Biochar enhances seedling growth and alters root symbioses and properties of sub-boreal forest soils

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Robertson, S. J., Rutherford, P. M., López-Gutiérrez, J. C. and Massicotte, H. B. 2012. **Biochar enhances seedling growth and alters root symbioses and properties of sub-boreal forest soils**. Can. J. Soil Sci. **92**: 329–340. Biochar application may enhance soil properties, improve plant productivity, and increase long-term carbon storage, but impacts of biochar on plant-microbe symbioses mediating plant nutrient uptake in temperate or boreal soils are not well known. We planted lodgepole pine (*Pinus contorta* var. *latifolia*) or sitka alder (*Alnus viridis* ssp. *sinuata*) seeds in pots containing field-collected forest soils (from central British Columbia) amended with 0, 5, or 10% (dry mass basis) biochar with and without urea fertilizer (150 mg N kg^{-1}). Pine seedlings were harvested at 4 mo and roots were assessed for abundance and diversity of ectomycorrhizal (ECM) morphotypes using light microscopy and DNA sequencing. Biochar raised soil pH, exchangeable cations and cation exchange capacity in some treatments in both soils. Pine had greater biomass in biochar + fertilizer treatments compared to control and fertilizer-only treatments; this corresponded in part to an increase in abundance of some ECMs. Alder seedlings were harvested at 2, 3 and 4 mo to measure N fixation in root nodules using acetylene reductase assay (ARA). Alder seedlings had greater shoot biomass when grown in biochar-amended soils compared with unamended control. Although mean ARA rates (at 4 mo) were greater in biochar-amended soils compared with controls, the data showed great variation and differences were not statistically significant ($P > 0.05$). This study showed that biochar addition can enhance soil properties and the early growth of pine and alder in some sub-boreal forest soils; small changes in ECM abundances may be expected.

Key words: Ectomycorrhizas, black carbon, N fixation, actinorhiza, *Pinus contorta*, *Alnus viridis* ssp. *sinuata*

Robertson, S. J., Rutherford, P. M., López-Gutiérrez, J. C. et Massicotte, H. B. 2012. **Le biochar favorise la croissance des plantules et modifie la symbiose et les propriétés des racines dans les sols forestiers subboréaux**. Can. J. Soil Sci. **92**: 329–340. L'application de biochar pourrait rehausser les propriétés du sol, rendre les plantes plus productives et accroître le stockage du carbone à long terme, mais on connaît mal l'incidence de ce produit sur la symbiose entre les microorganismes et les plantes qui concourt à l'absorption des éléments nutritifs, dans les sols des régions tempérées et boréales. Les auteurs ont planté des graines de pin tordu latifolié (*Pinus contorta* var. *latifolia*) et d'aulne de Sitka (*Alnus viridis* ssp. *sinuata*) dans des pots remplis de sol forestier prélevé sur le terrain (dans le centre de la Colombie-Britannique) et bonifié avec 0, 5 ou 10 % (poids sec) de biochar, enrichi ou pas avec de l'urée (150 mg de N par kg). Les plantules de pin ont été récoltées à l'âge de quatre mois et on en a évalué les racines afin de déterminer l'abondance et la diversité morphologique des ectomycorhizes (ECM) par microscopie photonique et séquençage de l'ADN. Le biochar relève le pH du sol, augmente la concentration des cations échangeables et rehausse la capacité d'échange cationique pour certains traitements, dans les deux sols. La biomasse du pin se développe davantage avec le traitement biochar + engrais, comparativement à ce qu'on observe avec le sol témoin et l'application d'engrais uniquement; on le doit en partie à la plus grande abondance d'ECM. Les plantules d'aulne ont été récoltées après deux, trois ou quatre mois, et on a mesuré les nodules fixateurs d'azote sur les racines par dosage à l'acétylène réductase (DAR). La biomasse des pousses d'aulne est plus importante quand ces dernières sont cultivées dans un sol amendé avec du biochar plutôt que dans le sol témoin non bonifié. Bien que les résultats du DAR (à quatre mois) soient plus élevés dans le sol bonifié au biochar que dans le sol témoin, les données révèlent une grande variabilité et des écarts statistiquement non significatifs ($P > 0{,}05$). Cette étude montre que l'addition de biochar peut rehausser les propriétés du sol et favoriser la croissance des plantules de pin et d'aulne dans certains sols forestiers subboréaux; on peut aussi s'attendre à de petites modifications dans l'abondance des ECM.

Mots clés: Ectomycorhizes, carbone noir, fixation du N, actinorhizes, *Pinus contorta*, *Alnus viridis ssp. sinuata*

Biochar is a form of black carbon produced by slow pyrolysis when organic materials are heated (maximum temperature typically ranges from $\sim$400 to $>$500°C) under conditions of low (or no) oxygen (Lehmann 2007a). It shares properties with natural black C (i.e., charcoal)

Abbreviations: **ARA**, acetylene reductase assay; **CEC**, cation exchange capacity; **ECM**, ectomycorrhizal; **PCR**, polymerase chain reaction

that is ubiquitous in soils and sediments; the term "biochar" is used to distinguish the material as black carbon specifically produced for environmental applications (Sohi et al. 2009). Black C has been described as a continuum from partially charred biomass materials that still retain their physical structure, to charcoal, soot, and ultimately graphite (Preston and Schmidt 2006). Biochar has been proposed as a soil amendment to provide long-term sequestration of C (due to its relative recalcitrance) and to improve soil properties (Laird 2007; Lehmann 2007a, b; Sohi 2009). While the putative benefits of biochar addition to soils include increased plant growth, nutrient availability, cation exchange capacity, water retention, increase in soil pH and reductions in release of some greenhouse gases (i.e., methane and nitrous oxide), there is incomplete evidence that this holds for all types of biochars or all types of receiving soils. Most positive reports are for acidic tropical soils of low C content and low native fertility (Lehmann 2007a; Sohi et al. 2009; Verheijen et al. 2009). Biochar has not been widely studied in Canadian soils. Western Canada, with its close proximity to wood waste materials and other organic residues, may be a promising location for the production and utilization of biochar for forest management in nursery, restoration and reforestation applications. Very little has been reported on the potential benefits or negative impacts of biochar addition to temperate or boreal forest soils. The impact of biochar addition on ectomycorrhizal (ECM) communities and other microbial groups that mediate C and nutrient cycling in temperate and boreal forest soils are largely unknown.

