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Biological, physicochemical and plant health responses in lettuce and strawberry in soil or peat amended with biochar

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ABSTRACT

Biochar, a solid coproduct of biomass pyrolysis, has recently been proposed as soil amendment in agriculture. We studied the effect of biochar on soil and substrate physicochemical properties, plant growth, disease susceptibility and rhizosphere microbiology in two contrasting cropping systems, i.e., lettuce grown in field soil and strawberry grown in white peat. For both systems, changes in the physicochemical properties of the plant growth media were observed. In the lettuce bio-assay, biochar addition had no effect on crop growth, crop health (*Rhizoctonia solani* infection test) and rhizosphere microbiology. In contrast, in the strawberry bioassay, addition of 3% biochar to peat resulted in (i) a higher fresh and dry plant weight, (ii) a lower susceptibility for the fungal pathogen *Botrytis cinerea* on both leaves and fruits, and (iii) changes in the rhizosphere microbiology, analysed by Phospholipid Fatty Acid (PLFA) profiling and 16S rDNA amplicon sequencing. Biochar addition led to an increase of bacterial diversity and a shift in composition of the rhizosphere microbiota. Extra inorganic plant nutrition and lime added to the peat reduced these effects of biochar on the strawberry plants. We conclude that in certain plant growth media, biochar amendment can result in chemical changes that induce multiple responses in the plant, including shifts in the rhizosphere microbiome. Biochar can be beneficial for plant growth, especially in conditions of limited nutrient availability.

1. Introduction

Biochar is the by-product of pyrolysis of biomass for biofuel production (Gravel et al., 2013). Biochar has the potential to reduce the CO_2 release into the atmosphere and can further be used for environmental remediation (Barrow, 2012; Xie et al., 2015), although application in agriculture remains one of the most common uses of biochar. Addition of biochar may change the physicochemical and biological properties of soils and substrates, which in turn may affect crop growth and health (Elad et al., 2011; Jeffery et al., 2011).

Biochar has a typical porous structure, high surface area and affinity for charged particles (Keech et al., 2005; Steiner et al., 2008). Thanks to these properties, biochar addition to soil can lead to an increase in soil water permeability and soil water retention (Asai et al., 2009; Laird et al., 2010) and an increase in soil pH (Chan et al., 2007; Rondon et al., 2007). Other commonly reported effects of biochar addition to soil are retention of nutrients and an increase of the organic carbon content (Lehman et al., 2003; Chan et al., 2007; Nelissen et al., 2015). Considering all these effects, biochar would have the potential to improve plant productivity. Additionally, biochar could affect plant productivity due to its nutrient content (Graber et al., 2010).

Several pot and field trials showed that biochar addition to the soil can enhance productivity and performance of crops (e.g. Chan et al., 2007; Asai et al., 2009; Graber et al., 2010;). However, also neutral or even negative effects of biochar addition to the soil on crop growth have been reported (e.g.Gravel et al., 2013; Nelissen et al., 2015).

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Various types of biochars were reported to act in disease suppression against fungal foliar pathogens. Elad et al. (2010) showed less susceptibility of pepper and tomato plants to *Botrytis cinerea* and *Oidiopsis sicula* when biochar was added to soil. Likewise, biochar amendment to substrate reduced the severity of three foliar diseases caused by *B. cinerea*, *Colletotrichum acutatum* and *Podosphaera apahanis* on strawberry plants (Meller Harel et al., 2012). In these studies with foliar pathogens and biochar added to the soil or substrate, there is no direct toxicity effect of biochar on the pathogens. This suggests that biochar can affect the plant-wide systemic response (Jaiswal et al., 2014). It has been shown that biochar induces disease related genes linked to induced systemic resistance (ISR) (Meller Harel et al., 2012; Mehari et al., 2015; Huang et al., 2015). It is known that ISR can be promoted by the colonization of plant growth promoting rhizobacteria (PGPR) and fungi (PGPF) (Mehari et al., 2015). These PGPR and PGPF are mainly present in the rhizosphere, i.e., the narrow zone of soil surrounding the plant root, and can therefore influence the growth, nutrition and health of plants (Philippot et al., 2013). Consequently, the positive effect of biochar on crop performance could also be indirect through the stimulation of plant beneficial rhizosphere microbes.

Previous studies examined generally only one or two of the above mentioned effects of biochar on agricultural properties (e.g. soil physicochemical and biological properties, crop productivity and performance, plant health or rhizosphere microbiology). However, we believe that, in order to define biochar as a positive or negative operator on the crop-soil/substrate system, all these individual factors need to be integrated in order to estimate the overall impact and to understand the underlying mechanism. Therefore, in this study, we selected two target crop systems for our bio-assays: lettuce and strawberry. Lettuce is typically well adapted for growth in field-soil, representing a complex environment for the plant roots. Strawberry grows well in a soilless system such as white peat, a well standardised environment with a low nutritional and microbial background. Lettuce is known to be responsive to nutrients (Upadhyay et al., 2014) and *Rhizoctonia solani* was chosen as pathogen system, as it is the predominant pathogen causing basal rot on lettuce (Van Beneden et al., 2009). Strawberry was infected with *Botrytis cinerea,* known as a serious pathogen reported to cause fruit losses up to 50% (Jarvis, 1962). However, the leaves are also very important in the infection cycle, as infection of leaves by *B. cinerea* may lead to increased inoculum production when leaves are senescing in a perennial growing system (Braun and Sutton 1988; Sutton and Peng, 1993). Biochar is expected to affect the composition of the plant growth media, such as pH, carbon content, nutrient availability, microbiology and water management and availability. It has previously been suggested that the effect of biochar on crop productivity would be dose and crop dependent (Gravel et al., 2013), but we realize that it should also be soil or substrate dependent. Therefore, in the present study the plant growth media were well characterized before and after the plant growth tests. The used field soil had an optimal pH and stored relevant nutrient concentrations for growth of lettuce. The white peat of the strawberry was confirmed to be low in plant nutrients and microbial diversity, and had a low pH. Based on previous reports, we expected that biochar has a neutralising effect on the peat pH and a fertilising effect for the plant (Carter et al., 2013). Effects beyond these two factors were tested by also combining biochar mixed in white peat with liming and extra addition of plant nutrient compounds.

This study aims to increase our understanding of the effect of biochar on the relation between the physicochemical properties of the plant growth media, crop growth, disease susceptibility and the rhizosphere microbial community. This kind of information is needed to fully appreciate the role of biochar as a soil or substrate amendment for agriculture.

