Short-term and Residual Effects of Laccase Application on Creeping Bentgrass Thatch Layer

Sudeep S. Sidhu¹

University of Florida, North Florida Research and Education Center, Quincy, FL 32351

Qingguo Huang, Robert N. Carrow, and Paul L. Raymer

Department of Crop and Soil Sciences, The University of Georgia, Griffin Campus, 1109 Experiment Street, Griffin, GA 30223

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Abstract. Organic layer formation in the form of thatch is a major problem in managed turfgrass systems. Biweekly application of laccase enzyme has been well-documented to facilitate the degradation of thatch and reduce the accumulation rate of organic matter in ‘Crenshaw’ creeping bentgrass (*Agrostis stolonifera* L.). A field experiment involving creeping bentgrass was conducted to evaluate the residual effects on thatch accumulation after ceasing laccase applications. A significant reduction in thatch layer thickness was observed at 6, 12, and 18 months after treatment initiation when laccase was applied at different rates and frequencies. Residual effects of laccase application were observed for thatch layer thickness, but no additional accumulation of thatch was observed 6 months after treatment cessation. At 18 months after treatment initiation, a significant increase in the thatch layer was observed where treatments had been ceased for 12 months, but no thatch accumulation was observed for laccase treatment for a second 6-month period during the second year. This information is critical to turf practitioners when developing laccase application protocols. Limiting laccase applications for a period of 6 months during 1 year was shown to be effective for thatch control.

Lignin is a plant cell wall constituent that acts as a protective matrix and limits the availability of readily biodegradable plant materials, such as cellulose and hemicelluloses, for microbial degradation (Ledeboer and Skogly, 1967). Lignin is formed in plants by oxidative coupling of monolignols 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; this process is known as lignification (Ledeboer and Skogly, 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 the 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). Other researchers have indicated a homogeneous structure of lignin based on studies suggesting lignin formation by repetitive units (Banoub and Delmas, 2003).

The formation of a thatch-mat layer at home lawn and recreational turfgrass sites, especially golf greens, is accelerated when organic matter production exceeds the degradation rate (Beard, 1973). Thatch, a layer of highly organic matter 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 generally below the thatch layer, where soil or sand is intermingled with thatch as a result of earthworm activity or cultural practices, such as core aeration and topdressing (McCarty, 2005). A thatch layer is often desirable to increase resilience and wear tolerance of the turfgrass surface, reduce surface hardness, and moderate soil temperature extremes (Beard, 1973). However, an excessive thatch or mat layer is undesirable in turfgrass because it leads to decreased saturated hydraulic conductivity (SHC), decreased movement of oxygen through the thatch or mat zone, 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 of core aeration, vertical mowing, grooming, and topdressing are often effective for reducing thatch, but they are known to adversely impact turf quality (Landreth et al., 2008; McCarty et al., 2007). Additionally, these practices have intensive requirements for labor, equipment, and energy (Barton et al., 2009; Landreth et al., 2008; McCarty et al., 2007), and they have shown contrasting results regarding reducing the organic matter content in the 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). Nondestructive biological and chemical attempts to enhance organic matter degradation in the thatch layer have included the usage of glucose, cellulase solutions (Ledeboer and Skogly, 1967), and commercial products containing mixtures of amino acids, microbial inocula, and fertilizers. These products target the degradation of cellulosic and hemicellulosic sugars in thatch biomass by improving conditions for microbial populations. However, the efficacy of these products have been inconsistent for reducing thatch in turfgrass (Lancaster et al., 1977; McCarty et al., 2005; Murdoch and Barr, 1976).

The rate of microbial decomposition is partially dependent on the lignin content of organic matter. Lignin degradation can act 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 the natural degradation of lignin by producing extracellular lignolytic enzymes, thus exposing cellulosic materials to further bacterial degradation in the environment (Blanchette, 1984; Kirk et al., 1975, 1976; Mester et al., 2004; Otjen and Blanchette, 1987). Lignolytic enzymes such as tyrosinases, catechol oxidases, laccase, catechol dioxygenases, and monophenol monooxygenase that use oxygen as an electron acceptor to oxidize phenolic compounds are likely present in environmental samples and provide estimates of the sum of activity from all or some combinations of these enzymes. Therefore, in most published work, the collective activity of these enzymes is referred to as phenol oxidase, which represents the activity of enzymes that use oxygen and oxidize phenols. Phenol oxidase activity in the top 7.5 cm of soil samples of turfgrass systems has been reported to range from 0.7 to 2.8 mmol/kg soil/h (Yao et al., 2009, 2011).

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

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¹Corresponding author. E-mail: s.sidhu@ufl.edu.

have reported reductions in cellulose content and total oxidizable organic matter of bermudagrass (*Cynodon dactylon* L.) and centipedegrass (*Eremochloa ophiuroides*) after inoculation with wood-decaying fungi (Sartain and Volk, 1984). However, field inoculation experiments involving bermudagrass showed no thatch degradation (Martin and Dale, 1980). Microbial inoculation under field conditions may be ineffective because it is difficult to maintain specific microbial activity for longer periods under turfgrass management systems (Yao et al., 2009, 2011).

Under greenhouse conditions, decreases in the rate of thatch layer build-up and accumulations of total organic matter in the top 2.5 cm of creeping bentgrass were reported in response to the direct application of laccase, an extracellular lignolytic enzyme produced from white-rot fungi *Trametes versicolor* (Sidhu et al., 2012). However, a net accumulation of organic matter in the thatch layer treated with laccase was observed over time with all treatments (Sidhu et al., 2012). A biweekly application of laccase enzymes on the thatch layer of dead creeping bentgrass verified the effectiveness of laccase for facilitating organic matter decomposition and the loss of the total sugar content of the thatch biomass. These results suggested that laccase application exposed cellulosic and hemi-cellulosic sugars to microbial degradation by opening the biomass structure (Sidhu et al., 2013a). Field studies conducted using creeping bentgrass, ultra-dwarf bermudagrass, and zoysiagrass verified the effectiveness of laccase for thatch management on different turfgrass species (Sidhu et al., 2013b, 2014). In other experiments, creeping bentgrass treated biweekly with laccase, core aeration, and sand topdressing had significant reductions in thatch accumulation (Sidhu et al., 2014). In previous studies, organic matter degradation in response to enzyme treatment was determined during and at the end of the application period (Sidhu et al., 2012, 2013a, 2013b, 2014).