In northern forests, most tree species (e.g., Pinaceae, Betulaceae, Salicaceae families) form ectomycorrhizal symbioses with a diverse array of soil filamentous fungi that obtain and transfer soil nutrients from mineral and organic sources to their plant hosts in exchange for photosynthetically derived C (Smith and Read 2008). Ectomycorrhizal mycelia form the dominant microbial biomass of the forest floor and exude labile organic compounds that are considered the major driving force (energy) for many microbial processes in the rhizosphere (Philippot et al. 2009). With respect to biochar amendment, the most important knowledge gaps concern the mechanisms by which biochar might affect the abundance and functioning of ECM fungi (Warnock et al. 2007). On one hand, ECM fungi and other rhizosphere microorganisms may benefit from changes in soil nutrient availability, which may alleviate growth limitations of fungi in nutrient poor soils (Treseder and Allen 2002; DeLuca et al. 2006) and increase root colonization (Warnock et al. 2007). In addition, biochar may serve as a source of reduced C compounds (e.g., labile C in biochar) or nutrients, or act as a refuge (protection from soil predators) for hyphae and bacteria that colonize biochar particles (or other porous materials) (Pietikainen et al. 2000; Warnock et al. 2007; Steinbeiss et al. 2009). Alternatively, biochar may negatively impact ECM fungi via interference with closely associated microorganisms such as mycorrhizal helper bacteria, phosphate-solubilizing bacteria, and plant growth promoting rhizobacteria, or with plant-fungus signaling (due to sorptive properties of biochar) during germination and early mycorrhizal establishment (Warnock et al. 2007).

Some plants (e.g., *Alnus* spp.) also form actinorhizal symbioses with soil-borne filamentous bacteria (*Frankia*) (Gaulke et al. 2006) and receive 70–100% of their nitrogen requirement via N fixation (Nickel et al. 2001; Myrold and Huss-Danell 2003). The actinorhizal symbiosis allows alders to colonize very poor or disturbed soils, thereby accelerating soil development and supporting microbial activities through addition of both N and C in the rhizosphere (Selmants et al. 2005; Roy et al. 2007). In this complex arrangement, the ECM symbiosis may help meet the high P demands of actinorhizal plants for nodule formation and N fixation; however, maintaining multiple root symbionts requires considerable energy from the plant (Roy et al. 2007). Biochar addition can induce changes in nutrient availability, which, in turn, can lead to enhanced N-fixation in soil (Rondon et al. 2007).

In this trial experiment, we grew lodgepole pine (*Pinus contorta* Dougl. Ex Loud. var. *latifolia* Engelm.) and sitka alder [*Alnus viridis* (Chaix) DC. ssp. *sinuata* (Regel) A. Löve & D. Löve] seedlings in pots containing forest soil amended with 0, 5 or 10% (by mass) biochar, with and without urea-N application. These two species are often used in reforestation and restoration, respectively; both grow quickly under current climate conditions and are expected to continue to thrive under predicted climate change (Hamann and Wang 2006). We hypothesized that biochar would enhance soil chemical properties, alter the richness and abundance of ECM communities (pine and alder), and promote greater N fixation by actinorrhizal symbionts (alder). We expected that biochar-induced changes in soil properties and root symbioses would improve growth of seedlings in subboreal forest soils.

MATERIALS AND METHODS

Biochar Preparation and Characterization

Biochar (Alterna Biocarbon Inc., Prince George, BC) was produced from softwood chips (mainly lodgepole pine) that first entered a pre-carbonization phase at $\sim$50°C prior to slow heating to 300°C over 50 min under oxygen limited conditions. The temperature was then raised to 410°C and held for $\sim$30 min. Biochar then proceeded to a cooling zone where a small amount of water was used to quench the material to a temperature of $\sim$70°C to prevent re-ignition when the biochar was removed from the pyrolysis unit. The material was pulverized using a hammer mill ($>$90% mass $<$1 mm diameter) prior to use in this study. Biochar properties and methods of characterization are presented in Table 1.

Table 1. Properties of the biochar used in this study. All concentrations are expressed on an oven-dry basis

Property	Units	Value[z]	Method or reference
pH (deionized H_2O)		8.91 (0.02)	2:1 solution: solid ratio
Total C	g 100 g^{-1}	80.2 (0.3)	Dumas combustion
Total N	g 100 g^{-1}	0.27 (0.01)	Dumas combustion
C/N	g g^{-1}	295 (10)	Mass ratio
H/C	mole $mole^{-1}$	0.44 (0.03)	Dumas combustion
O/C[y]	mole $mole^{-1}$	0.123 (0.004)	Dumas combustion
Total S	g 100 g^{-1}	0.023 (0.002)	Dumas combustion
Total P	g 100 g^{-1}	0.0525 (0.008)	EPA digestion 3052, ICP-OES[x]
Total Ca	g 100 g^{-1}	1.087 (0.183)	EPA digestion 3052, ICP-OES
Total Mg	g 100 g^{-1}	0.179 (0.003)	EPA digestion 3052, ICP-OES
Total K	g 100 g^{-1}	0.672 (0.003)	EPA digestion 3052, ICP-OES
Mehlich 3 P	mg kg^{-1}	58.9 (1.9)	Ziadi and Tran (2008)
Mehlich 3 Ca	mg kg^{-1}	3856 (87)	Ziadi and Tran (2008)
Mehlich 3 Mg	mg kg^{-1}	473 (31)	Ziadi and Tran (2008)
Mehlich 3 K	mg kg^{-1}	3450 (180)	Ziadi and Tran (2008)
Volatile matter	g 100 g^{-1}	17.0 (0.04)	ASTM Method D1762-84[w]
Ash	g 100 g^{-1}	3.4 (0.1)	ASTM Method D1762-84
Water content	g 100 g^{-1}	3.16 (0.05)	Dried at 105°C to constant mass

[z]Mean with standard error in parentheses ($n = 4$).
[y]Oxygen is calculated; it is the result of the summation percentages of carbon, hydrogen, sulphur, nitrogen and ash subtracted from 100% (ASTM E 870-82, ASTM 2002).
[x]Multi-acid (HF, HNO_3, HCl) microwave digestion, followed by inductively coupled plasma optical emission. spectrometry (ICP-OES).
[w]ASTM (2007).