2. Materials and methods

2.1. Biochar and plant growth medium

Biochar was prepared from holm oak at 650 °C for 12–18 h and was kindly provided by Proininso S.A. (Malaga, Spain). This biochar consists of 72.4% dry matter (DM) (%/fresh), 77.8% organic matter (%/DM) and 74.2% C (%/DM) and was previously used and fully characterized by Vandecasteele et al. (2014, 2016),

Field soil used in the lettuce assay was sampled from the arable layer 0–20 cm of an ongoing field experiment at ILVO (BOPACT; D'Hose et al., 2016, 225) and its chemical properties at the beginning and end of the experiment were measured as described below (Sections 2.2 and 2.3) and are listed in Supplemental (S) Table S1a and Table S1b, respectively. This sandy loam soil (pH-KCl = 5.79; clay = 5.3%; silt = 37.7%; sand = 57.0%) was sieved (1 cm), air-dried (99% dry matter/fresh), and stored at room temperature until use.

Peat used in the strawberry assay was NOVOBALT white peat 100% (AVEVE Lammens, Wetteren, Belgium). The chemical properties of the 'NOVOBALT peat' at the beginning (week 1) and end (week 13) of the experiments are listed in Supplemental Table S1c and Table S1d, respectively.

2.2. Chemical characterization of soil and amended soil

Methods for the chemical characterisation of soil and peat are based on European Standards developed by the European Committee for standardization (CEN) or by the International Organization for Standardization (ISO). European Standard EN numbers or ISO numbers refer to the specific standards.

Soil was sampled at the start and the end of the lettuce experiment for chemical analysis. At the start of the experiment, 1 l of thoroughly mixed soil was sampled after one week of pre-incubation. At the end of the experiment the soil that remained after sampling for rhizosphere microbiology (see Section 2.6 and 2.7) was used (± 1 l).

Prior to chemical analysis, the soil samples were thoroughly mixed and divided into three sub-samples. The first sub-sample was used immediately for pH-KCl, Electrical Conductivity (EC) and soil mineral N (NO_3^--N + NH_4^+-N) determination. Soil dry matter (DM) content was determined by oven drying at 105 °C. The pH was measured potentiometrically in a 1:5 soil:KCl (1 M) extract according to ISO 10390. The EC was measured by means of a temperature compensating conductivity meter (E SK 10B electrode, 25 °C) in a 1:5 soil:H_2O extract according to EN 13038. Soil mineral N was determined in a 1 M KCl extract according to ISO TS14256-1:2003 with a Skalar San++ mineral N analyzer. The second and third sub-sample were oven dried at 45 °C and 70 °C, respectively. The samples were ground in a mortar and passed through a 2 mm and 250 μm sieve, respectively, prior to analysis of chemical soil properties. Ammonium lactate (AL) extractable elements were assessed on the second sub-sample by extracting plant-available concentrations of P, K, Ca, Mg, Fe, Mn and Na with ammonium lactate (extraction ratio 1:20) in dark polyethylene bottles, shaken for 4 h (Egnér et al., 1960). The suspension was filtered in dark polyethylene bottles that were stored at 4 °C until analysis. Elements were analysed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Varian Vista-Pro) with an axial torch. Total organic carbon (TOC) was measured on the third sub-sample by dry combustion at 1050 °C using a Skalar Primacs SLC TOC analyser (ISO 10694).

2.3. Chemical characterisation of peat and amended peat

The substrate was sampled at the start and the end of the strawberry experiment. At the start of the experiment, 1 l of thoroughly mixed peat was sampled after one week of pre-incubation. At the end of the experiment all the remaining peat after sampling for studying the rhizosphere microbiology (see Section 2.6 and 2.7) was used (± 1 l).

Dry matter content was determined according to EN 13040. EC (EN 13038) and pH_{H2O} (EN 13037) were measured in a 1:5 soil to water (v/v) suspension. Determination of organic matter content and ash was done according to EN 13039. Extraction (1:5 v/v) of water soluble nutrients and elements (NO_3-N, NH_4-N, Cl, Na, SO_4 and PO_4-P) was done according to EN 13652, and measured with a Dionex DX-600 IC ion chromatography (Dionex, Sunnyvale, CA), and for NH_4—N with a Skalar San++ mineral N analyzer. Plant-available concentrations of P, K, Ca, Mg, Fe and Mn were extracted (1:5 v/v) in ammonium acetate buffered at pH 4.65, and measured by CCD simultaneous ICP-OES (VISTA-PRO, Varian, Palo Alto, CA).

2.4. Lettuce-soil bioassay

Air-dried field soil was mixed with biochar (BC) to a concentration of 0% (1323 g field soil) 1% (18 g BC + 1310 g field soil) and 3% (54 g BC + 1273 g field soil) and pre-incubated for one week at 15 °C. Initial moisture content was set to 40% water-filled pore space (WFPS), and bulk density was adjusted to 1400 g l^{-1}. Subsequently, the mixed field soil was put in 1.5 l pots. Per pot, one 1 month old butterhead lettuce (*Lactuca sativa*, cultivar Alexandria) seedling was planted and placed in a growth chamber at 20 °C, 16 h/8 h day-night light regime and 80% relative humidity. Seven plants were grown per treatment and the experiment was done twice. So, in total, fourteen biological replicates were grown for each treatment in which pots were placed in the growing chamber according to a completely randomised design. For each replicate separately, the soil moisture was adjusted weekly to 40% WFPS on the basis of measured mass loss and the supplied amount of water was recorded. No fertilizers were added during the experiment.

After 8 weeks, the lettuce heads were harvested and weighed (fresh weight and dry weight (2 days at 70 °C)).

The detached leaf test of Van Beneden et al. (2009) was conducted to determine the susceptibility of the lettuce plants towards *Rhizoctonia solani* infection. Four detached leaves of five lettuce plants were inoculated with two mycelial plugs (4 mm) of the *R. solani* AG1-1B isolate S014-22, one on each side of the leaf. Control leaves were inoculated with sterile potato dextrose agar (PDA) plugs. The leaves were placed in sealable plastic containers with moistened tissue paper and incubated at 20 °C in the dark. The resulting lesions on the leaflets were recorded using a 0 – 4 disease scale with 0 = no symptoms, 1 = about 1–25% of the leaf area is affected, 2 = about 50% of the leaf area is affected, 3 = more than 50% of the leaf are is affected, 4 = the whole leaf is affected. An example of this disease scale on strawberry leaves is given in Fig. S1. In each experiment, five plants were inoculated, thus in total 80 lesions per treatment were scored (5 plants x 4 leaves x 2 plugs x 2 experiments).

To differentiate between the evaporation from the soil surface and transpiration by the lettuce plant, a separate experiment was run in the growth chamber with an identical set-up as described above but without lettuce grown in the pots. For each replicate separately, the soil moisture was adjusted weekly to 40% WFPS on the basis of measured mass loss and the supplied amount of water was recorded.

