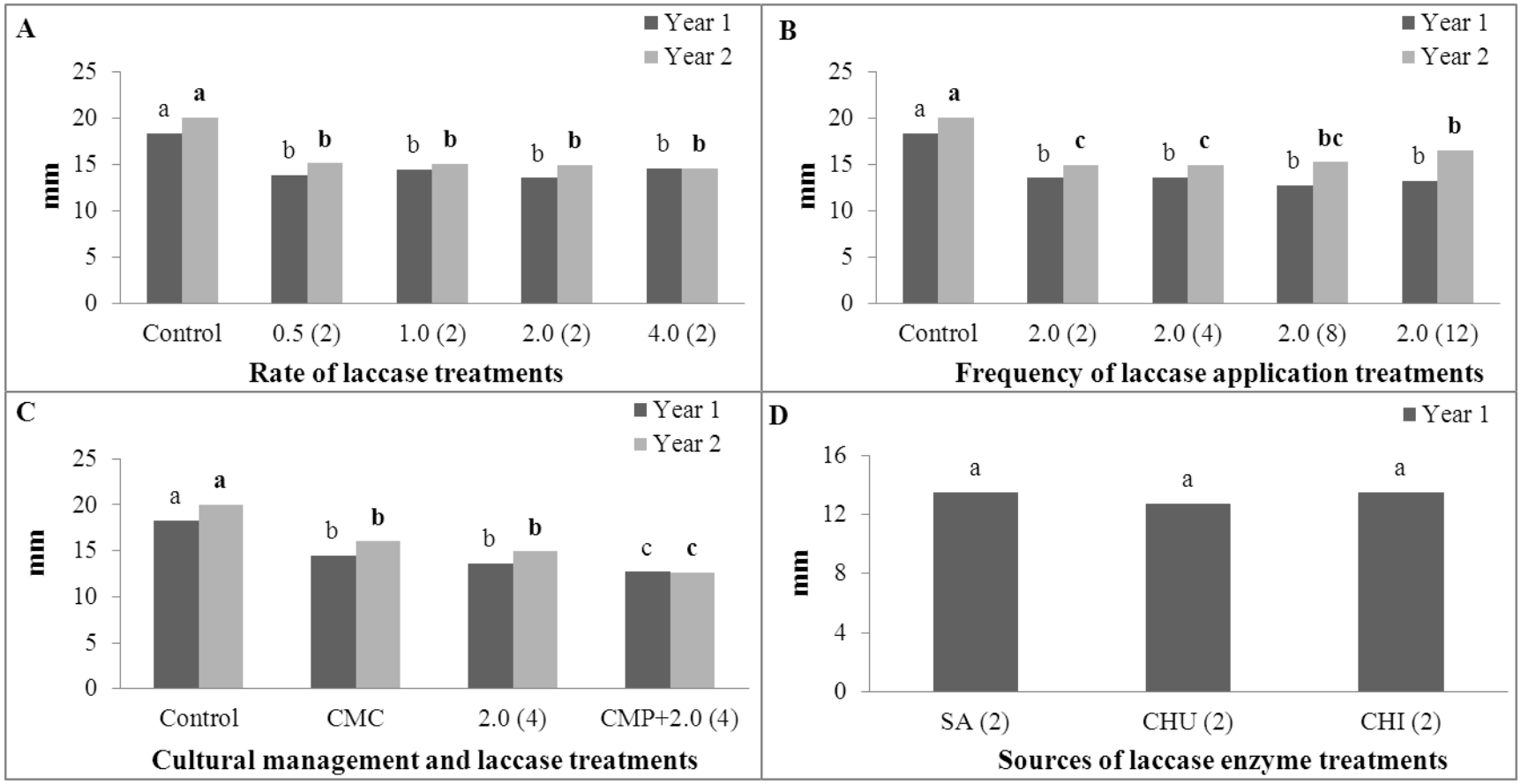

Fig. 5.1 Thatch layer thickness (TLT) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.1A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.1B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with 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 $\alpha = 0.05$.