Molar ratios of hydrogen/carbon and oxygen/carbon, volatile matter and ash contents (ASTM Method D1762-84 in American Society for Testing and Materials 2007) have been used to characterize black C materials (Joseph et al. 2009; Deenik et al. 2010). Although not usually applied to non-soil materials, the Mehlich 3 extraction has been used to characterize biochar materials (Major et al. 2010) and was used here to obtain an approximation of the available elements contained with biochar.

Biochar was basic (pH 8.91) and had a relatively high C content (80.2 g 100 g^{-1}) and high C:N ratio (295) (Table 1). Approximately 11, 36, 26 and 51% of the total P, Ca, Mg and K, respectively, was "available" as estimated by the Mehlich 3 procedure. Available Ca and K were relatively high (>3000 mg kg^{-1}) compared with Mg (473 mg kg^{-1}) and P (59 mg kg^{-1}) (Table 1). The volatile matter and ash contents in biochar were 17.0 and 3.4 g 100 g^{-1}, respectively.

The biochar used in this study had similar C content, C/N (mass) ratio, H/C (molar) ratio and O/C (molar) ratio to other wood-derived biochars (Krull et al. 2009). The volatile matter content of the biochar used in this study was 17%, which is within the range reported by Joseph et al. (2009) for industrial charcoals. The ash content and total elemental concentrations of N, P, and K found in this study fall within the ranges reported in the literature for wood and green waste biochars (Chan and Xu 2009). Mehlich 3 extractable P and Ca were lower, extractable Mg was higher, and extractable K was approximately the same as values reported by Major et al. (2010) for biochars produced from mango tree prunings.

Soil Collection and Characterization

Soils (organic horizons and upper ~15 cm of mineral layers) were collected from under lodgepole pine (*Pinus contorta* Dougl. Ex Loud. var. *latifolia* Engelm.) and sitka alder [*Alnus viridis* (Chaix) DC. ssp. *sinuata* (Regel) A. Löve & D. Löve] dominated stands of local forests in the sub-boreal spruce (SBS) zone of central BC in October 2008. At each of the two sites, soil samples were collected at five randomly selected locations within a 50 m × 50 m area; a composite sample was created for each of the two sites. Gray Luvisols are typical in the areas where the pine and alder soils were collected. Soils were sieved (1 cm^2 screen) and homogenized to mix mineral and organic horizons. Total C and N content was determined using <100-mesh soil samples (air-dried, then ground in a Model MM200 ball mill; Retsch, Haan, Germany) via dry combustion using a Model 1500 NC Elemental Analyzer (Fisons, Milan, Italy). The pH was measured in double deionized H_2O using a 2:1 (solution:soil) ratio (Hendershot et al. 2008a). Particle size distribution (proportions of sand, silt and clay) was determined on the original untreated soils using the hydrometer method following oxidation of organic material (Kroetsch and Wang 2008).

Soil pH (in water) was neutral in the pine soil (7.16) and slightly acidic in the alder soil (6.38). The pine soil had 1.8-fold more total C and 1.6-fold more total N compared with the alder soil (Table 2). Based on the proportions of sand, silt and clay, soil texture was silty loam and sandy loam for the pine and alder soils, respectively (Table 2).

Table 2. Selected initial properties of soils used in this study[z]

Soil	pH (2:1 H_2O)	Total C (g 100 g^{-1})	Total N (g 100 g^{-1})	Sand (g 100 g^{-1})	Silt (g 100 g^{-1})	Clay (g 100 g^{-1})
Pine	7.16 (0.02)	5.05 (1.16)	0.230 (0.044)	16.0 (0.42)	63.6 (0.42)	20.5 (0.03)
Alder	6.38 (0.02)	2.74 (0.02)	0.139 (0.002)	61.8 (0.02)	29.3 (0.02)	8.9 (0.03)

[z]Means with standard errors in parentheses ($n = 4$ except for sand, silt and clay where $n = 3$).

Greenhouse Setup

Pine soils were divided into six treatment groups ($n = 4$) and amended with biochar (0, 5 or 10% by mass-dry basis) and/or urea fertilizer (150 mg N kg^{-1} dry-soil basis; granules mixed into soil) as follows: (1) control (0% biochar + no urea); (2) 0% biochar + urea; (3) 5% biochar; (4) 5% biochar + urea; (5) 10% biochar, (6) 10% biochar + urea. Alder soils were prepared the same way but were divided into four treatment groups ($n = 4$) as follows: (1) control (0% biochar + no urea); (2) 0% biochar + urea; (3) 10% biochar, and (4) 10% biochar + urea. Deepots™ (D40, Stuewe and Sons, Inc., Oregon) were filled with 140 g of soil treatment, with clay pellets at the bottom to minimize soil loss through drainage. Surface-sterilized (30% H_2O_2 for 30 min) pine seeds (Tree Seed Center, Surrey, BC) were planted into four replicate containers for each of the six pine soil treatments; surface-sterilized (30% H_2O_2 for 5 min) alder seeds (collected adjacent to UNBC campus) were planted into four replicate pots for each of the four alder soil treatments. Seedlings were established in the greenhouse under standard temperature and light regimes (20/16°C day/night temperature, and 16 h photoperiod).

Plant and Soil Sampling

Pine and alder systems ($n = 4$) were destructively sampled at 4 mo for plant (shoot, root, and nodule) biomass, ECM morphotype analyses and soil properties. Replicate ($n = 4$) pots of alder systems were also harvested at 2, 3, and 4 mo for N fixation analysis. Plant shoot and nodule biomasses were determined after drying for 48 h at 75°C. Pine root systems were heavily sampled for ECM characterization and DNA sequencing, and were therefore excluded from biomass measurements. Soil pH, total C and total N were determined as above. Exchangeable cations and cation exchange capacity (CEC) were determined in control and biochar-amended soils using the ammonium acetate method at pH 7.0 (Hendershot et al. 2008b).