2.5. Strawberry-peat bioassay

Peat was used as growing medium and mixed with BC to a concentration of 0% (298 g peat) 1% (3.2 g BC + 295 g peat) and 3% (9.4 g BC + 289 g peat). In a second experiment, we additionally added 1.33 g l^{-1}fertilizer (PGMix, Peltracom, Ghent, Belgium) or 3 g L^{-1} lime (Dolokal extra, Ankerpoort NV, Maastricht, the Netherlands) or a combination of both fertilizer and lime to the peat and to the peat/3% biochar mixture. Both experiments were done twice and statistical analysis was done on both repetitions. In two additional experiments, either fertilizer or lime was added to the peat/3% biochar mixtures (supplementary material Appendix A in Supplementary materials). These experiments were not repeated (Table 1).

Each substrate type was wetted to obtain 40% WFPS, and bulk density was adjusted to 200 g L^{-1}. Each mixture was put in a closed bag and pre-incubated at 15 °C for one week. Subsequently, 1.5 l pots were filled with the mixed peat and one cold-stored bare-root strawberry (*Fragaria* x *ananassa*, cultivar Elsanta) transplant was planted per pot. For each experiment, twenty strawberry plants were grown per treatment. The plants were grown in the greenhouse at 20 °C for 13 weeks, and placed in the greenhouse according to a completely randomised design. Every week, the moisture content of the substrate was adjusted to 40% WFPS on the basis of mass loss and the supplied amount of water was recorded.

Botrytis cinerea leaf inoculation was done 12 weeks after planting based on the method of Meller Harel et al. (2012). Briefly, the *B. cinerea* isolate 895 (Debode et al., 2013) was cultured on PDA at 20 °C for four days. Agar disks (4 mm) containing pathogen mycelium and conidiophores were cut out from the colony edge and placed, mycelium side down, on the surface of three young fully expanded strawberry leaves, with one disk per leaflet. Similar, the remaining plants were inoculated with sterile PDA plugs. In each experiment, 12 plants per treatment were inoculated, thus in total 216 lesions per treatment were scored (12 plants × 3 leaves × 3 plugs per leaf × 2 experiments).

The inoculated plants were sprayed with water and maintained for one week in high humidity by covering each 10 l pot with a plastic box. The resulting lesions on the leaflets were recorded using a 0–4 disease scale with 0 = no symptoms, 1 = about 1 – 25% of the leaf area is affected, 2 = about 50% of the leaf area is affected, 3 = more than 50% of the leaf are is affected, 4 = the whole leaf is affected seen and sporulation was observed. An example of this disease scale on strawberry leaves is given in Fig. S1. Fruits were

Table 1
Experimental set-up of the strawberry experiments.

	Lime (3 g/L substrate)	Fertilizer (1.33 g/L substrate)	Biochar dose (% on dry matter basis)
Experiment 1			0%, 1%, 3%
Experiment 2	+	+	0%, 3%
Experiment 3 (Appendix A in Supplementary materials)[a]	+		0%, 3%
Experiment 4 (Appendix A in Supplementary materials)[a]		+	0%, 3%

[a] Experiments were not repeated.

harvested, weighted and infected with *Botrytis cinerea* isolate 895 based on the method of Bhaskara Reddy et al. (2000). Briefly, individual ripe strawberry fruits were inoculated with 20 μl conidial suspension (2×10^5 conidia ml^{-1}) and incubated at 11 °C in humid conditions. When the first symptoms appeared, the strawberries were evaluated daily and spoiled fruits were discarded to avoid secondary infection.

Thirteen weeks after planting, the strawberry plants were collected and weighed (fresh weight (FW) and dry weight (DW, 2 days at 70 °C)).

2.6. Rhizosphere microbiology: phospholipid fatty acid (PLFA) analysis

Soil samples (+/− 50 g) were taken from each pot and stored at −20 °C and then freeze-dried. In total 4 biological repetitions within the lettuce bioassay and 5 repetitions per treatment within each experiment of the strawberry bioassay were used for PLFA analysis. Total lipids were isolated from 6 g of freeze-dried soil or 1 g of freeze-dried substrate in a phosphate buffer:chloroform: methanol mixture (0.9:1:2). Neutral, glycol- and phospholipids were separated by solid phase extraction, eluting, respectively, chloroform, acetone and methanol. Phospoholipids were saponified to obtain free fatty acids, which were subsequently methylated using 0.2 M methanolic KOH to form fatty acid methyl esters (FAMEs). FAMEs were analysed with a capillary gas chromatograph-flame ionisation detector (Perkin Elmer Clarus 600, Perkin Elmer, Waltham, USA) with a col-elite-2560 column (100 m length x 0.25 mm ID, 0.25 μm film thickness, Perkin Elmer). The temperature program started at 75 °C, followed by a heating rate of 10 °C min^{-1} up to 180 °C and followed by a final heating rate of 2 °C min^{-1} up to 240 °C. PLFA's were identified and quantified from the retention time and response factor of each FAME in the external FAME and BAME mix (Sigma Aldrich, St Louis, MO, USA). The C values were corrected using a working standard C19:0.

The abundance of individual PLFAs was calculated in absolute C amounts (PLFA-C, C_x [nmol g^{-1}]) based on the concentrations in the liquid extracts using the following formula:

$$C_x[\text{nmol g}^{-1}] = \frac{A_x \times c_i[\mu g] \times 1000}{A_i \times W[g] \times M\left[\mu g\ \mu mol^{-1}\right]}$$

where C_x is the concentration of the fatty acid studied, A_x is the peak area of the fatty acid studied, A_i is the peak area of the internal standard, c_i is the absolute amount of internal standard in the vial [μg], W is the amount of soil [g], M is the molecular weight of the fatty acid [μg μmol^{-1}].

Twenty PLFAs were selected because of their use as biomarker fatty acids for six distinct microbial groups: Gram-positive bacteria (i-C15:0, a-C15:0, i-C16:0, i-C17:0), Gram-negative bacteria (C16:1c9, C16:1t9, C17:0cy, C18:1c11, C19:0cy), bacteria (non-specific) (C14:0, C15:0, C16:0, C17:0, C18:0), actinomycetes (10Me-C16:0, 10Me-C17:0, 10Me-C18:0), fungi (C18:1c9, C18:2n9,12) and mycorrhiza (C16:1c11). PLFAs were used as markers for specific bacterial or fungal groups according to Kroppenstedt et al. (1984), Brennan (1988), O'Leary and Wilkinson (1988), Frostegard and Baath (1996), Stahl and Klug (1996), Zelles (1997) and Olsson (1999).