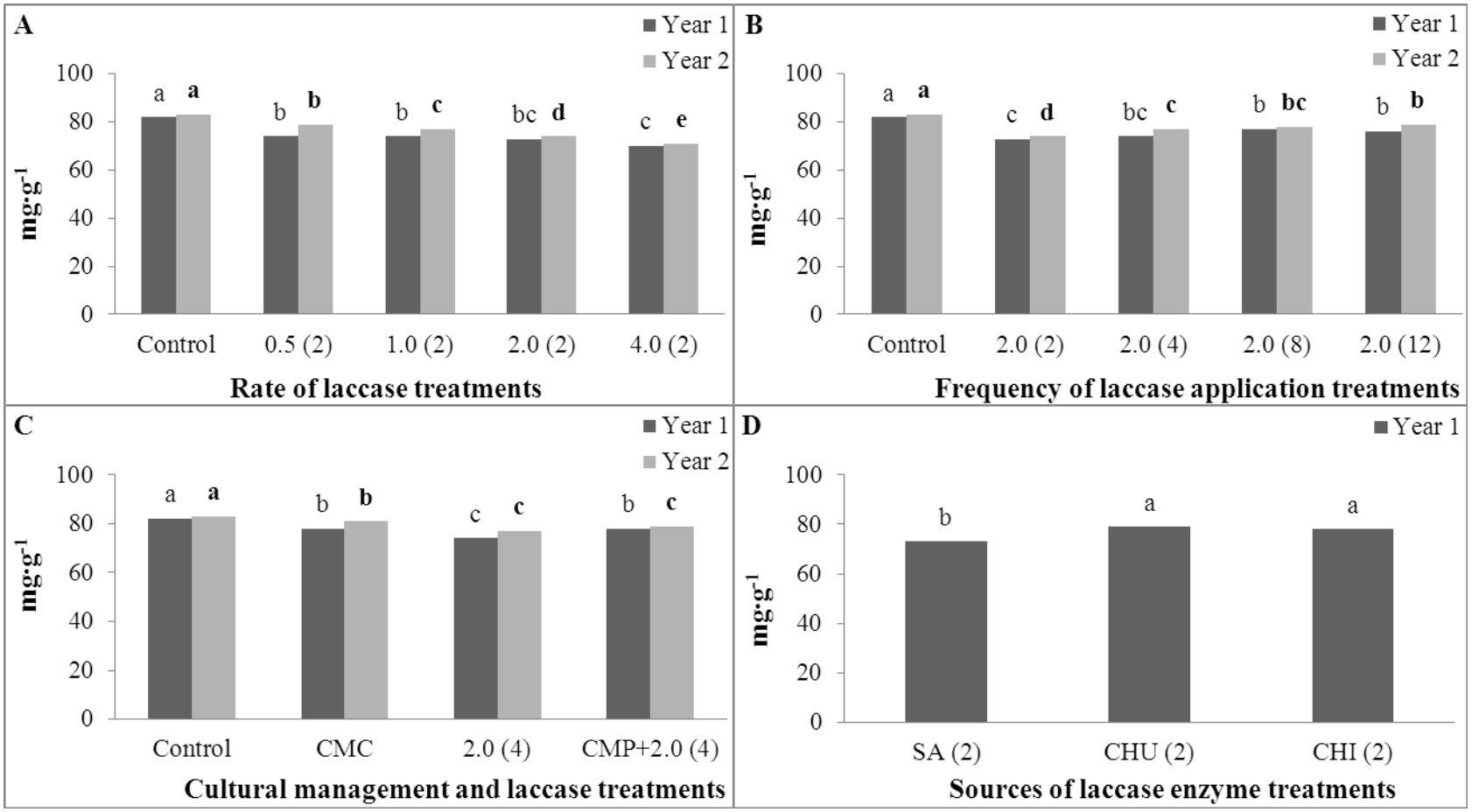

Fig. 5.2 Extractive-free acid-soluble lignin content (L_S) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.2A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.2B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks 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