Ectomycorrhizas

Ectomycorrhizas were assessed on roots of pine and alder (200 root tips per plant) after 4 mo of growth using standard microscopy techniques (Agerer 1987–2002; Ingleby et al. 1990; Goodman et al. 1996). ECM morphotypes were described and grouped according to color, texture, lustre, dimensions, tip shape, branching pattern, and presence or absence of rhizomorphs (mycelial strands). Root squash mounts were examined at 400–1000 × magnification and descriptions of mantle features, emanating hyphae, rhizomorphs, and other distinguishing features were used to further differentiate ECM morphotypes. Root tips that appeared uncolonized or lacked a well-developed mantle were categorized as non-mycorrhizal (NM). Most alder ECMs were scored as unidentified ECM morphotypes or non-mycorrhizal due to the high proportion of lightly colonized (i.e., thin mantle) root tips.

To confirm the identity of ECM morphotypes, DNA from individual root tips was extracted using the DNeasy Plant Mini Kit (MoBio, Carlsbad, CA) and amplified using polymerase chain reaction (PCR). The ITS region of fungal rDNA was targeted using the universal primer ITS1 (White et al. 1990) and the fungal-specific primer NLB4 (Martin and Rygiewicz 2005). PCR reactions consisted of 10 × PCR buffer, 2 mM dNTPs, 50 μM $MgCl_2$, 10 μM of each primer (Proligo, CO), 0.5 mg mL^{-1} of bovine serum albumin and 0.7 U Platinum Taq DNA polymerase (Invitrogen Life Technologies) in a 27 μL volume, to which 3 μL of extracted DNA (diluted 1:10) was added. Positive and negative controls were run simultaneously. Thermocycler conditions included an initial denaturation for 3 min at 95°C followed by 35 cycles of denaturation (95°C for 30 s), annealing (54°C for 30 s) and extension (72°C for 30 s) and final extension at 72°C for 10 min. PCR products were cleaned using the Agentcourt AMPure XP purification system (Beckman Coulter, Beverly, MA) and sequenced in the fragment analysis and DNA sequencing service at the University of British Columbia–Okanagan using a 3130xl Genetic Analyzer (Applied Biosystems). Forward and reverse sequences were aligned and manually corrected using the sequence analysis software Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI) to generate consensus sequences that were then compared with the NCBI GenBank (http://www.ncbi.nlm.nih.gov) database. Sequences were submitted to NCBI GenBank under accession numbers JF298203-JF298210.

Acetylene Reduction Assay

Two-, three- and four-month alder seedlings from the four treatment groups ($n = 4$) were removed from soil and sealed in 250 mL Kerr™ glass jars. Air in the jar was replaced with acetylene gas (10% vol/vol) through a rubber septum in the jar lid. Gas samples (0.7 cm^3) were taken after 4 h of incubation in the dark (20°C) and analyzed for ethylene using a gas chromatograph

(SRI 8610A Wennick Scientific Corporation, Ottawa, ON) with a flame ionization detector and fitted with a Porapak Column (Alltech Canada, Guelph, ON). The column was maintained at 68°C and the carrier gas (26 psi pressure) was hydrogen. Ethylene levels were obtained using PeakSimple 3.74 Chromatography Software (SRI Inc. 2008). The ARA measurements were expressed on a per nodule dry-mass basis.

Statistical Analyses

All analysis of variance (ANOVA) comparisons were conducted at an alpha of 0.05 and using the Fisher Least Significant Difference (LSD) post-hoc test to pinpoint significant differences (SYSTAT version 8.0, 1998, SPSS Inc.). Pine and alder soil properties (C, N, C:N and pH) were analyzed within biochar and fertilizer treatment groups using two-way ANOVA. Exchangeable cations (Ca, K, Mg and Na) and CEC were analyzed in biochar-amended (non-fertilized) pine and alder soils using one-way ANOVA. Plant biomass was compared in biochar and fertilizer treatment groups at 4 mo for pine (two-way ANOVA) and across the 2-, 3- and 4-mo sampling points for alder (three-way ANOVA). For pine ECMs, the frequency, relative abundance (based on a random sample of 200 root tips per plant) and Shannon and Simpson's diversity indices (Magurran 2004) values were compared in biochar and fertilizer treatment groups using two-way ANOVA. Mean ARA values were also compared across biochar and fertilizer treatment groups at each harvest time using two-way ANOVA.

RESULTS

Plant Biomass

For pine (Fig. 1A), shoot biomass was significantly greater ($P = 0.007$) in 5 and 10% biochar treatments with fertilizer, as compared with non-biochar treatments. For alder (Fig. 1B), shoot biomass increased significantly from 2 to 4 mo ($P < 0.001$) in all treatment groups. At 4 mo, alder shoot biomass was significantly greater in the 10% biochar treatments ($P = 0.038$) compared with non-biochar treatments, regardless of fertilizer application. The alder shoot: root ratio (data not shown) was approximately one for all treatments except after 4 mo, when values for fertilized seedlings in 10% biochar were significantly greater than non-fertilized seedlings ($P = 0.004$).

Soil Properties

Biochar addition increased the pH of both pine and alder soils at the beginning of the experiment (data not shown). Mean soil pH ($n = 4$; SE in parentheses) of the non-fertilized pine soil at time 0 was 7.16 (0.02), 7.50 (0.02) and 7.80 (0.03) for the 0, 5 and 10% biochar treatments, respectively; means varied significantly ($P < 0.001$). Soil pH of the non-fertilized alder soil at time 0 was 6.38 (0.02) and 7.77 (0.03) for the 0 and 10% biochar treatments, respectively; means varied significantly ($P < 0.001$).

With respect to non-fertilized pine soil harvested at 4 mo, CEC ($P = 0.004$) and exchangeable Ca ($P = 0.031$), K ($P = 0.005$), Mg ($P < 0.001$) and Na ($P = 0.006$) were significantly greater in the 10% biochar treatment compared with the non-biochar control (Table 3). Pine soil amended with 5% biochar did not exhibit significant differences from the non-biochar control. Non-fertilized alder soil amended with 10% biochar had significantly greater exchangeable Ca ($P = 0.018$), K ($P < 0.001$) and Na ($P = 0.013$), but the CEC was not greater than the non-biochar control (Table 3). Pine soils had greater exchangeable cations and CEC than alder soils.