2.7. Rhizosphere microbiology: NGS analysis

2.7.1. Sampling and DNA extraction

The rhizosphere was sampled from all strawberry and lettuce roots at the end of the plant bio-assay experiments. This was done according to Lundberg et al. (2012). Briefly, roots were placed in a sterile 50 ml tube containing 25 ml phosphate buffer. Tubes were vortexed at maximum speed for 15 s, which released most of the rhizosphere soil from the roots and turned the water turbid. The turbid solution was then filtered through a 100 μm nylon mesh cell strainer into a new 50 ml tube to remove plant parts and large sediment particles. The turbid filtrate was further centrifuged for 15 min at 3200 g to form a loose pellet containing fine sediment and microorganisms. These pellets (250 mg) were immediately used for DNA extraction with the PowerSoil DNA isolation kit (Mo Bio, USA), according to the manufacturer's instructions, or treated further for storage and later use. For storage, 1 ml subsamples were transferred to 1.5 ml microfuge tubes, spun at 10,000 g for 5 min to form tight pellets. These rhizosphere pellets, averaging 25 mg, were flash-frozen in liquid nitrogen and stored at −80 °C until DNA extraction. Within the lettuce experiment, five biological replicates for each treatment were selected to be used for 16S rDNA (V3-V4) amplicon sequencing. Similarly, within the strawberry experiments, five or six biological replicates were selected within each treatment in the first and second experiment respectively (Table 1) for sequencing 16S rDNA (V3-V4). Within the second strawberry experiment (Table 1), we also used these six biological replicates for sequencing the ITS2 DNA fragment, providing information on the fungal composition of the rhizosphere.

2.7.2. 16S and ITS2 rDNA amplicon sequencing

Illumina amplicon sequencing of the bacterial rhizosphere populations was done on the V3-V4 fragment of the 16S rRNA gene. V3-V4 was amplified using the primers S-D-Bact-0341-b-*S*-17 and S-D-Bact-0785-a-A-21, as described by Klindworth et al., (2013), extended with illumina specific adaptors. Following PCR conditions were used: initial denaturation at 95 °C for 3 min, followed by 25 cycles consisting of denaturation (95 °C for 30 s), annealing (55 °C for 30 s) and extension (72 °C for 30 min) and a final extension step at 72 °C for 5 min. To amplify the fungal rDNA-ITS2 region an adapted forward primer of fITS7bis from Ihrmark et al. (2012) (GTGAATCATCRAATYTTTG) and the ITS4NGSr reverse primer (Tedersoo et al., 2014) were used, both extended with Illumina specific adaptors. The ITS2-PCR conditions were as above, except for 30 cycles with an annealing time of 1 min. A second PCR was done to attach dual indices and sequencing adaptors to all fragments, using the Nextera XT index kit (Illumina, San Diego, CA, USA). Same PCR conditions were used as in the first PCR, but 8 cycles were used instead of 25 or 30 PCR cycles. Mastermixes for all PCRs were prepared using the Kapa HiFi Hotstart ReadyMix (Kapabiosystems, Wilmington, MA, USA) according to the manufacturer's instructions and total reaction volumes were 25 and 50 μl for the first and second PCR, respectively. Each PCR was followed by a PCR product clean-up using the HighPrep PCR reagent kit (MAGBIO, Gaithersburg, MD). The final libraries were quality controlled using the Qiaxcel Advanced, with the Qiaxcel DNA High Resolution kit (QIAGEN, Germantwon, MD, USA), and concentrations were measured using the Quantus double-stranded DNA assay (Promega, Madison, WI, USA). The final barcoded libraries of each sample were diluted to 10 nM and pooled in equal amounts. Resulting libraries were sequenced using Illumina MiSeq v3 technology (2×300 bp, paired-end) by Macrogen, South-Korea, using 30% PhiX DNA as spike-in.

Demultiplexing of the amplicon dataset and deletion of the barcodes was done by the sequencing provider. The raw demultiplexed sequence data is available in NCBI's Sequence Read Archive under the submission PRJNA294259 for the bacterial sequences and PRJNA317548 for the fungal sequences. Trimmomatic v0.32 was used for removing the primers (Bolger et al., 2014). Raw Illumina forward and reverse reads were merged using the program PEAR v.0.9.8. Length cut-off values for the merged sequences were set between 400 and 450 bp for the V3-V4 and between 200 and 480 bp for the ITS2 region. A minimum overlap size of 120 bp and quality score threshold of 30 were used for all

sequences (Zhang et al., 2014). ITSx v.1.0.11 was used to extract the ITS2 sequences (Bengtsson-Palme et al., 2013). In the following steps, different programs of the Usearch software v7.0.1090 were used (Edgar, 2014). Merged sequences were quality filtered with a maximum expected error of 3 with the "fastq_filter" option. Next, sequences of all samples that needed to be compared to each other were merged, dereplicated and sorted by size. In total we retained 2,566,864 sequences after processing (around 80% of original set), resulting in an average of 58,338 sequences per sample. Clustering the reads into Operational Taxonomic Units (OTUs) was done using Uparse, with an identity level of 97% for V3-V4 and 98.5% for ITS2 (Edgar, 2014; Ihrmark et al., 2012). In the case of V3-V4 sequences, chimeras were removed using "uchime_ref" with the RDP Gold database as a reference (Edgar et al., 2011). Finally, sequences of individual samples were mapped back to the representative OTUs using the "usearch_global" algorithm at 97% identity, and converted to an OTU table (McDonald et al., 2012).

We also sequenced two negative controls. Both gave a very low number of sequences (1198 and 1665 sequences respectively) after quality filtering, indicating that probably no contamination of the samples occurred during the sample preparation.

2.8. Downstream data analysis and statistics

Chemical soil and substrate properties and water use were analysed as a one-way ANOVA with treatment (i.e.% biochar) as a factor using SPSS 16.0 software. Significant differences ($p < 0.05$) between means were determined by Scheffe's test.

The plant properties data was analysed using Statistica (v. 11; Statsoft). For the plant growth data, multiple comparison of the means was done with an ANOVA post-hoc Tukey HSD test in which treatment (% biochar) was used as factor. If the experiment was repeated, repetition was used as a blocking factor. Three dependent variables were studied: plant fresh weight, dry weight and disease susceptibility of lettuce or strawberry leaves. For the strawberry fruit inoculation experiment with *B. cinerea*, the effect of biochar addition (factor) was studied using a repeated measures ANOVA, as fruit rot was evaluated over 3 time points.

Statistical differences in the PLFA profiles between the different treatments were determined using a MANOVA analysis for the absolute abundances. Statistical differences of the relative abundances of these PLFA data were determined using Anova analyses by the Statistical Analysis of Metagenomic Profiles (STAMP) program (Parks and Beiko, 2010). Correction of multiple testing was done using the Benjamini-Hochberg False Discovery Rate method.

