

A mycorrhizal fungus grows on biochar and captures phosphorus from its surfaces



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ABSTRACT

Biochar application to soils has potential to simultaneously improve soil fertility and store carbon to aid climate change mitigation. While many studies have shown positive effects on plant yields, much less is known about the synergies between biochar and plant growth promoting microbes, such as mycorrhizal fungi. We present the first evidence that arbuscular mycorrhizal (AM) fungi can use biochar as a physical growth matrix and nutrient source. We used monoxenic cultures of the AM fungus *Rhizophagus irregularis* in symbiosis with carrot roots. Using scanning electron microscopy we observed that AM fungal hyphae grow on and into two contrasting types of biochar particles, strongly attaching to inner and outer surfaces. Loading a nutrient-poor biochar surface with nutrients stimulated hyphal colonization. We labeled biochar surfaces with ³³P radiotracer and found that hyphal contact to the biochar surfaces permitted uptake of ³³P and its subsequent translocation to the associated host roots. Direct access of fungal hyphae to biochar surfaces resulted in six times more ³³P translocation to the host roots than in systems where a mesh prevented hyphal contact with the biochar.

We conclude that AM fungal hyphae access microsites within biochar, that are too small for most plant roots to enter (<10 μm), and can hence mediate plant phosphorus uptake from the biochar. Thus, combined management of biochar and AM fungi could contribute to sustainable soil and climate management by providing both a carbon-stable nutrient reservoir and a symbiont that facilitates nutrient uptake from it.

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1. Introduction

Biochar is a carbon rich residue of pyrolyzed biomass (combusted under low oxygen conditions) that as a soil amendment can improve soil fertility. Many governments have recently become interested in investigation of biochar because of its potential in climate change mitigation (Laird, 2008; Sohi, 2012) because charcoal decomposes very slowly in the soil (estimates range from 1000 to 10,000 years; Skjemstad et al., 1998; Krull and Skjemstad, 2003).

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Soil organic carbon is globally the largest organic carbon reservoir, even when both the biosphere and the atmosphere are included (Lal, 2008). Transfer of carbon from the atmosphere to soil could thus have a large impact on the global carbon balance. Combining biochar production with bioenergy production could even result in a CO₂-negative balance (Lehmann, 2007a).

There are sites in South America where charcoal mixed with feces and bones has been added to soils over a period of several thousand years, resulting in e.g. the Amazonian Dark Earth, or *terra preta* soils. These soils have significantly higher fertility compared with nearby soils that lack charcoal, and the charcoal is considered at least partly responsible for this (Glaser et al., 2001).

Both in field observations and controlled experiments, plant yield has been observed to respond positively to biochar addition, especially in acidic and coarse textured soils (Jeffery et al., 2011).

However, much less is known about the response of soil organisms to biochar addition (Lehmann et al., 2011), despite their importance for nutrient cycling and as plant symbionts. Mycorrhiza is a symbiosis between the majority of land plants and root endophytic fungi (Smith and Read, 2008). It is an ancient and ubiquitous mutualistic interaction that is important for plant biomass production. Among other functions in ecosystems (Rillig, 2004), mycorrhizal fungi provide their host plants with mineral nutrients and receive photosynthetically derived carbohydrates in return. Arbuscular mycorrhizal (AM) fungi are especially important for acquiring phosphorus (P), which has low mobility in soil and is often a poorly accessible plant nutrient because it is immobilized on soil colloids (Smith and Read, 2008).

The few studies of the effects of biochar on mycorrhiza have mainly considered root colonization and show diverging results. Some researchers report root colonization rates to be strongly enhanced by biochar (Ishii and Kadoya, 1994; Blackwell et al., 2010), whereas others present evidence that colonization decreases (Birk et al., 2009; Warnock et al., 2010). Some of the negative effects on root colonization could be explained by plant mediated feedback mechanisms, i.e. soils amended with high-ash biochar content could release and provide elevated amounts of available nutrients (Lehmann et al., 2003; Mukherjee and Zimmermann, 2013) and a nutrient-saturated host plant decreases its mycorrhizal root symbionts (Gryndler et al., 2006). It is likely that there are other, direct effects of biochar on mycorrhizal fungi that are poorly understood. To disentangle the direct effects from the indirect host-feedback effect, it is necessary to experimentally restrict the access to biochar to only the fungus.

Addition of biochar affects multiple soil properties that all can directly influence AM fungi (Warnock et al., 2007), such as: modified soil pH and its feedback on nutrient availability and microbial community structure (Lehmann, 2007b); altered nutrient release, retention or immobilization (Blackwell et al., 2010; Mukherjee and Zimmermann, 2013); changed water retention capacity (Glaser et al., 2002; Basso et al., 2013); and provision of shelter against fungivore grazing (Thies and Rillig, 2009; Ogawa and Okimori, 2010). The last could lead to a relative increase of viable hyphae inside biochar because intense grazing by soil animals decreases the amount of hyphae in the soil matrix. In the absence of grazing, increased hyphal density in the biochar must be a result of increased productivity, a foraging strategy in response to chemical properties of the material. It is known that AM fungi proliferate in both organic and inorganic nutrient rich patches (Hodge et al., 2001; Hammer et al., 2011).

Nutrients are taken up by AM fungi via active parts of the external mycelium, including the region immediately behind the growing hyphal tips (Bago, 2000). Older parts of the mycelium are also likely to be involved in nutrient uptake through lateral hyphal walls (Schnepp et al., 2007). Phosphorus is taken up as orthophosphate by high-affinity transporters in the external mycelium (Harrison and Van Buuren, 1995; Maldonado-Mendoza et al., 2001; Benedetto et al., 2005). Close proximity of the hyphae to the nutrient sources is important for uptake efficiency, especially for ions with low mobility such as phosphate (Barber, 1995).