For pine soil harvested at 4 mo, pH ($P < 0.001$), total C ($P < 0.001$), total N ($P = 0.029$) and C:N ratio ($P < 0.001$) were all significantly greater in 10% biochar treatments compared with non-biochar treatments, however, no significant differences were seen due to fertilizer (Table 4). For alder soil harvested at 4 mo, pH ($P < 0.001$), total C ($P < 0.001$) and C:N ratios ($P < 0.001$) were significantly greater in 10% biochar treatments compared to non-biochar treatments.

Ectomycorrhizas

Pine and alder root tips hosted distinct communities of ECM fungal morphotypes: nine ECM morphotypes were identified with pine and four ECM morphotypes were identified with alder (Table 5). There was minimal overlap in fungal community composition between the two hosts, as confirmed by standard microscopy and sequencing.

Pine

The ECM community associated with 4-mo pine roots included *Cenococcum*, *Hebeloma*, *Rhizopogon-Suillus* 1 and 2, *Tomentella*, *Amphinema*, E-strain, MRA and Russulaceae (Tables 5 and 6). Root systems, regardless of biochar and/ or fertilizer treatment, were generally dominated by E-strain (~29–59%) and Russulaceae (~16–33%) ECMs. Non-mycorrhizal (or partially colonized) root tips were detected in 40% of all pine root systems ($n = 4$), usually in low relative abundance (<10% of all root tips). Pines grown in control (biochar-free) soils tended to have a greater abundance of non-mycorrhizal root tips (Table 6), but differences were not statistically significant ($P > 0.05$).

The abundance of some individual morphotypes (e.g., *Rhizopogon-Suillus* 2, E-strain and *Cenococcum*) on pine root systems varied between biochar and fertilizer treatment groups (Table 6). The abundance of *Rhizopogon-Suillus* 2 was greatest in the fertilized 5% biochar treatment compared to 0% biochar treatments. *Cenococcum* abundance was significantly greater in the 5% biochar treatment ($P = 0.018$) compared with 0 and 10% biochar treatments, which were not different from each other. For E-strain, a significant interaction ($P = 0.035$) was found between biochar and fertilizer treatments.

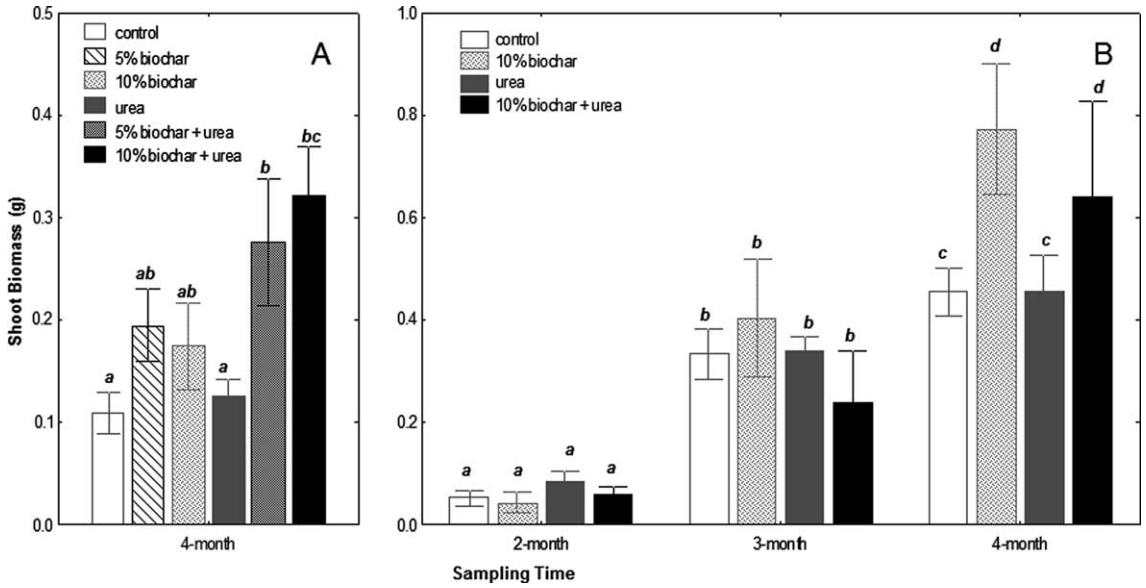

Fig. 1. Comparison of shoot biomass for pine (A) and alder (B) for biochar and fertilizer treatment groups in 2-, 3- and 4-mo sampling times. Different letters within plant type indicate significant treatment differences at $\alpha = 0.05$.

Thus, in soil treatments lacking biochar amendment, E-strain abundance was significantly greater ($P = 0.045$) in fertilized compared to unfertilized soils, but fertilizer treatment in 10% biochar-amended soils resulted in significantly lower ($P = 0.042$) abundance compared with 0% biochar (+ fertilizer) soil. Other ECM morphotypes did not vary in abundance with biochar or fertilizer treatment. ECM community diversity, as described by the Shannon (H') and Simpson's (1/D) diversity indices, did not vary significantly between any treatments.

Alder

Four-month alder included *Tomentella*-like, *Cenococcum* and other unidentified morphotypes, including basidiomycetous (presence of clamped hyphae) ECM (Table 5). Alder ECMs could not be morphotyped precisely as they were generally less well-developed and exhibited a higher proportion of partially colonized roots compared with pine. The closest matches for the ECM root tip sequences in Genbank corresponded to ECM sequences previously described for *Alnus* (Tedersoo et al. 2009) in alder root tips, and to previously described ECM sequences for *Pinus* (Walbert et al. 2010) as well as other hosts (Haug 2002; Hartmann et al. 2009; Moreau et al. 2006) in pine root tips.

N Fixation Rates

There were no differences in ARA rates (μmol g^{-1} h^{-1}, based on nodule dry weight) between treatment groups at 2 and 3 mo (Fig. 2). At 4 mo, mean ARA rates appeared to be lower in non-biochar treatments, compared with 10% soil treatments; but, large variations in the data resulted in non-significant differences ($P > 0.05$) between treatment means. At the end of the study, nodules continued to fix N (as measured by ARA) in biochar-treated systems, whereas those associated with plants in non-biochar treatments (i.e., 3 of 4 replicates) did not. The addition of fertilizer had no effect on ARA rates at 2, 3 or 4 mo. The number and dry weight of nodules (data not shown) did not vary with treatment.