30 to be statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

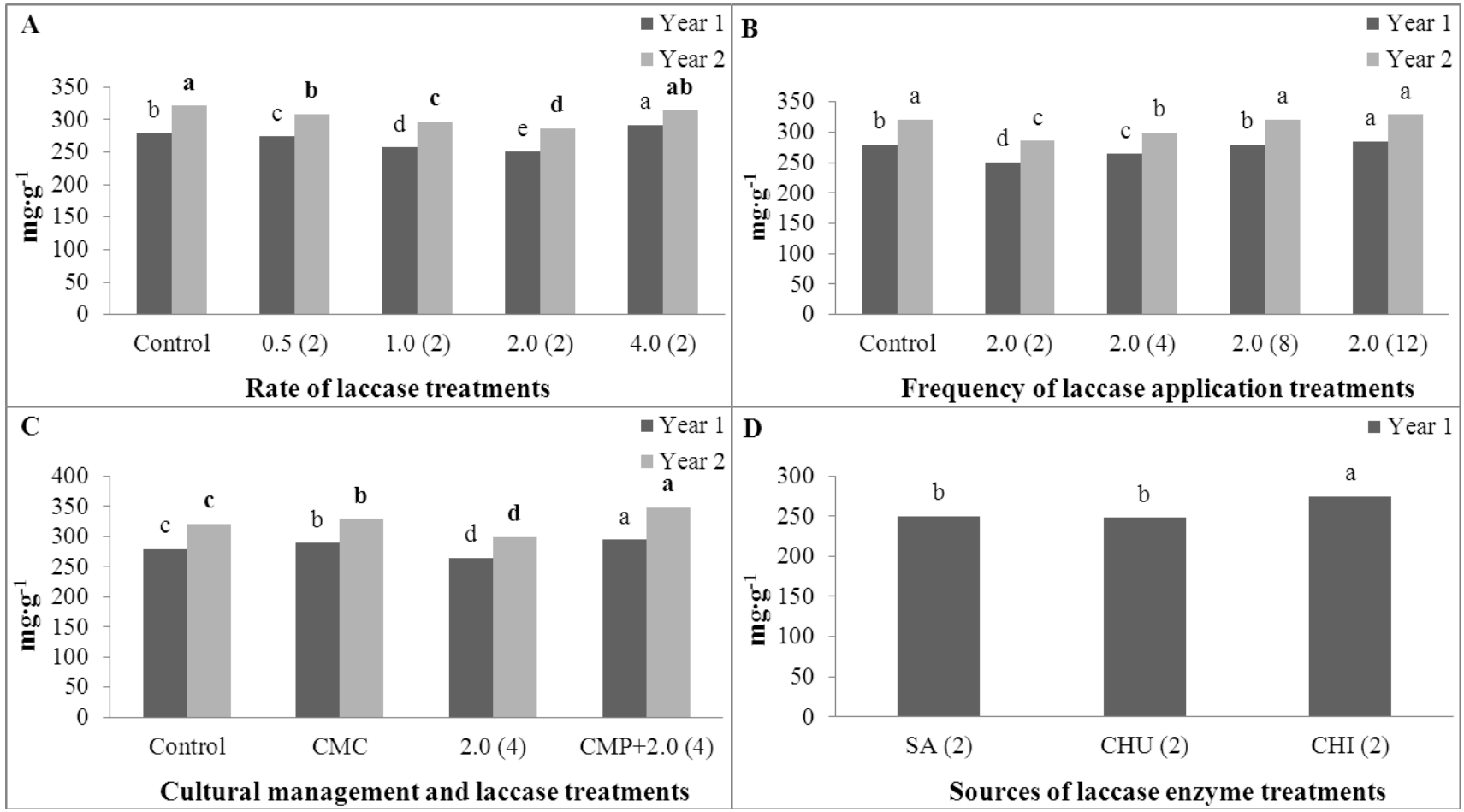

Fig. 5.3 Extractive-free acid-insoluble lignin content (L_I) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.3A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.3B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks 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 $\alpha = 0.05$.