The fate of naturally occurring laccase enzymes depends on the interaction with soil, which is composed of mineral constituents and organic matter. These soil constituents can adsorb extracellular enzymes and provide surfaces for enzymatic reactions. Adsorption of enzymes to soil constituents can immobilize laccase enzymes (Giaveno et al., 2010), change their efficacy (Ahn et al., 2007; Gianfreda and Bollag, 1994; Zimmerman et al., 2004), and change their stability and denaturation (Rao et al., 2000; Yan et al., 2010). Gianfreda and Bollag (1994) observed that montmorillonite and Kollinite adsorbed 71% and 64% of laccase, respectively. However, compared with free enzymes, the kinetic parameters of laccase were improved when immobilized on montmorillonite. Wu et al. (2014) also observed similar results when laccase was adsorbed on iron and aluminum minerals for 18 h. However, the long-term residual impacts of laccase under field conditions, particularly the presence of

thatch and mat layers, requires investigation. The current study was designed to expand the results of previous studies by investigating the residual effects of laccase application on organic matter degradation. Knowledge of any residual effects would lead to turf management and economic implications. Therefore, the major objectives of this study were to determine the residual effects of laccase application on physical and chemical properties of the thatch layer of creeping bentgrass and to compare the residual effects of laccase with and without repeated applications.

Materials and Methods

Experimental design

A field experiment was conducted on 'Crenshaw' creeping bentgrass (Engelke et al., 1995) *Agrostis stolonifera* L. at The University of Georgia, Griffin Campus (Griffin, GA), 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) per the recommendations of the United States Golf Association (U.S. Golf Association Green Section Staff, 1973). Fertilizer applications for 2010 and 2011 consisted of 50 kg·ha⁻¹ granular fertilizer 24-4-10 (N-P₂O₅-K₂O) (Lesco Inc., Strongsville, OH) during the third weeks of March, September, and October, and 2 kg·ha⁻¹ soluble 20-20-20 fertilizer (JR Peters Inc., Allentown, PA) every 2 weeks starting the third week of April through September. Bentgrass plots were mowed three times per week with a Toro Greensmaster 3100 (The Toro Company, Bloomington, MN) and maintained at a height of 4.2 mm.

The field study was conducted on plots (0.305 × 0.61 m) with 12 treatments replicated four times in a completely randomized block design. A priori comparisons of the rate of application, frequency of application, influence of cultural management practices (core aeration and topdressing), and sources of laccase groups of treatments of the experiment were used for the evaluations (Table 1). Laccase was applied for 6 months from July 2010 to Dec. 2010 for treatments T1 to T12. Laccase applications were repeated only for treatment T11 from July 2011 to Dec. 2011 (Table 1). All plots were sampled at either 6, 12, and 18 months after treatment initiation or 0, 6, and 12 months after the end of the initial treatment application period to observe the potential residual effects of laccase application. Laccase treatments were sprayed using a flat fan nozzle and portable CO₂ sprayer system as 410 mL of solution in a 2-L bottle. Laccase enzymes from *Trametes versicolor*, a white-rot fungus, were purchased from Sigma-Aldrich (product 53739; Sigma Aldrich Inc., St. Louis, MO) and applied at activity levels of 0 (control), 0.5, 1.0, 2.0, and 4.0 units·cm⁻² every 2 weeks and at a laccase activity level of 2.0 units·cm⁻² every 2, 4, 8, and 12 weeks to optimize the rate and frequency of laccase application. Plots receiving cultural manage-

ment treatments were core-aerated and sand-topdressed twice yearly in April and September. Core aeration was accomplished using a Ryan Greensaire 24 Aerator (Ryan Inc., Johnson Creek, WI) fitted with 1.27-cm tines with a spacing of 5.0 × 5.0 cm and adjusted to penetrate to a depth of 6.25 cm. Immediately following core aeration, sand topdressing with 1134 g of sand (Quikrete Premium Play Sand, Atlanta, GA) per plot was accomplished using a Scotts Precision Green Spreader (Scotts Miracle-Gro, Marysville, OH). Laccase was applied at 2.0 units·cm⁻² every 4 weeks on plots core-aerated and sand-topdressed twice per year to observe the effectiveness of laccase in combination with the cultural management practice. Hereafter, rate and frequency treatments are presented as the rate of the laccase activity level followed by the frequency of application in parentheses; for example, "2.0 (4)" denotes treatments involving laccase with an activity of 2.0 units·cm⁻² applied at 4 weeks (Table 1).

Laccase from two different sources was compared for its effectiveness on thatch management. Laccase from the *Pycnoporus* genus was procured from Jiangnan University, China [CHU (2)], and from a commercial industrial wholesale supplier in China [CHI (2)]; it was applied at an activity level of 2.0 units·cm⁻² every 2 weeks (Table 1). The CHU (2) treatment (i.e., T12) was applied from July 2010 to Dec. 2010 and from July 2011 to Dec. 2011 to compare the effects of continued application of laccase every year for 6 months with the residual effects of one 6-month period of laccase application (Table 1).

Measurements

The residual effects of laccase application on the physical and chemical properties of the thatch layer were determined at 6, 12, and 18 months after the initiation of treatment application. Variables measured included the total organic matter content at a depth of 0 to 2.5 cm (OM_U), a depth of 2.5 to 5.0 cm (OM_L), a depth of 0 to 5.0 cm (OM), thatch layer thickness (TLT), and SHC. Similarly, the extractive-free acid-soluble lignin (L_S) and acid-insoluble lignin (L_I) contents were determined to observe the impact of treatment applications on the chemical composition properties of the thatch layer biomass. The total lignin content (L_T) was calculated after the addition of the L_S and L_I contents.