DISCUSSION

In this greenhouse study, we investigated the impact of forest soil amendment with biochar on plant growth and

Table 3. Exchangeable cations and cation exchange capacity (CEC) for non-fertilized pine and alder soils at 4 mo[z]

Soil	Biochar (%)	ex Ca (cmol+ kg^{-1})	ex K (cmol+ kg^{-1})	ex Mg (cmol+ kg^{-1})	ex Na (cmol+ kg^{-1})	CEC (cmol+ kg^{-1})
Pine	0	19.33 (0.69)*b*	0.51 (0.01)*b*	2.50 (0.03)*b*	0.124 (0.008)*b*	20.00 (0.65)*b*
	5	19.49 (0.31)*b*	0.58 (0.02)*b*	2.78 (0.08)*b*	0.114 (0.005)*b*	19.73 (0.35)*b*
	10	21.43 (0.46)*a*	0.68 (0.02)*a*	3.38 (0.10)*a*	0.153 (0.006)*a*	23.13 (0.64)*a*
Alder	0	8.12 (0.23)*b*	0.32 (0.02)*b*	2.32 (0.05)	0.070 (0.003)*b*	10.09 (0.19)
	10	10.49 (0.70)*a*	0.56 (0.03)*a*	2.55 (0.17)	0.128 (0.016)*a*	12.70 (1.33)

[z]Means with standard errors in parentheses ($n = 4$); buffered (pH 7) ammonium acetate method (Hendershot et al. 2008b).
a, *b* Different letters within soil type indicate significant treatment differences at $\alpha = 0.05$.

Table 4. Total C, total N, C:N (by mass) and pH for pine and alder soils at 4 mo[z]

Soil	Biochar (%)	Urea Fertilizer	pH (2:1 water)	Total C (g 100 g^{-1})	Total N (g 100 g^{-1})	C:N Ratio
Pine	0	−	7.27 (0.03)*b*	3.60 (0.25)*c*	0.178 (0.010)*b*	20.2 (0.9)*c*
		+	7.28 (0.05)*b*	3.25 (0.20)*c*	0.170 (0.009)*b*	19.2 (1.0)*c*
	5	−	7.51 (0.04)*a*	7.16 (1.64)*b*	0.206 (0.033)*b*	33.7 (4.5)*b*
		+	7.36 (0.01)*a*	7.43 (0.84)*b*	0.213 (0.009)*b*	34.7 (4.9)*b*
	10	−	7.48 (0.04)*a*	14.25 (2.69)*a*	0.286 (0.054)*a*	50.1 (6.6)*a*
		+	7.48 (0.03)*a*	10.73 (0.94)*a*	0.218 (0.007)*a*	49.2 (7.2)*a*
Alder	0	−	6.86 (0.02)*b*	2.94 (0.82)*c*	0.115 (0.008)*c*	24.6 (9.8)*b*
		+	6.76 (0.02)*b*	1.61 (0.11)*c*	0.069 (0.004)*b*	23.7 (6.0)*b*
	10	−	7.56 (0.07)*a*	12.23 (1.10)*a*	0.159 (0.017)*a*	77.2 (3.4)*a*
		+	7.51 (0.06)*a*	7.86 (0.55)*b*	0.113 (0.002)*c*	69.6 (12)*a*

[z]Means with standard errors in parentheses ($n = 4$).
a–c Different letters within soil type indicate significant treatment differences at $\alpha = 0.05$.

soil properties, ectomycorrhizal community structure (pine) and actinorhizal N fixation (alder). Pine and alder growth was increased at four months in biochar-treated soils. In addition to associated effects of increased soil pH, exchangeable cations, and cation exchange capacity, biochar amendment did not greatly impact ECM communities or *Frankia*-mediated N-fixation.

Plant Growth and Soil Properties

Shoot biomass of alder at 4 mo was significantly greater in biochar treatments compared with unamended soil (no biochar, no fertilizer) and to soil receiving only N application. For pine, treatments receiving both biochar and fertilizer were significantly greater than unamended soil and soil receiving only urea fertilizer. Soil amended with 10% biochar generally had higher levels of extractable cations and CEC (pine soil only) compared with unamended soil, findings supported by other studies (Van Zwieten et al. 2010). Although unamended soils in this study were not strongly acidic, the increased pH, exchangeable cations and CEC following biochar addition may have contributed to greater plant nutrient availability (or nutrient use efficiency) and subsequent growth. Observed shifts in the abundance of some ECMs (discussed below), may also have influenced nutrient acquisition by pine seedlings.

Other studies have shown increased plant growth following biochar addition to soil; however, reports are variable and decreases in plant growth have also been cited (Glaser 2002; Deenik 2010). Increasing evidence suggests that the addition of some biochars to agricultural soils may cause a spike in microbial activity resulting in net N immobilization, in some cases reducing plant-available N and plant yields (Kolb et al. 2009; Deenik et al. 2010; Verheijen et al. 2010). Although the majority of the C in biochar is expected to be recalcitrant and relatively non-bioavailable, a small labile component may be bioavailable and stimulate microbial uptake of soil mineral N. Recently, Deenik et al. (2010) reported that the addition of biochar high in volatile matter content (22%) stimulated microbial activity, caused net N immobilization and reduced yields of lettuce and corn. Addition of biochar low in volatile matter content (6.3%) had lower spikes in microbial activity and net N immobilization. In the current study, a high rate of biochar addition (up to 10% by mass), containing relatively high volatile matter content (17%), did not negatively impact pine or alder growth at 4 mo. Biochar

Table 5. Ectomycorrhizal (ECM) morphotypes identified from pine *(Pinus contorta)* and alder *(Alnus crispa)* seedlings grown in this study

ECM morphotype	Host	GenBank accession number	Match in GenBank	Base pair similarity (%)
Cenococcum	Pine	JF298206	*Cenococcum geophilum* EU057125.2	480/490 (97)
Hebeloma	Pine	JF298207	*Hebeloma ammophilum* AY948190.1	736/738 (99)
Rhizopogon-Suillus 1	Pine	JF298208	*Rhizopogon* FJ554251.1	668/670 (99)
Rhizopogon-Suillus 2	Pine	JF298209	*Rhizopogon* GQ267482.1	681/685 (99)
Tomentella	Pine	JF298210	*Tomentella* AF430289.1	764/773 (99)
Amphinema[z]	Pine			
E-strain[z]	Pine			
MRA[z]	Pine			
Russulaceae[z]	Pine			
Tomentella-like	Alder	JF298203	Uncultured ectomycorrhiza (Thelephorales) FM993226.1	809/815 (99)
Unknown-yellow	Alder	JF298204	Uncultured ectomycorrhiza FM993124.1	748/819 (92)
Unknown-white	Alder	JF298205	Uncultured ectomycorrhiza (*Alnicola*) FM993256.1	815/825 (99)
Cenococcum[z]	Alder			

[z]Identified morphologically.