The goals of our study were to investigate: a) whether AM fungal hyphae grow on and into ash-rich and ash-poor biochar and if this depends on the surrounding nutrient conditions; b) whether attachment and uptake structures are established on the biochar surfaces; and c) if AM fungi can acquire nutrients, especially P that is associated with biochar and thus whether biochar can serve as a nutrient reservoir. We used *in vitro* mycorrhizal cultures and added biochar in a separate compartment, to which hyphae, but not the host plant roots, had access. By using these gel/water-based cultures we could observe

morphological patterns in the growth medium that otherwise are obscured in soil.

2. Materials and methods

2.1. Cultures and biochar

Experiments 1–3 were performed with sterile root organ cultures of *Daucus carota* L. inoculated with *Rhizophagus irregularis* Schenk and Smith (DAOM 197198; Biosystematics Research Center, Ottawa, Canada), recently recommended to be renamed from *Glomus intraradices* (Stockinger et al., 2009). Because of the biotrophic nature of AM fungi, sterile experiments need to be performed in connection with a host plant. To achieve this, transformed carrot roots with inhibited shoot formation were grown in Petri plates. The transformed *D. carota* roots originated from a clone of the DC1 line, transformed with T-DNA from the Ri plasmid of *Agrobacterium rhizogenes* (Becard and Fortin, 1988), originally established by StArnaud et al. (1996). Maintenance was accomplished by propagating AM fungi colonized cultures on a minimal nutrient medium (M-medium; Becard and Fortin, 1988; Table S1), including 10 g l⁻¹ sucrose, low phosphorus concentration of 35 μM (4.8 mg l⁻¹ KH₂PO₄) and 0.3% Phytigel™ for stabilization (Sigma Chemical Co., St. Louis, MO, USA). Experiments 1–3 used 2-compartmented Petri plates, where one side served as a root compartment (RC) including roots and the AM fungus, and the other served as a root free hyphal compartment (HC). The RC was filled with 15 ml M-medium containing phytigel and sucrose. Cylindrical plugs of colonized roots from four month old cultures were transferred into a hole in the fresh gel. After an establishment period of 30 days, the HC was filled either with 15 ml liquid M-medium lacking sucrose and phytigel, or with MilliQ water. Plates were produced in excess and only those were used that showed hyphal growth toward or over the barrier after 30 days, because a mycelium in the HC was the prerequisite for the start of the study. Roots passing over the barrier to the hyphal compartment were periodically removed.

We used a nutrient and ash poor wood biochar for Experiments 1–3. It was made from wood pellets, a mixture of spruce and pine wood without bark or any adhesive agents that are manufactured for household energy production (purchased from Bioenergi Skandinavien AB, Sweden). Pellets were cylindrical with a diameter of 0.7 cm and length of ~1.3 cm. They typically had an ash content of 0.3% and an N content of <0.1%. Biochar was produced by pyrolyzing the wood pellets at 550 °C under low oxygen conditions in a muffle furnace (Nabertherm, Wilhelm Tham AB, St Anna, Sweden) for 12 h. The pellet parent material was filled into metal containers, surrounded and covered by sand, and loosely sealed with aluminum foil. The resulting wood biochar was 0.5 cm in diameter and ~1 cm long; mass yield was 32% of the dry parent material. The wood biochar contained 85% C, 0.1% N (Euro EA Elemental Analyzer, HekaTech, Germany) and 0.15% P (acid digested in a 4:1 mixture (v:v) of 65% nitric–70% perchloric acids; total P was determined by the molybdate blue method (Murphy and Riley, 1962, using Auto-Analyzer 3, Bran+Luebbe, Norderstedt, Germany). Ash content was 1.4%, determined gravimetrically after combustion at 600 °C. The pH of the wood biochar at equilibrium with water (1:5 w/w) was 7.6.

In Experiment 1, we also tested a nutrient- and ash-rich biochar that was produced from chicken manure (from Bauhaus, Econova Garden AB, S-61621 Åby, Sweden) under the same conditions as the wood pellet biochar. The chicken manure biochar product is coarsely granular, intermixed with sand particles and contains 37% C, 2.5% N and 0.5% P. Ash content was 44% and pH was 10.8. Biochar used in all experiments was derived from the

same production batch. It was autoclaved prior to addition to the sterile cultures.

2.2. Experimental design and method of analysis

2.2.1. Experiment 1: small-scale physical interactions between hyphae and biochar surfaces

We investigated the morphological response of AM fungal mycelia to two contrasting biochars, with low (wood) or high (chicken manure) ash and nutrient content, using electron microscopy. Wood or chicken manure biochar was added to the hyphal compartment in either MilliQ water or M-medium (always without sugar and gel) in a full factorial design ($n = 5$). After 2.5 months, the plates were destructively sampled. We analyzed both outer and inner parts of the biochar; for the latter we physically broke open the particles with a scalpel. To determine the location, contact surface and morphology of fungal hyphae on the external surfaces and inside the pores of the biochar, we used scanning electron microscopy (SEM) in three different imaging modes: low vacuum environmental, cryo and traditional high vacuum (Goldstein et al., 2003; Balogh-Brunstad et al., 2008a; Dohnalkova et al., 2011).

To minimize artifacts of traditional biological sample preparation, low vacuum environmental SEM (Quanta FEG 3D; FEI, Eindhoven, The Netherlands) was used (Balogh-Brunstad et al., 2008a). After a small section was cut with a sharp scalpel to reveal surfaces within the biochar particle, the samples were mounted on stubs with double sticky carbon tape and transported to the microscopy laboratory in their original solution. Each sample was imaged in low vacuum mode at 5 keV accelerating voltage using the gaseous secondary electron detector. Exposure in the environmental SEM still dehydrated the fungal hyphae, decreasing resolution.