OTU tables of the 16S amplicon sequencing were analysed using the QIIME software package (v1.9.0) (Caporaso et al., 2010a). Taxonomy was assigned with the script "assign_taxonomy.py" using the uclust method considering maximum 3 database hits, with the Silva v119 97% rep set (as provided by QIIME) as reference. Representative bacterial OTU sequences were aligned to the SILVA 97% rep set using the PyNast algorithm with QIIME default parameters (Caporaso et al., 2010b; Quest et al., 2012). Similarly, taxon assignments of fungal OTU sequences were done using the UNITE database (version 7.0) (Kõljalg et al., 2013). Rarefaction analysis was done using the "alpha_rarefaction.py" script. Rarefaction depth of the bacterial and fungal OTUs was reached at 10,000 sequences. Two samples (1 sample of the lettuce 1%BC treatment, 1 sample of the lettuce 3%BC treatment) contained a lower number of bacterial sequences than the established rarefaction depth and were therefore deleted for further analysis. Multivariate analysis was done using the specific R package vegan (version 2.0–10) (Oksanen et al., 2010). The OTU tables were normalized by removing those OTUs with an abundance lower than 0.01% in all samples. Dissimilarity matrices (based on the Bray-Curtis dissimilarity index) were calculated from the OTU tables of both the lettuce and strawberry experiments. Effect of biochar addition on the bacterial and fungal communities was studied by doing a PERMANOVA and a principal coordinate analysis (PCoA) on these dissimilarity indices. The STAMP analysis software was used to study individual differences in the bacterial groups (Parks and Beiko, 2010). For each experiment, ANOVA analyses were done on a species table to determine the effect of biochar addition on the individual groups (phyla, species). To correct for multiple testing, we used the Benjamini-Hochberg False Discovery Rate method.

Shannon-Wiener diversity indices were calculated using the "alpha_diversity.py" script in the QIIME software package, and used to estimate the within sample diversity. The number of observed OTUs, which represents the bacterial and fungal richness, were determined on a rarefaction depth of 10,000 sequences. To study differences among mean richness and diversity indices, ANOVA analysis was done. Tukey HSD test was used to find the mean richness and diversity indices that are significantly different from each other. Both analyses were done using R (version 3.1.0) (R core team, 2015). To correlate chemical data with the bacterial OTU tables, a distance-based redundancy analysis was done, using Bray-Curtis as dissimilarity index. Analysis was done using the function "capscale" in the R package vegan (Oksanen et al., 2010).

3. Results

3.1. Effect of biochar on physicochemical properties of plant growth media

Changes in chemical properties and water use of the plant growth media that were caused by the addition of biochar were measured at the beginning and end of each bio-assay. Results are listed in Table S1a and Table S1b for the field soil used for lettuce growth and Table S1c and Table S1d for the peat used for strawberry growth. Only those properties in which we expected changes to occur during the short period of the bio-assay were measured at the end of the experiment too (Nelissen et al., 2015).

Addition of 3% biochar to the field soil significantly increased the plant-available concentrations of the macronutrients P, K, Ca and Mg. Potassium concentrations also increased in the 1% biochar/field soil variant. Moreover, addition of biochar in a 1% or 3% concentration increased pH and TOC content, which was still observed at the end of the experiment. A lower mineral N concentration was observed for both biochar dosages at the start of the experiment. This mineral N concentration was reduced at the end of the experiment with no significant differences in concentration between soils treated with different biochar dosages (Table S1a). At the end of the experiment, the EC value increased for soils treated with 3% biochar (Table S1b). Water use was poorly correlated with the fresh lettuce biomass, but was significantly lower for the 1 and 3% biochar treatment when compared to the 0% biochar control. Biochar addition reduced the evaporation from the soil rather than affecting the water use by the plants, as shown by the similar decline (12%) in water use by the pot mixes without lettuce plants (0% biochar: 611 ml, 3% biochar: 536 ml).

Similarly, chemical properties of the biochar amended and unamended peat were determined for each experimental set-up. In experiment 2 statistical variations between the peat/fertilizer/lime and peat/fertilizer/lime/biochar at the beginning of the experiment could not be determined due to a too low number of replications ($n = 2$). This was also the case for the 1% biochar application on peat without fertilization and lime addition (Table S1c).

For both experimental set-ups, the total amount of mineral N was depleted after 13 weeks (mineral N concentrations < 5 mg/l

Table 2
Fresh and dry weights and disease resistance (mean ±standard error) for the lettuce (n = 14) and the strawberry bio-assays (n = 40).

(a) Lettuce			
	Plant properties[a]		
	Fresh weight (g plant material)	Dry weight (g plant material)	Disease resistance (leaf inoculation)[b]
Lettuce in soil			
0% biochar	38.56 ± 0.32	3.88 ± 0.08	2.16 ± 0.30
1% biochar	36.88 ± 0.47	3.62 ± 0.09	2.02 ± 0.27
3% biochar	34.26 ± 0.34	3.20 ± 0.06	2.70 ± 0.23
(b) Strawberry			
	Plant properties[a]		
	Fresh weight (g plant material)	Dry weight (g plant material)	Disease resistance (leaf inoculation)[b]
Strawberry in peat			
0% biochar	2.21 ± 0.06	0.63 ± 0.01	1.96 ± 0.07
1% biochar	2.24 ± 0.17	0.66 ± 0.03	2.07 ± 0.08
3% biochar	**5.89±0.15**	**1.35±0.04**	**1.19±0.08**
Strawberry – peat + lime + fertilizer			
0% biochar + lime + fertilizer	39.22 ± 1.54	14.90 ± 0.60	0.50 ± 0.08
3% biochar + lime + fertilizer	42.04 ± 1.37	15.77 ± 0.72	0.44 ± 0.07

[a] Values marked in bold are statistically different from the control treatment (= 0% biochar).
[b] Lesions of *Rhizoctonia solani* on lettuce and *Botrytis cinerea* on strawberry using a disease scale of 0–4.

peat). Application of biochar in unfertilized peat (experiment 1; Table 1) significantly raised the water-extractable K, Ca and Mn concentrations and the water-soluble P concentrations. However values remained extremely low compared to the reference values. Additionally the substrate with biochar had a significantly lower organic matter (OM) content and Cl concentration at the beginning of the experiments (Table S1c). Peat that was additionally fertilized and limed (experiment 2; Table 1) still showed an increase in Ca and Mn concentrations due to biochar application, but this could not statistically be proven. The increase of water dissolved P due to biochar addition was still detected at the end of the unfertilized peat experiments (Table S1d), but compared to the experiment where peat was fertilized and limed, these concentrations are extremely low. In contradiction with the lettuce growth in the field soil/biochar mixes, water use in the strawberry experiment was strongly positively correlated with the plant biomass.

Similar analyses on the physiochemical composition of peat after biochar addition were done for peat which was fertilized or limed (experiment 3 and 4; Table 1). For both experiments, a significant increase in pH was observed. Addition of biochar to limed peat increased the amount of K, Ca and Mn and the concentration of water-soluble phosphorus and reduced the mineral N content. More information on these data can be found in the supplementary material (Appendix A in Supplementary materials).