Table 6. Mean (SE) frequency, abundance and Shannon (H') and Simpson's (1/D) Diversity values of pine ECMs for biochar and fertilizer treatment groups compared by two-way ANOVA ($n=4$)

ECM morphotype	Freq (%)	Abund *P* value	No Fertilizer			Fertilizer		
			0% biochar	5% biochar	10% biochar	0% biochar	5% biochar	10% biochar
Amphinema	67	NS	5.9 (2.7)	1.9 (1.4)	6.5 (3.2)	4.4 (2.3)	1.4 (0.4)	15.4 (8.3)
Hebeloma	33	NS	4.3 (4.3)	3.4 (2.1)	7.1 (7.1)	0.7 (0.5)	1.3 (1.1)	3.5 (2.4)
Russulaceae	80	NS	30.9 (12.6)	32.4 (7.6)	22.0 (10.9)	16.1 (5.0)	32.8 (12.9)	22.5 (11.6)
Rhizopogon-Suillus 1	63	NS	4.4 (2.8)	8 (5.1)	3.9 (2.8)	14.0 (5.9)	9.8 (5.1)	12.1 (3.1)
Rhizopogon-Suillus 2	33	**0.037**	0*a*	5.3 (3.0)*ab*	4.9 (3.7)*ab*	0*a*	15.6 (7.9)*b*	2.0 (2.0)*a*
Tomentella	20	NS	0.2 (0.2)	0	2 (1.5)	0	1.3 (1.3)	15 (13.1)
E-strain	100	**0.035**	29.1 (10.9)*a*	43.4 (8.7)*ab*	51.8 (14.1)*ab*	59.1 (3.8)*b*	32.3 (11.0)*ab*	28.7 (8.7)*a*
Cenococcum	70	**0.018**	1 (0.8)*a*	2.7 (0.9)*b*	1.8 (0.5)*a*	1 (0.4)*a*	3.6 (1.4)*b*	0.2 (0.1)*a*
MRA	7	NS	0	0.2 (0.2)	0	0	0.2 (0.2)	0
non-mycorrhizal	40	NS	24.3 (14.6)	2.7 (1.4)	0	4.6 (2.9)	1.7 (1.2)	0.6 (0.4)
Shannon Diversity (H')		NS	0.96 (0.23)	1.06 (0.12)	0.93 (0.24)	0.92 (0.11)	1.19 (0.09)	1.14 (0.07)
Simpson's Diversity (1/D)		NS	2.86 (0.52)	2.86 (0.35)	2.40 (0.40)	2.37 (0.14)	2.64 (0.32)	2.69 (0.24)

a, *b* Within each row, *a* and *b* denote significant differences between treatments at $\alpha=0.05$.

addition did not appear to limit N availability in these soils because shoot biomass in biochar-only treatments was equal to, or better than, the controls or treatment receiving only fertilizer N. Seedling ECM are proficient at acquiring N and most likely not negatively impacted via competition with saprotrophic microflora for mineral N or organic sources of N (Allen et al. 2003). Biochar-induced transformation of soil N may also differ between agricultural and forest soils. For example, Kolb et al. (2010) found that conifer forest soil had the greatest total extractable N following high rates of biochar addition, whereas temperate arable soils exhibited reductions in extractable N with increasing rates of biochar addition. Increased net N mineralization in black carbon-treated forest soils has been attributed to declines in inhibitory phenolic compounds or due to increased sorption of available C (DeLuca et al. 2002, 2006; Berglund et al. 2004).

Ectomycorrhizas

To our knowledge, this is the first study to examine the impacts of biochar amendment on indigenous ECM communities in sub-boreal forest soils that are typical of northern regions of western Canada. Biochar amendment did not influence the diversity of the ECM community associated with pine, but biochar amendment (5 or 10% by weight) increased the abundance of *Rhizopogon-Suillus 2* (only fertilized soil) and *Cenococcum* (fertilized

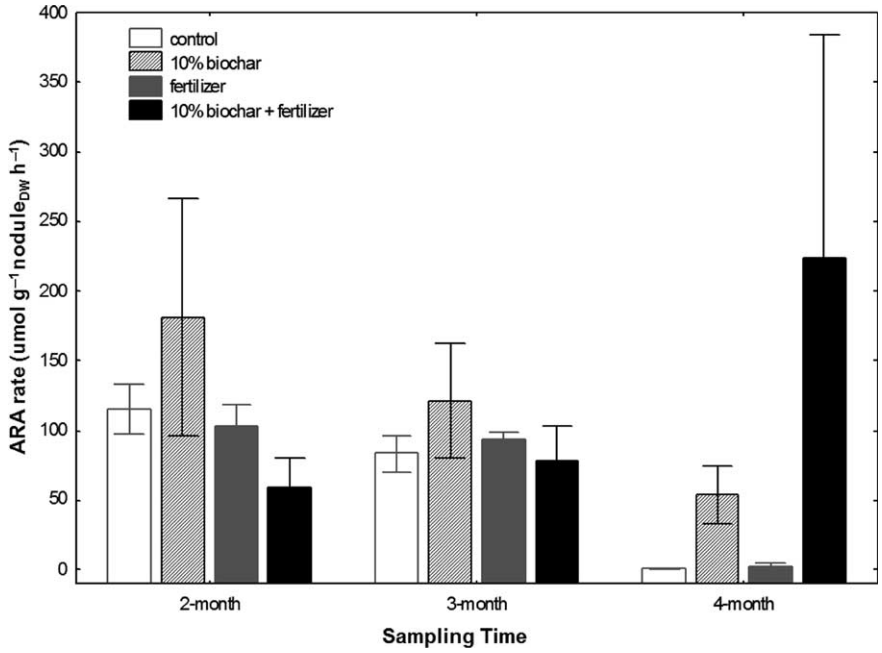

Fig. 2. Acetylene reduction assay (ARA) values (μmol g^{-1} h^{-1}) for alder nodules at 2, 3 and 4 mo by treatment group ($n=4$).