To enhance resolution, we used cryoSEM on samples in a nearly hydrated state (Dohnalkova et al., 2011). The cut samples were secured on stubs with liquid carbon glue for about 1 min, plunged into liquid nitrogen inside the Quorum cryopreparation stage (Polaron, Quorum Technologies, United Kingdom) then each sample was removed and transferred to the cryopreparation chamber. The upper layer of the amorphous water was sublimated for 3–5 min at $-95\text{ }^{\circ}\text{C}$ to emphasize the three dimensional structure of the biochar, fungal hyphae and extracellular exudates. Then the temperature was decreased back to $-160\text{ }^{\circ}\text{C}$. The sample was sputter coated with a few nanometers of Pt in the cryopreparation chamber and transferred to the cooled stage of the Quanta FEG 3D (FEI, Eindhoven, The Netherlands) SEM for secondary electron imaging.

The third method was traditional, high vacuum SEM using an XL 30 FEG SEM (FEI, Eindhoven, The Netherlands) to provide high resolution images both of the outside and the inside parts of the biochar (Goldstein et al., 2003). The samples were air dried, cut with a sharp scalpel, mounted on stubs with double sticky carbon tape, gold coated (100 nm) and imaged in high vacuum at 2 keV. This method provided best resolution of surface details. In total, 108 images were taken from biochar samples from 9 plates; four of each wood biochar in water or M-medium, and one plate of chicken manure biochar in M-medium.

The growth direction of hyphal tips was determined from 35 hyphal tip occurrences found within all of the SEM images and hyphal attachment was determined on 511 hyphae found on 37 different locations within all SEM images. In addition, all plates ($n = 5$) of wood and chicken manure biochar in water or in M-medium were imaged with stereo microscopy. Biochar macropore size was also measured on 22 randomly selected SEM images of cut biochar.

2.2.2. Experiment 2: mycelium foraging for biochar under different nutrient regimes

We measured pattern and density of AM fungal colonization of wood biochar with two levels of nutrient content relative to the surrounding reference medium with either high or low nutrient content.

Three treatments were applied to the HC of the plates: untreated biochar placed in MilliQ water; untreated biochar placed in M-medium; or M-medium loaded biochar placed in MilliQ water ($n = 5$). The M-medium loaded biochar was produced by soaking biochar in M-medium overnight, followed by rigorously washing five times with MilliQ water prior to autoclaving. Ninety days after the addition of the biochar to the HC, the plates were analyzed *in situ*, in the HC, for hyphal length (Tennant, 1975) and for the number of contact points of hyphae to the biochar (Fig. 1A). A 2 mm wide band around the longitudinal midsection of biochar particle running horizontally to the plate bottom (Fig. 1A) was selected for quantification, where contact points were easily visible. Base and wall area of the cylindrically shaped pellets were measured separately and counts were expressed per outer geometric surface area. Screening of a 2 mm wide band on each particle provided a representative surface area that allowed us to determine if hyphae were distinctly touching the surface of the biochar or entering its pore network (Fig. 1B) or simply growing parallel to the outer biochar surfaces without touching it (Fig. 1C).

2.2.3. Experiment 3: access of AM fungal hyphae to adsorbed ^{33}P from biochar surfaces

We quantified recovered ^{33}P in roots that were connected through AM fungal mycelia to a root free compartment with biochar surface that had been labeled with ^{33}P (Fig. 5A,B). In one case, this biochar was freely available for AM fungal hyphae; in the other case, a fine mesh prevented AM fungal hyphae from direct contact with its surface. The wood biochar surface had been labeled with radioactive phosphorus (^{33}P) as phosphate, to be used as tracer for the extent of uptake of the adsorbed phosphorus. Biochar (20 g, approx. 50 ml) was labeled for 6 h in 100 ml of 1 mM PO_4^{3-} solution that contained 5 MBq of ^{33}P . After the labeling period, the biochar was thoroughly rinsed in MilliQ water, through four cycles of shaking on a table shaker, for 30 min each, then for a final period of 3 h. Samples of the labeling solution were taken before and after addition of the biochar and after each MilliQ water rinse. The ^{33}P labeling solution contained 49.6 kBq ml^{-1} before addition of 20 g biochar and 47.9 kBq ml^{-1} after removal of the biochar. Thus, the biochar sorbed 2.02% of the radioactivity and with this, 2.02 $\mu\text{mol P}$. Radioactivity that diffused out of the biochar decreased exponentially during the rinsing cycles and the last rinse contained less than 200 Bq ml^{-1} , or 0.37% of the added radioactivity (Fig. S1).

The ^{33}P labeled biochar was added into triangular shaped mesh bags of either 25 μm (accessible for AM fungal hyphae) or 1 μm mesh size (inaccessible for AM fungal hyphae). Each mesh bag contained 500 mg biochar, labeled with a radioactivity of 2.5 kBq and 50.4 nmol absorbed P as phosphate. To initiate the experiment, the mesh bags were placed into the HC in 15 ml of MilliQ water of one-month-old root organ cultures. Roots passing over the barrier to the HC were eliminated and the RC was amended with 4 ml glucose solution (50 mg ml^{-1}). Plates were sealed and cultured at 24 $^{\circ}\text{C}$.

The plates were destructively harvested 69 days after addition of the ^{33}P labeled biochar. The mesh bags were opened and checked for hyphal colonization. Of 8 original replicates per treatment, two mesh bags with each treatment did not produce the intended treatment effect (i.e. present or absent hyphal colonization in the inaccessible or accessible mesh bags because there was no hyphal growth or penetration of the 1 μm mesh). These samples were not

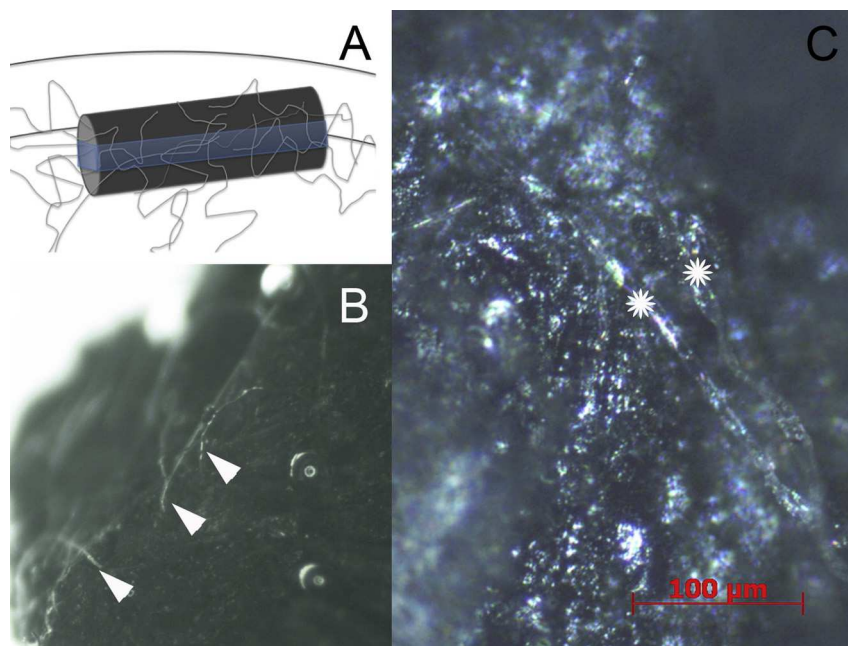


Fig. 1. Visualization of the method for quantification of hyphal contact to biochar. A dissecting light microscope was used for examining biochar particles to determine the hyphal contact points on the surfaces. (A): schematic drawing of a light blue band around the particle shows the area that was selected for quantification. (B): The arrows on the image show hyphal entry points into the pores of biochar that were detected by light microscopy. (C): Many hyphae also run parallel to the biochar surface, as shown by the stars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

analyzed so that the final replication was six samples per treatment ($n = 6$). The biomass in the RC was collected after solubilizing the phytigel in 10% sodium citrate, then it was freeze dried and the dry weight was recorded. Internal mycelium and residues of the external mycelium of the fungus in the RC were included in this sample. The roots were ashed at 500 °C, then acid digested in 1 M HCl (Lekberg et al., 2010), mixed with Packard Ultima Gold scintillation cocktail, and radioactivity was quantified on a Beckmann LS 6500 Scintillation Analyzer.

2.3. Statistical analysis

Differences of the results between treatments in each experiment were analyzed by ANOVA and the posthoc test, Tukeys HSD, performed with JMP 9 (SAS Institute Inc., Cary, USA). Results are presented as mean \pm SE.

3. Results

3.1. Experiment 1: small-scale physical interactions between hyphae and biochar surfaces

The SEM investigation revealed that dense mycelia surrounded the outer surface of the wood biochar and its close proximity (Fig. 2A). In MilliQ water, hyphal coverage was patchy on the outer surface and more pronounced at the base of the cylindrical pellets, where up to 16 μm hyphae crossing a surface of 100 μm^2 could be observed. However, a larger part of the biochar surface remained uncolonized. Biochar in sugar-free M-medium was fully covered by mycelium on the outer surface. Hyphae had grown inside the biochar samples and could be observed on the exposed surface of a broken biochar particle (Fig. 2B). We found hyphae that had entered small openings or pores (Fig. 2C) and ~80% of the hyphae near the surface were firmly attached to surfaces over parts of their length (stable in vacuum; Fig. 2D). About 89% of the observed hyphal tips and putative adsorbing structures (Fig. 2E,F) were directed

toward the biochar surfaces and were firmly attached to it. With cryo-SEM, putative hyphal exudates were detected (Fig. 2F; arrows). These were visible as amorphous structures on the biochar and they were only found directly beneath hyphae or hyphal tips (Fig. 2F,G). The wood biochar morphology and macropore size (between 1 and 15 μm in diameter, Fig. 2H) allowed hyphal colonization because the observed hyphal diameters ranged from 1 to 10 μm .

The chicken manure biochar had a different matrix than the wood biochar; it lacked defined pore structure, crumbled more easily and exhibited larger heterogeneity than the wood biochar. However, hyphae grew through interstices also in these samples (Fig. 3A,B). Hyphae grew on and into chicken manure biochar both when placed into M-medium (Fig. 3C) and MilliQ water (Fig. 3D).

3.2. Experiment 2: mycelium foraging for biochar under different nutrient regimes

Biochar loaded with M-medium had more than three times as many hyphae growing on or into it than untreated biochar in water or M-medium, relative to the total amount of hyphae in the HC (Fig. 4A, $F = 12.7$, $p = 0.0005$). This was also macroscopically visible in the HC (Fig. 4B–D). For M-medium loaded biochar in MilliQ water, a few runner hyphae connected the mycelium of the RC to biochar particles, while the rest of the plate remained unexplored by the fungus (Fig. 4B). Once in contact with the biochar surface, hyphae proliferated on and into the biochar (Fig. 4B). Untreated biochar pieces placed into MilliQ water also attracted the mycelium as runner hyphae connected to the particles but fewer hyphal attachments and entry points were found on the surface (Fig. 4C). In hyphal compartments containing M-medium, hyphal density was generally very high and covered the whole plate, irrespective of whether hyphae were in contact with a piece of biochar or not (Fig. 4D). Hyphae grew on and into the biochar frequently but the greater number of contact and entry points was a function of a higher total hyphal length in the entire plate. The number of hyphal

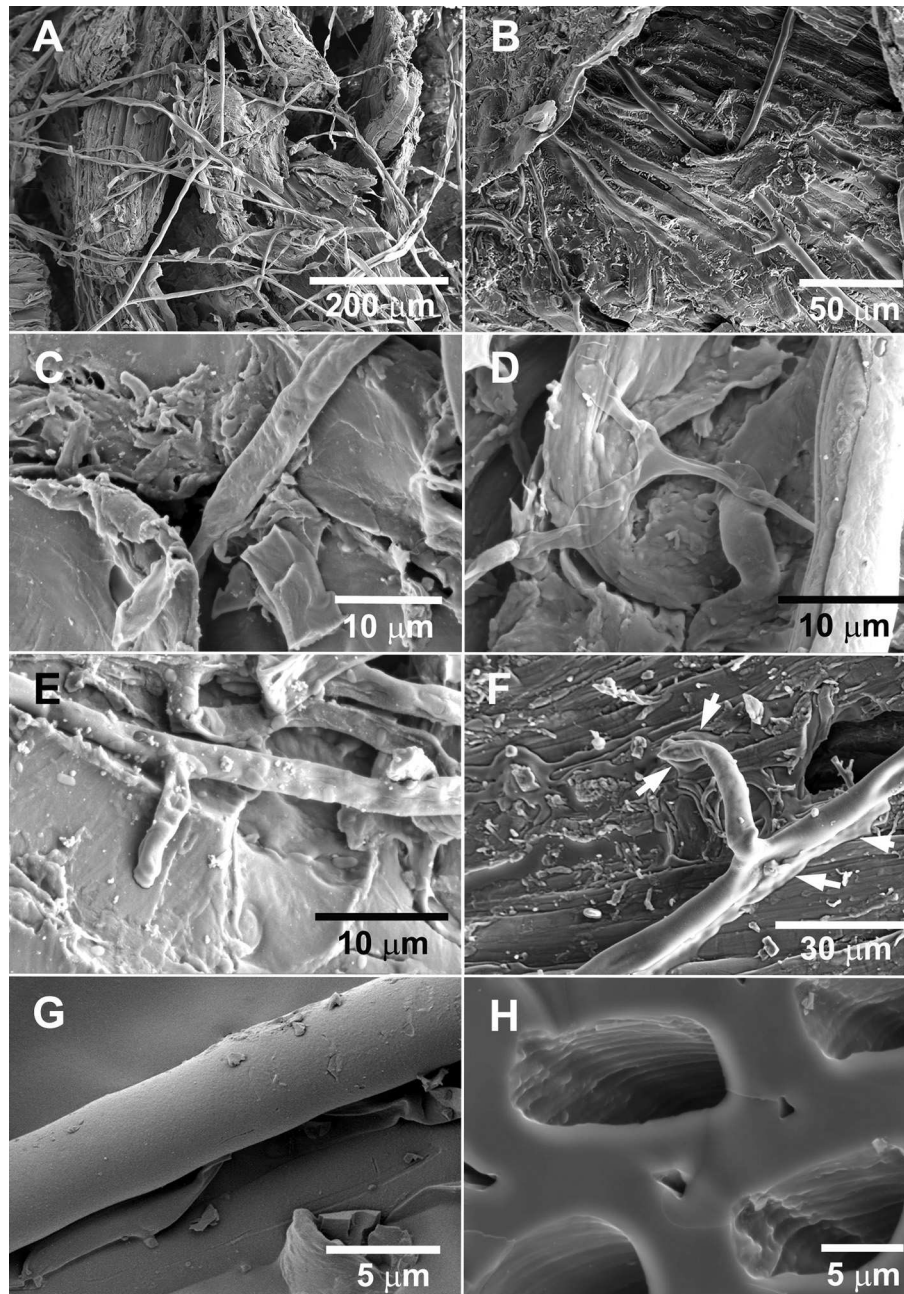


Fig. 2. Scanning electron microscopy (SEM) images of colonized wood biochar from Expt. 1. Wood biochar was covered with hyphae (elongated structures) on the outer surfaces in M-medium (A) and inside pores in MilliQ water (B). Hyphae were able to enter pores (C) and strongly attach to surfaces (D). Branched fine structures, putative uptake structures, were observed (E). Cryo-SEM revealed exudates beneath the hyphae (F, arrows). Few well preserved hyphae were observed with traditional high vacuum SEM, showing dried putative exudates beneath (G). Pore size diameter distribution of wood biochar is commonly between 5 and 15 μm (H). Images A–F were obtained by cryo-SEM, and H was obtained by environmental SEM.

touching points was $4.1 \pm 0.53 \text{ mm}^{-2}$ biochar surface for biochar loaded with M-medium placed in water, $0.28 \pm 0.12 \text{ mm}^{-2}$ biochar surface for untreated biochar in water, and $1.96 \pm 0.55 \text{ mm}^{-2}$ biochar surface for untreated biochar placed into M-medium.