Laccase activity assay. The activity of laccase was quantified by a calorimetric assay using a Beckman DU 640B spectrophotometer (Beckman Instruments Inc., Fullerton, CA). One activity unit of laccase corresponded to the amount of enzymes causing 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, which is a specific substrate for laccase, in citrate-phosphate buffer at pH 3.8 (Park et al., 1999).

Total organic matter content. The total organic matter content was determined by the

Table 1. Description of laccase treatments applied on creeping bentgrass. Treatments are presented as laccase source followed by activity levels (units·cm⁻²) followed by application frequency (weeks) in parenthesis.

Treatment	Volume of solution	Laccase activity level (units·cm ⁻²)	Application frequency (wk)	Cultural management practice	Source of laccase	Designation	Application timeframe
T1: Control	410 mL	0	2	No	NA	Control	0–6 mo., 12–18 mo.
Rate of application							
T2: SA ^a 0.5 (2)	410 mL	0.5	2	No	Sigma Aldrich	0.5 (2)	0–6 mo.
T3: SA 1.0 (2)	410 mL	1.0	2	No	Sigma Aldrich	1.0 (2)	0–6 mo.
T4: SA 2.0 (2)	410 mL	2.0	2	No	Sigma Aldrich	2.0 (2)	0–6 mo.
T5: SA 4.0 (2)	410 mL	4.0	2	No	Sigma Aldrich	4.0 (2)	0–6 mo.
Application frequency							
T6: SA 2.0 (4)	410 mL	2.0	4	No	Sigma Aldrich	2.0 (4)	0–6 mo.
T7: SA 2.0 (8)	410 mL	2.0	8	No	Sigma Aldrich	2.0 (8)	0–6 mo.
T8: SA 2.0 (12)	410 mL	2.0	12	No	Sigma Aldrich	2.0 (12)	0–6 mo.
Cultural management							
T9: CMC ^b	410 mL	0	0	Yes	NA	CMC	
T10: CMC+SA 2.0 (4)	410 mL	2.0	4	Yes	Sigma Aldrich	CMC+2.0 (4)	0–6 mo.
Laccase sources							
T11: CHI 2.0 (2)	410 mL	2.0	2	No	Industrial-China	CHI (2)	0–6 mo.
T12: CHU 2.0 (2)	410 mL	2.0	2	No	University-China	CHU (2)	0–6 mo., 12–18 mo.

^aSA denotes laccase procured from Sigma Aldrich; CHU denotes laccase procured from China (Jiangnan University); CHI denotes laccase procured from China (industrial supplier).

^bCMC denotes cultural management control.

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

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

Saturated hydraulic conductivity. The SHC was measured by a constant hydraulic heat method using a Marriott tube apparatus (McCarthy, 1934). An intact core (diameter, 4.7 cm; 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 CaCl₂ solution. Steady-state flow through the samples was established by flowing 0.05 N CaCl₂ through the core for 10 min. After 10 min, the volume of water that passed through the core was measured for 1 min and repeated three times. SHC was calculated using Darcy's equation (Olsen, 1966).

Extractive-free lignin content. The 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 using a rotary shaker at 200 rpm, and then passed through a series of sieves with an 841-µm sieve at the top and a 177-µm sieve at the bottom. The material retained by the 177-µm

sieve 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% USP grade) ethyl alcohol, respectively. The L_S and L_I contents in the thatch layer were determined using a two-step acid-hydrolysis procedure according to the laboratory analytical procedure developed by the National Renewable Energy Laboratory (2008). Acid-soluble lignin is primarily low-molecular-mass phenolic compounds. During the first step, extractive-free thatch samples were hydrolyzed for 60 min with 72% H₂SO₄ at 30 °C. During the second step, H₂SO₄ was diluted to 4%; the samples were autoclaved at 121 °C for 1 h and then vacuum-filtered. 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 to calculate the acid-insoluble lignin content using the weight difference. Acid-soluble lignin was determined using this hydrolysis liquid at a wavelength of 240 nm 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 effects; this consisted of 11 treatments, three levels of treatment duration, and four replications. The CHU treatment (i.e., T12) was repeated from July to Dec. 2011, and it was not considered in the full model. Treatments were combined to form the following: a rate of application group [control, 0.5 (2), 1.0 (2), 2.0 (2), and 4.0 (2)]; an application frequency group [control, 2.0 (2), 2.0 (4), 2.0 (8), and 2.0 (12)]; a cultural management group [control, 2.0 (2), CMC, and CMC+2.0 (4)]; a laccase source group [SA 2.0 (2), CHU 2.0 (2), and CHI 2.0 (2)]; and a 2-year application group [SA 2.0 (2) and CHU 2.0 (2)]. An analysis of variance (ANOVA) was performed to evaluate the main effects of treat-

ment duration, treatments, and interaction effects of duration × treatment using a general linear model (GLM) (SAS Institute Inc., 1994). Treatments were combined into five groups and analyzed as repeated measures to evaluate the effects of treatment, treatment duration, and the interaction effects of treatment × treatment duration. Fisher's protected least significant difference test with $\alpha = 0.05$ was used to determine 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 2). Strong duration (time after treatment applications was initiated) effects ($P \leq 0.001$) were observed for OM_U, TLT, and all lignin content measurements (Table 2), indicating the residual effects of laccase applications on these parameters. No duration effects were observed for SHC and OM_L. Interaction effects of duration × 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 lignin and total lignin.

Rate of application. The rate of laccase application significantly affected ($P \leq 0.001$) TLT, L_I, and L_T (Table 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 2). Interaction effects ($P \leq 0.001$) of duration × treatment were observed for L_I and L_T contents. After 6 months of treatment, no differences were observed for OM_U, OM_L, or OM with any of the treatments. Samples obtained at 12 months after treatment initiation showed that OM_U at a laccase activity level of 4.0 units·cm⁻² decreased by 21.5 mg·g⁻¹ when compared with control (Table 3). No differences were observed for OM_L and OM in the 12-month sampling.