3.2. Effect of biochar on plant growth and disease susceptibility

The effects of biochar on lettuce and strawberry growth and disease susceptibility are reported in Table 2a and 2b. Overall, biochar addition to the field soil had no effect on the growth of lettuce, expressed as fresh and dry shoot weight (Table 2a). In contrast, 3% biochar application raised the strawberry plant weight significantly, with 166.5% in fresh weight and 114.3% in dry weight. Supplemental addition of lime and fertilizer to the soil reduced the growth stimulating effect of the biochar itself, although a distinct but not significant increase in fresh weight due to biochar in the mix was still noted (Table 2b).

Biochar addition did not affect lettuce resistance to *R. solani*. No remarkable differences in lesion sizes could be observed on the biochar/field soil grown lettuce leaves (Table 2a), while biochar addition to peat did reduce the susceptibility of the strawberry plants to *B. cinerea*. A significant reduction of lesion sizes was observed for the leaves of plants grown in peat treated with 3% biochar compared to the control group (0% biochar). However, this effect of biochar was absent when the peat was supplemented with lime and fertilizer. A trend towards a lower infection rate was still seen, but was not significant. It should be noted that during these experiments an overall low infection rate was observed (Table 2b), which may explain the non-significant effect. In addition to the infection of the strawberry leaves, the strawberry fruits were infected with *B. cinerea* and the number of symptomatic fruits was recorded over time for the experiment where peat was treated with fertilizer and lime (Fig. 1). Due to a low number of fruits developed in the strawberry grown in peat which was unfertilized and not limed, fruit inoculation was excluded in this experiment. Strawberry fruits produced on peat/biochar (3%) were less susceptible to the grey mold pathogen *B. cinerea*, the infection

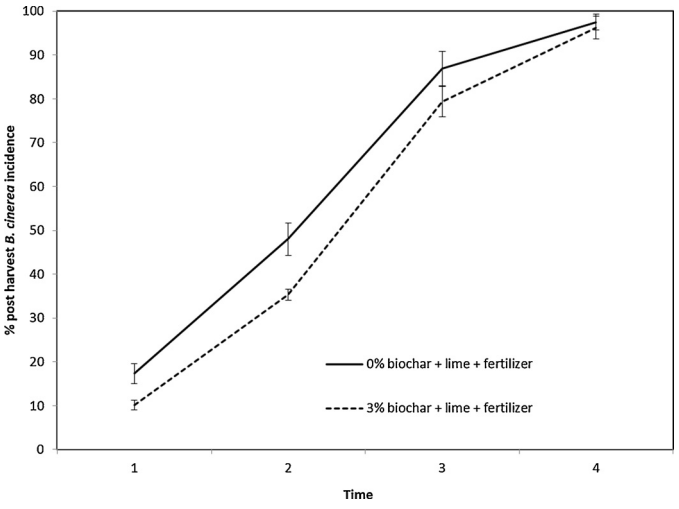

Fig. 1. Effect of 3% biochar treatment on the postharvest decay of strawberry fruit caused by *Botrytis cinerea*. Both lime and PGMix were added to the peat. Data were pooled across 2 plant experiment repetitions and 4 picks per experiment. In total, 109 fruits were inoculated per treatment. (Time: number of days measured since the first symptoms of infection).

process was slowed down, as indicated by a reduced number of symptomatic fruit at time point 1 and 2 ($p = 0.05$) (Fig. 1).

Similar analyses were done in strawberry experiment 3 and 4 (Table 1). For both experiments, no significant effect on plant growth and disease susceptibility was measured. More information can be found in the supplementary material (Appendix A in Supplementary materials).

3.3. Effect of biochar on the rhizosphere microbiology

3.3.1. Rhizosphere microbiology: PLFA

Biochar applied at 1% or 3% in the field soil had no significant effect on the microbial groups, except for the stimulation of arbuscular mycorrhizae, represented by one biomarker (C16:1c11) (data not shown).

In the peat substrate, the biochar addition did not change the total microbial biomass (Table S2) and the absolute number of fatty acids per microbial group, but induced some specific changes in the microbial content. The relative abundances of six biomarkers were significantly different in the peat/biochar mix compared to the control (0% biochar), representing a decrease in Gram negative bacteria (Anova, $p = 0.047$) and non-specific bacteria (Anova, $p = 5.57 \times 10^{-3}$) and a significant increase in fungi (Anova, $p = 0.042$) (Table S3). These shifts in microbial relative abundances due to biochar application were absent in peat enriched with fertilizer and lime (Table S3), whereas 3% biochar applied to peat that is supplemented with fertilizer or lime (experiment 3 and 4; Table 1) did slightly change the relative PLFA abundances (Appendix A in Supplementary materials).

3.3.2. Rhizosphere microbiology: 16S and ITS2 amplicon sequencing

Differences in bacterial community composition of the lettuce rhizosphere grown in field soil with variable dosage of biochar (0%, 1% and 3%) were studied using PERMANOVA analysis. No significant differences were observed between the treatments ($p = 0.127$), indicating that biochar application did not influence the bacterial community composition in the lettuce rhizophere.

For the bacterial community composition of the rhizosphere of strawberry plants grown in peat, an interaction effect between the addition of lime/fertilizer and of biochar was present (PERMANOVA, $p = 0.035$). Therefore the effect of biochar on the bacterial community composition was analysed individually for the two experiments. There were significant differences between the rhizospheres bacterial communities developed in peat versus peat/biochar (PERMANOVA, $p = 0.01$), and also in peat/fertilizer/lime versus peat/fertilizer/lime/biochar (PERMANOVA, $p = 0.019$). These differences due to biochar addition were visualised using principal coordinates analysis (PCoA) (Figs. S2, S3). The effect of biochar on the rhizosphere's bacterial community thereby seems to be less clear when additional fertilization and liming of the soil occurred.

To determine the bacterial groups influenced by the addition of biochar and giving rise to the separate clustering in the PCoA plots (Figs. S2, S3), the taxonomic profiles of the strawberry rhizosphere bacteria were studied. Both a significant reduction in the relative abundance of Proteobacteria (Anova, corrected p-value = 0.020) and a significant increase in the relative abundance of Planctomycetes (Anova, corrected p-value = 0.024) resulted from addition of biochar to the peat substrate (Table S4). Furthermore a relatively large, but not significant reduction in Acidobacteria and increase in Actinobacteria occurred upon addition of 3% biochar to peat. The rhizospheres of peat/lime/fertilizer and peat/lime/fertilizer/biochar differed significantly in only two, relatively small bacterial groups: the Armatimonadetes (reduction) and the Cyanobacteria (increase) (Table S4). Furthermore, significant shifts on genera level were observed. Strawberry rhizospheres in peat differed from those in peat/biochar by the relative abundance of eleven species of the 190 (relative abundance at least 0.01%), among which three genera known to harbour plant-beneficial agents, two with microbial N cycling and one plays a major role in plant growth promotion (Table 3a). For the strawberry rhizosphere of peat/lime/fertilizer, an additional treatment of biochar gave significant changes in the relative abundances of thirteen genera of the 148, of which two are described in literature as potential biocontrol agents (increase) and one is involved in N cycling (increase) (Table 3b).