3.3. Experiment 3: access of AM fungal hyphae to adsorbed ^{33}P from biochar surfaces

We recovered 11.5% of the initially added ^{33}P (2.5 kBq) from *D. carota* roots connected to AM fungal hyphae that had direct physical access to ^{33}P loaded biochar surfaces. This is six times more

than from roots connected to AM fungal mycelia that did not have direct physical contact with labeled biochar and therefore could only acquire ^{33}P through diffusion (Fig. 5C, $F = 16.78$; $p = 0.0022$; Fig. 5D). From the amount of ^{33}P , we calculated that 5.8 nmol P was transferred to roots by hyphae that had direct physical contact with the biochar, compared with 0.97 nmol P for those that only had access to ^{33}P through diffusion from the surrounding solution. In both treatments, the solution in the fungal compartment surrounding the biochar mesh bags contained similar amounts of desorbed ^{33}P of about 450 Bq per compartment ($F = 0.027$; $p = 0.87$, Fig. 5D). The amount of the desorbed radioactivity in the solution of

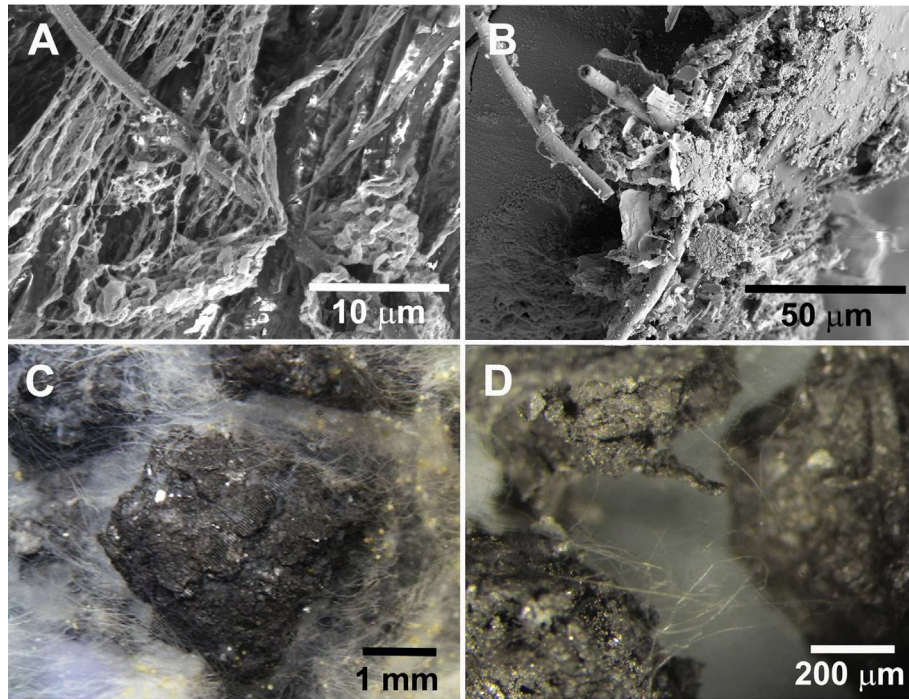


Fig. 3. Microscopy images show the interaction of AM fungal hyphae and chicken manure biochar particles. Chicken manure biochar was successfully colonized by AM fungi (A: surface shot, B: inner part of the biochar) as shown by cryoSEM. *In situ* dissecting microscope images of chicken manure biochar show complete hyphal coverage in M-medium (C) and distinct hyphal connection of particles in MilliQ water (D).

Hyphal touching points on biochar per hyphal length in HC

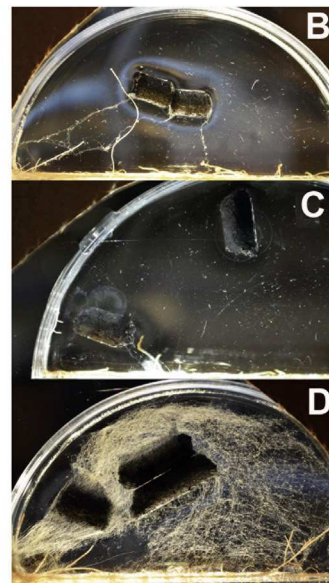
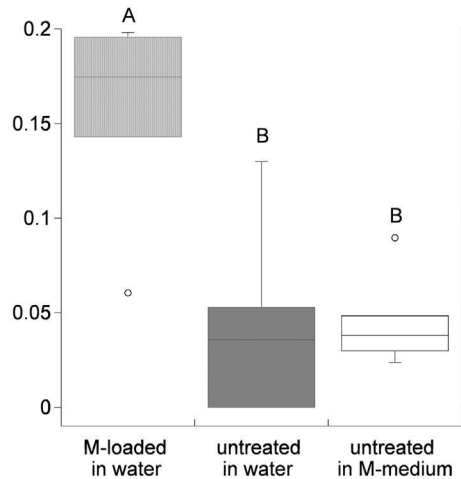


Fig. 4. Relative number of hyphal attachment points on the biochar surfaces in water or M-medium and on M-medium loaded biochar in water, Expt. 2 (A). The data were normalized to the total hyphal length present in the hyphal compartment. Different letters (a, b) denote statistically significant differences, $n = 5$. Photographs of mycelium growing around biochar loaded with M-medium in water (B), untreated biochar in water (C), or in M-medium (D).

the hyphal compartment (HC) was not related to the amount of radioactivity in the root compartment (RC; linear regression, $F = 0.035$; $p = 0.85$). Hyphal lengths in the HC outside the mesh bags did not differ among the treatments ($F = 0.0064$; $p = 0.94$).