Table 2. Analysis of variance (ANOVA) table showing the effects of laccase treatments, treatment duration, and duration $\times$ treatment interactions on creeping bentgrass.

Source of variation	df	Organic matter	Organic matter	Organic matter	Thatch layer thickness	Saturated hydraulic conductivity	Acid-soluble	Acid-insoluble	Total lignin L _T
		(OM ₀) (0–2.5 cm)	(OM _L) (2.5–5.0 cm)	(OM) (0–5.0 cm)			lignin L _S	lignin L _I	
Full model									
Repetition	3	1,429	973	779	5	123	282	60	167
Treatment	10	2,583**	316	515	40***	131***	37	2,065***	1,966***
Error A (repetition × treatment)	30	699***	317*	247**	2	24	30	21	56
Duration	2	7,934***	15	792**	85***	110	1205***	69,963***	79,960***
Duration × treatment	20	316	181	106	2	24	36	970***	1,127***
Error	66	221	194	105	2	39	66	26	123
Rate of application									
Repetition	3	4,105	1,368	1,747	4	108	182	52	141
Treatment	4	400	139	201	53***	3	55	3,029***	2,980***
Error A (repetition × treatment)	12	313	175***	146**	0.6	4	32	19	42
Duration	2	2,900***	112	120	29***	35	507**	37,614***	43,511***
Duration × treatment	8	225	36	46	2	4	31	1,696***	1,982***
Error	30	249	37	40	1	23	59	25	101
Application frequency									
Repetition	3	1,333	1,134	522	3	55	73	19	41
Treatment	4	322	488	380	53***	4	59	2,170***	1,641***
Error A (repetition × treatment)	12	663**	464	302	1	5	43	35	78
Duration	2	4,108***	173	404	64***	25	690**	35,405***	38,414***
Duration × treatment	8	496	290	95	1	4	47	2,113***	1,439***
Error	30	230	346	158	2	17	85	29	168
Cultural management									
Repetition	3	194	122	58	2	75	110	36	31
Treatment	3	4,865*	252	821	49***	272*	28	1,668***	1,388**
Error A (repetition × treatment)	9	906**	195***	217**	2	57	28	22	41
Duration	2	1,343**	132*	436***	23**	138	226**	13,999***	15,053***
Duration × treatment	6	275	65	114	0.7	44	23	206***	317***
Error	24	225	38	47	3	74	32	15	52
Laccase sources									
Rep	3	531	510	418	8	55	92	10	43
Treatment	2	1,088*	86	281	1	12*	23	2,909***	2,703***
Error A (repetition × treatment)	6	337	224	207	0.8	12	7	7	5
Duration	1	34	94	87	1	65*	83	46,837***	42,974***
Duration × treatment	2	37	20	1	0.1	2	13	1,360***	1,622***
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	4,150**	780	1,520	15	75	57	1,447***	926**
Error A (repetition × treatment)	3	120	381**	330*	3	56	7	2	12
Duration	2	3,337*	105	460*	8*	113	284*	31,421***	34,666***
Duration × treatment	2	97	174	100	4	41	10	2,142***	2,362***
Error	12	526	56	74	2	125	60	49	105

***. **Significant at $P = 0.05$, 0.01, or 0.001, respectively.

*, **, ***Significant at $P = 0.05$, 0.01, or 0.001, respectively.

Table 3. Total organic matter content at three depths of 0–2.5 cm (OM_U), 2.5–5.0 cm (OM_L), and 0–5.0 cm (OM) at 6, 12 and 18 mo. after initiation of different laccase treatments applied on creeping bentgrass. Treatments are presented as laccase source followed by activity levels (units·cm⁻²) followed by application frequency (weeks) in parenthesis.

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

^zMeans within a column in a treatment group (treatment effect) followed by the same lowercase letter are not significantly different according to the least significant difference (LSD) at $\alpha = 0.05$

^yMeans within a row in a treatment group (duration effect) followed by the same uppercase letter are not significantly different according to the LSD at $\alpha = 0.05$.

^xSA denotes laccase procured from Sigma Aldrich; CHU denotes laccase procured from China (Jiangnan University); CHI denotes laccase procured from China (industrial supplier); CMC denotes cultural management control.

Samples obtained at 18 months after the start of the experiment showed a 10.4-mg·g⁻¹ increase in the organic matter content for the 1.0 (2) treatment compared with control. The organic matter content (0–2.5 cm) increased by 19.5 mg·g⁻¹ at 0.5 units·cm⁻² when sampled between 6 and 18 months after treatment initiation. Significant reductions of 6.2 and 8.0 mg·g⁻¹ in the OM_L contents from 6 to 18 months for treatments 2.0 (2) and 4.0 (2), respectively, were observed, and a reduction in organic matter (8.5 mg·g⁻¹) from 6 to 12 months was observed for treatment 4.0 (2), suggesting the residual effects of laccase.

Laccase treatments at different activity levels were equally effective; after 6 months of treatment application, TLT was lowered by 3.8 to 4.8 mm compared with control (Fig. 1A). Twelve months after the start of treatment, TLT was lowered by all activity levels of laccase. However, treatments with laccase at activity levels of 2.0 and 4.0 units·cm⁻² showed significant reductions in TLT values compared with nontreatment and treatments involving 0.5 and 1.0 units·cm⁻² of laccase activity. A reduction in TLT was observed for all treatments compared with control when sampled 18 months after the initiation of treatment. Applications of laccase at 0.5, 1.0, and 2.0 units·cm⁻² were effective for maintaining the TLT up to 6 months after treatment completion; however, with 4.0 units·cm⁻² laccase treatment, TLT was lowered from 14.5 mm at 6 months after treatment initiation to 13.3 mm at 12 months after treatment initiation. A significant in-

crease in TLT occurred when laccase was applied at 0.5 and 1.0 units·cm⁻² over the course of the three sampling dates.

Laccase activity levels had no effect on SHC at the time of any of the sampling dates (Table 4). After 6 months of treatment, laccase applications up to 2.0 units·cm⁻² lowered L_S by 7.8 to 8.9 mg·g⁻¹ compared with control (Table 4). Acid-soluble lignin with treatment involving 4.0 cm⁻² of laccase activity was reduced by 12.2 mg·g⁻¹ when compared with control at the end of treatment (6 months) (Table 4). No differences in L_S were observed at the sampling times of 12 and 18 months after treatment initiation.