Additionally, the bacterial composition of pure biochar was studied to see if biochar could serve as an additional habitat for micro-organisms. Eighty-two different genera (with relative abundance >0.01%) were detected, and three of these genera (*Bauldia*, *Devosia*, *Opitutus*) were also among the bacterial types enriched by adding biochar to peat (Table S5).

Similarly, the effect of biochar on the fungal community composition of the rhizosphere was studied for the second strawberry experiment. However, no significant effect of biochar addition to the peat/lime/fertilizer medium was seen on the fungal community (PERMANOVA). This could be mainly due to an overrepresentation of Basidiodendron which comprises around 42% of the relative abundance within the peat/lime/fertilizer and peat/lime/fertilizer/biochar treatments. The fungal genera which

Table 3a
Relative abundances of genera (mean ± standard error) of the strawberry rhizosphere (no lime or fertilizer added), which are significantly changed by the addition of biochar. Functions of the genera related to promotion of plant growth and biocontrol, are listed as previously described in literature.

Genus	Peat			Function	References
	Control	1% biochar	3% biochar		
Acidocella (%)	9.85 ± 3.15	4.78 ± 2.62	2.55 ± 0.94	/	
Bdellovibrio (%)	0.05 ± 0.02	0.04 ± 0.01	0.11 ± 0.05	Biocontrol agent gram – bacteria	Dori-Bachash et al. (2008) and Jurkevitch et al. (2000)
Devosia (%)	0.15 ± 0.12	0.2 ± 0.12	0.42 ± 0.07	Microbial N cycling	Rivas et al. (2002) and Hoque et al. (2011)
Haliangium (%)	0.05 ± 0.01	0.13 ± 0.09	0.25 ± 0.04	Possible biocontrol agent (production haliangicin-antifungal)	Fudou et al. (2001) and Kundim et al. (2003)
Marmoricola (%)	0.01 ± 0.01	0.04 ± 0.02	0.07 ± 0.03	/	
Phenylobacterium (%)	0.25 ± 0.13	0.44 ± 0.10	0.62 ± 0.17	/	
Rhizobium (%)	0.04 ± 0.04	0.06 ± 0.02	0.15 ± 0.05	Microbial N cycling	Courty et al. (2015) and Meng et al. (2015)
Schlesneria (%)	0.30 ± 0.10	0.38 ± 0.07	0.65 ± 0.11	/	
Singulisphaera (%)	0.16 ± 0.04	0.15 ± 0.03	0.23 ± 0.04	/	
Sorangium (%)	0.04 ± 0.03	0.07 ± 0.02	0.11 ± 0.03	Potential biocontrol agent (antifungal activity)	Ligon and Hill (2001) and Kim and Yun (2011)
Variovorax (%)	0.02 ± 0.01	0.05 ± 0.04	0.13 ± 0.03	Plant growth promotion	Chen et al. (2013) and Zhang et al. (2013)

Table 3b
Relative abundances of genera (mean ± standard error) of the strawberry rhizosphere (lime and fertilizer added), which are significantly changed by the addition of biochar. Functions of the genera related to promotion of plant growth and biocontrol, are listed as previously described in literature.

Genus	Peat + lime + fertilizer		Function	References
	control	3% biochar		
Aquicella (%)	0.33 ± 0.08	0.25 ± 0.05	/	
Bauldia (%)	0.41 ± 0.03	0.26 ± 0.04	/	
Devosia (%)	0.60 ± 0.04	0.87 ± 0.03	Microbial N cycling	Rivas et al. (2002) and Hoque et al. (2011)
Haliangium (%)	0.30 ± 0.11	0.98 ± 0.09	Possible biocontrol agent (production haliangicin-antifungal)	Fudou et al. (2001) and Kundim et al. (2003)
Inquilinus (%)	0.07 ± 0.01	0.15 ± 0.02	/	
Nocardia (%)	0.34 ± 0.01	0.08 ± 0.01	/	
Opitutus (%)	0.34 ± 0.12	1.10 ± 0.07	/	
Planctomyces (%)	0.05 ± 0.01	0.12 ± 0.01	/	
Prosthecobacter (%)	0.30 ± 0.02	0.16 ± 0.04	/	
Pseudolabrys (%)	0.31 ± 0.07	0.94 ± 0.07	/	
Reyranella (%)	1.22 ± 0.01	0.13 ± 0.02	/	
Rhodanobacter (%)	1.20 ± 0.25	3.01 ± 0.12	Possible biocontrol agent	De Clercq et al. (2006)
Taibaiella (%)	0.02 ± 0.00	0.10 ± 0.03	/	

contribute for at least one percent in the strawberry rhizosphere are represented in Fig. S4.

To have insight in the complexity of the rhizosphere bacterial communities of both the lettuce and strawberry rhizospheres, community richness (number of observed OTUs) and diversity (Shannon-Wiener diversity indices) were estimated in the different treatments (Table 4). Significant differences in the number of observed species (ANOVA, $p = 5.8 \times 10^{-11}$) and in the Shannon-Wiener diversity indices (ANOVA, $p = 2 \times 10^{-16}$) were observed. Highest richness and diversity were measured in the lettuce rhizosphere grown in field soil. Richness and diversity of the strawberry rhizosphere microbiome grown in peat substrate, were significantly lower. Addition of biochar however raised both the number of observed OTUs as the diversity of the rhizosphere microbiome. This effect was significant for the strawberry microbiome developed in peat, but not in peat/fertilizer/lime. Nonetheless, a trend towards a higher diversity and richness in the rhizosphere microbiome due to biochar was observed.

Similarly this was done for the fungal community of the rhizosphere within the second strawberry experiment (Tables 1 and 4). No change in the rhizosphere richness or diversity were observed in the fungal communities due to the addition of biochar.

In conclusion, biochar altered the rhizosphere of strawberry when the plants were grown in peat, but this biochar effect declined when the peat substrate was supplemented with plant fertilizer and lime. In contrast, the rhizosphere of lettuce grown in field soil contained a high bacterial diversity and was not influenced by the addition of biochar.

3.4. Relation of microbiome community and plant-soil/substrate properties

As shown above, biochar had an effect on the physicochemical composition of the substrate of the strawberry bio-assay. These changes could be correlated with the shifts in the rhizosphere microbiome observed when peat was treated with biochar. Therefore we did a distance-based redundancy analysis to correlate the physicochemical parameters with the bacterial communities on the data of the strawberry rhizosphere obtained from plants grown in unfertilized peat (Fig. 2). This figure illustrates that the microbial communities of unfertilized peat without lime addition are correlated with changes in the concentration of Mn, Ca, P and Cl when biochar was added to the peat. A similar analysis could be done for the limed and fertilized peat, but would be less meaningful because of the less clear clustering (Fig. S2). An analysis for the lettuce experiment was not done, because no significant effects of biochar amendment were observed on the bacterial composition, as described above.