4. Discussion

Biochar was an attractive substrate for the external mycelium of AM fungi. We found hyphae growing on and into its pores,

especially under low nutrient conditions. This was true for each of two different biochar types. The fungi grew both into the ash-poor wood biochar as well as into the high pH, and ash-rich chicken manure biochar. We demonstrated that when the mycelium has direct contact with the biochar surfaces, it is able to capture adsorbed phosphates. Under P limiting growth conditions, such an extra nutrient source could constitute an important competitive advantage for the fungus itself, as well as for the associated host plant. This direct access to a P source in biochar is limited to soil

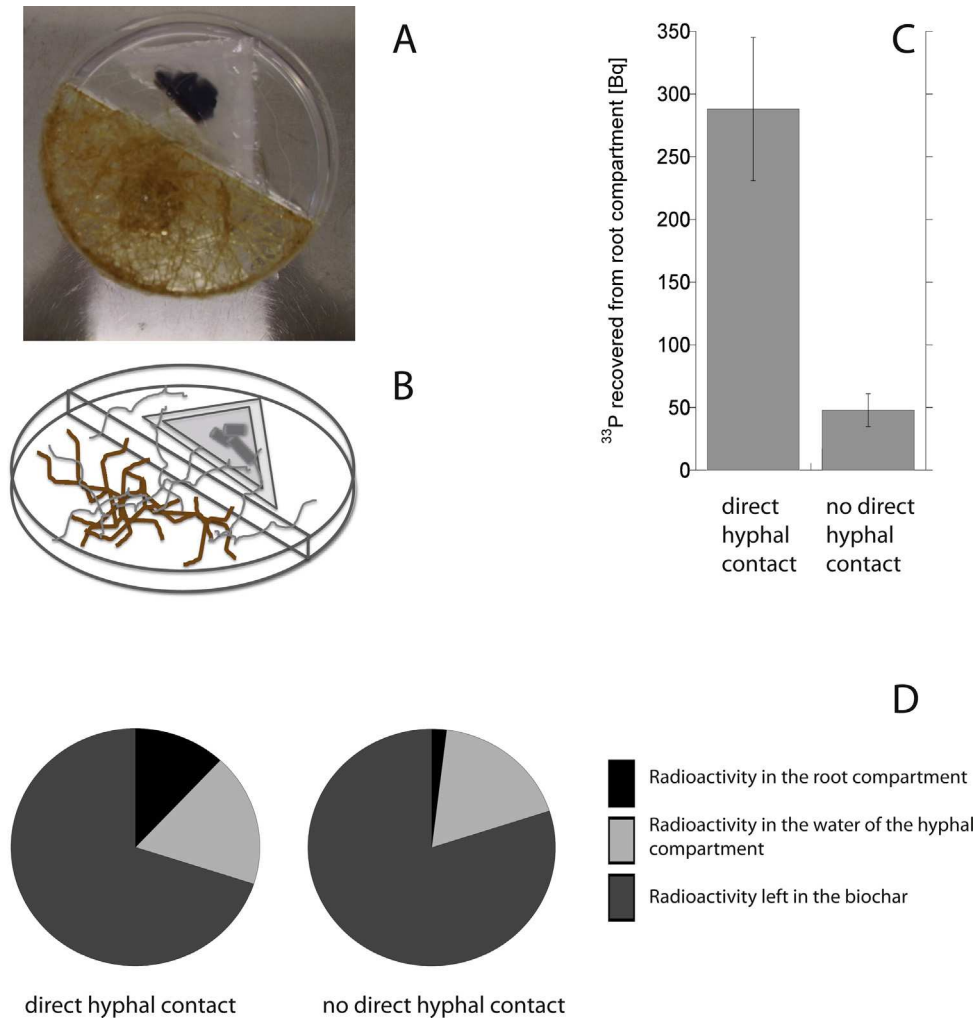


Fig. 5. P uptake from biochar, Expt. 3. Photograph (A) and sketch (B) of the experimental design. Amount of ^{33}P , derived from the surface of the biochar in the hyphal compartment, recovered in roots from the root compartment. Error bars represent SE ($n = 6$). AM fungal hyphae either had direct access to the biochar surface or not, determined by the mesh size of the bags enclosing the biochar (C). Distribution of ^{33}P within different fractions at the end of Expt. 1: ^{33}P recovered from the biomass in the RC (black), liquid medium in the HC (light gray), left in the biochar and extraradical mycelium in the HC (dark gray) (D).

microorganisms because the small pore size suggests that the majority of the inner surface area is not root accessible. Root hairs might have similar diameter as exploiting hyphae but would be unable to explore the interior of the solid substrate because of their limited length (80–1500 μm ; Marschner, 1995). The fine hyphae of AM fungi can connect the roots to those pore spaces. Studies on the effect of nutrient loaded biochar on mycorrhizal and non-mycorrhizal plants in soil could further demonstrate the potential of biochar as a mycorrhiza-mediated plant fertilizer.

Biochar and different types of charcoal attract AM fungal hyphae not only under sterile conditions but also in natural soil (Gavito and Olsson, 2003; Ogawa and Okimori, 2010). AM fungal hyphae attaching to outer and inner surfaces of biochar particles and producing exudates have, to our knowledge, not been reported before; to be certain that hyphae are of AMF origin, these kind of studies need to be conducted under sterile conditions where all other fungi are excluded. Attachment of AM fungal hyphae to biochar indicates that AM fungi use biochar as a physical support for growth. The results of Experiment 3 demonstrate that they are able to access nutrients during exploration of the surfaces. We observed fine-branched hyphae, with diameter as small as 1 μm , attached on the outer and inner surfaces of the biochar, that are likely involved in

nutrient foraging and acquisition. These hyphae are finer than those of branched absorbing structures of *R. irregularis* (Bago et al., 1998). A tree shaped branched absorbing structure expands typically over several hundred μm and is difficult to observe if the hyphae involved grow around particle edges in a complex matrix such as biochar. The structures shown (Fig. 2D–F) might constitute the innermost tips of typical branched absorbing structures. Images of lower magnification (Fig. 2A,B) show fine hyphal ramifications growing into different pores of the biochar. Close hyphal attachment facilitates nutrient uptake, because P-transporters or other nutrient transporters of the extraradical mycelium are in direct contact with the ions to be absorbed and exudates would not be lost by diffusion into the soil solution, or as in this experiment, into the growth medium. Hyphae growing into biochar pores are likely able to take up ions that otherwise could be resorbed to the biochar surface before they could exit the pore system of the biochar.