The extractive-free L_I content was lowered compared with control when treated with laccase up to 2.0 and 1.0 units·cm⁻² at 6 and 12 months after initiating treatment, respectively (Table 4). At the end of the treatment application, L_I was higher with 4.0 units·cm⁻² of treatment compared with control. Similarly, in samples obtained 12 months after treatment initiation, the L_I content was higher than that of the control when treated with 2.0 and 4.0 units·cm⁻² of laccase activity, suggesting the residual effects of laccase. Laccase treatments showed higher L_I content compared with control at 18 months after treatment initiation (Table 4). An increase in the L_I content was observed with all treatments (Table 4). Variations in L_T content followed trends that were similar to those for L_I content with different laccase activity levels (Fig. 2A).

Frequency of application. Laccase application frequency effects ($P \leq 0.001$) were observed for TLT, L_I, and L_T (Table 2). Strong duration effects ($P \leq 0.001$) were observed for OM_U, TLT, and L_T contents (Table 2). Interaction effects of duration $\times$ treatment were observed for L_I ($P \leq 0.001$) and L_T ($P \leq 0.001$) (Table 2). No differences in OM_U, OM_L, and OM were observed between laccase treatments and control at 6 and 12 months after treatment initiation. However, increases in OM_U and OM contents were noted for the 2.0 (12) treatment compared to control at 18 months after the start of the treatment application. An accumulation of 52.3 mg·g⁻¹ in OM_U was obtained with the 2.0 (12) treatment at sampling dates between 6 and 18 months (Table 3).

The TLT was lowered by 4.7 to 5.6 mm, 4.1 to 5.6 mm, and 3 to 4.8 mm with laccase treatments at different frequencies compared with control when sampled at 6, 12, and 18 months, respectively (Fig. 1B). No differences among application frequencies were observed at any sampling date. An increase in the TLT was observed for all treatments at the time of the 18-month sampling compared with TLT after 6 months of treatment. No significant change in TLT was observed after 12 months (Fig. 1B).

All laccase treatments lowered the L_S content of the nontreated control at 6 months after treatment initiation (Table 4). However, the decrease in the L_S content compared with control was greater with the 2.0 (2) treatment than with the 2.0 (8) and 2.0 (12) treatments

Fig. 1. Thatch layer thickness (TLT; in mm) at 6, 12, and 18 months after treatment initiation on creeping bentgrass. (A) Laccase application rates. (B) Frequency of application of laccase. (C) Cultural management and laccase treatments. (D) Laccase sources. The stacked bars in (C) represent the depths of the thatch layer and sand deposition on plots after topdressing. Treatments are presented as the laccase source followed by the activity level (units-cm⁻²) and the application frequency (weeks) in parentheses. Values are the means of four replicates. The same letter within the bars (6 months = lowercase standard; 12 months = lowercase bold; 18 months = lowercase italics) and the same letter on top of the bars (duration effect = uppercase bold) are not considered statistically different according to Fisher's protected least significant difference (LSD) at $\alpha = 0.05$. CMC, cultural management control; SA, laccase procured from Sigma Aldrich; CHU, laccase procured from China (Jiangnan University); CHI, laccase procured from China (industrial supplier).

(Table 4). This suggested that frequent applications of laccase effectively lowered the L_S content relative to the nontreated control. No differences in L_S values were observed when sampled at 12 and 18 months after starting the experiment. After 6 months of treatment, a reduction in the L_I content was observed for laccase treatments every 2 weeks (29.6 mg·g⁻¹) and 4 weeks (15.5 mg·g⁻¹) compared with control (Table 4). The L_I content increased by 5.8 mg·g⁻¹ with the 2.0 (12) treatment compared with control. An increase in the L_I content was observed after 12 months when laccase was applied every 2, 8, and 12 weeks. However, a 10.4-mg·g⁻¹ reduction in L_I content was observed after 12 months compared to nontreatment following applications of laccase every 4 weeks. All laccase treatments showed an increase in the L_I content from nontreatment when sampled at 18 months (Table 4). A significant increase in the L_I content was observed for all treatments when sampled over time (Table 4). Similar trends were observed for the L_T content with laccase applications at different frequencies (Fig. 2B).

Cultural management. The cultural management treatment group showed treatment effects for OM_U ($P \leq 0.05$), TLT ($P \leq 0.001$), SHC ($P \leq 0.05$), L_I ($P \leq 0.001$), and L_T (Table 2). Significant duration effects were observed for the organic matter content (0–2.5 cm, 2.5–5.0 cm, and 0–5.0 cm), TLT,

and L_S , L_I , and L_T extractive-free lignin contents. Duration $\times$ treatment interaction effects ($P \leq 0.001$) were observed for L_I and L_T (Table 2).

No differences were observed for OM_U , OM_L , and OM contents at 6 months after treatment initiation (Table 3). When sampled 12 months after treatment initiation, the OM_U content had decreased by 50 and 40.7 mg·g⁻¹ with the CMC and CMC+2.0 (4) treatments, respectively, compared with the control. Significant duration effects were observed for OM_L and OM contents, which were evident from the slight increases of 18.4 and 18.7 mg·g⁻¹ in OM_L and OM contents compared with control when sampled 18 months after treatment initiation (Table 3). No duration effect was observed for OM_U (Table 3). After 6 months, TLT decreased from 18.3 mm for the control to 13.6 and 15.7 mm for the 2.0 (4) and CMC+2.0 (4) treatments, respectively (Fig. 1C). Measurements of the thatch layer that received treatment with core aeration and sand topdressing indicated 3 to 4 mm of sand additions, and this was included in the calculations. A TLT reduction of 4.1 mm was observed for the 2.0 (4) treatment when compared with control after 12 months. After 18 months, TLT was reduced from 21.0 mm for the control to 16.2 and 17.9 mm for the 2.0 (4) and CMC+2.0 (4) treatments, respectively (Fig. 1C). Significant duration effects were observed for the control and laccase treatment of 2.0 (4) for

TLT, and no duration effects were observed for treatments including cultural management practices (Fig. 1C).