4. Discussion

In the present research, we studied the effect of biochar on two different crop-soil/substrate systems: lettuce grown in soil and strawberry grown in peat. Changes in the physicochemical properties of the soil and substrate were observed for both the lettuce and strawberry assays. Adding biochar to the field soil affected the carbon content, the pH, the soil water evaporation and

Table 4
Effect of biochar addition on the number of observed OTUs (calculated at a rarefaction depth of 10.000 sequences) and Shannon-wiener diversity indices (mean ± standard error) for the strawberry rhizosphere (experiment 1 and 4), the lettuce rhizosphere and the strawberry rhizosphere at the start of the bio-assay (strawberry t = 0).

BACTERIA		Number of observed OTUs	Shannon-Wiener diversity index
Strawberry – peat	Control	901 ± 60 (a)	7.63 ± 0.21 (a)
	1% biochar	993 ± 36 (ab)	8.07 ± 0.12 (ab)
	3% biochar	1198 ± 74 (b)	8.61 ± 0.14 (bc)
Strawberry – peat + lime + fertilizer	Control	857 ± 29 (a)	7.69 ± 0.21 (a)
	3% biochar	946 ± 34 (a)	8.13 ± 0.08 (ab)
Lettuce – soil	Control	1642 ± 33 (c)	9.29 ± 0.09 (cd)
	1% biochar	1726 ± 83 (c)	9.31 ± 0.18 (cd)
	3%biochar	1706 ± 54 (c)	9.41 ± 0.10 (d)
FUNGI		Number of observed OTUs	Shannon-Wiener diversity index
Strawberry – peat + lime + fertilizer	Control	462 ± 23 (a)	5.59 ± 0.23 (a)
	3% biochar	446 ± 38 (a)	5.66 ± 0.08 (a)

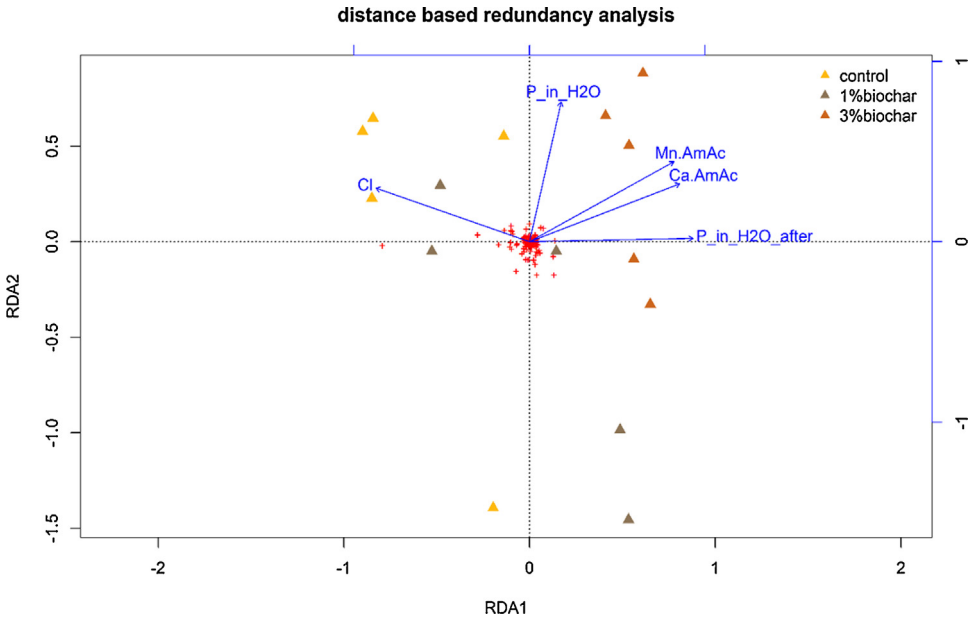

Fig. 2. Distance based redundancy analysis of the 16S amplicon sequencing data shows differential taxonomic composition in the strawberry rhizosphere upon biochar addition to substrate. Soil physicochemical parameters that are significantly different (Table S1c; Table S1d) are fitted to the plot. Red crosses represent the OTUs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the nutrient availability. In contradiction with the lettuce growth in the field soil/biochar mixes, water use in the strawberry experiment was strongly positively correlated with the plant biomass. The water use in the substrate was thus driven by plant growth with a minor or undetectable effect of the biochar amendments on the evaporation from the substrate. Adding biochar to the peat thus affected the nutrient availability, but had in this case only small effect on pH or soil water evaporation.

Moreover, biochar addition induced major changes in (i) rhizosphere microbiology, (ii) plant growth and (iii) plant health exclusively in the strawberry bio-assays, especially when no additional fertilization or liming of the peat was done.

We observed shifts in the composition and diversity of the microbial community of the strawberry rhizosphere due to the addition of biochar. Previously, it has been described that biochar can alter the microbial composition of bulk soil, but the factors that drive these changes in microbial composition are still unknown (Graber et al., 2014a). The following hypotheses have been suggested: (1) Biochar could provide an additional habitat for bacteria and fungi (Ezawa et al., 2002; Thies and Rillig, 2009) and may provide places of refuge for fungal grazers for microbes (Warnock et al., 2007), (2) Biochar may interfere with microbial intercellular signalling (Masiello et al., 2013), (3) Due to its chemical composition, biochar could have an effect on microbial composition. Biochar borne organic chemicals may suppress some members of the microbial community and promote others (Kolton et al., 2011), (4) Biochar may change the physicochemical properties (e.g. pH, EC), which could have an effect the microbial communities (Graber et al., 2014b). Based on our observations, we suggest two additional hypotheses: (5) An effect of biochar on the microbial community due to its effect on the nutrient composition of soil and substrate. The biggest changes in rhizosphere microbial composition and diversity were observed when peat was not limed and fertilized. Additional application of fertilizer and lime reduced the effects on the strawberry rhizosphere microbiology. Complementary, no effects of biochar application on the lettuce rhizosphere microbiology were observed in nutrient rich soil. This strengthens our hypothesis that in nutrient limiting conditions, biochar provides an additional nutrient source for the present microbial community, which can alter the proportion and composition of microbial communities. (6) Biochar could serve as a source of micro-organisms. We showed that from the 82 bacterial species present on pure biochar, at least three species were found in the strawberry rhizosphere after 12 weeks of growth in peat-biochar mixtures. Adding biochar to soil or substrate can therefore enhance species to the habitat.

Biochar addition promoted plant growth solely in the strawberry assay with unfertilized peat that was not limed. This made us suspect that biochar only promotes plant growth in nutrient limiting conditions. Following explanations for this assumption are proposed: (1) