Biochar restructures plant-soil-microbe relationships in a woody cropping system

Jake A. Nash[1]*, Jessica R. Miesel[2,3], Gregory M. Bonito[2], Monique L. Sakalidis[2,3], Han Ren[4], Daniel Warnock[2], Lisa K. Tiemann[2]

[1]Department of Biology, Duke University, Durham, NC, USA 27708

[2]Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, Michigan, USA 48824

[3]Department of Forestry, Michigan State University, East Lansing, Michigan, USA 48824

[4]College of Life and Environmental Sciences, Wenzhou University, Wenzhou, Zhejiang, 325035, China

*Corresponding author 413-822-4490; Email address: akejashn12@gmail.com

Core Ideas

- Biochar application increased soil extracellular enzyme activities up to five-fold
- Dissolved organic carbon and soil moisture were increased by biochar
- Decreases to nitrogen availability suggest biochar induced nitrogen immobilization
- Biochar led to less diverse root fungal communities and dominance by *Wilcoxina*
- The growth and/or survival of two conifer trees was decreased by biochar

Abstract

Biochars are porous charcoal-like materials that can enhance soil health and plant growth, but their use has not been adequately evaluated in woody cropping systems. To fill this knowledge gap, we investigated the effects of two slow pyrolysis pine biochars on plant

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/saj2.20334.

performance, soil physicochemical properties, extracellular enzyme activities, and root-associated fungal community composition in an experimental Christmas tree plantation over three years. Both biochars stimulated the activities of five extracellular enzyme activities, between 67% and 446%, but appeared to reduce nitrogen availability. Structural equation modelling identified increased soil moisture as a potential mechanism of biochar's effects on all measured enzyme activities, while increased dissolved organic carbon was a possible mechanism of biochar's effect on N and P targeting enzymes. This finding suggests that biochar-induced increases to dissolved organic carbon have a specific effect on nutrient targeting enzymes. Biochar was found to negatively impact tree growth and survival, but impacts varied between tree species and biochar type. High-throughput sequencing showed that biochar decreased the diversity of root-associated fungal communities, with the ectendomycorrhizal species *Wilcoxina mikolae* becoming hyper-dominant on balsam fir in response to one of the biochars. Changes to root-associated fungal communities may have been partially responsible for negative effects on conifer performance. Although our study identified negative effects of biochar on plant performance and fungal diversity, we also found widespread changes to soil chemistry and microbial function which might be leveraged in systems with more acidic soils or different crops to increase plant performance.

Key Words: Microbes; ectomycorrhizal fungi; extracellular enzyme activities; *Picea pungens*; *Abies balsamea*

Abbreviations: ANCOM, analysis of composition of microbiomes; ANOVA, analysis of variance; BG, β-glucosidase; CBH, cellobiohydrolase; DOC, dissolved organic carbon; ECM, ectomycorrhizal; EDX, energy dispersive X-ray spectroscopy; EEA, extracellular enzyme activity; ITS, internal transcribed spacer; LAP, leucine aminopeptidase; NAG, β-N-acetyl glucosaminidase; OTU, operational taxonomic unit; PCR, polymerase chain reaction; PER,

peroxidase; PERMANOVA, permutational multiple analysis of variance; PHEN, phenoloxidase; PHOS, acid phosphatase; PRS, plant root simulator; TDN, total dissolved nitrogen

1. Introduction

Biochar is a porous charcoal soil amendment produced by pyrolyzing plant or animal biomass (Lehmann and Joseph, 2009). Because of its relative chemical recalcitrance, biochar acts as a stable carbon (C) sink and has been proposed as means to offset anthropogenic CO_2 emissions (Lehmann et al., 2006). Biochars have highly variable physical and chemical properties depending on the feedstock material and manufacturing specifications (Enders et al., 2012; Gundale and DeLuca, 2006; Sohi et al., 2010). A biochar's physical and chemical properties determine its effect on soil properties such as pH, water-holding capacity, cation exchange capacity, bulk density, and nutrient availability (Dai et al., 2020; Gundale and DeLuca, 2007; Jeffery et al., 2011). Biochar has also been shown to impact plant- and soil-associated microbial communities (Lehmann et al., 2011; Zhang et al., 2018). In an ideal scenario biochar can significantly increase plant resistance to pathogens and climate change induced stresses by increasing the benefits of associating with root colonizing fungal symbionts and growth-promoting bacteria (Ali et al., 2017; Ishii and Kadoya, 1994; Matsubara et al., 2002; Robertson et al., 2012).

Biochar's effects on soil physicochemical properties and plant-microbe interactions can result in increased plant growth and crop yields (Major et al., 2010; Robertson et al., 2012; Rondon et al., 2007). However, neutral or negative effect of biochar on plant performance and plant-soil interactions have also been documented (Biederman and Harpole, 2013; Bonanomi et al., 2017; Elzobair et al., 2016a; Gaskin et al., 2010; Hagner et al., 2016; Lehmann et al., 2011; Van Zwieten et al., 2010; Warnock et al., 2007; Warnock et al., 2010).

These studies highlight the context-dependency of the effects of biochar application on plant-soil relationships and stress the importance of understanding the outcome of specific biochar-crop-soil combinations as a prerequisite to widespread use. Most studies on biochar have been in annual cropping systems and less is known about how biochar may affect crop performance and soil biology in short rotation woody cropping systems and orchards. Most studies on biochar's effects on woody plant growth and survival have been in the context of forestry, with generally positive effects of biochar on tree growth, though this effect may be more pronounced in angiosperms than conifers (Bieser and Thomas, 2019; Pluchon et al., 2014; Sarauer et al., 2019; Thomas and Gale 2015). However, short rotation cropping systems and orchards differ from forests in that they are typically plowed and fertilized and are often established on fallow agricultural fields (Bruckman and Pumpanen, 2019). Thus, the combination of crop species and soil management strategies used make short rotation woody cropping systems and orchards distinct from both annual agricultural fields and managed forests. Studies in short rotation woody cropping systems and orchards have found mixed effects of biochar on plant performance and more study is needed in these systems (Eyles et al., 2015; Glisczynski et al., 2016; Sorrenti et al., 2016; Rockwood et al., 2019). Many of the benefits of biochar are likely to be the most pronounced in well-drained sandy soils, which are the most prone to drought and nutrient leaching (Jeffery et al., 2011; Liu et al. 2013). In well-drained sandy soils, biochar could be effective at reducing irrigation costs during increasingly frequent periodic droughts by increasing soil water-holding capacity (Ali et al., 2017). Furthermore, biochar could decrease requirements for fertilizer application by limiting nutrient losses from leaching out of sandy soils and stimulating the ectomycorrhizal (ECM) fungi that associate with many trees and which can increase the nutrient-acquisition capabilities of their plant hosts (Lehmann et al., 2011; Robertson et al., 2012; Warnock et al., 2007).

Soil microorganisms mineralize organic matter using secreted extracellular enzymes including phosphatases, aminopeptidases, cellulases and chitinases, which liberate smaller and more labile forms of C and nutrients (Burns et al., 2013; Grandy et al., 2008; Sinsabaugh et al., 2008). Once liberated by the action of extracellular enzymes, the more labile forms of C and nitrogen (N) are readily available for uptake by plants and soil biota. Positive effects of biochar on soil extracellular enzyme activities (EEAs) documented in laboratory and greenhouse studies (Al Marzooqi and Yousef, 2017; Elzobair et al., 2016a; Masto et al., 2013; Paz-Ferreiro et al., 2014; Paz-Ferreiro et al., 2012) suggest that biochar amendments may be a useful approach for increasing soil microbial community activity rates and thus for increasing soil health and crop yields (Luo et al., 2018). However, as with the plant growth and soil fungal responses mentioned above, instances of neutral and negative effects of biochars on individual EEAs have also been reported (Elzobair et al., 2016b; Lammirato et al., 2011; Paz-Ferreiro et al., 2012) and the few field studies using woody crops have found mixed results (Ren et al. 2019; Zhou et al. 2020). Effects of biochar on EEAs might be different in systems with woody plants than in annual agricultural systems because of the inputs of tree litter and less frequent tillage (Bergstrom et al. 2000; Zhao et al. 2017). Despite the frequently observed increases of EEAs in response to biochar, few studies have attempted to elucidate the mechanisms of biochar's effects on EEAs (Bailey et al., 2011; Khadem and Raiesi, 2017; Khadem and Raiesi, 2019). Biochar may increase EEAs by increasing soil microbial biomass (Khadem and Raiesi, 2017) or by sorbing enzymes and stabilizing them (Khadem and Raiesi, 2019). A mechanistic understanding of biochar's effects on EEAs could help predict which types of biochar will result in particular changes to EEAs.

We conducted two studies to investigate the effects of two different conifer wood-derived biochars on soil, plants, and microbes in a coniferous Christmas tree plantation

featuring two commonly grown Christmas trees, blue spruce (*Picea pungens*) and balsam fir (*Abies balsamea*). The first study tested the hypotheses that the biochars 1) increase EEAs by altering specific soil physicochemical properties, 2) improve soil properties including nutrient concentrations and moisture, and 3) improve plant performance. The second study tested the hypothesis that biochar would modify root-associated fungal communities and increase ectomycorrhizal fungal diversity and abundance.

2. Methods

Experimental setup

2.1 Site description

We established the field experiment in May of 2016 in a previously fallow field at Michigan State University's Tree Research Center in Lansing, Michigan, USA (42°40'28.9"N 84°28'01.8"W). Soils are Marlette series (fine-loamy, mixed, semiactive, mesic Oxyaquic Glossudalfs) with slopes ranging from 0 to 3.0%. Mean annual air temperature at the field site is approximately 9.6℃, the mean annual precipitation is 87.1 cm, and the average annual snowfall is 91.4 cm (NOAA, https://www.ncdc.noaa.gov/oa/climate/normals/usnormals.html). The herbaceous layer is dominated by non-native weeds, including *Setaria viridis* (green foxtail), *Solidago* spp. (goldenrod) and *Conyza canadensis* (horseweed).

2.2 Biochar manufacturing processes and characteristics

Our studies evaluated two slow pyrolysis biochars (hereafter, BGR and USB). The BGR biochar was produced from woody pine biomass residue (woodchips, sawdust, limbs, etc.) from forestry operations in the upper peninsula of Michigan (primarily *Pinus resinosa* and *P. banksiana*) via pyrolysis at 650°C for 30 minutes in a rotary reactor system. The USB biochar was made from waste wood pallets (southern yellow pine species) and produced in a continuous carbonizer at 550°C for 18 min. Carbon (C) and nitrogen (N) contents of the

biochars were determined on an elemental analyzer (Costech Analytical Technologies, Inc., Valencia, CA, USA) while the concentrations of other elements in the surface layer of the biochars were determined by scanning electron microscopy with energy dispersive X-ray spectroscopy taken on three replicate subsamples (described in detail in section 2.5; see Table 1). The particle size distribution, pH, electrical conductivity, and ash content of the biochars were determined on three replicate subsamples of both biochars following the protocols

Table 1. Physical and chemical properties of BGR and USB biochars are reported.

recommended in the International Biochar Initiative's biochar standards (IBI 2015). Notably, the BGR biochar had higher Ca, Cu, Ni, Al, pH, electrical conductivity, ash content, and percentage of particles greater than 1 mm in size, but lower Si and N content than the USB biochar (see table 1).

2.3 Study design and establishment

The experimental design included both biochars applied at two rates (25 Mg ha^{-1}, and 75 Mg ha^{-1}; hereafter referred to as the low rate and high rate) as well as a control for a total of five biochar treatments (control, USB low rate, USB high rate, BGR low rate, and BGR high rate) and seedlings of two common North American Christmas tree species – balsam fir (*Abies balsamea*) and Colorado blue spruce (*Picea pungens*). We chose the low rate to be near the predicted optimal biochar dose for increasing plant performance, while the high rate was chosen to be on the upper end of potential biochar dosages (Major 2010) to test whether higher biochar application rates can be used for carbon sequestration objectives without negatively impacting soil processes and crop performance. To implement these treatments in a fully factorial design, we divided the study site into two units, each of which was further subdivided into six rows of 30 m length and 1 m width, with 1m wide buffer strips between rows for a total of 12 treatment areas (see Fig. S1 for a map of study layout). Three rows in each unit were planted with each tree species. Each treatment area consisted of fifteen 2 m ×

1 m plots. Note that each treatment was only applied to a single one of these treatment areas (i.e. as a single block). We applied the biochars on May 8 (BGR) and May 9 (USB), 2016 using a turf spreader followed by discing to a depth of 15 cm to incorporate the biochar. We then transplanted three bareroot seedlings into each plot with 0.5 m between seedlings within each plot. This planting rate yielded an initial count of 540 seedlings. The close spacing provided extra seedlings that could be destructively harvested while leaving the remaining seedlings at a typical spacing (2 m) for Christmas tree plantations (Landgren et al., 2003). To control competition from weeds, we applied the herbicides Simazine, Flumioxazin, Glyphosate, Clethodim, Pendimethalin, and 2,4-D at various points throughout 2016, 2017, and 2018, following standard practice for managing new conifer plantations (Table S1). We irrigated seedlings during periods of drought with a tractor sprayer. No fertilization or other soil amendments were applied other than the biochar. We then conducted two separate but complementary studies on the effects of biochar on the agroecosystem – Study 1 investigated the effects of biochar on soil properties, microbial nutrient cycling, and plant performance, while Study 2 focused on the assembly of root-associated fungal communities. The trees that we destructively sampled to examine root-associated fungal communities in Study 2 were taken from separate plots than those used in study 1 to avoid disturbing soil processes. Thus, we did not attempt to statistically relate the soil data from study 1 with the fungal community data from Study 2.

Study 1: Effects of biochar on soil properties, enzyme activities, and plant growth.

2.4 Field sampling

We sampled soil multiple times per year during growing seasons (June through October) in 2016, 2017, and 2018 (Table S2). For each soil sample, we took three cores (0-15 cm) from within each sampled plot and composited them into a single sample to reduce fine scale variability in soil properties. This sampling procedure was repeated five times per

treatment to yield 50 total samples for most sampling timepoints (5 biochar treatments x 2 tree species x 5 replicates; Table S2). We also installed cation and anion plant root simulator (PRS) probes (Western Ag, Saskatoon, SK, Canada) in five plots per treatment from July 17, 2018 to September 13, 2018 to measure the bioavailability of the following 15 ions over the growing season – NO_3^-, NH_4^+, Ca, Mg, K, PO_4, Fe, Mn, Cu, Zn, B, S, Pb, Al, and Cd. PRS probes bind ions from the soil solution and provide an integrated index of the availability of those ions over the deployment period.

We quantified weed biomass by clipping all herbaceous plant (weed) tissues at the soil surface within a 0.25 m^2 quadrat adjacent to one of the seedlings within each sampled plot (n=5). We separated live plant shoots into grasses and forbs, dried them to a constant mass and then weighed samples for the total dry mass of grasses and forbs. We surveyed tree seedling growth (stem diameter at the root collar, and seedling height) and survival in Spring and Fall of 2016, 2017, and 2018. We calculated a biomass proxy for each seedling by multiplying the basal area (cm^2) by the height (cm). This metric approximates the volume of the central stem of each seedling.

2.5 Laboratory soil analyses

Soil properties assumed to be dynamic (dissolved nutrients, microbial biomass, enzyme activities) were measured at multiple timepoints and soil properties that are relatively stable (bulk density, total C/N) were measured at a single timepoint (Table S2). Following sampling, we sieved soils to 5 mm to allow larger biochar particles to pass through and stored at 4° C for a maximum of one week before freezing them at -20°C or processing them for analysis. Soil samples collected in 2016 were analyzed for P, K, Mg, S, pH, and effective cation exchange capacity at the University of Maine (Orono, Maine, USA). We determined total soil C and N concentration via dry combustion (Costech ECS 4010 CN analyzer;

Costech, Valencia, CA USA). We measured soil bulk density by collecting 5.0 cm × 15.0 cm soil cores with an impact corer, weighing the volume of soil sampled, and using a subsample to measure gravimetric soil moisture content. For soil bulk density, we sampled five cores across each biochar treatment, combining the plots planted with blue spruce and balsam fir trees because we did not expect tree species identity to affect soil structure after only three seasons. We determined soil microbial biomass, dissolved organic carbon (DOC), total dissolved nitrogen (TDN), NO_3^-, and NH_4^+ on samples taken from multiple dates in 2016, 2017, and 2018 (Table S2), which were extracted by shaking 8 g of fresh soils with 40 mL 0.5m K_2SO_4 at >250 rpm for 60 minutes and then filtering through Whatman #1 filter paper. We stored filtrates at -20° C until analysis. We used a chloroform fumigation-extraction method (Vance et al., 1987) to determine soil microbial biomass C and N, with both fumigated and unfumigated extracts analyzed for DOC/TDN on a varioTOC analyzer (Elementar, Langenselbold, German). Microbial biomass C and N were calculated as the differences in extractable C and N in fumigated and unfumigated extracts, using efficiency factor conversions of 0.45 and 0.54, respectively (Brookes et al., 1985; Jenkinson et al., 2004). We quantified soil NO_3^- concentration using a modified version of the enzyme reduction method described in detail by Wittbrodt et al. (2015), with absorbance measured at 540 nm on a Biotek synergy H1 microplate reader (Winooski, VT, USA). We measured soil NH_4^+ concentrations in extracts by using the microplate protocol described by Sinsabaugh et al. (2000).

We used microplate-format colorimetric and fluorimetric assays (Saiya-Cork et al., 2002) to determine seven soil EEAs involved in C, N, and P cycling – β-glucosidase (BG), cellobiohydrolase (CBH), leucine aminopeptidase (LAP), β-N-acetyl glucosaminidase (NAG), peroxidase (PER), phenoloxidase (PHEN), and acid phosphatase (PHOS), with

fluorescence and absorbance measured on a Biotek synergy H1 microplate reader (Winooski, VT, USA). Briefly, we prepared soil slurries by homogenizing 1 g (wet weight) of soil in 125 mL of water with an immersion blender. We chose to suspend soil samples in water rather than a buffer to allow assays to proceed at the soil's natural pH (German et al., 2011). We used methylumbelliferyl-linked substrates for BG, CBH, NAG, and PHOS assays, a methylcoumarin-linked substrate for the LAP assay, and L-DOPA as a substrate for PHEN and PER assays. All fluorimetric assays were run with substrates at 40 μM concentration. We incubated assays for approximately 18 hours. We included homogenate blanks, substrate blanks, and buffer blanks in all assays.

We investigated whether biochar underwent physical or chemical changes following application by using scanning electron microscopy with energy dispersive X-ray spectroscopy (EDX) at Michigan State University's Center for Advanced Microscopy. We recovered biochar particles from soil cores sampled in October 2018 by dispersing ~15 mL of soil in 400 mL of deionized water and picking them out of the water with forceps. This method was intended to remove loosely adhering debris, while allowing tightly adhering soil particles to remain. We also selected biochar particles that had not been applied to the field and had been stored for three years to represent samples without field aging. We prepared biochar particles for mounting by drying them at 60° C for two days, after which we mounted them to stubs with quick curing epoxy and coated them with osmium (≈10 nm thickness) in an NEOC-AT osmium chemical vapor deposition coater (Meiwafosis Co., Ltd., Osaka, Japan). We performed scanning electron microscopy using either a JEOL 6610LV or JEOL 7500F scanning electron microscope (JEOL Ltd., Tokyo, Japan) equipped with an Oxford Instruments AZtec system (Oxford Instruments, High Wycomb, Bucks, England) for EDX. We performed EDX on unaged biochars and on portions of aged biochars that were free of

encrusted soil particles to avoid confounding the elemental composition of adhering soil with that of the biochar.

2.6 Statistical analyses

For variables that were measured at multiple timepoints, we tested the effects of biochar application using linear mixed models with the nlme package in R (Pinheiro et al., 2013), with biochar treatment, sampling date, and their interaction included as fixed effects, and plot included as a random effect. Biochar treatments were coded as five separate treatments – control, BGR low, BGR high, USB low, and USB high – and entered into models as a single variable, rather than using biochar type and rate as separate variables. We inverse or log transformed data to meet the assumptions of normality when absolute values of skewness exceeded two or those of kurtosis exceeded seven (Kim, 2013). We used pairwise t-tests or Mann-Whitney U tests (when neither the original or transformed variable met the assumption of normality) to evaluate the significance of differences between biochar treatments and the control at individual sampling dates and did post-hoc tests that averaged the effects of biochar application across all sampling dates using the emmeans package in R (Lenth et al., 2018). We used chi-square tests to test for differences in tree survival between treatments. Because pairwise tests were only performed between the control and each of the biochar treatments, we calculated Bonferroni corrections by multiplying P-values by four (2 biochars x 2 rates = 4 comparisons). We conducted structural equation modelling using the R package lavaan (Rosseel, 2012) to test which soil physicochemical characteristics could be identified as potential mechanisms of biochar's effects on EEAs. We identified modifications to DOC, inorganic N, and soil moisture *a priori* as potential mechanisms of biochar's effects on EEAs because these variables were correlated with both biochar application and EEAs for at least one of the biochars. In the structural equation models, we first selected a subset of the

sampling dates for which we had matched data for DOC, inorganic N, soil moisture, and EEAs. This subset contained data from six sampling dates – one from 2017 and five from 2018 – and 297 data points. Then, we accounted for the effect of sampling date by first fitting linear models to EEAs and the soil physicochemical variables with sampling date as the only explanatory variable. We used the residuals from these models in all structural equation models so that we could test for relationships between variables while controlling for variation due to sampling date. We used these three physicochemical variables in structural equation models with paths leading from biochar to these variables, and also from these variables to EEAs. We also included a direct path from biochar to EEAs. We calculated net coefficients representing the importance of each mechanism of biochar's effects on EEAs by multiplying the coefficient leading from biochar application to each soil physicochemical property and the coefficient leading from each soil physicochemical property to each enzyme. We calculated these coefficients as standardized coefficients which vary from -1 to 1. We used bootstrapping to calculate the standard errors and to test for significance of these coefficients. In total, we fit ten different structural equation models to the data to test for the effects of the two biochars on the five hydrolytic enzymes. We did not include oxidative enzymes in the structural equation models because they were not significantly affected by biochar application.

Study 2: Effects of biochar on root-associated fungal communities

2.7 Sequencing of root-associated fungal communities

We profiled root-associated fungal communities by performing high-throughput amplicon sequencing of the fungal ITS region. In late October of 2017, we destructively sampled seedlings from across all treatments (N = 5) and froze intact root systems at -80°C until processing. We prepared root samples for DNA extractions by thawing them, washing

them free of loosely adhering soil with deionized water, and then drying them at 35°C. Tightly adhering soil was not washed from the root system and was included in the DNA extraction because it is likely to contain rhizosphere and root-associated fungi. Dried root samples were gently crushed by hand in a paper bag and fine roots that easily broke off from the root system were collected and ground by hand between two sheets of paper into a fine powder (Benucci et al., 2016). We extracted DNA from 50 mg of ground root material using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. We prepared libraries for sequencing using a three step PCR that has been described previously (Benucci et al., 2019; Chen et al., 2018) with the primers ITS1F and ITS4 (Gardes and Bruns, 1993; White et al. 1990). In the first step, we used the primers without adapters to amplify the ITS region for ten cycles. In the second step, we used primers modified with frameshifts and Illumina adaptors for ten cycles of PCR (Lundberg et al., 2013). In the final step, we ligated barcodes and sequencing adaptors with 15 PCR cycles. We normalized PCR products to a concentration of 1-2 ng/µl using the SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, United States) and then pooled the PCR products in equimolar volumes. We used Agencourt AMPure XP magnetic beads to remove small fragments and primer dimers (Beckman Coulter, United States). We sequenced samples on an Illumina MiSeq at Michigan State University's Genomics Core Facility.

2.8 Sequence Analysis and Statistics

We used FastQC to quality control sequences (Andrews, 2010) and demultiplexed sequences in QIIME 2 (Bolyen et al., 2019). We removed primers and conserved regions from sequences using Cutadapt v2.6 and USEARCH v10 (Edgar, 2016; Edgar and Flyvbjerg, 2015; Martin, 2011). We clustered sequences into operational taxonomic units (OTUs) at a 97% similarity threshold using the UPARSE algorithm of USEARCH (Edgar, 2013) and

discarded singletons. We assigned taxonomy to representative sequences using the naïve Bayesian "feature-classifier" plugin in QIIME 2 (Bokulich et al., 2018; Bolyen et al., 2019) trained on the February 2, 2020 release of the UNITE database (Kõljalg et al., 2005). We assigned taxonomically identifiable OTUs to guilds using FUNGuild (Nguyen et al., 2016). We conducted downstream analyses on the OTU matrix using the phyloseq and vegan packages in R (Ihaka and Gentleman, 1996; McMurdie and Holmes, 2013; Nguyen et al., 2016). We performed alpha and beta diversity analyses on two different OTU matrices. In the first OTU matrix, representing the full dataset, we rarefied samples to an even sequencing depth of 13,472, which excluded three samples with lower sequencing depth from analysis. In the second OTU matrix, we filtered to only include OTUs which were designated as ectomycorrhizal (ECM) by FUNGuild. We then calculated the relative abundance of ECM OTUs as their proportion among all ECM OTUs without rarefaction. We used this second dataset to test for effects on the composition and diversity of the ECM community specifically. We calculated the Shannon and Simpson diversity indices and the number of observed OTUs as measures of alpha diversity, which we tested for significance with ANOVA and pairwise t-tests with Bonferroni corrections. We calculated beta diversity with the Bray-Curtis dissimilarity metric, plotted with principal coordinates analysis (PCoA), tested for homogeneity of variance, and tested for significance with permutational multiple analysis of variance (PERMANOVA). We tested for effects of tree species and biochar treatment on the abundance of individual taxa that occurred in at least 40% of samples in each comparison using analysis of composition of microbiomes (ANCOM) on unrarefied, non-relativized OTU matrices, with a W statistic cutoff of 0.6 used for significance (Mandal et al., 2015). For all analyses on the fungal community data, we combined the low and high application rates for each biochar into a single grouping to increase statistical power.

Representative sequences, OTU tables, OTU taxonomy assignments, and sample metadata are available in supplemental files 2-6.

3. Results

Study 1: Effects of biochar on soil properties, enzyme activities, and plant growth.

3.1 Soil physicochemical properties

The application of BGR at the highest rate resulted in strong and persistent increases to dissolved organic C (DOC, Fig. 1a; LMM, $P < 0.0001$), with significant effects observed at four out of six sampling dates (t-test, $P < 0.0001$), and an increase of 58% averaged across all sampling dates (pairwise test, $P < 0.0001$). At the lower application rate, BGR increased DOC by 9% averaged across all sampling points (pairwise test, $P = 0.07$), while USB treatments did not alter DOC. Biochar had less clear-cut effects on total dissolved nitrogen (TDN), which demonstrated either increases, decreases, or a lack of an effect in response to biochar treatments, depending on the sampling date (Fig. 1b). Across all sampling dates, there were no consistent effects of biochar on TDN. Together, these two patterns show that BGR increased the DOC pool without modifying the TDN pool, which led to predictable increases in the DOC:TDN ratio at both application rates (pairwise test, low rate $P = 0.02$, high rate $P < 0.0001$). Microbial biomass C and N were minimally affected by biochar treatment. At the lower application rate, BGR increased microbial biomass N relative to the control (pairwise test, $P = 0.03$), but this effect was not observed at the higher application rate of BGR or in the USB treatments.

Generally, both types of biochar positively affected soil moisture content in all three years ($P < 0.0001$), with greater effects at the higher application rate (Fig. S2). Although USB-amended plots generally had higher soil moisture than control plots, pairwise t-tests for

each sampling date were never significant while the effects of BGR on soil moisture were much more pronounced and often significant, especially at the higher application rate (Fig. S2). However, these effects were not entirely consistent across sampling dates. For example, in 2016 there was a significant interactive effect between biochar treatment and sampling date on soil moisture (LMM, $P = 0.017$), with negative effects of USB low rate ($P = 0.034$), USB high rate ($P = 0.036$), and BGR low rate ($P = 0.006$) on July 29. This sampling date came five days after a rainstorm in which 22 mm of precipitation fell in a single day. These negative effects of biochar on soil moisture content contrast with positive effects of both types of biochar on soil moisture that were observed on the day after the same storm (July 25). Throughout 2017 and 2018, there were no significant negative effects of any biochar treatments on soil moisture, although on some dates there was an absence of any positive effect.

In addition to its effects on soil moisture, biochar modified a number of soil physicochemical properties. At the end of the first growing season, both biochars had increased soil Mg and P, while BGR at the high rate had increased Ca, K, B, Mn, Na, S, and effective cation exchange capacity (pairwise t-test, Bonferroni $P < 0.05$). USB decreased Al and Fe contents (pairwise t-test, Bonferroni $P < 0.05$). Total Zn and Cu were not affected by biochar application. Total soil N was not affected by biochar application two months after the establishment of the experiment (July 19, 2016), although we did not analyze soils for total N at later timepoints and cannot rule out longer term effects. BGR decreased soil bulk density by 10.1% ($P = 0.099$) and 20.7% ($P < 0.001$) relative to the control treatment at the low rate and high rate respectively, while USB had no effect on bulk density at either application rate (Bonferroni-corrected $P = 1$). Two months post-application, biochar had resulted in shifts in soil pH from 5.81 (in the control plots) up to 6.72 ($P = 0.007$) and 7.12 ($P < 0.0001$) at the

high rate for USB and BGR respectively with weaker, though still significant effects at the low rate (Fig. S3).

Plant root simulator (PRS) resin strips installed in 2018 showed that both types of biochar decreased the bioavailability of NO_3^-, Fe, Mn, Cu, and Pb and increased the bioavailability of NH_4^+ and K (ANOVA, $P < 0.05$) during the 2018 growing season (Fig. S4). Although biochar increased the bioavailability of NH_4^+, the vast majority of the resin-bound inorganic N was in the form of NO_3^-, which exhibited a strong negative response to biochar application, overwhelming the positive response of NH_4^+. For almost all ions that were significantly affected by biochar application, effects of BGR were greater than those of USB (Fig. S4). Effects of biochar on other ions were less consistent between biochar types. Resin-bound phosphate decreased at USB low rate and increased at BGR high rate, while Mg increased with USB and decreased with BGR at both application levels. The bioavailability of Ca, Zn, B, SO_4, Al, and Cd were not affected by either biochar. Biochar decreased the bioavailability of the three heavy metals Fe, Cu, and Pb by around 56% and 84% for USB and BGR, respectively, applied at the higher rate.

We collected data on extractable soil inorganic N data across 2016, 2017, and 2018 which largely support the patterns revealed by the PRS probes but also demonstrates the potential for sporadic disruptions to the general trend of lower N availability in the biochar treatments (Fig. S5). Immediately following the establishment of the experiment (July 7, 2016) inorganic N content decreased significantly ($P < 0.0001$) in all biochar treatments (11.8-13.6 μg N/g dry soil) relative to the control (32.4 μg N/g dry soil). Treatment effects quickly became less pronounced, but still with generally lower concentrations of inorganic N (Fig. S5). There was an exception to this pattern on August 21, 2018 when BGR-amended

plots had greater soil inorganic N than control plots at both biochar application rates ($P <$ 0.0001).

3.2 Enzyme activities

Averaged across all sampling dates in the first growing season (2016), biochar induced significant increases to the soil extracellular enzyme activities (EEAs) of BG, CBH, LAP, NAG and PHOS which were mostly observed in the high rate treatments of both USB and BGR treatments. Yet this pattern was not entirely consistent and there were significant treatment by date interactions for all enzymes (LMM), indicating temporal instability (Fig. 2a-e). However, in the two following seasons (2017 and 2018), patterns in hydrolytic EEAs became much more stable, with positive and application rate-dependent effects of both biochars observed at almost all sampling dates (Fig. 2f-k; LMM, P <0.0001). Across 2017 and 2018, effect sizes were generally greatest in the BGR high rate treatment, which resulted in increases of 95%, 67%, 446%, 83%, and 183% for BG, CBH, LAP, NAG, and PHOS activities respectively. Effects of the two biochars at the low rate on enzyme activities were not always significant, but effects at the high rate were highly significant for all hydrolytic enzymes in 2017 and 2018 (Fig. 2f-k). This pattern was generally stable throughout these two years, with date by treatment interaction terms only significant for CBH (LMM, $P < 0.0001$) and PHOS ($P = 0.02$), although this was driven by changes in the relationship of activities between some of the BGR and USB treatments, rather than by their relationship to the control treatment which almost always had the lowest mean EEA for the hydrolytic enzymes (Fig. 2f-k). Among the five hydrolytic enzymes, effect sizes of biochar application were highest for LAP, averaging a 197% increase in activity in 2017 and 2018 relative to the control across all biochar treatments. LAP was also the only enzyme that showed somewhat consistent increases in activity in response to biochar in 2016. There was never an effect of

biochar on peroxidase activity, while phenoloxidase activity was negligible (often negative) and was not tested for significance.

Based on correlations observed in the linear mixed models, we identified DOC, soil moisture, and inorganic N as potential mechanisms of biochar's effects on EEA and further investigated their roles using structural equation modelling (Fig. 3a, b displays model structure). Structural equation modelling revealed that BGR increased the activities of all five hydrolytic enzymes both indirectly by increasing soil moisture (which then stimulated EEA) and directly via unmeasured mechanisms (Fig. 3a, c). BGR also had indirect positive effects on LAP and PHOS (but not BG, CBH, or NAG) by increasing DOC content (Fig. 3a, c). USB had direct effects on all EEAs, but few indirect effects (Fig. 3b, d) largely because USB had weaker effects on the soil variables that would have mediated such effects. However, there was a significant, weakly positive indirect effect of USB on BG activity mediated by a negative effect on inorganic N (Fig. 3d).

3.3 Electron microscopy investigation of biochar structure

In scanning electron microscope images, the microporous structure of the pine wood feedstock was readily apparent with visible pores and pits (Fig. 4). Images taken on field-aged biochar showed that soil particles had encrusted areas of both types of biochar, often filling the pores (Fig. 4 c, d), and that a filamentous fungus had colonized the surface of BGR (Fig. 4c), though it is unclear if this represents selective microhabitat usage or incidental colonization. There was no obvious evidence of physical degradation of biochar structure from visual assessment, and no substantial changes in the surface chemistry of either biochar after field aging (data not shown).

3.4 Plant performance

BGR significantly increased mortality of blue spruce (Fig. 5a) and balsam fir (Fig. 5b) trees and decreased the growth of balsam fir (Fig. 5d). For blue spruce, the BGR low rate resulted in decreased survival that was immediately apparent by the first sampling date (chi-square test, $P < 0.05$) and persisted throughout the experiment, with survival of 7% of blue spruces in the BGR treatment compared to survival of 38% of blue spruces in the control by the end of the experiment (Fall 2018). BGR applied at 75 Mg ha^{-1} had no effect on blue spruce survival (Fig. 5a). Balsam fir survival decreased in both BGR treatments, starting in September of the first growing season (2016) and persisting throughout the experiment (chi-square test, $P < 0.05$, Fig. 5b), with survival averaging 39% in the BGR treatments compared to 70% in the control treatment by the end of 2018. Balsam fir displayed lower growth in the BGR treatments beginning in the spring of 2017 (though it was sometimes only significant in the low rate treatment), with biomass proxy values in the BGR treatments approximately half of those in the control treatment by the end of the experiment (pairwise t-test, $P < 0.05$). There was generally no effect of USB on the growth or survival of either tree species. In 2016, weed biomass was higher in all biochar treatments than in the control, though the only significant difference was with the BGR low rate which had 94% greater weed biomass than the control (pairwise t-test, Bonferroni $P < 0.05$). There were no significant differences in weed biomass in 2018.

Study 2: Effects of biochar on root-associated fungal communities

3.5 Fungal communities

Root-associated fungal communities had an average of 357 unique fungal OTUs per sample and 3,407 total OTUs across all 50 samples, although ectomycorrhizal (ECM) taxa

constituted a small fraction of this diversity, with an average of only 6.4 ECM OTUs per sample and a total of 51 ECM OTUs (Fig. 6a, b). Although ECM OTUs made up only 1.5% of the observed OTUS of the root associated fungal community, they constituted 7.5% of the total number of sequenced reads. ECM sequence relative abundance modestly increased in the USB treatment relative to the control, though the effect was not significant (t-test, Bonferroni $P = 0.14$). ECM OTU richness on balsam fir roots decreased from a mean of 10.6 OTUs in the control down to 4.8 and 5.4 OTUs in the USB and BGR treatments respectively (ANOVA, $P = 0.002$; t-test, $P = 0.01$, 0.018), while total OTU richness decreased from a mean of 430 OTUs in the control down to 348 and 315 OTUs i n the USB and BGR treatments respectively (Fig. 6a, b; ANOVA, $P = 0.0007$; t-test, $P = 0.027$, 0.002). Saprotroph OTU richness declined in all treatments relative to the control, though this effect was only significant in the BGR treatment on balsam firs (ANOVA, $P = 0.01$). For balsam fir ECM fungal communities, both biochar treatments resulted in significant declines in Shannon diversity (ANOVA, $P = 0.001$; t-test, $P = 0.006$ and $P = 0.08$ for USB and BGR, respectively), which incorporates species evenness, while total fungal communities had similar Shannon diversity across all biochar treatments (Fig. 6a, b). ECM and total fungal OTU richness on blue spruce roots were somewhat lower in biochar treatments than in the control, but differences were not significant. The decrease in ECM species richness and Shannon index on balsam fir indicates that biochar treatment favored ECM communities that were dominated by a low number of dominant species. ECM communities in all treatments were dominated by a single OTU identified as *Wilcoxina mikolae* which occurred in 49 out of 50 samples (Fig. S6). *W. mikolae* increased in relative abundance (calculated as a percentage of ECM reads) from 60% in the control to 93% in the USB treatment on balsam fir roots (Fig. S6; ANCOM, $W > 0.6$). *W. mikolae* was similarly abundant in the USB treatment on blue spruce at 92% of ECM reads, though it was also dominant in the blue spruce control

treatment (83% of ECM reads) and thus the effect of USB treatment was not significant (Fig. S6). *W. mikolae* did not respond to BGR application on either balsam fir or blue spruce. Two other OTUs from the Pyronemataceae (of which *Wilcoxina* is also a member) that were both classified as *Pulvinula* spp. were generally in low abundance (mean of 4%) but were dominant in three samples from the BGR treatment (36-84% relative abundance among ECM sequences), though they were observed too infrequently to test for differential abundance. Tests on the full fungal community indicated that the genera *Atractospora* and *Coprinellus* were in higher and lower abundance, respectively, in the fir BGR treatment compared to the control. Patterns in beta diversity show that total root-associated fungal communities were primarily structured by tree species (PERMANOVA, $P = 0.0001$), with little effect of biochar treatment (Fig. 7a). Balsam fir and blue spruce total fungal communities were separated from each other along the first axis of the PCoA (Fig. 7a). There was little effect of tree species on ECM community composition, but a significant effect of biochar treatment (Fig. 7b; PERMANOVA, $P = 0.0001$), though this significant result may have been due to large differences in beta dispersion between groups ($P < 0.0001$).

4. Discussion

We hypothesized that biochar would 1) increase tree growth and survival, 2) improve soil physicochemical properties including soil moisture and nutrient concentrations, 3) improve soil microbial community functionality including extracellular enzyme activities (EEAs) and soil microbial biomass, and 4) stimulate ectomycorrhizal (ECM) colonization. As expected, our study found that biochar improved some physicochemical properties including soil moisture, phosphate availability, and the concentrations of multiple cations and also stimulated most EEAs. However, biochar also decreased nitrogen availability and ECM diversity, and reduced seedling growth and survival. Furthermore, there was not an effect of

biochar on soil microbial biomass as we had predicted. Although biochar has the potential to increase plant productivity by ameliorating unfavorable soil physicochemical conditions (Ali et al., 2017; Major et al., 2010), it is likely that the specific combination of biochars, crop species, and soil type were responsible for the negative outcomes that we observed.

4.1 Differences between biochar types

Many of the soil variables in this study were more strongly affected by BGR than by USB, which raises questions about which properties of the biochars were responsible for their different effects. The BGR biochar had higher pH, electrical conductivity, ash content, and concentrations of certain heavy metals (Al and Cu). The positive effect of BGR on DOC may have been due to its higher pH and larger effect on soil pH than USB (Fig. S3), which can lead to the deprotonation and subsequent liberation of endogenous (not biochar-derived) DOC (Smebye et al., 2016). Alternatively, the increase in DOC in the BGR-amended soils may have been due to higher DOC in the biochar itself, although we did not quantify DOC content or composition of the biochars. Typically, higher pyrolysis temperatures decrease biochar DOC (Liu et al., 2019), but in our study the BGR biochar produced at 650° C had a greater effect on soil DOC than the USB biochar produced at 550° C. Although biochar is considered very stable in soils, with turnover times on the order of centuries to millennia (Lehmann et al., 2012), recent work suggests that biochar can contain a portion of C that is relatively labile and cycles on timeframes relevant to our three-year field experiment. For example, up to around 5% of the C present in biochar can be immediately extracted as DOC (Liu et al., 2019; Liu et al., 2015; Mukherjee and Zimmerman, 2013; Wu et al., 2018), and 0.20% of the total charred C in wildfire-derived charcoal can be extractable as DOC 100 years after its formation (Hockaday et al. (2007), demonstrating that biochar leachates can contribute directly to the DOC pool in both the short- and long-term.

4.2 Mechanisms of biochar's effects on enzyme activities

The observed differences in DOC content between biochar treatments likely had cascading effects on soil microbial community functioning, characterized by an increase in EEAs involved in the liberation of N and P, but not C. Through structural equation modelling, we found that BGR increased EEAs involved in N and P cycling (Fig. 2) by increasing DOC, though there was no DOC-mediated effect of biochar on EEA for any of the enzymes involved in C acquisition (Fig. 3c). The DOC mechanism may have contributed to the very large effects of biochar on LAP and PHOS activity, which exhibited greater responses to biochar than did any C targeting EEA. Biochar volatile matter is relatively available to microbes and can induce nutrient limitation by acting as an available carbon source (Deenik et al., 2010). Thus, the increased DOC pool associated with BGR may have led to immobilization of N and P, which can stimulate the production of enzymes that target those nutrients (Allison and Vitousek, 2005). With regards to N, this finding is supported by the decreases in both extractable (Fig. S5) and resin-bound (Fig. S4) inorganic N, which provides evidence for decreased N mineralization that may be due to DOC-induced N limitation. This mechanism was only observed in response to BGR application because USB did not have an effect on DOC. However, there was only a single structural equation model (USB's effect on BG) in which biochar increased EEA by decreasing inorganic N. Thus, the positive effects of DOC on EEAs in BGR-treated soils could also be due to the DOC relieving C limitation of microbial enzyme synthesis, rather than by inducing N limitation. BGR also increased the activities of all hydrolytic enzymes (including C-targeting enzymes) via its positive effect on soil moisture (Fig. 3), which likely provided more favorable conditions for microbial metabolism and enzyme production (Xiao et al., 2018). Total N and exchangeable calcium are also potential controls on enzyme activities in biochar-amended

soils (Wange et al., 2015). Biochar can also have no effects (Elzobair et al., 2016b) or negative effects (Wu et al., 2013) on extracellular enzyme activities.

Despite our findings that indirect effects mediated by DOC, moisture, and inorganic N are possible mechanisms for biochar's effects on EEA, we still found that direct effects explained the greatest proportion of biochar's effects on EEAs in all models (Fig. 3 c, d). This suggests that the soil variables measured in our experiment did not fully capture the mechanisms of biochar's effect on EEAs. Biochar may have altered EEAs through its positive effect on pH (Pokharel et al., 2020) or by sorbing enzymes and slowing their turnover in the soil (Bailey et al., 2011; Elzobair et al., 2016a). The observed direct effects of biochar on EEAs may also be due to microbial community dynamics. Three hypotheses of biochar's effects on EEA that involve the microbial community are possible: 1) increase of soil microbial biomass in response to biochar while enzyme production per unit microbial biomass remains constant, 2) alteration of soil microbial community composition, resulting in a higher EEA production per unit microbial biomass and 3) alteration of soil microbial community physiology, also resulting in a higher EEA production per unit microbial biomass. Hypothesis 1 can be ruled out because we did not observe changes in microbial biomass C in response to biochar application, though microbial biomass-mediated effects of biochar on EEAs are possible in other systems (Khadem and Raiesi, 2017). Hypotheses 2 and 3, however, represent viable explanations for biochar's positive effects on EEAs. Hypothesis 2 is supported by significant changes to root-associated fungal diversity and composition (Figs. 6, 7). These changes were characterized by a decrease in total fungal, saprotroph, and ECM OTU alpha diversity on balsam fir trees in response to biochar (Fig. 6). This pattern suggests that lower diversity fungal communities can be associated with higher rates of enzyme production. Lu et al. (2015) documented a similar pattern, finding that biochar decreased

fungal diversity (measured by denaturing gradient gel electrophoresis), while increasing the activity of urease, invertase, and phosphatase. However, we did not sequence bulk soil fungal communities which might be more responsible for soil EEAs than are root-associated fungal communities. Hypothesis 3 is also certainly possible, though our data do not provide us with the necessary evidence to distinguish between microbial physiological and compositional mechanisms of biochar's effects on EEAs.

4.3 Effects of biochar on fungal communities

Wilcoxina mikolae sequences were present in almost all of our root samples, often as the dominant ECM species. USB resulted in higher relative abundances of *W. mikolae* to the point of hyperdominance of ECM communities, which may have prevented colonization by other ECM fungi and hindered the development of a diverse ECM community. *Wilcoxina* spp. are considered to be pyrophilic ("fire loving") ECM taxa because of their frequent occurrence on tree roots following wildfire (Jones et al., 2010). The frequent presence of *Wilcoxina* in recently burned forests has been attributed to both its affinity for disturbance and the thermotolerance of its propagules (Baar et al., 1999). Our data provide evidence that in addition to previously demonstrated linkages with disturbance and soil heating, the presence of pyrogenic carbon in soil may be another factor that favors *Wilcoxina*. An E-strain fungus (likely *Wilcoxina*) increased its colonization in response to biochar and constituted the majority of ECM root tips when 10% biochar was amended into soil (Robertson et al., 2012). Our finding that *Wilcoxina* sequence abundance is increased by USB suggests that effects of biochar application on microbial community composition may be relevant to understanding the assembly of microbial taxa following wildfires or prescribed fires in non-agricultural systems, thus linking the role of pyrogenic carbon in natural and agricultural systems. *Wilcoxina* is classified as an ectendomycorrhizal fungus, meaning that it colonizes

intracellularly in addition to the characteristic extracellular Hartig net and mantle (Trevor et al., 2001). The positive responses of *Wilcoxina* to biochar may be due to biochar's liming effect as ectendomycorrhizal fungi are known to form abundant mycorrhizae at slightly alkaline soil pH (Danielson and Pruden, 1989; Taylor and Finlay, 2003; Theodorou and Bowen, 1969), while most ECM fungi prefer moderately acidic soils (Erland et al., 1990; Hung and Trappe, 1983). The negative effects of biochar on ECM diversity in balsam fir (Fig. 6b) may explain the greatly decreased tree growth, which was not observed in blue spruce (Fig. 5c, d). Although results are mixed from studies measuring the effect of ECM diversity on tree growth and nutrient uptake, there is promising evidence that ECM diversity has positive effects on plant fitness (Baxter and Dighton, 2001; Jonsson et al., 2001; Köhler et al., 2018). Additionally, the high abundance of *Wilcoxina* sequences may have hindered tree performance as *W. mikolae* has previously been associated with negative plant responses and may be a less beneficial mutualist than other ECM fungal taxa (Mikola 1988).

4.4 Effects of biochar on tree performance and weed productivity

The reduced survival and growth of seedlings in response to BGR may have been due to the negative effects of the biochar on N availability, as we observed decreases in inorganic N and increases in the DOC:TDN ratio relative to the control. Although conifer seedlings responded negatively to BGR, this is not evidence of a general inhibitory effect of biochar on plant growth, because we observed positive effects of BGR on weeds, with up to a 94% increase in weed biomass. In a greenhouse experiment by our group, these same two biochars had no effect on the growth of the aggressive weed *Abutilon theophrasti* grown in a pure sand medium (O'Neil et al., 2021). The contrast between the responses of the weeds and the conifer seedlings to biochar is similar to the findings of a meta-analysis of the effect of biochar on plant productivity (Biederman and Harpole, 2013), which found that on average

annual plants responded positively to biochar, while perennial plants responded slightly negatively (though non-significantly) to biochar application. This increase in weed growth in the biochar plots may have contributed to the decrease in ECM fungal diversity as fast-growing herbaceous plant species have been previously shown to decrease ECM colonization of conifers (Wolfe et al., 2008). Higher weed biomass likely also exerted a competitive effect on the tree seedlings for water, nutrients, and light and may be related to the decrease in inorganic N. The problem of competition from weeds may have been compounded by interference of biochar with the pre-emergent herbicides that we applied (Graber et al., 2012; Spokas et al., 2009; Yu et al., 2006; Zheng et al., 2010). Our study adds to past work demonstrating that biochar can have the unintended consequence of increasing pressure from weeds (Major et al., 2005). It is also worth noting that herbicide application may have affected microbial processes including EEAs and mycorrhizal colonization. However, our goal was to manage this experiment similarly to a typical Christmas and/or landscape tree plantation. Thus, because herbicides are widely used in the industry (Landgren et al. 2003), they should be considered as part of this type of agroecosystem.

Phytotoxicity from contaminants in the biochar is another mechanism that may have reduced tree growth and survival. Biochar can accumulate organic compounds created during pyrolysis and also concentrate metalloids that were present in the feedstock, both of which can negatively affect plants if present in high enough concentrations. The BGR biochar was more inhibitory to plant growth and survival than the USB biochar and also had higher concentrations of Cu and Al. BGR biochar contained 0.30% Cu which is at the higher end of the allowable range of 0.014 to 0.6% set by the International Biochar Initiative (IBI 2015), while there are no published guidelines for Al in biochar. However, the total concentrations of these elements in soil were not increased by either of the biochars and resin-bound Cu was

actually decreased by BGR biochar, suggesting that the metal sequestration ability of biochar may counteract the endogenous metals contained in the biochar (Zhang et al. 2013). Although toxicity by heavy metals is unlikely in our study, we cannot rule out the possibility that organic compounds such as polycyclic organic hydrocarbons or volatile organic compounds may have had phytotoxic effects (Dutta et al. 2017). Unfortunately, we did not measure the concentrations of either of these classes of organic compounds in the biochar or soil and cannot comment further on this possibility.

Although our study found that biochar negatively affected the performance of conifer seedlings on a well-drained fine sandy loam soil, we observed wide ranging effects on soil physical, chemical, and biological properties that could be harnessed to improve conditions in other systems with different combinations of crops and soil types. When applied to our near-neutral pH soils, biochar resulted in a soil pH that was higher than the optimal level for the growth of blue spruce and balsam fir (Hart et al., 2009). In contrast, this increase in pH may be beneficial in more acidic soils, which are common for Christmas tree plantations that have been through multiple rotations and have been subject to the acidifying effects of conifer litter and fertilizer (Hornung, 1985). Indeed, a recent meta-analysis found that soils with a lower initial pH were more likely to result in greater crop yields with biochar application (Jeffery et al., 2017). Both biochars also exhibited the potential to sequester heavy metals (Fig. S4), which could improve tree performance in contaminated soils or reduce tree performance if availability is reduced to levels that limit plant growth. Likewise, the increase we observed in soil moisture with biochar application may have greater importance in more arid climates where soil moisture is a stronger constraint on tree growth and survival. Further investigations may provide a predictive framework for understanding which types of biochar will result in soil physicochemical closest to the optima of a given crop species or desired

microbially mediated process (e.g. nitrification, mycorrhizal colonization), opening up the possibility for "designer biochars" to ameliorate specific soil quality issues (Novak et al., 2014).

5 Conclusions:

Despite the overwhelming interest in biochar application as a strategy to increase crop productivity and modify soil biological processes, there are still relatively few studies on woody crops, and even fewer on those that associate with ectomycorrhizal fungi. Our study, which sought to pursue a comprehensive assessment of biochar's effects on an ectomycorrhizal cropping system, provides strong evidence that a much greater understanding of the effects of biochar on soil biology and crop performance is needed before biochar is applied widely in cropping or forestry systems with ectomycorrhizal plants. The increased extracellular enzyme activity, lower ectomycorrhizal fungal diversity, decreased tree performance, and modifications to a number of soil physicochemical properties suggest that complex interactions between plants, microbes, and soils need to be considered when predicting the effects that biochar will have on a given cropping system. Despite these possible pitfalls, the potential for biochar to be economically viable in woody cropping is strong because of the relatively long rotation periods which may justify the greater upfront investment in biochar if there are proven benefits. A greater mechanistic understanding of the context-dependencies that determine biochar's effects on crop performance will help develop science-based recommendations on biochar application for growers.

Data Availability Statement

Data related to the fungal sequencing is made available in supplemental files 2-6.

Conflict of Interest Statement

The authors declare no conflict of interest.

Acknowledgments

We thank Randy Klevickas and Paul Bloese for management of the field experiment. We thank Chase O'Neil, Chase Kasmerchak, Eion Riley, Kristen Przano, Ethan Rocklin, and Charity Moore for assistance in field sampling and laboratory work. We thank Reid Longley for preparing DNA libraries and doing initial sequence data processing. We thank Midhun Gelder for conducting analyses of biochar physiochemical properties. We thank Darian Smercina and Andrew Curtright for advising on certain procedures. We thank Biogenic Reagents for providing BGR biochar. This project was supported by the Michigan Christmas Tree Association, Michigan State University Project GREEEN, the Michigan Department of Agriculture and Rural Development Specialty Crop Block Grant Program, and by the USDA National Institute of Food and Agriculture, McIntire Stennis project 1006839

Author Contributions

Jake Nash – Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing-original draft, Writing-review & editing

Jessica Miesel – Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing-review & editing

Gregory Bonito – Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Writing-review & editing

Monique Sakalidis – Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Writing-original draft, Writing-review & editing

Han Ren – Conceptualization, Data curation, Investigation, Methodology, Writing-review & editing

Daniel Warnock – Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing-original draft

Lisa Tiemann – Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing-review & editing

Figure captions

Table 1. Physical and chemical properties of BGR and USB biochars are reported.

Macronutrients (%)			Micronutrients (%)			Particle Size Distribution (%)			Other Properties		
Element	USB	BGR	Element	USB	BGR	Size (mm)	USB	BGR		USB	BGR
C	84.2 ± 1.9	84.2 ± 2.3	Cl	0.13 ± 0.03	0.13 ± 0.13	>16	3.0 ± 1.3	0	pH	9.6 ± 0.08	11.2 ± 0.04
N	0.26 ± 0.01	0.002 ± 0.002	Cu	0.00 ± 0.00	0.30 ± 0.15	8-16	1.8 ± 0.6	0	EC (uS/cm)	221.2 ± 12.5	869.4 ± 27.9
O	13.19 ± 1.49	12.01 ± 6.55	Fe	0.00 ± 0.00	0.06 ± 0.03	4-8	2.6 ± 0.8	0.3 ± 0.2	% Ash	2.8 ± 0.14	4.8 ± 0.25
P	n.d.	n.d.	Mn	0.00 ± 0.00	0.05 ± 0.05	2-4	7.3 ± 0.4	2.0 ± 0.4	Bulk Density (g/mL)	0.30	0.19
K	0.09 ± 0.02	0.28 ± 0.28	Ni	0.00 ± 0.00	0.39 ± 0.20	1-2	18.6 ± 1.0	76.0 ± 5.9			
S	0.03 ± 0.03	0.09 ± 0.03	Na	0.00 ± 0.00	0.03 ± 0.03	0.5-1	35.6 ± 1.5	20.7 ± 6.3			
Ca	0.30 ± 0.09	1.30 ± 1.13	Si	0.60 ± 0.00	0.15 ± 0.1	<0.5	31.3 ± 2.5	1.8 ± 0.6			

				1	5		
Mg	0.14 ± 0.04	0.10 ± 0.10	Al	0.3 1 ± 0.0 1	4.3 7 ± 2.4 2		

Figure 1. Mean concentrations and standard errors of dissolved organic carbon (a) and total dissolved nitrogen (b) are plotted in each treatment group at six sampling dates in 2017 and 2018 with the standard error displayed. Note that the location of points along the x-axis are offset slightly to minimize overlap and improve legibility.

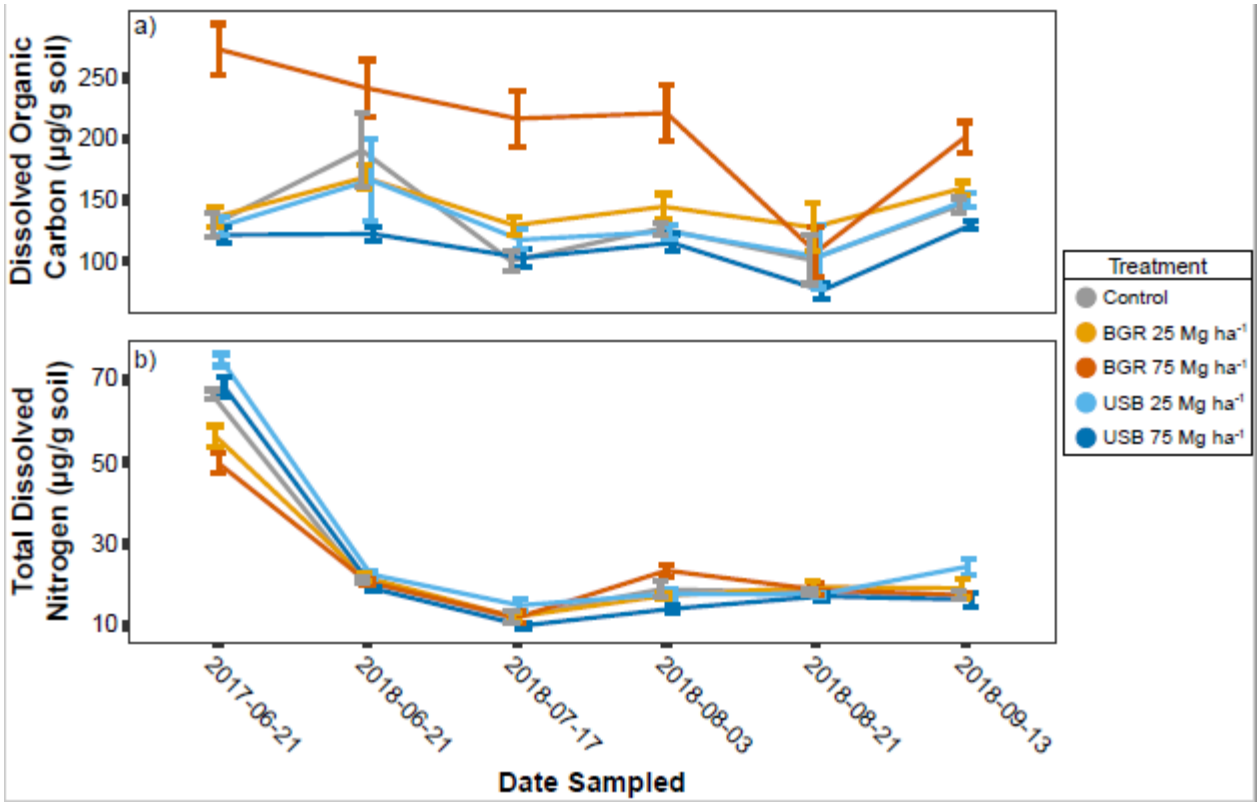


Figure 2. The mean extracellular enzyme activities (EEAs) of six enzymes in each of the treatments are plotted across 13 sampling dates in 2016 (a-e) and in 2017/2018 (f-k). Note that EEA data from 2016 and from 2017/2018 are plotted on different scales and that we do not have data on peroxidase activity from 2016.

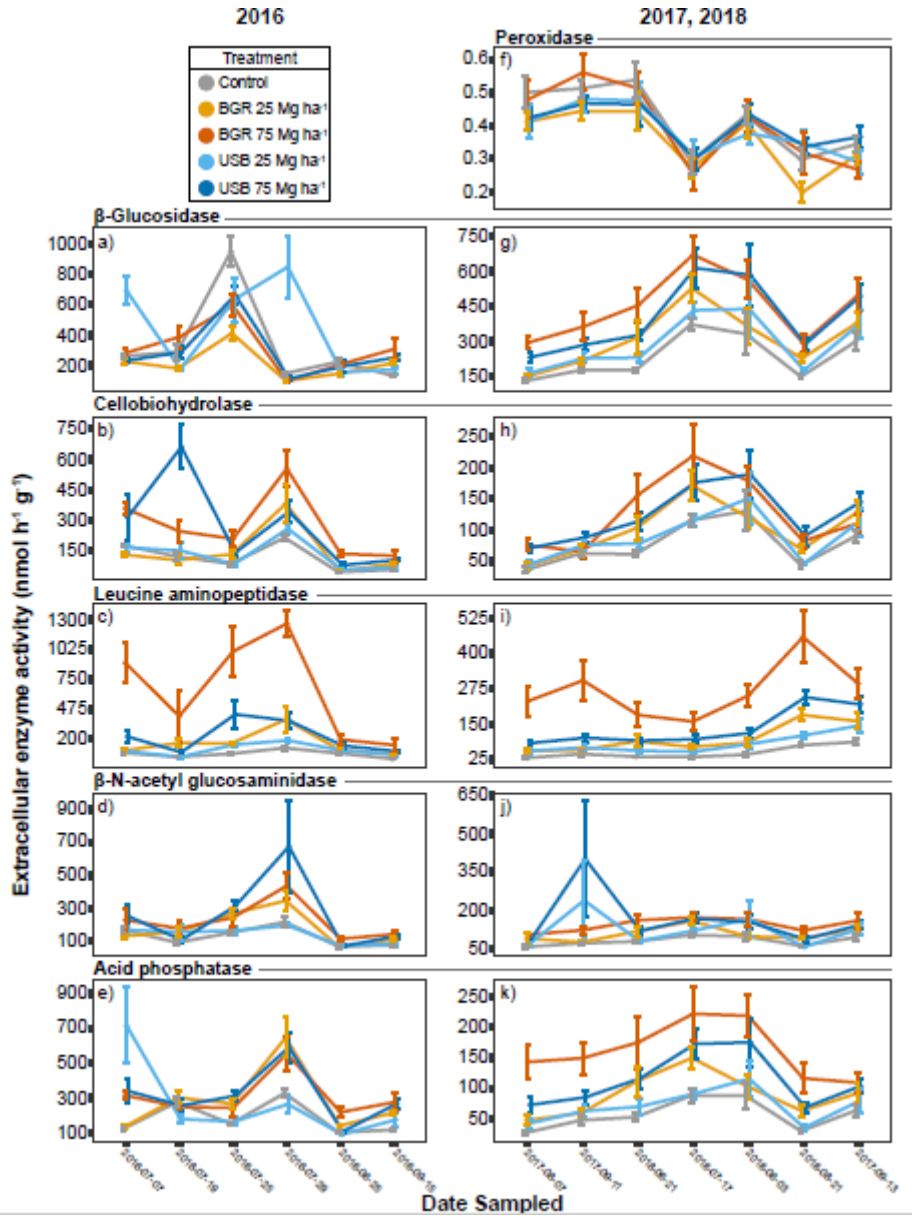


Figure 3. The results of ten structural equation models (5 enzymes x 2 biochars) that predicted extracellular enzyme activities (EEAs) based on direct effects of biochar and indirect effects mediated throughout effects on dissolved organic carbon (DOC), inorganic nitrogen, and soil moisture. (a,b) Model coefficients can be grouped into three categories (labelled in panel a) – response arrows (response of soil variables to biochar), effect arrows (effect of soil variables on extracellular enzyme activities), and the direct effect of biochar on extracellular enzyme activities. Standardized coefficients for the response arrows are

displayed for each biochar, with asterisks indicating statistical significance at $P < 0.05$. Coefficients of the effect arrows are not displayed because these were different for each of the five enzymes. Mechanisms of biochar's effect on extracellular enzyme activities are color-coded to match the plots in panels c and d. (c,d) Standardized coefficient estimates (with a range of between -1 and 1) of the direct and indirect effects of biochar on each of the five extracellular enzyme activities are plotted with standard errors displayed. The indirect effects were calculated by multiplying the coefficients of the response arrows by those of the effect arrows (marked in panel a). Asterisks indicate statistical significance of effects at $P < 0.05$.

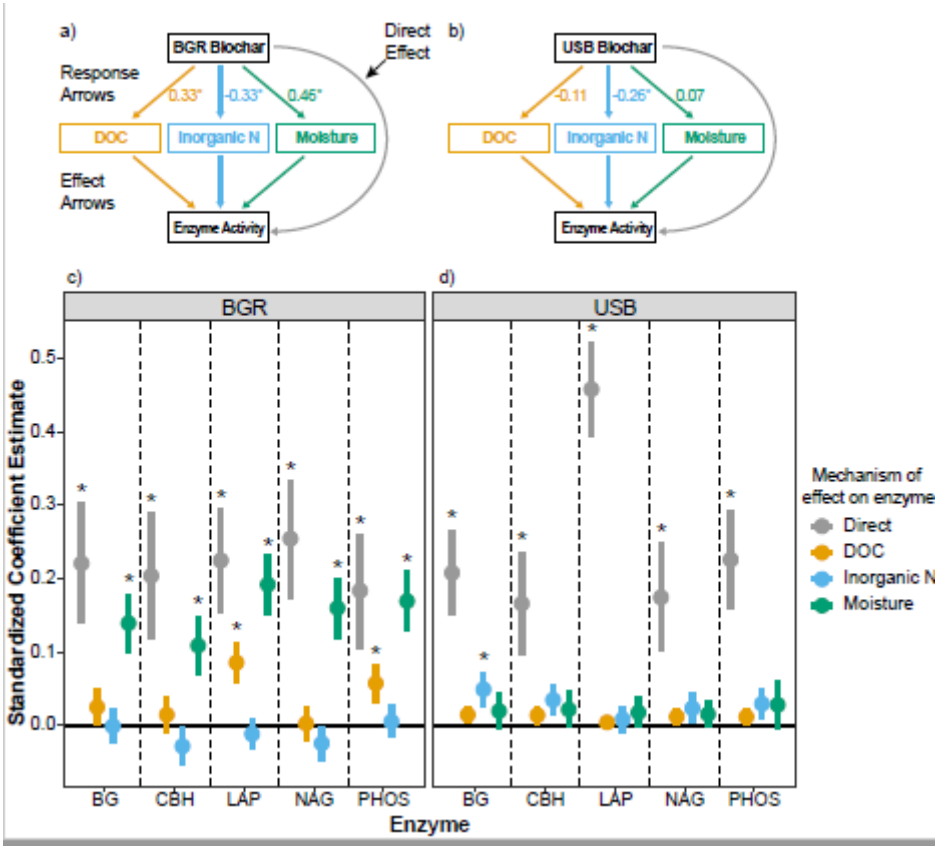


Figure 4. Scanning electron microscopy demonstrates possible interactions that biochar may have had with soil and microbes. (a) a piece of BGR biochar that was recovered from field soil three years after application still had a relatively intact pore structure. (b) a piece of USB

biochar still had noticeable pits in its xylem cell walls. (c) a piece of BGR biochar after three years in the field was crusted in soil particles and colonized by what appears to be a fungal hypha. (d) a piece of USB biochar after three years in the field was almost entirely covered in soil particles and may be serving as a nucleus for soil aggregation.

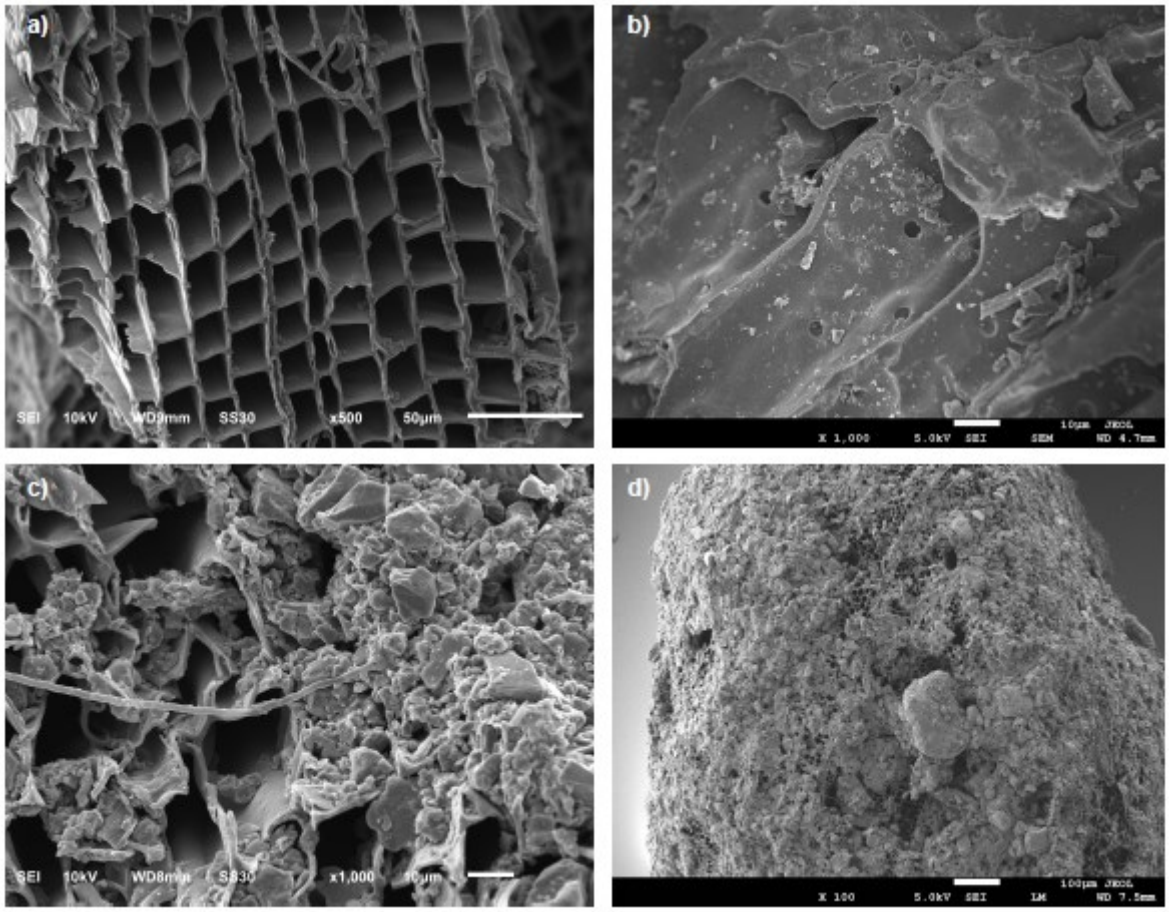


Figure 5. The percent surviving trees at six sampling dates across 2016, 2017, and 2018 is plotted for blue spruce (a) and balsam fir (b). A proxy of tree biomass (calculated by multiplying tree basal area by tree height) is plotted for blue spruce (c) and balsam fir (d) trees. In all plots, significant differences (Bonferroni-corrected $P < 0.05$) of biochar treatments from the control treatments is indicated by asterisks that are color coded to match the legend. Chi-square tests were used to test significant differences in tree survival and t-tests were used to test significant differences in the biomass proxy.

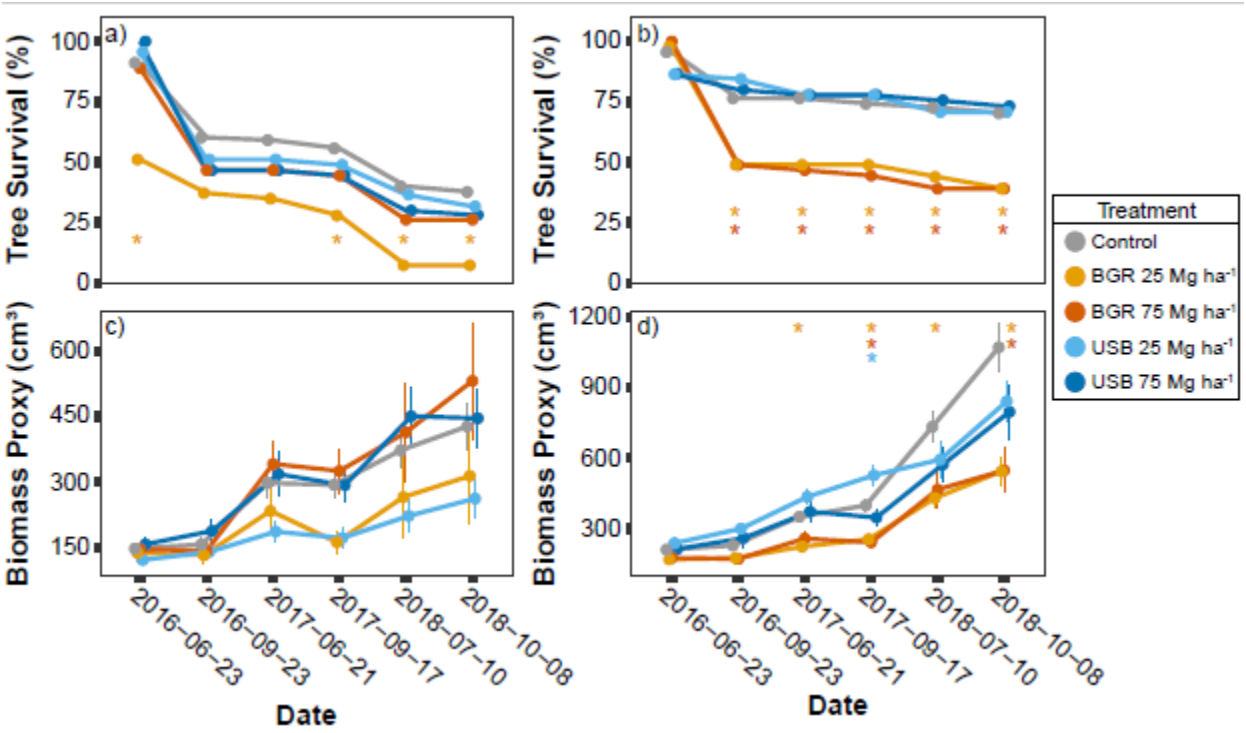


Figure 6. Patterns in root-associated fungal alpha diversity are plotted for the full fungal community (a) and the ectomycorrhizal fungal community (b). The number of observed OTUs and the Shannon Index were calculated as measures of alpha diversity. Error bars represent standard deviations. Asterisks indicate significant differences between the biochar treatments and the control based on pairwise t-tests with Bonferroni-corrected p-values.

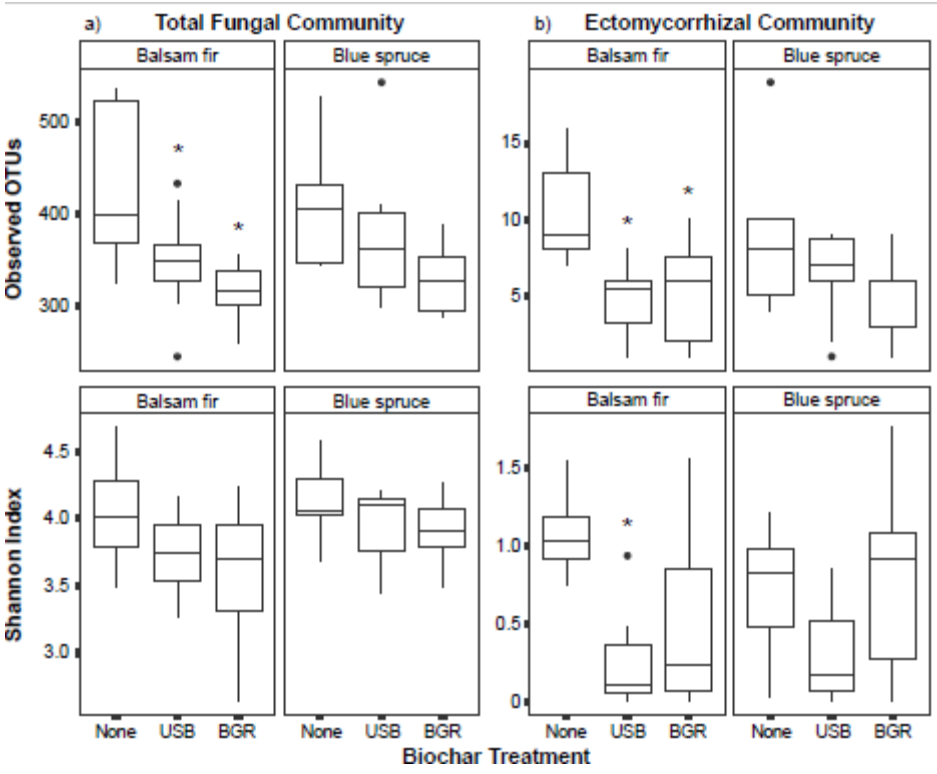
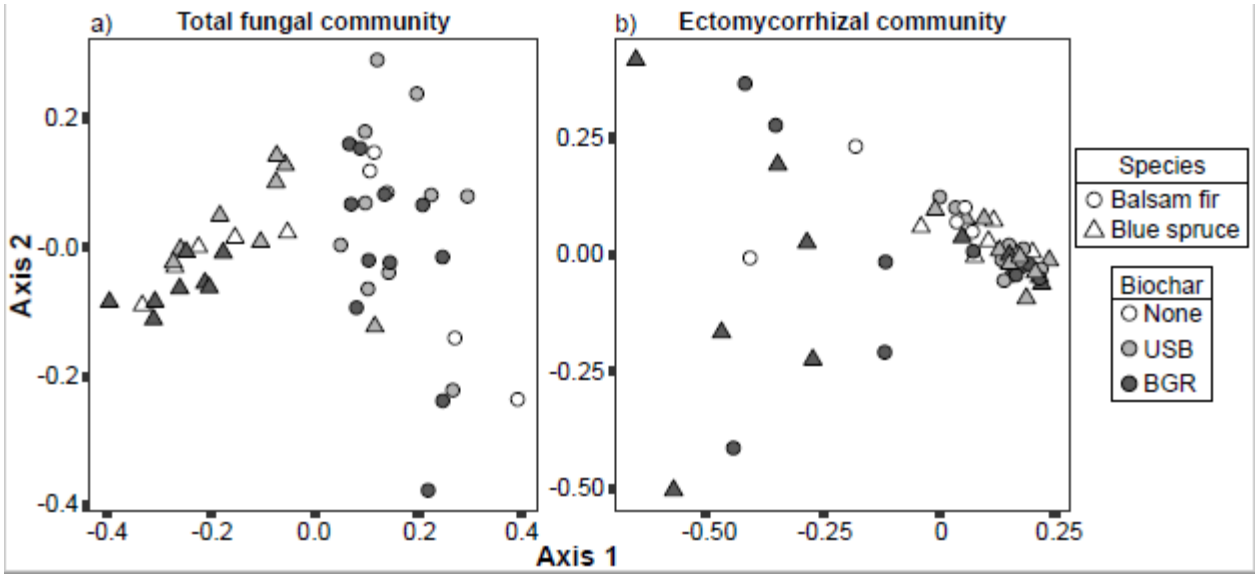


Figure 7. Principal coordinate analyses are plotted for the full fungal community (a) and the ectomycorrhizal fungal community (b) based on sequencing of ITS2 DNA amplicons prepared from root and rhizosphere samples. Points are colored by the biochar treatment and have different shapes based on the tree species.



Literature Cited:

Al Marzooqi, F., and L.F. Yousef 2017. Biological response of a sandy soil treated with biochar derived from a halophyte (*Salicornia bigelovii*). Appl. Soil Ecol. 114:9-15.

Ali, S., M. Rizwan, M.F. Qayyum, Y.S. Ok, M. Ibrahim, M. Riaz, M.S. Arif, F. Hafeez, M.I. Al-Wabel, and A.N. Shahzad. 2017. Biochar soil amendment on alleviation of drought and salt stress in plants: a critical review. Environ. Sci. Pollu. R. 24:12700-12712.

Allison, S.D., and P.M. Vitousek. 2005. Responses of extracellular enzymes to simple and complex nutrient inputs. Soil Biol. Biochem. 37:937-944.

Andrews, S. 2010. FastQC: a quality control tool for high throughput sequence data [Online]. Available at http://www.bioinformatics.babraham.ac.uk/projects/fastqc.

Baar, J., T.R. Horton, A. Kretzer, and T.D. Bruns. 1999. Mycorrhizal colonization of *Pinus muricata* from resistant propagules after a stand- replacing wildfire. New Phytol. 143:409-418.

Bailey, V.L., S.J. Fansler, J.L. Smith, and H. Bolton Jr. 2011. Reconciling apparent variability in effects of biochar amendment on soil enzyme activities by assay optimization. Soil Biol. Biochem. 43:296-301.

Baxter, J.W., and J. Dighton. 2001. Ectomycorrhizal diversity alters growth and nutrient acquisition of grey birch (*Betula populifolia*) seedlings in host–symbiont culture conditions. New Phytol. 152:139-149.

Benucci, G.M.N., C. Lefevre, and G. Bonito. 2016. Characterizing root-associated fungal communities and soils of Douglas-fir (*Pseudotsuga menziesii*) stands that naturally produce Oregon white truffles (*Tuber oregonense* and *Tuber gibbosum*). Mycorrhiza 26:367-376.

Benucci, G.M.N., R. Longley, P. Zhang, Q. Zhao, G. Bonito, and F. Yu. 2019. Microbial communities associated with the black morel *Morchella sextelata* cultivated in greenhouses. PeerJ. 7:e7744.

Bergstrom, D., C. Monreal, A. Tomlin, and J. Miller. 2000. Interpretation of soil enzyme activities in a comparison of tillage practices along a topographic and textural gradient. Can. J. Soil Sci. 80:71-79.

Biederman, L.A., and W.S. Harpole. 2013. Biochar and its effects on plant productivity and nutrient cycling: a meta- analysis. GCB bioenergy. 5:202-214.

Bieser, J.M., and S.C. Thomas. 2019. Biochar and high-carbon wood ash effects on soil and vegetation in a boreal clearcut. Can. J. For. Res. 49:1124-1134.

Bokulich, N.A., B.D. Kaehler, J.R. Rideout, M. Dillon, E. Bolyen, R. Knight, G.A. Huttley, and J.G. Caporaso. 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome. 6:90.

Bolyen, E., J.R. Rideout, M.R. Dillon, N.A. Bokulich, C.C. Abnet, G.A. Al-Ghalith, H. Alexander, E.J. Alm, M. Arumugam, and F. Asnicar. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37:852-857.

Bonanomi, G., F. Ippolito, G. Cesarano, B. Nanni, N. Lombardi, A. Rita, A. Saracino, and F. Scala. 2017. Biochar as plant growth promoter: better off alone or mixed with organic amendments? Front. Plant Sci. 8:1570.

Brookes, P., A. Landman, G. Pruden, and D. Jenkinson. 1985. Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biol. Biochem. 17:837-842.

Bruckman, V.J. and J. Pumpanen. 2019. Biochar use in global forests: Opportunities and challenges. P 427-453. *In* Busse, M., C.P. Giardina, D.M. Morris, and D.S. Page-Dumroese (ed.) Developments in Soil Science. Elsevier, Amsterdam, NL.

Burns, R.G., J.L. DeForest, J. Marxsen, R.L. Sinsabaugh, M.E. Stromberger, M.D. Wallenstein, M.N. Weintraub, and A. Zoppini. 2013. Soil enzymes in a changing environment: current knowledge and future directions. Soil Biol. Biochem. 58:216-234.

Chen, K.H., H.L. Liao, A.E. Arnold, G. Bonito, and F. Lutzoni. 2018. RNA- based analyses reveal fungal communities structured by a senescence gradient in the moss *Dicranum scoparium* and the presence of putative multi- trophic fungi. New Phytol. 218:1597-1611.

Dai, Y., H. Zheng, Z. Jiang, and B. Xing. 2020. Combined effects of biochar properties and soil conditions on plant growth: A meta-analysis. Sci. Total Environ. 713:136635.

Danielson, R., and M. Pruden. 1989. The ectomycorrhizal status of urban spruce. Mycologia. 81:335-341.Deenik, J.L., T. McClellan, G. Uehara, M.J. Antal, and S. Campbell. 2010. Charcoal volatile matter content influences plant growth and soil nitrogen transformations. Soil. Sci. Soc. Am. J. 74:1259-1270.

Dutta, T., E. Kwon, S.S. Bhattacharya, B.H. Jeon, A. Deep, M. Uchimiya, and K.H. Kim. 2017. Polycyclic aromatic hydrocarbons and volatile organic compounds in biochar and biochar- amended soil: a review. GCB Bioenergy. 9:990-1004.

Edgar, R.C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10:996-998.

Edgar, R.C. 2016. UCHIME2: improved chimera prediction for amplicon sequencing. BioRxiv. 074252.

Edgar, R.C., and H. Flyvbjerg. 2015. Error filtering, pair assembly and error correction for next-generation sequencing reads. Bioinformatics. 31:3476-3482.

Elzobair, K.A., M.E. Stromberger, and J.A. Ippolito. 2016a. Stabilizing effect of biochar on soil extracellular enzymes after a denaturing stress. Chemosphere. 142:114-119.

Elzobair, K.A., M.E. Stromberger, J.A. Ippolito, and R.D. Lentz. 2016b. Contrasting effects of biochar versus manure on soil microbial communities and enzyme activities in an Aridisol. Chemosphere. 142:145-152.

Enders, A., K. Hanley, T. Whitman, S. Joseph, and J. Lehmann. 2012. Characterization of biochars to evaluate recalcitrance and agronomic performance. Bioresour. Technol. 114:644-653.

Erland, S., B, Söderström, and S. Andersson. 1990. Effects of liming on ectomycorrhizal fungi infecting *Pinus sylvestris* L. II. Growth rates in pure culture at different pH values compared to growth rates in symbiosis with the host plant. New Phytol. 115:683-688.

Eyles, A., S.A. Bound, G. Oliver, R. Corkrey, M. Hardie, S. Green, and D.C. Close. 2015. Impact of biochar amendment on the growth, physiology and fruit of a young commercial apple orchard. Trees. 29:1817-1826.

Gardes, M., and T.D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes — application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113-8.

Gaskin, J.W., R.A. Speir, K. Harris, K. Das, R.D. Lee, L.A. Morris, and D.S. Fisher. 2010. Effect of peanut hull and pine chip biochar on soil nutrients, corn nutrient status, and yield. Agron. J. 102:623-633.

German, D.P., M.N. Weintraub, A.S. Grandy, C.L. Lauber, Z.L. Rinkes, and S.D. Allison. 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol. Biochem. 43:1387-1397.

Glisczynski, F.V., R. Pude, W. Amelung, and A. Sandhage- Hofmann. 2016. Biochar-compost substrates in short- rotation coppice: Effects on soil and trees in a three-year field experiment. J. Plant Nutr. Soil Sci .179:574-583.

Graber, E., L. Tsechansky, Z. Gerstl, and B. Lew. 2012. High surface area biochar negatively impacts herbicide efficacy. Plant Soil. 353:95-106.

Grandy, A.S., R.L. Sinsabaugh, J.C. Neff, M. Stursova, and D.R. Zak. 2008. Nitrogen deposition effects on soil organic matter chemistry are linked to variation in enzymes, ecosystems and size fractions. Biogeochemistry. 91:37-49.

Gundale, M.J., and T.H. DeLuca. 2006. Temperature and source material influence ecological attributes of ponderosa pine and Douglas-fir charcoal. For. Ecol. Manage. 231:86-93.

Gundale, M.J., and T.H. DeLuca. 2007. Charcoal effects on soil solution chemistry and growth of *Koeleria macrantha* in the ponderosa pine/Douglas-fir ecosystem. Biol. Fertil. Soils 43:303-311.

Hagner, M., R. Kemppainen, L. Jauhiainen, K. Tiilikkala, and H. Setälä. 2016. The effects of birch (*Betula* spp.) biochar and pyrolysis temperature on soil properties and plant growth. Soil Tillage Res. 163:224-234.

Hart, J.M., C.G. Landgren, R.A. Fletcher, M.C. Bondi, B.A. Whithrow-Robinson, and G.A. Chastagner. 2009. Christmas tree nutrient management guide: western Oregon and Washington. Agric. Exp. Stn., Oregon State University, Corvallis.

Hockaday, W.C., A.M. Grannas, S. Kim, and P.G. Hatcher. 2007. The transformation and mobility of charcoal in a fire-impacted watershed. Geochim. Cosmochim. Acta. 71:3432-3445.

Hornung, M. 1985. Acidification of soils by trees and forests. Soil Use Manag. 1:24-27.

Hung, L.L., and J.M. Trappe. 1983. Growth variation between and within species of ectomycorrhizal fungi in response to pH in vitro. Mycologia. 75:234-241.

Ihaka, R., and R. Gentleman. 1996. R: a language for data analysis and graphics. J. Comput. Graph. Stat. 5:299-314.

IBI (2015). *Standardized product definition and product testing guidelines for biochar that is used in soil*. (IBI biochar standards V.2.1). IBI.

Ishii, T., and K. Kadoya. 1994. Effects of charcoal as a soil conditioner on citrus growth and vesicular-arbuscular mycorrhizal development. J. Japanese Soc. Horticult. Sci. 63:529-535.

Jeffery, S., D. Abalos, M. Prodana, A.C. Bastos, J.W. van Groenigen, B.A. Hungate, and F. Verheijen. 2017. Biochar boosts tropical but not temperate crop yields. Environ. Res. Lett. 12:053001.

Jeffery, S., F.G. Verheijen, M. van der Velde, and A.C. Bastos. 2011. A quantitative review of the effects of biochar application to soils on crop productivity using meta-analysis. Agric., Ecosyst. Environ. 144:175-187.

Jenkinson, D.S., P.C. Brookes, and D.S. Powlson. 2004. Measuring soil microbial biomass. Soil Biol. Biochem. 36:5-7.

Jones, M.D., B.D. Twieg, V. Ward, J. Barker, D.M. Durall, and S.W. Simard. 2010. Functional complementarity of Douglas- fir ectomycorrhizas for extracellular enzyme activity after wildfire or clearcut logging. Funct. Ecol. 24:1139-1151.

Jonsson, L.M., M.C. Nilsson, D.A. Wardle, and O. Zackrisson. 2001. Context dependent effects of ectomycorrhizal species richness on tree seedling productivity. Oikos. 93:353-364.

Khadem, A., and F. Raiesi. 2017. Influence of biochar on potential enzyme activities in two calcareous soils of contrasting texture. Geoderma. 308:149-158.

Khadem, A., and F. Raiesi. 2019. Response of soil alkaline phosphatase to biochar amendments: Changes in kinetic and thermodynamic characteristics. Geoderma. 337:44-54.

Kim, H.-Y. 2013. Statistical notes for clinical researchers: assessing normal distribution using skewness and kurtosis. Restorative Dentistry and Endodontics. 38:52-54.

Köhler, J., N. Yang, R. Pena, V. Raghavan, A. Polle, and I.C. Meier. 2018. Ectomycorrhizal fungal diversity increases phosphorus uptake efficiency of European beech. New Phytol. 220:1200-1210.

Kõljalg, U., K.H. Larsson, K. Abarenkov, R.H. Nilsson, I.J. Alexander, U. Eberhardt, S. Erland, K. Høiland, R. Kjøller, and E. Larsson. 2005. UNITE: a database providing web- based methods for the molecular identification of ectomycorrhizal fungi. New Phytol. 166: 1063-1068.

Lammirato, C., A. Miltner, and M. Kaestner. 2011. Effects of wood char and activated carbon on the hydrolysis of cellobiose by β-glucosidase from *Aspergillus nige*r. Soil Biol. Biochem. 43:1936-1942.

Landgren, C.G., R.A. Fletcher, M.C. Bondi, D.L. Barney, and R.L. Mahoney. 2003. Growing Christmas trees in the Pacific Northwest. Agric. Exp. Stn., Oregon State University, Corvallis.

Lehmann, J., J. Gaunt, and M. Rondon. 2006. Bio-char sequestration in terrestrial ecosystems–a review. Mitig. Adapt. Strateg. Glob. Chang. 11:403-427.

Lehmann, J., and S. Joseph. 2009. Biochar for environmental management: an introduction. Biochar for environmental management: Science and technology. 1:1-12.

Lehmann, J., M.C. Rillig, J. Thies, C.A. Masiello, W.C. Hockaday, and D. Crowley. 2011. Biochar effects on soil biota–a review. Soil Biol. Biochem. 43:1812-1836.

Lehmann, J., C. Czimczik, D. Laird, and S. Sohi. 2012. Stability of biochar in soil. p. 215-238. *In* J. Lehmann and S. Joseph (ed.) Biochar for environmental management. Routledge, New York.

Lenth, R., H. Singmann, J. Love, P. Buerkner, and M. Herve. 2018. Emmeans: Estimated marginal means, aka least-squares means. R package version 1, 3.

Liu, C.H., W. Chu, H. Li, S.A. Boyd, B.J. Teppen, J. Mao, J. Lehmann, and W. Zhang. 2019. Quantification and characterization of dissolved organic carbon from biochars. Geoderma. 335:161-169.

Liu, P., C.J. Ptacek, D.W. Blowes, W.R. Berti, and R.C. Landis. 2015. Aqueous leaching of organic acids and dissolved organic carbon from various biochars prepared at different temperatures. J. Environ. Qual. 44:684-695.

Liu, X., A. Zhang, C. Ji, S. Joseph, R. Bian, L. Li, G. Pan, and J. Paz-Ferreiro. 2013. Biochar's effect on crop productivity and the dependence on experimental conditions—a meta-analysis of literature data. Plant and Soil 373:583-594.

Lu, H., M.S. Lashari, X. Liu, H. Ji, L. Li, J. Zheng, G.W. Kibue, S. Joseph, and G. Pan. 2015. Changes in soil microbial community structure and enzyme activity with amendment of biochar-manure compost and pyroligneous solution in a saline soil from Central China. Eur. J. Soil Biol. 70:67-76.

Lundberg, D.S., S. Yourstone, P. Mieczkowski, C.D. Jones, and J.L. Dangl. 2013. Practical innovations for high-throughput amplicon sequencing. Nat. Methods. 10:999-1002.

Luo, G., L. Li, V.P. Friman, J. Guo, S. Guo, Q. Shen, and N. Ling. 2018. Organic amendments increase crop yields by improving microbe-mediated soil functioning of agroecosystems: A meta-analysis. Soil Biol. Biochem. 124:105-115.

Major, J. 2010. Guidelines on Practical Aspects of Biochar Application to Field Soil in Various Soil Management Systems. IBI. 23p.

Major, J., A. DiTommaso, J. Lehmann, and N.P. Falcao. 2005. Weed dynamics on Amazonian Dark Earth and adjacent soils of Brazil. Agric., Ecosyst. Environ. 111:1-12.

Major, J., M. Rondon, D. Molina, S.J. Riha, and J. Lehmann. 2010. Maize yield and nutrition during 4 years after biochar application to a Colombian savanna oxisol. Plant Soil. 333:117-128.

Mandal, S., W. van Treuren, R.A. White, M. Eggesbø, R. Knight, and S.D. Peddada. 2015. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microb. Ecol. Health Dis. 26:27663.

Martin, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal. 17:10-12.

Masto, R.E., S. Kumar, T. Rout, P. Sarkar, J. George, and L. Ram. 2013. Biochar from water hyacinth (*Eichornia crassipes*) and its impact on soil biological activity. Catena. 111:64-71.

Matsubara, Y., N. Hasegawa, and H. Fukui. 2002. Incidence of *Fusarium* root rot in asparagus seedlings infected with arbuscular mycorrhizal fungus as affected by several soil amendments. J. Japanese Soc. Horticult. Sci. 71:370-374.

McMurdie, P.J., and S. Holmes. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one 8:e61217.

Mikola, P. 1988. Ectendomycorrhiza of conifers. Silva Fennica. 22:19-27

Mukherjeem, A., and A.R. Zimmerman. 2013. Organic carbon and nutrient release from a range of laboratory-produced biochars and biochar–soil mixtures. Geoderma. 193:122-130.

Nguyen, N.H., Z. Song, S.T. Bates, S. Branco, L. Tedersoo, J. Menke, J.S. Schilling, and P.G. Kennedy. 2016. FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild. Fungal Ecol. 20:241-248.

Novak, J.M., K.B. Cantrell, D.W. Watts, W.J. Busscher, and M.G. Johnson. 2014. Designing relevant biochars as soil amendments using lignocellulosic-based and manure-based feedstocks. J. Soils Sed. 14:330-343.

O'Neil, C.M., J. Nash, L.K. Tiemann, and J.R. Miesel. 2021. Mycorrhizal Symbioses Enhance Competitive Weed Growth in Biochar and Nutrient-Amended Soils. Front. Agron. 3.

Paz-Ferreiro, J., S. Fu, A. Méndez, and G. Gascó. 2014. Interactive effects of biochar and the earthworm *Pontoscolex corethrurus* on plant productivity and soil enzyme activities. J. Soils Sed. 14:483-494.

Paz-Ferreiro, J., G. Gascó, B. Gutiérrez, and A. Méndez. 2012. Soil biochemical activities and the geometric mean of enzyme activities after application of sewage sludge and sewage sludge biochar to soil. Biol. Fertil. Soils. 48:511-517.

Pinheiro, J., D. Bates, S. DebRoy, D. Sarkar, and R Core Team. 2013. nlme: Linear and nonlinear mixed effects models. R package version 3:111.

Pluchon, N., M.J. Gundale, M.C. Nilsson, P. Kardol, and D.A. Wardle. 2014. Stimulation of boreal tree seedling growth by wood- derived charcoal: effects of charcoal properties, seedling species and soil fertility. Funct. Ecol. 28:766-775.

Pokharel, P., Z. Ma, and S.X. Chang. 2020. Biochar increases soil microbial biomass with changes in extra-and intracellular enzyme activities: a global meta-analysis. Biochar 2:65-79.

Ren, H., C. Lv, V. Fernández-García, B. Huang, J. Yao, and W. Ding. 2019. Biochar and PGPR amendments influence soil enzyme activities and nutrient concentrations in a eucalyptus seedling plantation. Biomass Convers. Bioref. 1-10.

Robertson, S.J., P.M. Rutherford, J.C. Lopez-Gutierrez, and H.B. Massicotte. 2012. Biochar enhances seedling growth and alters root symbioses and properties of sub-boreal forest soils. Can. J. Soil Sci. 92:329-340.

Rockwood, D.L., M.F. Ellis, R. Liu, F. Zhao, P. Ji, Z. Zhu, K.W. Fabbro, Z. He, and R.D. Cave. 2019. Short rotation Eucalypts: opportunities for biochar. Forests 10:314.

Rondon, M.A., J. Lehmann, J. Ramírez, and M. Hurtado. 2007. Biological nitrogen fixation by common beans (*Phaseolus vulgaris* L.) increases with bio-char additions. Biol. Fertil. Soils. 43:699-708.

Rosseel, Y. 2012. Lavaan: An R package for structural equation modeling and more. Version 0.5–12 (BETA). J. Stat. Softw. 48:1-36.

Saiya-Cork, K., R. Sinsabaugh, and D.R. Zak. 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biol. Biochem. 34:1309-1315.

Sarauer, J.L., D.S. Page- Dumroese, and M.D. Coleman. 2019. Soil greenhouse gas, carbon content, and tree growth response to biochar amendment in western United States forests. GCB Bioenerg. 11:660-671.

Sinsabaugh, R., H. Reynolds, and T. Long. 2000. Rapid assay for amidohydrolase (urease) activity in environmental samples. Soil Biol. Biochem. 32:2095-2097.

Sinsabaugh, R.L., C.L. Lauber, M.N. Weintraub, B. Ahmed, S.D. Allison, C. Crenshaw, A.R. Contosta, D. Cusack, S. Frey, and M.E. Gallo. 2008. Stoichiometry of soil enzyme activity at global scale. Ecol. Lett. 11:1252-1264.

Smebye, A., V. Alling, R.D. Vogt, T.C. Gadmar, J. Mulder, G. Cornelissen, and S.E. Hale. 2016. Biochar amendment to soil changes dissolved organic matter content and composition. Chemosphere. 142:100-105.

Sohi, S.P., E. Krull, E. Lopez-Capel, and R. Bol. 2010. A review of biochar and its use and function in soil. p. 47-82. *In* D. Sparks (ed.) Advances in agronomy. Vol. 105. Elsevier, Amsterdam, NL.

Sorrenti, G., M. Ventura, and M. Toselli. 2016. Effect of biochar on nutrient retention and nectarine tree performance: A three- year field trial. J. Plant Nutrit. Soil Sci. 179:336-346.

Spokas, K., W. Koskinen, J. Baker, and D. Reicosky. 2009. Impacts of woodchip biochar additions on greenhouse gas production and sorption/degradation of two herbicides in a Minnesota soil. Chemosphere. 77:574-581.

Taylor, A.F., and R.D. Finlay. 2003. Effects of liming and ash application on below ground ectomycorrhizal community structure in two Norway spruce forests. Water Air Soil Pollut. 3:63-76.

Taylor, D.L., W.A. Walters, N.J. Lennon, J. Bochicchio, A. Krohn, J.G. Caporaso, and T. Pennanen. 2016. Accurate estimation of fungal diversity and abundance through improved lineage-specific primers optimized for Illumina amplicon sequencing. Appl. Environ. Microbiol. 82:7217-7226.

Theodorou, C., and G. Bowen. 1969. The influence of pH and nitrate on mycorrhizal associations of *Pinus radiata* D. Don. Aust. J. Bot. 17:59-67.

Thomas, S.C. and N. Gale. 2015. Biochar and forest restoration: a review and meta-analysis of tree growth responses. New Forests. 46:931-946.

Trevor, E.Y., K.N. Egger, and L.R. Peterson. 2001. Ectendomycorrhizal associations–characteristics and functions. Mycorrhiza 11:167-177.

van Zwieten, L., S. Kimber, S. Morris, K. Chan, A. Downie, J. Rust, S. Joseph, and A. Cowie. 2010. Effects of biochar from slow pyrolysis of papermill waste on agronomic performance and soil fertility. Plant Soil. 327:235-246.

Vance, E.D., P.C. Brookes, and D.S. Jenkinson. 1987. An extraction method for measuring soil microbial biomass C. Soil Biol. Biochem. 19:703-707.

Wang, X., D. Song, G. Liang, Q. Zhang, C. Ai, and W. Zhou. 2015. Maize biochar addition rate influences soil enzyme activity and microbial community composition in a fluvo-aquic soil. Appl. Soil Ecol. 96:265-272.

Warnock, D.D., J. Lehmann, T.W. Kuyper, and M.C. Rillig. 2007. Mycorrhizal responses to biochar in soil–concepts and mechanisms. Plant Soil. 300:9-20.

Warnock, D.D., D.L. Mummey, B. McBride, J. Major, J. Lehmann, and M.C. Rillig. 2010. Influences of non-herbaceous biochar on arbuscular mycorrhizal fungal abundances in roots and soils: results from growth-chamber and field experiments. Appl. Soil Ecol. 46:450-456.

White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. p. 315-322. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White (ed.) PCR Protocols, a guide to methods and applications. Academic Press, San Diego, CA.

Wittbrodt, B.T., D. Squires, J. Walbeck, E. Campbell, W. Campbell, and J.M. Pearce. 2015. Open-source photometric system for enzymatic nitrate quantification. PloS one. 10:e0134989.

Wolfe, B.E., V.L. Rodgers, K.A. Stinson, and A. Pringle. 2008. The invasive plant *Alliaria petiolata* (garlic mustard) inhibits ectomycorrhizal fungi in its introduced range. J. Ecol. 96:777-783.

Wu, F., Z. Jia, S. Wang, S.X. Chang, and A. Startsev. 2013. Contrasting effects of wheat straw and its biochar on greenhouse gas emissions and enzyme activities in a Chernozemic soil. Biol. Fertil. Soils 49:555-565.

Wu, H., X. Dong, and H. Liu. 2018. Evaluating fluorescent dissolved organic matter released from wetland-plant derived biochar: effects of extracting solutions. Chemosphere. 212:638-644.

Xiao, W., X. Chen, X. Jing, and B. Zhu. 2018. A meta-analysis of soil extracellular enzyme activities in response to global change. Soil Biol. Biochem. 123:21-32.

Yu, X.Y., G.G. Ying, and R.S. Kookana. 2006. Sorption and desorption behaviors of diuron in soils amended with charcoal. J. Agric. Food Chem. 54:8545-8550.

Zhang, L., Y. Jing, Y. Xiang, R. Zhang, and H. Lu. 2018. Responses of soil microbial community structure changes and activities to biochar addition: a meta-analysis. Sci. Total Environ. 643:926-935.

Zhang, X., H. Wang, L. He, K. Lu, A. Sarmah, J. Li, N.S. Bolan, J. Pei, and H. Huang. 2013. Using biochar for remediation of soils contaminated with heavy metals and organic pollutants. Environ. Sci. Pollut. Res. 20:8472-8483.

Zhao, Q., A.T. Classen, W.W. Wang, X.R. Zhao, B. Mao, and D.H. Zeng. 2017. Asymmetric effects of litter removal and litter addition on the structure and function of soil microbial communities in a managed pine forest. Plant and Soil. 414:81-93.

Zheng, W., M. Guo, T. Chow, D.N. Bennett, and N. Rajagopalan. 2010. Sorption properties of greenwaste biochar for two triazine pesticides. J. Hazard. Mater. 181:121-126.

Zhou, C., K. Heal, M. Tigabu, L. Xia, H. Hu, D. Yin, and X. Ma. 2020. Biochar addition to forest plantation soil enhances phosphorus availability and soil bacterial community diversity. For. Ecol. Manage. 455:117635.