After 6 months of application, treatment involving core aeration and sand topdressing showed an increase of 13.5 cm·h⁻¹ in SHC compared to nontreatment (Table 4). Laccase treatment with or without cultural management resulted in no differences in SHC (Table 4). No changes in SHC were observed during a comparison with the control for other sampling durations. No duration effects were recorded for SHC in this group of treatments (Table 4). Reductions of 3.6, 7.8, and 3.8 mg·g⁻¹ in the L_S contents were recorded for the CMC, 2.0 (4), and CMC+2.0 (4) treatments, respectively, when compared with control at the 6-month sampling. However, no differences in the L_S contents were observed at the time of sampling at 12 and 18 months after treatment initiation. A slight but significant duration effect was observed for the L_S content with the 2.0 (4) treatment (Table 4). Extractive-free L_I and L_T contents 6 months after treatment application were lowered with laccase treatment alone and increased with the CMC and CMC+2.0 (4) treatments when compared with control (Table 4, Fig. 2C). A similar trend was recorded for L_I at the time of sampling conducted at 12 months after treatment initiation. Acid-insoluble contents of all treatments increased compared to the control at 18 months after treatment initiation. Significant

Table 4. Saturated hydraulic conductivity (SHC), extractive-free acid-soluble lignin (L_S), and extractive-free acid-insoluble lignin (L_I) at 6, 12, and 18 mo. after initiation of different laccase treatments applied on creeping bentgrass. Treatments are presented as laccase source followed by activity levels (units·cm⁻²) followed by application frequency (weeks) in parenthesis.

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

^zMeans within a column in a treatment group (treatment effect) followed by the same lowercase letter are not significantly different according to the least significant difference (LSD) at $\alpha = 0.05$.

^yMeans within a row in a treatment group (duration effect) followed by the same uppercase letter are not significantly different according to the LSD at $\alpha = 0.05$.

^{*}SA denotes laccase procured from Sigma Aldrich; CHU denotes laccase procured from China (Jiangnan University); CHI denotes laccase procured from China (industrial supplier); CMC denotes cultural management control.

duration effects were observed for L_I and L_T , with increases in the lignin content occurring with all treatments over time (Table 4, Fig. 2C).

Sources of laccase enzyme. Laccase enzymes procured from different sources were similarly effective for organic matter content (OM_U, OM_L, and OM) (Table 3), TLT (Fig. 1D), and SHC (Table 4). Slight differences in L_S and L_I contents were observed for treatments involving laccase from different sources (Table 4). Six months after treatment initiation, the L_S content was lower for the 2.0 (2) treatment compared with the other two laccase enzymes. Samples obtained at 12 and 18 months showed no differences in L_S contents with different laccase enzymes. When treated with CHI (2) for 6 months, the L_I content was slightly higher than that of the other two laccase enzymes. However, when sampled after 12 months, the L_I content was higher with 2.0 (2), followed by CHI (2) and CHU (2). No differences in L_S and L_I contents were observed in treatments involving different laccase sources at 6 months after the start of treatment (Table 4). Significant increases in L_I and L_T contents occurred in samples obtained between 6 and 18 months (Table 4, Fig. 2D).

Application duration. Strong effects of the laccase application duration were observed for OM, TLT, and L_T in samples obtained 18 months after treatment initiation (Table 3, Figs. 3 and 4). An increase in organic matter was observed 18 months after the initiation of treatment CHU (2), which

was applied for 6 months during year 1 and 6 months during year 2, compared with the application of laccase 2.0 (2) for only 6 months during year 1 (Table 3). The baseline measurement of TLT was 17.2 mm. The TLT of the control continued to increase with time, whereas for the 2.0 (2) and CHU (2) treatments, TLT was significantly reduced after 6 months. At 12 months after treatment initiation, TLT was slightly lower than it was after 6 months. However, TLT with the 2.0 (2) treatment was 3.3 mm higher than it was with CHU (2) after 18 months (Figs. 3 and 4).

Discussion

Nondestructive methods used to manage thatch are desired, but they are often ineffective. Commercial microbial inoculums, such as Biodethatch, Thatch-Away, and Earth Anew, on bermudagrass, creeping bentgrass, and annual bluegrass have been reported to be ineffective for reducing the thatch layer depth (Gilbeault et al., 1976; Lancaster et al., 1977; Murdoch and Barr, 1976). Similarly, applications of the biological granular supplement Thatch-X (Lebanon Seaboard Corporation, Lebanon, PA) on creeping bentgrass (McCarty et al., 2007) and of wetting agents Aqua-Gro (Aquatrols Corp of America, Paulsboro, NJ), Milogranite (Milogranite, Milwaukee, WI), and activated sewage sludge on Kentucky bluegrass (Murray and Juska, 1977) were ineffective for decreasing the organic matter content of the thatch layer. One of the possible reasons

for the inconsistent results of organic matter decomposition was the emphasis on the degradation of cellulosic and hemi-cellulosic sugars instead of lignin. The lignin protective matrix must be removed to open the biomass structure and increase access to readily decomposable structural carbohydrates (Ledebor and Skogly, 1967).

Direct microbial inoculation of turfgrass systems have been ineffective for managing thatch (Martin and Dale, 1980). Different microbial populations require a specific microclimate with particular moisture and temperature regimes for growth. The inability to maintain a microbe-specific preferred micro-environment for prolonged durations under turfgrass management systems may lower the possibility of maintaining specific microbial activities over time.