and non-fertilized soil); abundance of E-strain was reduced with 10% biochar treatment (only fertilized soil). Biochar amendment did not influence the abundance of other ECM morphotypes and did not significantly decrease the proportion of uncolonized (non-mycorrhizal) root tips. Previous studies have reported a greater number of colonized root tips (Harvey et al. 1976) as well as earlier onset of mycorrhizal formation (Herrmann et al. 2004) in association with low levels (usually less than 2%) of activated charcoal amendment. Enhanced early ECM establishment may contribute to greater plant productivity, with increased biomass of fungal mycelia providing access to a greater supply of nutrients (Smith and Read 2008). In contrast, Choi et al. (2009) found a dramatic reduction in ECM colonization of *Pinus densiflora* and *Larix kaempferi* roots grown in brown forest soils amended with 30% biochar (by volume) compared with controls. These findings suggest that there may be an upper limit to the amount of biochar that can be added to soil without disrupting the formation of ECMs.

Little is understood regarding the importance of species assemblages of ECM communities for mycorrhizal functioning or how small changes in relative abundance may impact plant nutrient uptake. It has been suggested that ECM fungal species that dominate host root systems may contribute more to mycorrhizal functions such as nutrient acquisition and C exudation than less abundant species (Allen et al. 2003). It then follows that E-strain and Russulaceae, which accounted for about 29–59% and 16–33% of ECM communities, respectively, contribute significantly to nutrient acquisition in 4-mo pine seedlings. Changes in the abundance of a less-abundant species such as *Cenococcum* may not have greatly influenced nutrient acquisition. However, the large increase in abundance of *Rhizopogon-Suillus 2* in the 5% biochar plus fertilizer treatment may have played a significant role in the nutrient acquisition of pine in this treatment. *Rhizopogon-Suillus* ECMs, which rarely dominate root systems, may exhibit extensive growth of extraradical mycelia and rhizomorphs and be functionally important for nutrient acquisition and cycling in some ecosystems (Agerer 2001; Genney 2006). Interestingly, *Rhizopogon-Suillus* species were absent from all control seedlings in the current study.

The role of E-strain in nutrient acquisition for young seedlings is unknown, but it may be an important early colonizer of young pine roots that is replaced through succession as the root system ages. Russulaceae ECMs, which are commonly associated with acidic, low-N soils, may be specialized to access organic sources of N directly via oxidative enzyme activity, which may give these fungi a competitive advantage over ECM fungi unable to acquire nutrients from organic substrates in nutrient-poor habitats (Lilleskov et al. 2002). The fact that biochar addition had no significant influence on the abundance of E-strain and Russulaceae ECMs in non-fertilized soils means that nutrient uptake through ECM-mediated processes was likely not greatly influenced by biochar in soil not receiving N-fertilizer additions. Given the fact that only three ECM morphotypes fluctuated in their abundance due to biochar addition, it is unclear if there was a direct link between ECM communities and the observed greater plant growth of pine in biochar-amended soils.

The generally limited ECM colonization of root tips in alder did not allow us to fully determine the effect of biochar-amendment on alder ECM community composition. However, molecular identification of the ECM fungi present on alder root tips agrees with the previously described trend of *Alnus* ECMs being distinct and less diverse than those of other hosts (Pritsch et al. 2009; Tedersoo et al. 2009; Kennedy and Hill 2010).

Biological N Fixation

Alder N fixation (ARA) rates did not vary with 10% biochar treatments at 2 and 3 mo; however, at 4 mo, N fixation was maintained in all four replicates of biochar treatments compared to none or negligible rates in the four replicates of non-biochar treatments. We believe that the lack of significant biochar treatment effect (at 4 mo) was in part due to the large variation in the ARA data and the small sample size ($n = 4$). This trend occurred concurrently with a significant increase in alder shoot biomass in biochar-amended soils, although there was no change in the shoot: root ratio (maintained at about 1) or in nodule number, biomass or condition. We do not have a definitive explanation for this trend. Fluctuations in N fixation have been reported to be transient in nature, resulting from aging nodules or variability of nitrogenase activity (Roy et al. 2007). For 2- to 3-yr-old red alder, N fixation fluctuated both annually and diurnally over a 7-mo growing season (Tripp et al. 1979). The number of nodules may be independent from effective N fixation, as some nodules are ineffective for N fixation (Roy et al. 2007).

Contrary to other studies (Coté et al. 1989; Voisin et al. 2002; Martin et al. 2003), urea-N had no effect on ARA (N fixation) rates or nodule frequency and size. Gaulke et al. (2006) reported that urea addition (140, 280 and 560 kg N ha^{-1}) inhibited N fixation (as ARA) and had a negative impact on *Alnus* seedling growth at the end of the first year. The N application rates in our study (equivalent to $\sim$200 kg N ha^{-1}) were comparable with the study reported by Gaulke et al. (2006). Perhaps the soil preparation methods used in the current study (i.e., mixing of organic and inorganic soil components) resulted in net N immobilization of available N in urea treatments.

Management and Conclusions

This short-term greenhouse study showed that biochar addition can enhance soil properties and the early growth of pine and alder in sub-boreal forest soils. It supports the idea that incorporation of biochar into nursery soil for seed germination and seedling growth

prior to outplanting may be an appropriate method for introducing biochar into forest ecosystems where benefits may contribute to sustainable forest management. Early colonization of pine by some ECMs appeared to be enhanced by biochar addition, but the abundance of the majority of ECMs were unaffected by biochar addition. There is need to further determine/explore the extent of this effect as well as the impact increased frequency and (or) shifts in ECM abundance might have on seedling growth. These soils and plant species may be particularly amenable to biochar treatment because these systems typically receive inputs of black carbon through historic wildfires (Schmidt and Noack 2000; Preston and Schmidt 2006). To date, there has been insufficient experimental research to determine the longer-term effects of biochar addition to forest soils, including the influence of biochar on N cycling, microbial ecology and potential environmental impacts.

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