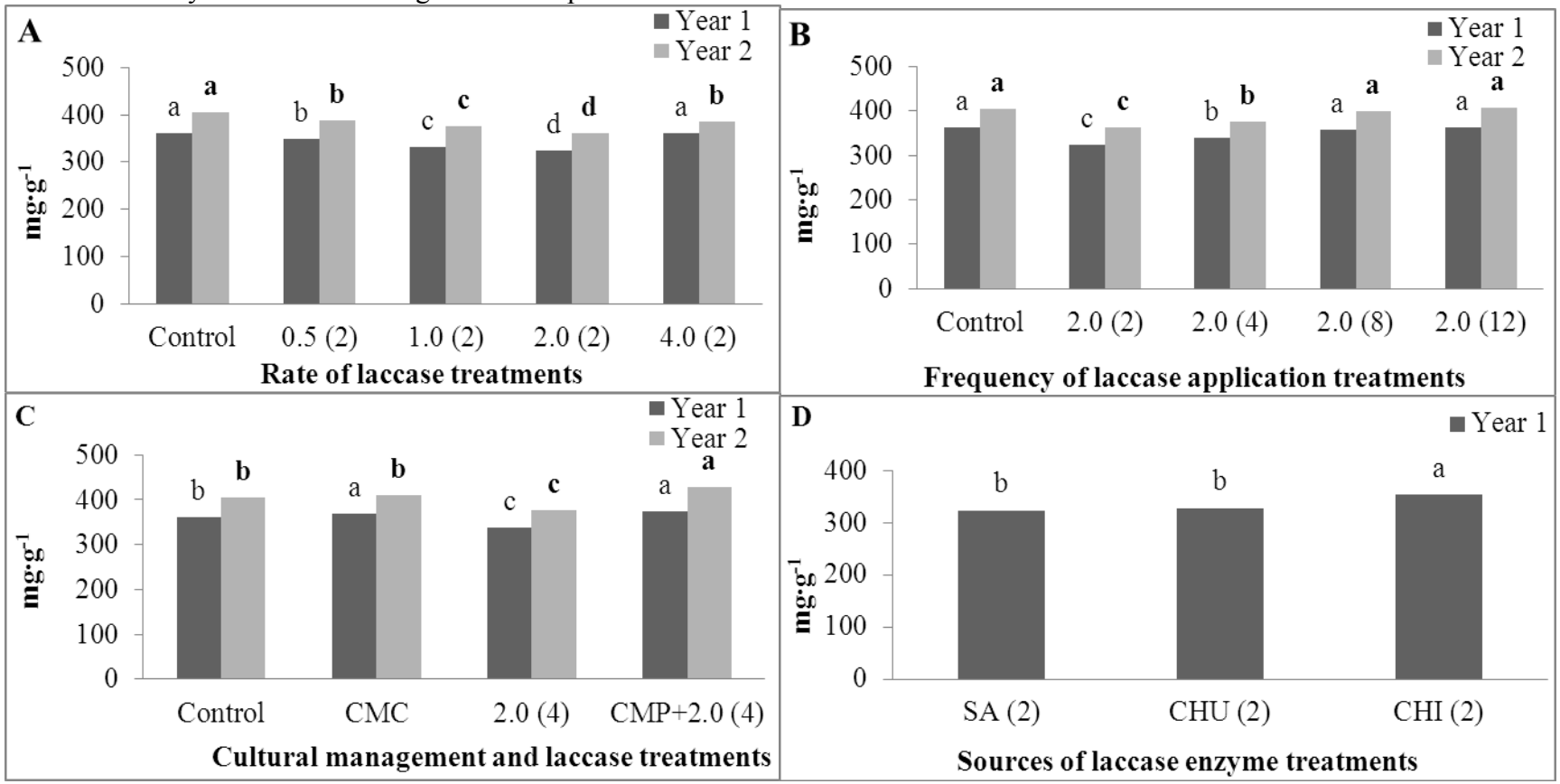

Fig. 5.4 Extractive-free total lignin content (L_I) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.4A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.4B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison 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 $\alpha = 0.05$.

