Soil Biology & Biochemistry 58 (2013) 248–254

Contents lists available at SciVerse ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Nitrogen, biochar, and mycorrhizae: Alteration of the symbiosis and oxidation of the char surface

Chase LeCroy [a,1], Caroline A. Masiello [a,*], Jennifer A. Rudgers [b,2], William C. Hockaday [c], Jonathan J. Silberg [d]

[a] Department of Earth Science, Rice University, Ms 126, 6100 Main St., Houston, TX 77030, USA
[b] Department of Ecology and Evolutionary Biology, Rice University, Ms 170, 6100 Main St., Houston, TX 77030, USA
[c] Department of Geology, Baylor University, One Bear Place #97354, Waco, TX 76798, USA
[d] Department of Biochemistry and Cell Biology, Rice University, Ms 140, 6100 Main St., Houston, TX 77030, USA

ARTICLE INFO

Article history:
Received 13 August 2012
Received in revised form
28 November 2012
Accepted 30 November 2012
Available online 25 December 2012

Keywords:
Biochar
Mycorrhizae
XPS
Charcoal
Black carbon

ABSTRACT

In some cases amending soil with biochar improves fertility, although the exact mechanisms through which biochar alters soil processes are not well understood. In other cases, however, biochar amendment can have no effect on plant growth, or can have negative effects. When crop benefits occur, simultaneous amendment with biochar and mineral nutrients causes results that are not additive, suggesting that biochar may be capable of improving the efficiency of nutrient uptake by plants, but the mechanisms of this synergy remain unknown. One possible mechanism that has not been fully explored is alterations to the plant–mycorrhizal fungus mutualism, a relationship that occurs in most land plants. In a 4 week greenhouse experiment, we investigated possible effects of the presence of biochar, mycorrhizal fungi, and nitrogen fertilizer on sorghum seedling growth. Results indicated that the combined treatment of biochar, mycorrhizal fungi, and high nitrogen decreased aboveground plant biomass by 42% relative to the mycorrhizae and high nitrogen treatment, while simultaneously promoting mycorrhizal root colonization. This is evidence for an induced parasitism of the mycorrhizal fungus in the presence of nitrogen and biochar within the 4 week timescale of our experiments.

Using x-ray photoelectron spectroscopy, we found evidence of increased surface oxidation on biochar particles over the 4 weeks of our trial, consistent with sorption of labile, plant derived dissolved organic matter or char oxidation, either via biotic or abiotic processes. Biochar in soils with mycorrhizae but without sufficient nitrogen showed more surface oxidation than other treatment combinations, and showed a significantly greater fraction of surface carbon present in carbonyl (–C=O) functionalities. Our results suggest that soil nitrogen acts as a switch controlling the ability of char to influence the mycorrhizal symbiosis and, in turn, the degree to which the fungi oxidize the char surface.

1. Introduction

Of the emerging renewable energy and biofuel technologies, biomass pyrolysis (thermal conversion and/or gasification) stands out as a particularly promising option because of its ancillary benefits, not the least of which is a non-traditional approach to carbon capture and sequestration. The thermal decomposition of biomass under the partial exclusion of oxygen is an exothermic reaction that yields heat, water, combustible gases, and organic liquids (bio-oils) (Bridgwater, 2003). About 30% of the initial biomass carbon can also be retained in the form of charcoal (called biochar if produced intentionally for C sequestration). The critical step in making biomass pyrolysis sustainable and carbon negative is the incorporation of the biochar into the soil, rather than its combustion for energy. The longevity of biochar carbon in soil is such that net carbon emissions for the process are negative over the timescale of centuries to millennia (Lehmann, 2007). Soil emissions in the form of carbon dioxide, methane, and nitrous oxide could be reduced by a theoretical maximum of 1.8 Pg CO_2–C equivalent per year, which would create a meaningful sink in comparison to current fossil fuel emissions of 8.7 Pg C per year (Boden et al., 2011; Woolf et al., 2010).

* Corresponding author.
E-mail address: masiello@rice.edu (C.A. Masiello).
[1] Now at: Department of Geography, UCLA, 1255 Bunche Hall, Box 951524, Los Angeles, CA 90095, USA.
[2] Now at: Department of Biology, University of New Mexico, Castetter Hall 286, MSC03 2020, Albuquerque, NM 87131-0001, USA.

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http://dx.doi.org/10.1016/j.soilbio.2012.11.023

Biochar's effect on plants and mycorrhizal fungi is poorly understood (but see Solaiman et al., 2010). The literature on this interaction is often contradictory: biochar has been shown to increase and decrease plant growth, pH, mycorrhizal colonization, and nutrient availability (Warnock et al., 2007), or simply have no effect (Habte and Antal, 2010). Biochar has been shown to improve crop productivity in some but not all circumstances (Bell and Worrall, 2011; Chan et al., 2007, 2008; Rajkovich et al., 2012; Schnell et al., 2012; Zhang et al., 2012a, 2012b). Also confounding is the variety of methods and materials used. For example, the effect of biochar on plant growth can depend on both the feedstock and pyrolysis process (Chan et al., 2008; Rajkovich et al., 2012). Potential mechanisms for generalized biochar improvement in plant growth include a reduction in soil tensile strength (Chan et al., 2007), improved plant access to soil nutrients (Warnock et al., 2007), improved soil water-holding capacity (Atkinson et al., 2010; Briggs et al., 2012; Chan et al., 2007; Glaser et al., 2002; Kinney et al., 2012; Liu et al., 2012; Novak et al., 2012; Revell et al., 2012), and/or changes in the efficiency of plant–fungal interactions (Atkinson et al., 2010; Thies and Rillig, 2009; Warnock et al., 2007). However, the mechanisms underlying effects of biochar on plant growth remain unresolved, making it difficult to predict the conditions under which biochar amendments to soil will promote plant productivity.

Other studies have shown that fungi not only respond to the presence of charcoal in soil, but also interact with it, altering it physically and chemically (Hockaday et al., 2006). Long-term environmental alterations of soil charcoal appear to be crucial in the development of an oxidized patina that delivers beneficial ecosystem services, such as high soil cation exchange capacity (Cheng et al., 2006, 2008). However, the timescale and mechanism of formation of this patina are poorly described.

Based on previous studies, we hypothesized that soil nitrogen availability would act to alter the plant–fungal relationship. We examined biochar-driven changes in both the plant–fungal relationship and the degree of char surface oxidation and tested whether these interactions vary as a function of nitrogen availability in the soil. We sought to determine if biochar alters plant interactions with mycorrhizal fungi in the presence of high versus low nitrogen fertilizer, and whether it is possible to determine how the relationship was altered by measuring (1) plant size, (2) mycorrhizal colonization, and (3) surface oxidation of the biochar particles. We addressed these questions through a 4 week greenhouse pot trial of sorghum plants grown with and without biochar, with and without mycorrhizal infection, and at high and low levels of nitrogen fertilization.

2. Methods

2.1. General approach to isolating mechanisms of biochar effects

Our experimental conditions were designed to focus only on biochar, nitrogen, and the plant–mycorrhizal relationship. Therefore, we selected a highly mycorrhizal plant (sorghum) for a growth trial in soil and hydrologic conditions intended to eliminate other hypothesized benefits of biochar. We selected sorghum because it is highly dependent upon fungal symbionts and because of its importance as an agricultural crop used for food and biofuel (Hata et al., 2010). We chose a soil matrix with a very low tensile strength (sand-enriched) to limit any potential tensile-strength reduction from biochar amendment. We also saturated the pots with water to insure that the presence of biochar did not introduce a bias by enhancing the soil water content. After four weeks, we quantified the levels of root colonization by arbuscular mycorrhizal fungi grown in conjunction with the sorghum and examined changes in the biochar surface chemistry using x-ray photoelectron spectroscopy (XPS).

2.2. Greenhouse experimental design

We grew sorghum ± biochar, ± ammonium nitrate fertilizer, and ± mycorrhizae in a sandy soil that was watered to saturation. Each treatment was replicated 30 times. We planted seeds in 115 ml Cone-tainer pots (Stuewe & Sons, model number SC7) filled with a 1:1 ratio of organic potting media (Metro-Mix SB 200, Sun Gro Horticulture Ltd.) and sand (Quikrete, Premium Play Sand) by volume. The Cone-tainers had holes at the bottom, allowing water to drain freely. We inoculated mycorrhizal fungi treatment pots with spores of *Glomus intraradices* (Mykos, Xtreme Gardening), an arbuscular mycorrhizal fungus, by adding 5 g of inoculum on top of the soil of each pot followed by a cap of sand to prevent contamination by splashing. Nitrogen treatment pots had ammonium nitrate (Aldrich, 99+% purity) added once per week at the rate of 34 kg N/ha in the high nitrogen treatment versus 6.7 kg N/ha in the low nitrogen. Biochar treatment pots had 2.6% biochar by weight added to the growing medium, equivalent to an agricultural amendment rate of 17.6 tons/ha applied at a tillage depth of 20 cm. We planted three sorghum seeds in each pot and at the first sign of growth above soil, we thinned the pots to one plant each. All pots were arranged in a fully randomized array in the greenhouse at 27–35 °C with no artificial lighting. Watering to saturation occurred every day for four weeks until the plants were harvested.

2.3. Biochar production

We produced biochar from apple wood (*Malus domestica*) sawdust (Allied Kenco, Houston, TX, USA). The wood fragments were 5–10 mm × 0.5–1 mm × 0.5–1 mm and the particle size decreased by 25–50% following pyrolysis. The sawdust was sealed within a 20 L batch reactor constructed from 306 stainless steel and heated in a propane-fired furnace. Pyrolysis gases were passively vented to a series of heat exchangers at ambient temperature (~30 °C) to remove condensable liquids and bio-oils – preventing their condensation into the biochar. Non-condensable gases were combusted in a secondary (venturi-style) burner used to heat the reactor. The reactor used in this experiment produces approximately 2 kg biochar per batch, and all of the biochar used in the pot trial was derived from the same batch. The initial heating rate was approximately 5 °C/min to a reactor temperature of ~400 °C. Total heating time was approximately 250 min. The reactor was allowed to cool to ambient temperature (overnight) before removing the biochar. When removed from the reactor, the biochar was odorless and visually homogenous. Biochar was manually mixed during removal from the reactor vessel to further ensure homogeneity.

2.4. Quantifying sorghum biomass

At harvest, we separated the aboveground and belowground portions and dried them in an oven at 45 °C for several days. Dry weights were taken for both portions.

2.5. Root colonization

We stained the roots of each plant using the ink and vinegar technique (Vierheilig et al., 1998). All stained roots were viewed through a Leica DM2500 microscope at 400× magnification. Thirty fields of view were observed for each sample and the number of views in which hyphae were present was recorded and converted into a percentage (McGonigle et al., 1990).

2.6. Changes in surface chemistry

We took advantage of the 4 week exposure period in our experiments to determine how the surface chemistry of char was altered by the rhizosphere environment. XPS was used because this method analyzes only the top 10 nm of the surface of particles. Initial reactions between biochar and its soil environment start at the surface, and XPS allows the sensitive detection of the onset of these reactions. We bulked and sieved the growth medium of each treatment to facilitate picking of biochar fragments using tweezers for XPS analysis on individual pieces of biochar. We used XPS to analyze 5 biochar fragments from each treatment as well as from fresh biochar.

We determined the bonding environments of carbon and oxygen atoms on the surface of biochar particles using a PHI Quantera XPS with an Al X-ray source at 1486.6 eV and 40.7 W. The beam diameter was 200.0 μm and the pass energy 26 eV. High resolution, low intensity scans were performed to focus on the bonding environments. The C1s electron was targeted between 280 and 290 eV with 40 scans. The O1s electron was targeted between 528 and 538 eV with 30 scans. We analyzed and deconvoluted spectra into their component functional groups using MultiPak data analysis software (MultiPak V7.0.1, 04 Mar 16, Ulvac-Phi, Inc., 1994–2004). Images of biochar particles before and after exposure to soil + plants are available in the Supplemental Material (Figs. S1 and S2).

2.7. Statistical analysis

We performed factorial ANOVA using SAS statistical software (SAS v. 9.2, SAS Institute, Inc., Cary, NC). ANOVA models included the response variables of biomass, mycorrhizal colonization rate, and bonding environment percentages and the fixed effects of the biochar treatment (+/−), mycorrhizal fungi treatment (+/−) and nitrogen treatment (high/low) as well as all possible interactions.