Laccase enzymes are stable over wide pH and temperature ranges (Baldrian, 2006; Munoz et al., 1997; Stoilova et al., 2010; Thurston, 1994). Laccase, a multicopper oxidase, is an extracellular 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 macromolecules and between lignin and structural sugars (Wong, 2009). This opens the biomass structure, leading to increased availability of easily degradable sugars by microbes. By using laccase enzymes, turfgrass managers could effectively manage thatch over wide ranges of

Fig. 2. Extractive-free total lignin content (L_T ; in $\text{mg}\cdot\text{g}^{-1}$) at 6, 12, and 18 months after treatment initiation on creeping bentgrass. (A) Laccase application rates. (B) Frequency of laccase application. (C) Cultural management and laccase treatments. (D) Laccase sources. Treatments are presented as the laccase source followed by the activity level ($\text{units}\cdot\text{cm}^{-2}$) and application frequency (weeks) in parentheses. Values are the means of four replicates. The same letter within the bars (6 months = lowercase standard; 12 months = lowercase bold; 18 months = lowercase italics) and the same letter on top of the bars (duration effect = uppercase bolded) are not considered statistically different according to Fisher's protected least significant difference (LSD) at $\alpha = 0.05$. CMC, cultural management control; SA, laccase procured from Sigma Aldrich; CHU, laccase procured from China (Jiangnan University); CHI, laccase procured from China (industrial supplier).

environmental conditions and improve their ability to use existing populations of soil microbes for the decomposition of organic matter. In previous studies, reductions in thatch/mat layers with laccase treatment were demonstrated during greenhouse and field research (Sidhu et al., 2012, 2014). However, the question of residual effects was not addressed.

Laccase rate and frequency. In this study, laccase treatments at different rates and frequencies of application were ineffective for reducing OM_U , OM_L , and OM contents after 6 months of application (Table 3). However, when laccase was applied at 4.0 $\text{units}\cdot\text{cm}^{-2}$ every 2 weeks, a significant reduction in OM_U occurred at 12 months after treatment initiation (Table 3). This observation indicated that laccase application for 6 months continued to slow the accumulation of OM_U over the next 6 months. As time progressed from 12 to 18 months after treatment initiation, the residual effects of laccase decreased and OM_U values began to increase in the treated plots in a manner similar to that of the control. A reduction in OM_L content was observed over the 18-month sampling period with the 2.0 and 4.0 $\text{units}\cdot\text{cm}^{-2}$ treatments (Table 3). This may indicate that continued application of laccase at a high rate (2.0 and 4.0 $\text{units}\cdot\text{cm}^{-2}$) may be effective for reducing the organic matter content of older putting greens that contain high organic matter contents in the thatch/mat layer.

All frequencies and rates of laccase were effective for lowering TLT compared with control after 6 months of treatment (Fig. 1A and B). Additionally, TLT readings after treatments with the various laccase rates and frequencies for 6 months were lower relative to the control, even after 12 and 18 months, suggesting strong residual effects of laccase (Fig. 1A). However, TLT readings increased significantly at the three highest laccase rates between samplings at 12 and 18 months after treatment initiation, suggesting that the residual effects of laccase in the thatch layer were effective for only 6 months after treatment cessation (Fig. 1A). Although laccase treatments did decrease TLT, there were only minor differences in SHC, and no apparent trend (Table 4).

During the sub-study involving laccase application for 6 months during year 1 and again for 6 months during year 2, a significant reduction in the thatch layer was observed when comparing laccase applications for 6 months during year 1 and samples at 18 months after treatment initiation (Fig. 3). After treatment cessation, residual effects of laccase reduced thatch layer build-up during the next 6 months. However, a significant increase in the thatch layer was observed when no laccase enzyme was applied during the second year. However, no thatch build-up was observed when laccase was applied during the second year, suggesting that annual applications of laccase for 6 months during the summer was effective for reducing

or stabilizing thatch accumulation. No further reduction in the thatch layer was observed during the second year, even with the application of laccase. This suggested a threshold level for thatch layer reduction with the application of laccase. Increased OM_U was observed where laccase was applied for 6 months during the second year when compared with laccase applied for 6 months during the first year (Table 3). This may be attributed to the tight stacking of thatch biomass due to removal of lignin bonds and the reduction of structural sugars leading to weak thatch biomass.

The extractive-free L_S content initially decreased with the application of laccase, as was evident from sampling conducted after the conclusion of treatments at 6 months (Table 4). The extent of reduction at 6 months was dependent on the amount of laccase applied and the rate and frequency of applications. Applications of laccase up to 2.0 $\text{units}\cdot\text{cm}^{-2}$ decreased L_S in the thatch biomass, but applications of laccase at 4.0 $\text{units}\cdot\text{cm}^{-2}$ showed an increase in the L_S content expressed as a proportion of the sample based on dry weight when compared with the control (Table 4).

The increased L_S content could have been attributed to the loss of excessive structural sugars with laccase treatment at 4.0 $\text{units}\cdot\text{cm}^{-2}$ (Sidhu et al., 2014). Three major components of plant biomass are cellulosic sugars, hemi-cellulosic sugars, and lignin. Therefore, with the application of laccase,

Fig. 3. Thatch layer thickness (TLT; in mm) at 6, 12, and 18 months after treatment initiation on creeping bentgrass. Laccase treatments 0 (control), SA 2.0 (2) for 6 months during year 1, and CHU 2.0 (2) for 6 months during year 1 and 6 months during year 2 are shown. Treatments are presented as the laccase source followed by the activity level (units·cm⁻²) and application frequency (weeks) in parentheses. Horizontal lines near the bottom of the graph represent periods of treatment application from 0 to 6 months and 12 to 18 months. Values are the means of four replicates. Treatments with the same letter within a sampling date (6 months = lowercase standard; 12 months = lowercase bold; 18 months = lowercase italics) are not considered statistically different according to Fisher's protected least significant difference (LSD) at $\alpha = 0.05$. SA, laccase procured from Sigma Aldrich; CHU, laccase procured from China (Jiangnan University).

Fig. 4. Extractive-free total lignin content (L_T ; in mg·g⁻¹) at 6, 12, and 18 months after treatment initiation on creeping bentgrass. Laccase treatments 0 (control), SA 2.0 (2) for 6 months during year 1, and CHU 2.0 (2) for 6 months during year 1 and 6 months during year 2 are shown. Treatments are presented as the laccase source followed by the activity level (units·cm⁻²) and application frequency (weeks) in parentheses. Values are the means of four replicates. The same letter within the bars (6 months = lowercase standard; 12 months = lowercase bold; 18 months = lowercase italics) and the same letter on top of the bars representing the duration effect (control = uppercase standard; SA 2.0 (2) = uppercase bold; CHU 2.0 (2) = uppercase italics) are not considered statistically different according to Fisher's protected least significant difference (LSD) at $\alpha = 0.05$. SA, laccase procured from Sigma Aldrich; CHU, laccase procured from China (Jiangnan University).

lignin bonds are broken, which leads to the opening of the biomass structure and makes sugars more available for microbial decomposition. As the sugar content is decreased, the lignin content is increased; this is because it is expressed as the proportion of the total dry weight. Decreased structural carbohydrate (cellulosic and hemi-cellulosic sugars) content in thatch biomass after laccase treatment was previously reported by Sidhu et al. (2013a, 2014).

A decrease in the structural sugar content was observed as the rate of laccase activity increased, indicating more availability of sugars for microbial degradation (Sidhu et al., 2014). Residual effects of laccase applications were observed between the 6-

and 18-month sampling dates in the proportion of lignin present in thatch biomass. Lignin continued to accumulate during this period, suggesting the continued loss of sugar content from the biomass, even after laccase treatments had ceased (Table 4). L_I is the major component of L_T , and a similar trend in L_T was observed with increasing laccase application rates (Fig. 2A). Maximum reductions in L_S , L_I , and L_T were observed at 6 months when laccase was applied every 2 weeks. The extent of this reduction decreased with the decreased laccase application frequency (Table 4, Fig. 2B), suggesting that the extent of lignin reduction was dependent on the amount of laccase applied.

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

A 10% reduction in the TLT was reported after core aeration four times annually on creeping bentgrass (McCarty et al., 2007) and three to six times per year on Tifgreen bermudagrass (McWhirter and Ward, 1976). Carrow et al. (1987) noted no effects of core aeration applied once or twice per year on the thatch-mat depth of Tifway bermudagrass, although a reduction in sand density was observed. Several studies have reported an increase in water infiltration in turfgrass fields after core aeration due to the formation of water channels and porous profiles (Bunnell et al., 2001; Canaway et al., 1986; McCarty et al., 2005).

During this study, organic matter content in the top 2.5 cm was lower at 12 months with cultural management practices when compared with the control. This may be attributed to the dilution effect created by sand topdressing on the surface layer because sand topdressing showed no effect on organic matter at a depth of 2.5 to 5.0 cm (Table 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 due to laccase activity in cellulose and hemi-cellulose sugars resulting in higher L_T contents, as seen at 18 months (Fig. 2C). As raw organic matter decomposes, such as in composting situations, lignin content and density in the resulting material increases.

Lignin dynamics were also apparent in TLT results; the application of laccase along with cultural management effectively decreased TLT at 6 and 18 months and increased L_T content at all three sampling times (Figs. 1C and 2C). The increase in L_T may be attributed to the change in the thatch biomass

structure caused by laccase, making structural sugars more available for decomposition and a better microclimate for microbial growth due to core aeration and sand top-dressing. Increased losses of structural sugars from the thatch biomass may be responsible for the increased levels of lignin in the remaining thatch material. A significant loss in structural sugars of creeping bentgrass thatch biomass was also observed with the application of laccase during greenhouse and field studies (Sidhu et al., 2013a, 2014).

SHC was higher at 6 months with CMC treatment (Table 4). At 18 months, SHC was higher when laccase was applied in combination with CMC when compared with the control and only laccase treatments. The increase in SHC may be attributed to core aeration, which creates channels for rapid water movement. The laccase and CMC data illustrated that the 2.0 (4) treatment of laccase was effective for reducing TLT at 6, 12, and 18 months when applied alone or with CMC. Laccase alone did not influence SHC; however, in combination with CMC, SHC increased relative to the control. These results suggested that laccase has positive effects on the management of thatch and can be used in conjunction with routine CMC.

Laccase source. Different sources of laccase enzymes were proven to be equally effective for reducing the TLT (Fig. 1D). However, slight differences in L_S and L_I were observed with different laccase sources (Table 4). The lignin content (L_S and L_I) was significantly lower in laccase procured from Sigma Aldrich compared with CHU and CHI 6 months after application, but no differences were observed in lignin content when sampled at 12 and 18 months after treatment initiation (Table 4). This suggested that laccase from Sigma Aldrich was initially more effective; however, the other laccase sources were proven to be equally effective for reducing thatch biomass over time.

In conclusion, this field research demonstrated the residual effects of laccase enzymes on creeping bentgrass; TLT was reduced by averages of 23.5, 25.7, and 23.9% across all laccase rates at the 6-, 12-, and 18-month sampling times, respectively (Fig. 1A). In a similar manner, the average TLT reductions across all frequencies of laccase applications were 27.8, 25.5, and 19.7% for the control at the time of the 6-, 12-, and 18-month samplings, respectively (Fig. 1B). Organic matter contents (OM_U , OM_L , and OM) were not appreciably affected by laccase rates or frequency at the time of any of the three sample dates. The total lignin content increased over time, suggesting that decomposition of cellulose and hemicellulose fractions resulted in a concentration of lignin. Therefore, the total OM may not change, but the composition did change over time. The 2.0 (4) treatment of laccase was effective for reducing TLT at 6, 12, and 18 months when applied alone or in combination with CMC. Laccase alone did not influence SHC; however, in combination with CMC, SHC increased relative to the control. Lac-

case from different sources was equally effective for organic matter decomposition and thatch layer reduction. Laccase applications for 6 months during the second year were effective for ceasing thatch layer build-up. The results of this study indicated that laccase, when applied at an optimum rate and/or frequency for 6 months (July to December) during 1 year, is effective for reducing the organic matter content and TLT. For golf course superintendents managing bentgrass putting greens, the application of laccase for 6 months each year could be an effective means of preventing thatch layer accumulation.

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