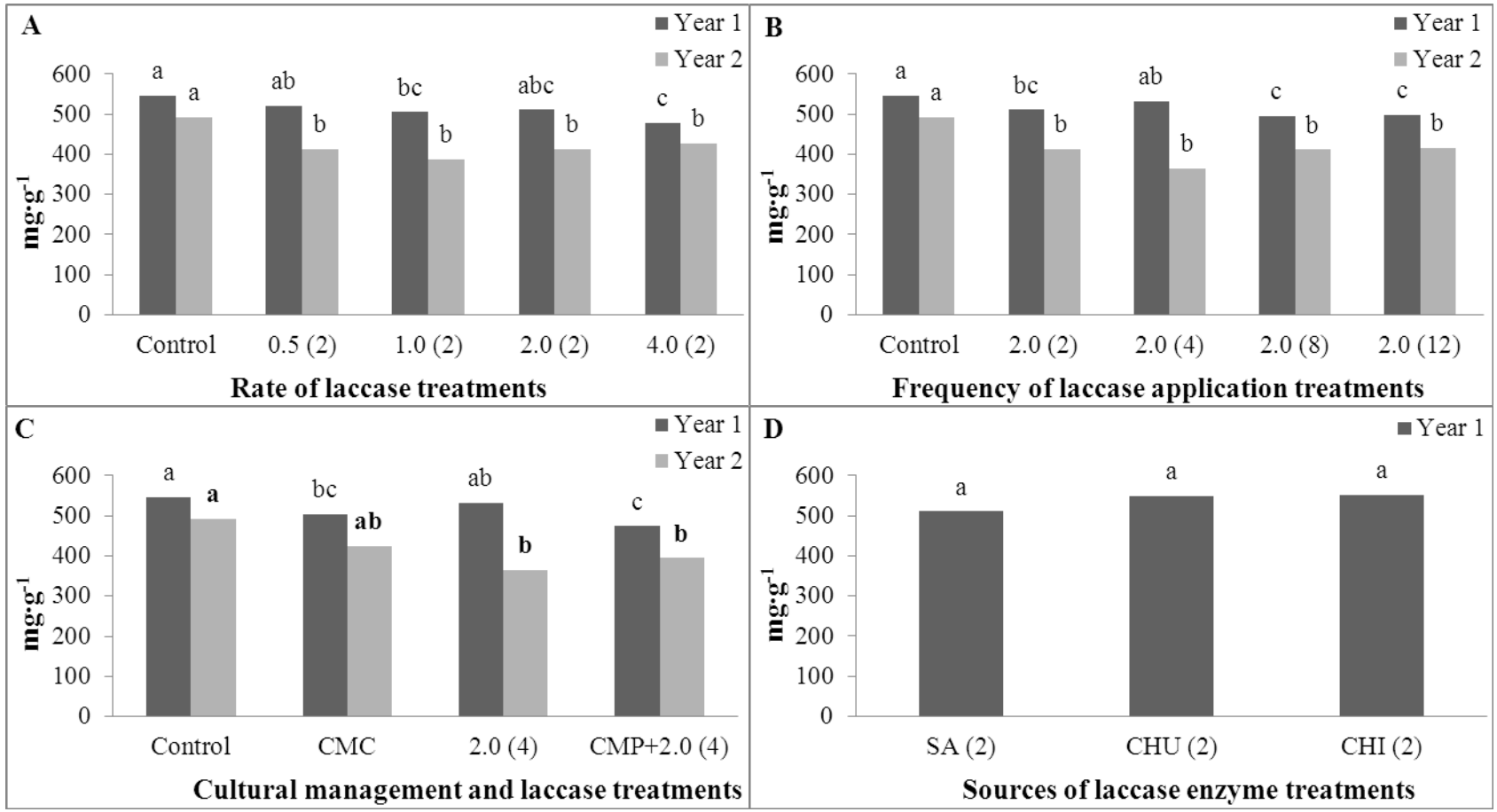

Fig. 5.5 Extractive-free total sugar content (S_T) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.5A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.5B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison 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 $\alpha = 0.05$.

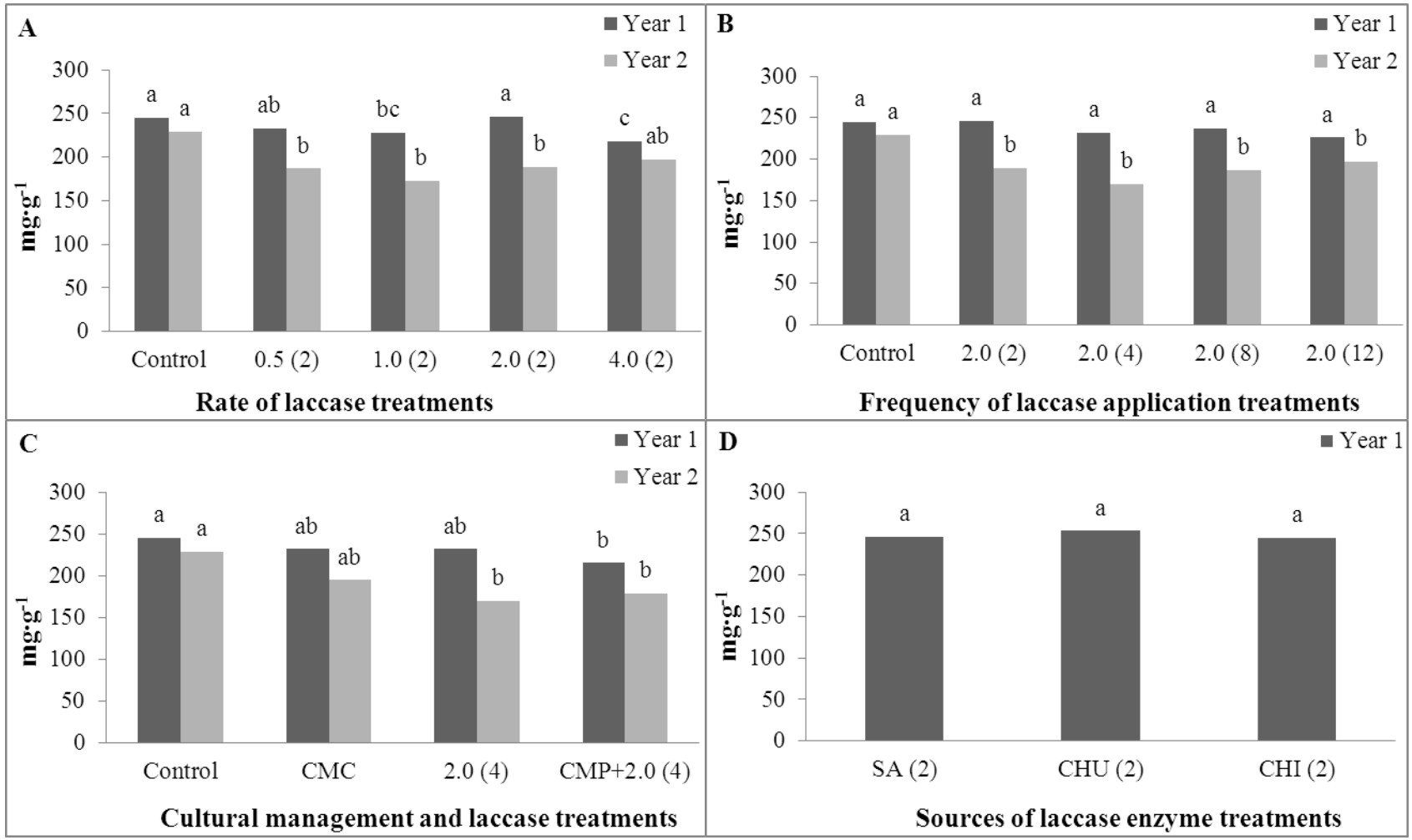

Fig. 5.6 Extractive-free glucose content (S_{GLU}) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.6A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.6B); laccase at 2.0 units cm^{-2} applied at a frequency

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 $\alpha = 0.05$.

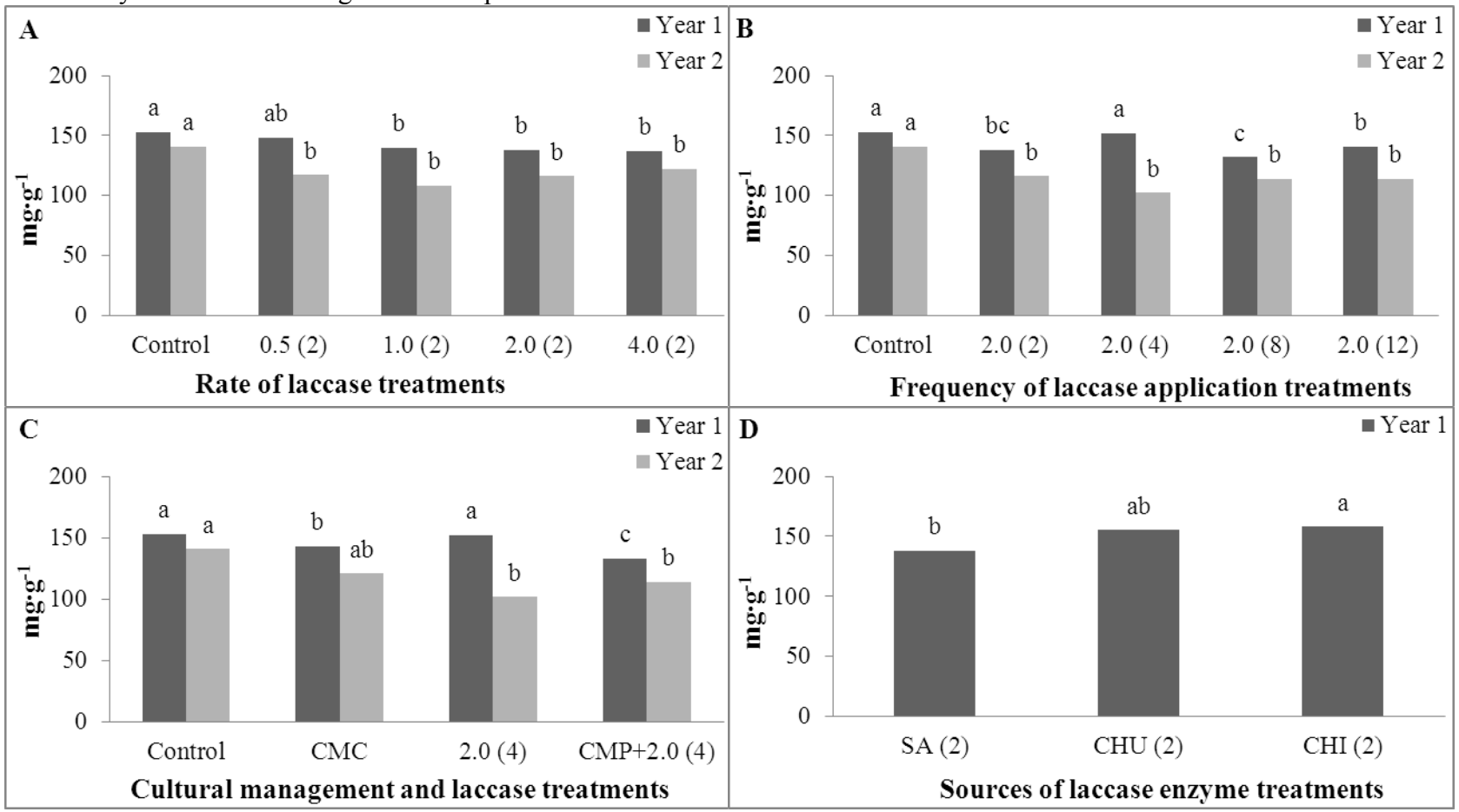

Fig. 5.7 Extractive-free xylose content (S_{XYL}) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.7A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.7B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice (Fig 5.7C); and laccase enzyme from different sources (Fig 5.7D).

63 Values are means of four replicates. Same letter on top of the bars (year one = standard, year two = bolded) are not considered to be
64 statistically different according to Fisher's protected LSD at $\alpha = 0.05$.

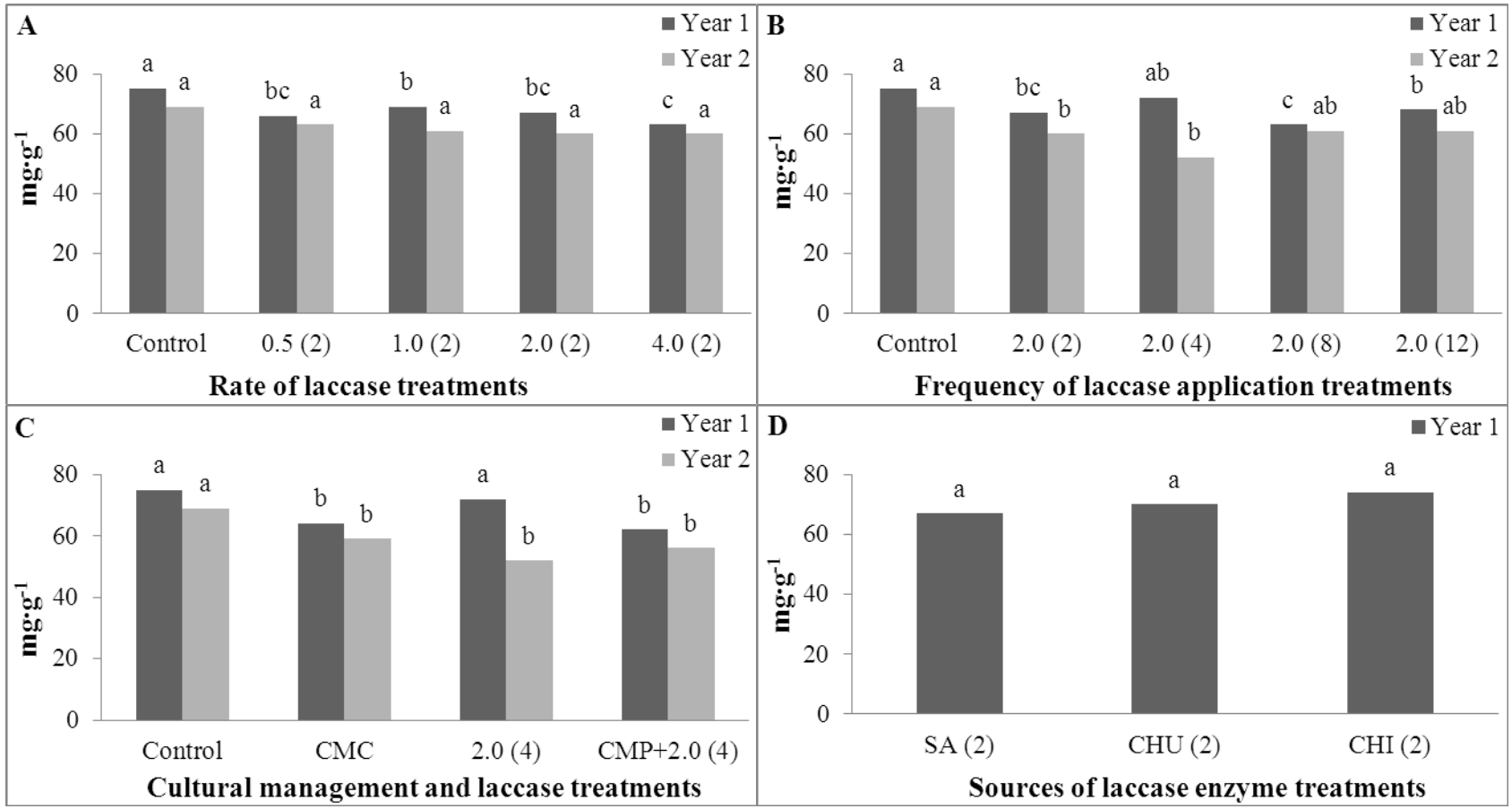

Fig. 5.8 Extractive-free galactose content (S_{GAL}) after treatment application on creeping bentgrass with: five different levels of laccase (Fig 5.8A); laccase activity level 2.0 units cm^{-2} applied at four frequencies (Fig 5.8B); laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in 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 $\alpha = 0.05$.