3. Results and discussion

3.1. Above- and below-ground biomass: evidence for biochar- and nitrogen-induced fungal parasitism

In the presence of high nitrogen, biochar appeared to promote parasitism by mycorrhizal fungi in the initial stages of plant growth explored by this experiment. This is suggested by the decline in biomass relative to other high nitrogen treatment combinations (Figs. 1–3). The increase in productivity due to nitrogen addition was immediately recognizable in the bimodal distribution of biomass (Figs. 1 and 2). The treatment of biochar, mycorrhizae, and high nitrogen caused an increase in root colonization (Fig. 3) and a decrease in aboveground plant biomass with an average that was low enough to approach the level of the unfertilized treatments. We attribute the decrease in plant biomass to an induced parasitism on the part of the mycorrhizae. If the biochar provides an environment where nitrogen is more readily available to the roots directly, the mycorrhizal hyphae receive the benefit of organic substrates from the plant without providing nitrogen in return. Corroborating this hypothesis is the observed stimulation of mycorrhizal colonization (+B, +M, high N, Fig. 3; see next section).

For belowground biomass (Fig. 2), statistical analysis revealed no significant effect other than the increase due to higher nitrogen levels. A marginal reduction in belowground biomass due to the presence of biochar was nearly significant ($p = 0.067$). This relationship could be driven by the same parasitism observed in the aboveground biomass, lending support to the idea that there is some mechanism by which biochar induces mycorrhizal parasitism at high levels of mineral nitrogen.

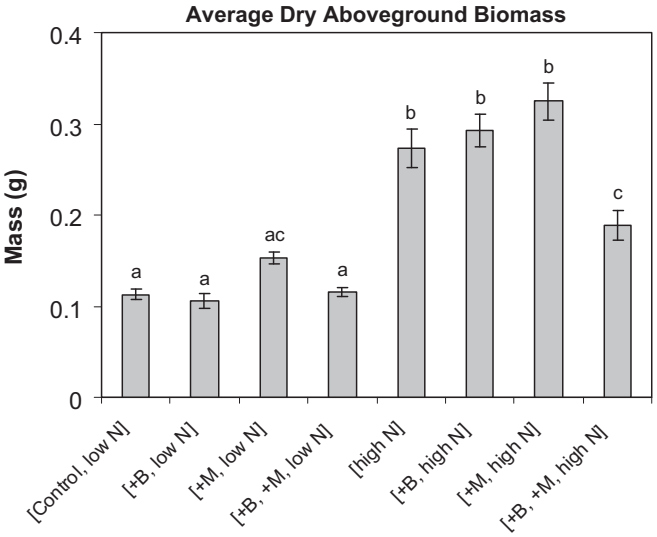

Fig. 1. Average mass of dried aboveground biomass from 30 plants per group. Different letters indicate values that are significantly different as determined by factorial ANOVA. Error bars represent standard error. ([+B]: Biochar added to soil, [+M]: Mycorrhizal inoculum added to soil, [low N]: fertilized with ammonium nitrate at 6.7 kg N/ha, [high N]: fertilized with ammonium nitrate at 34 kg N/ha.)

Both increases and decreases in mycorrhizal abundance have been observed in conjunction with biochar amendments, although an increase is more common (Lehmann et al., 2011). One of the mechanisms suggested by Lehmann et al. (2011) was a reduced utility of the symbiosis with high nutrient and water availability, but our study shows the opposite pattern: mycorrhizae flourished with high levels of nitrogen fertilizer and water. Solaiman et al. (2010) also observed an increase in mycorrhizal colonization in a biochar amendment wheat field trial, but in that case, increased mycorrhizal colonization was associated with positive cropping outcomes (an increase in grain yield). In another study, Elmer and Pignatello (2011) observed a linear trend of increasing mycorrhizal colonization with biochar amendment rate in asparagus plants. Their result reflects the colonization levels we observed in inoculated soils without biochar (+M, low N) and with biochar (+M, high N), but does not explain the inflated colonization that

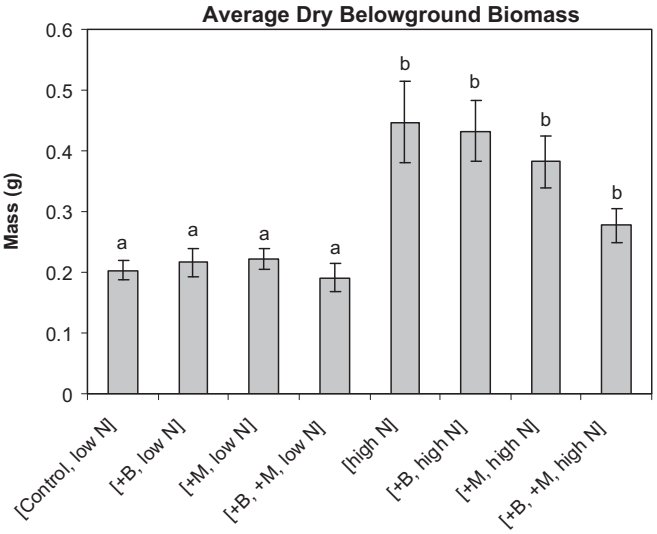

Fig. 2. Average mass of dried belowground biomass from 30 plants per group. Different letters indicate values that are significantly different as determined by factorial ANOVA. Error bars represent standard error.

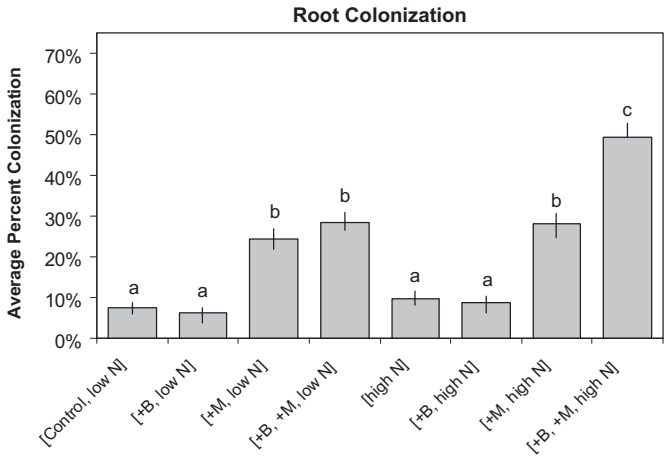

Fig. 3. Root colonization rates for ~25 plants per treatment. Different letters indicate values that are significantly different as determined by factorial ANOVA. Error bars represent one standard error. The [+B, +M, high N] treatment significantly increased colonization while decreasing aboveground and total plant biomass relative to other high N treatments.

occurred with the addition of both high nitrogen and biochar. Nitrogen additions have been observed to increase rates of colonization by mycorrhizal fungi in other studies, including studies on *G. intraradices* (e.g. (Hodge and Fitter, 2010; Smith and Smith, 2011)), and high levels of nutrients commonly cause mycorrhizal fungi to shift from plant mutualists to plant parasites (e.g. (Antunes et al., 2012; Johnson et al., 1997)). In our study, biochar accelerated the transition to parasitism in the presence of high nitrogen.

3.2. Biochar stimulates mycorrhizal colonization

The treatments with biochar and mycorrhizal inoculation had greater root colonization by the fungus, suggesting that the presence of biochar does stimulate mycorrhizal colonization of roots (Fig. 3). Fig. 4 shows the microscope view of a normal root (a) and a highly colonized root (b). The blue strands of hyphae show good staining and are easily visible against the cleared root. As expected, the treatments with mycorrhizae inoculation had significantly greater colonization. Contamination within the greenhouse (via spore transport) is likely the cause of the treatments without mycorrhizal inoculation showing some colonization; the growing media were sterilized and the pots were either new or thoroughly washed and bleached.

3.3. Oxidation of the biochar surface measured by XPS

Few studies have used XPS to analyze the surface chemistry of biochar, and none have previously been able to control for exposure to mycorrhizal fungi. XPS separates surface carbon functional groups into four categories: −COOR, −C═O, −COR, and a fourth category that includes −CH, C−C, and C═C functional groups (Fig. 5). Previous work has shown an increase in the oxidation of biochar with weathering, but causes of the oxidation have not been clear. Time scales and methods in previous studies also cover a wide range. Yao et al. (2010) performed artificial weathering experiments on biochar produced from sewage sludge, using a modified Soxhlet reactor to repeatedly wet the biochar with water at 30 °C and with and without humic acids for 300 h. They observed an increase in the −COOH and −C═O groups, but it could not be determined whether the oxidation was biotic or abiotic. Cheng et al. (2006) and Zimmerman (2010) attributed an observed increase in oxidation of biochar to abiotic processes in incubation. Nguyen et al. (2008) recovered biochar ranging in age from 2 to 100 years in soils that had been subjected to slash-and-burn agriculture. Their time series showed a period of rapid oxidation in the form of an increase in the ratio of oxidized to reduced carbon followed by a subsequent constant oxidation level. However, the time period of reaching this static level was poorly constrained due to the 100 year range of biochar age. Zimmerman (2010) observed a leveling-off of biochar remineralization rates after a one year laboratory incubation, but the oxidative environment was substantially different from that of the char studied by Nguyen et al. (2008).