AM fungal hyphae also grow into patches of fresh organic matter (Mosse, 1959; Hodge et al., 2001; Hammer et al., 2011), which are rich in readily degradable carbon compounds. However, AM fungi are not likely to use this carbon as an energy source because they are obligate biotrophs, meaning that they are dependent on their host plant to supply them with energy compounds. Instead, it has

been suggested that fungi are attracted by available mineral nutrients contained in or adsorbed on the organic matter (Ravnskov et al., 1999; Hammer et al., 2011). Similarly, in our second experiment, the M-medium loaded biochar that contained a mixture of nutrients was clearly more attractive than untreated biochar (Fig. 4). The charcoals of the *terra preta* soils in Amazonia were traditionally mixed or composted with organic, nutrient rich materials such as feces or bones (Schaefer et al., 2004; Birk et al., 2011) so they were also loaded with mineral nutrients. Such practices ensured that the sorption sites of the biochar surface would already be saturated with nutrients and an initial immobilization of soil solution nutrients by the biochar surface is prevented.

Only a few studies have examined the response of extraradical mycelium of AM fungi to mineral nutrients; mycelia were found to respond (Gavito and Olsson, 2008; Hammer et al., 2011) or not respond (Gavito and Olsson, 2003), with increased growth in mineral nutrient patches. Uptake and attachment structures of AM fungi on interfaces are rarely studied. In contrast, the direct attachment of ectomycorrhizal fungi to solid materials, such as minerals has a role beyond simple physical stabilization. For example, ectomycorrhizal fungi are able to release nutrients from minerals by weathering (Wallander and Wickman, 1999; Balogh-Brunstad et al., 2008a, b) and clearly prefer or “biosense” P-rich minerals under P limited conditions (Berner et al., 2012; Leake et al., 2008).

The mode of adsorption of phosphate on the biochar used in this study was not determined. Fresh biochars can have large amounts of positive surface charge (Cheng et al., 2008), which could cause rather strong adsorption of phosphate. However, within a year of exposure to air and water, this positive charge largely disappeared as oxidation proceeded (Cheng et al., 2008) and initially immobilized phosphates would be released. Biochars commonly have negative surface charges and thus, high exchange capacity for base cations such as Ca^{2+} , Mg^{2+} and K^{+} (Liang et al., 2006). This exchange capacity increases with age. Accumulation of positive ions enhances phosphate adsorption (De Luca et al., 2009; Yao et al., 2011) so one could expect it to be found in a secondary anion layer. The AM fungi might “biosense” these phosphate groups adsorbed on biochar surfaces. It requires less energy to release adsorbed P than to extract P from primary minerals (Leake et al., 2008).

Our experiment likely underestimates the potential effect of AM fungi on phosphate uptake from biochar in a natural soil system. In MilliQ water, hyphae were able to access one sixth of the phosphate from solution compared with the amount accessed directly from biochar surface, which became available by diffusion into solution from the biochar. Phosphate diffusion into the soil solution, in contrast, is restricted by immobilization on soil colloids and binding to organic matter, thus the access to especially the inner surface area of biochar could contribute an important additional nutrient reservoir. Biochar particles can contain fertile microsites with high P concentration in small pores that would be inaccessible for direct contact with P-immobilizing Fe, Al and Ca minerals. Mobilized P is protected in pore networks that have long capillary distances and more importantly, by locally increased pH (Lehmann, 2007b) that prevents dissolution of Fe- and Al-oxides and oxyhydroxides. Plants can profit from these fertile microsites because their associated AM fungi can link their roots to the biochar pore surfaces, enabling nutrient uptake.

The excessive P fertilization in some modern agricultural practices leads to P leaching into the groundwater, eutrophication of rivers and lakes and in the end, irreversible loss to the oceans, depleting P resources. There is no doubt that global P resources are limited (Cordell et al., 2009) and recycling and responsible use of the remaining supplies is of critical importance for sustainability of

agriculture and food security of humankind. Biochar has proven to be a very efficient agent for phosphate removal from test solutions, wastewaters and soil leachates (Laird et al., 2010; Yao et al., 2011, 2013; Morales et al., 2013). Thus, applying biochar in agriculture would reduce P loss from fertilized soils and with help of AM fungi this would help close the nutrient cycle. The wood biochar we used in our experiments was ash- and thereby cation-poor. Hence the amount of P adsorbed to it was rather low. Further, we likely underestimate hyphal P uptake from biochar because we did not take into account P that originated in the biochar feedstock. Better efficiency and productivity, i.e. higher amounts of P absorbed and transferred to plants, would be expected with use of a substrate that is richer in cations. Biochar could be optimized for its nutrient content and sorption capacity by optimizing feedstock, pyrolysis temperature and processing time (Chan and Xu, 2009; Morales et al., 2013; Yao et al., 2013). Such optimized biochar could prevent excessive leaching of P and other nutrients and keep important nutrients in the soil system. Mycorrhizal fungi, either already present in the soil or applied with biochar, could access the adsorbed nutrients and provide them to plants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2014.06.012>.

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