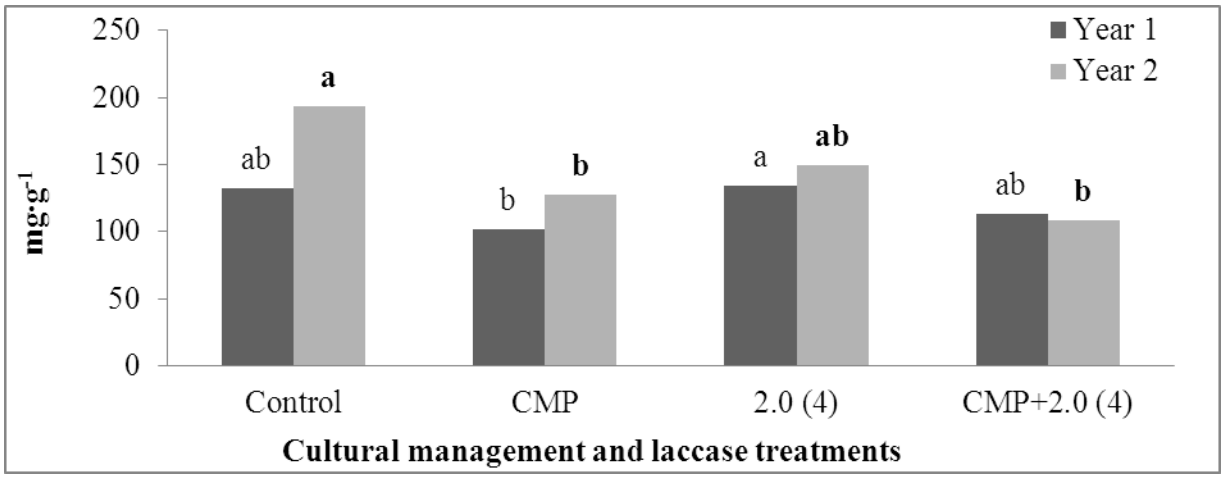

Fig. 5.9 Organic matter content in the 0-2.5 cm surface layer (OM_U) after treatment application on creeping bentgrass with laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice. Values are means of four replicates. Same letter on top of the bars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

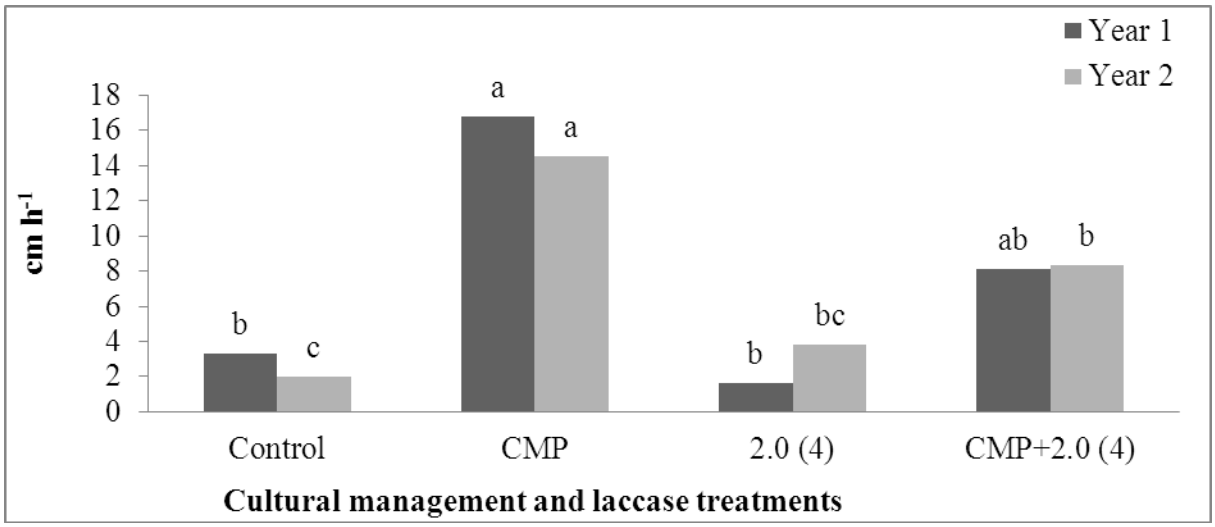

Fig. 5.10 Saturated hydraulic conductivity (SHC) after treatment application on creeping bentgrass with laccase at 2.0 units cm^{-2} applied at a frequency of 4 weeks in comparison with cultural management practice. Values are means of four replicates. Same letter on top of the bars (year one = standard,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