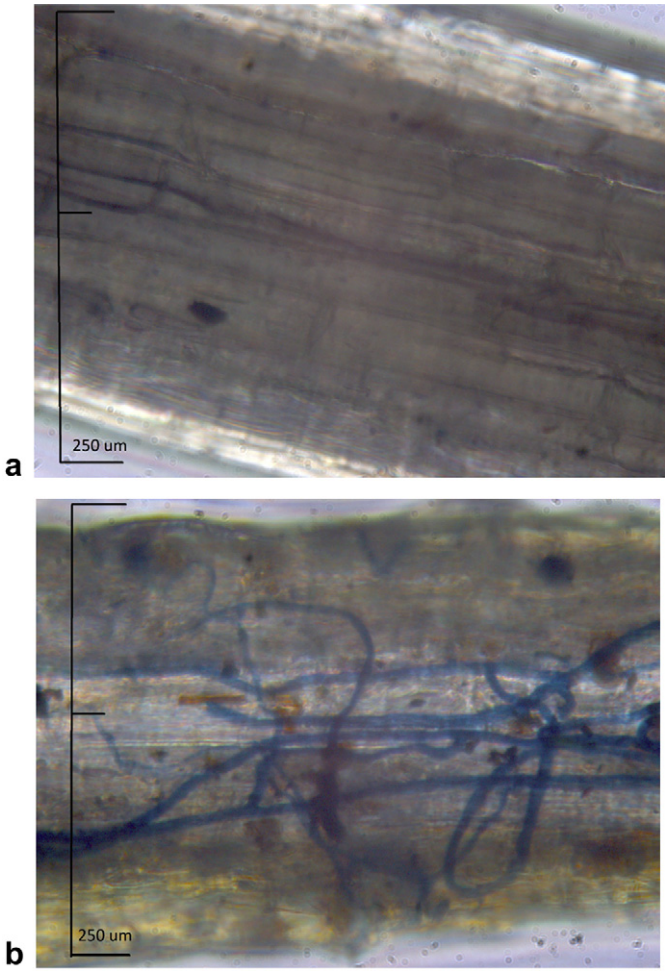

Fig. 4. Sorghum roots at 400× magnification. 4a) uninfected root. 4b) highly colonized root; the blue ribbons are mycorrhizae growing into and around the root.

We observed the largest effect on biochar surface chemistry simply from its addition to the plant−soil environment. All biochar exposed to soil + plants for four weeks showed more surface carbon oxidation in the form of increased percentages of −COR, −C═O, and −COOR groups (Table 1; Fig. 6). It is theoretically possible that the XPS measurements occasionally sampled hyphae fragments on the surface of the biochar rather than the biochar itself, but if this had affected our measurements, it would have been seen in larger standard deviation for the +mycorrhizae treatments (Table 1). This is not the case, so we can be confident that the biochar surface was sampled, not the mycorrhizal hyphae. Our results suggest that the first stage of biochar patina development involves sorption of dissolved organic compounds in soil, leading to a generalized increase in surface carbon oxidation.

Additionally, one treatment showed small, but statistically significant differences in biochar surface oxidation compared to

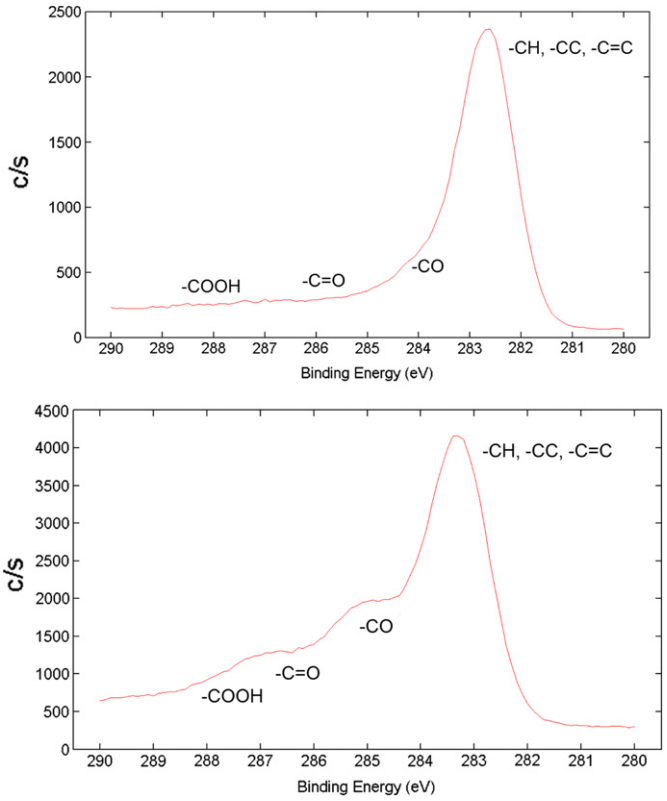

Fig. 5. XPS spectra of the C1s electron for biochar. Above, fresh biochar; below, biochar grown with fertilizer and mycorrhizal inoculation. The fresh biochar has one dominant peak representing C–C bonds. After being in soil, the biochar becomes more oxidized as seen by the extra peaks at higher binding energies.

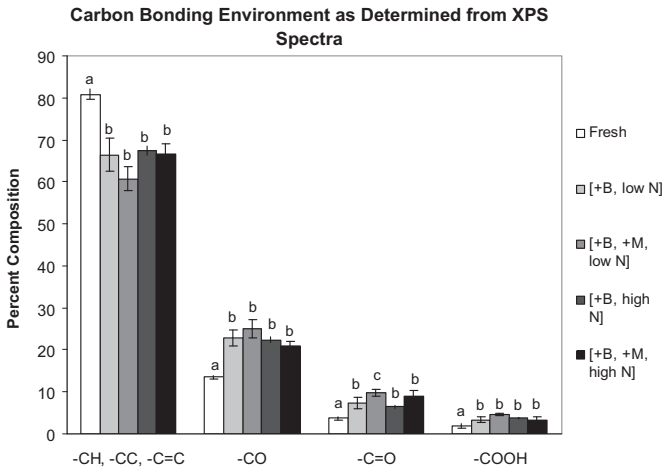

Fig. 6. XPS spectra of Carbon 1 s electron, resolved into bonding environment using MultiPak data analysis software. Each group is averaged from five replicate samples that were picked from their respective group of bulked soil. Different letters indicate values that are significantly different as determined by factorial ANOVA. Error bars represent one standard deviation; instrument error is <5%. Increased oxidation is shown by all the biochars that were added to soil. The treatment with mycorrhizae but low nitrogen fertilizer shows an increase in the percentage of carbon in carbonyl functionalities.

others. The soils with mycorrhizae but low nitrogen caused more oxidation of the biochar surface than the other treatment combinations, and showed a significantly greater fraction of carbon present as carbonyl groups (–C=O, Fig. 6). This treatment had significantly less mycorrhizal colonization than the combined treatment of biochar, mycorrhizae, and high nitrogen, suggesting that the greater oxidation is not due to the quantity of mycorrhizae, but perhaps to their behavior. Fungi have been shown to degrade aromatic structures (Ascough et al., 2010; Hedges et al., 1988; Schreiner et al., 2009; Wengel et al., 2006), and the fungal oxidation of lignin produces both aromatic carbonyl (–C=O) and aromatic acid (–COOH) functionalities, with carbonyl groups preferentially enriched in the early stages of decomposition (Goni et al., 1993; Hedges et al., 1988). Our observed increase in carbonyl (–C=O) and lack of increase in carboxyl carbon (–COOH) detected by XPS is consistent with the early stages of fungal oxidation of aromatic materials. It is possible that our low-nitrogen treatment stimulated the metabolism of mycorrhizal fungi, driving the hyphae to more actively seek nutrients and resulting in more extensive oxidation of biochar via extracellular cometabolism. Likewise, the mycorrhizae could have limited their nutrient-seeking efforts if they were not necessary in the combined biochar, mycorrhizae, and high nitrogen treatment, leading to the observed decreased biochar surface oxidation.

One possible explanation for the transformation of mycorrhizal fungi from symbionts to parasites in the +biochar, +mycorrhizae, high N treatment is biochar sorption of molecules used for cellular signaling, a hypothesis that has been suggested by others (Lehmann et al., 2011; Thies and Rillig, 2009; Warnock et al., 2007). To monitor cell density, fungi release non-polar compounds and use the density-dependent accumulation of these extracellular signaling molecules to coordinate decisions within a population (Albuquerque and Casadevall, 2012; Hogan, 2006), such as spore germination (Macko et al., 1970), the transition between different morphological forms (Oh et al., 2001), and growth rate (Chen et al., 2004). In mycorrhizal fungi, coordinated growth during an infection could represent an evolutionary strategy to maintain the symbiotic relationship with plants and maximize their reproductive fitness in the environment. Unfortunately, the molecular details and fitness consequences of cell–cell signaling are not well understood for any mycorrhizal fungi, including the fungus examined here, *G. intraradices*. There is evidence that *G. intraradices* is exposed to elevated levels of a signaling molecule, tyrosol, during its infection of plants. Legumes infected with *G. intraradices* accumulated tyrosol within the cell walls of their roots, whereas plants lacking an infection do not accumulate this fungal signaling molecule (Schliemann et al., 2008). Tyrosol is a phenolic compound (4-(2-hydroxyethyl) phenol) that has been studied in the human fungal pathogen *Candida albicans*, in which it regulates growth (Chen et al., 2004). Soils containing biochar may deplete tyrosol and other fungal

Table 1
Bonding environment percentages averaged for 5 replicates in each group. Data from analysis using MultiPak software.

Group	–COOR average	Standard deviation	–C=O average	Standard deviation	–C-or average	Standard deviation	–CH, C–C, C=C average	Standard deviation
Fresh biochar	1.96	1.17	3.69	1.03	13.52	0.88	80.83	2.66
–M, –N	3.36	1.58	7.35	3.29	22.83	4.48	66.46	8.75
+M, –N	4.60	0.81	9.73	1.72	24.93	4.80	60.73	6.52
–M, +N	3.75	0.36	6.45	0.88	22.37	1.45	67.42	2.30
+M, +N	1.82	3.15	9.03	2.80	21.07	2.21	66.75	4.90

signaling compounds from plant roots, such as farnesol, farnesoic acid, and dimethoxycinnamate. These fungal signaling compounds are non-polar, like compounds previously shown to adsorb to charcoal and activated carbon (Zackrisson et al., 1996). Biochar interference in the chemical communication between sorghum and mycorrhizal fungi could underlie the transition of mycorrhizal fungi from symbionts to parasites, although more research is needed to document and understand this transformation.

4. Conclusion

We showed that biochar induced mycorrhizal parasitism in juvenile sorghum plants in the presence of high levels of nitrogen fertilizer. The increased root colonization rates in the treatment with biochar and nitrogen fertilizer along with the much lower aboveground biomass provide supporting evidence for this conclusion. While this interaction significantly reduced plant growth in the first 4 weeks, longer studies will be necessary to determine if an effect can be observed in crop yields.

Along with biochar effects on the plant–fungal relationship, we also explored the changes in biochar after exposure to plants, fertilizer, and mycorrhizal fungi. When compared to fresh biochar, we observed changes in the surface chemistry of biochar that had spent four weeks in soil with sorghum. The percentage of carbon in each of the functional groups –CO, –C=O, and –COOH increased when watered and exposed to the soil environment, regardless of the experimental treatment. We also observed treatment-specific surface chemistry effects. Biochar in the +mycorrhizae, low nitrogen treatment had an increase in the percentage of carbon in the carbonyl fraction versus other soil-exposed biochars. This treatment had significantly less mycorrhizal colonization than the +mycorrhizae, high nitrogen treatment indicating that this form of oxidation is not correlated with the quantity of mycorrhizae. Given that the only difference between the treatments was the presence of mycorrhizae, biotic oxidation is the likely cause for the increase in the carbonyl fraction. It is possible that the mycorrhizae in the +mycorrhizae, low nitrogen treatment were more active in their search for nutrients due to their nitrogen-limited environment and thus oxidized the biochar further.

Acknowledgments

We appreciated the help of Bo Chen with XPS measurements and Prudence Sun and Liz Yin in the greenhouse, and we acknowledge the Rice Shared Equipment authority, which provided access to the XPS. Rebecca Barnes and Krystle Hodge helped with plant harvesting. Xtreme Gardening generously provided the mycorrhizae used here. We acknowledge support from NSF EAR 0911685, NSF EAR 0949337 and from the Rice University Shell Center for Sustainability.

Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2012.11.023.

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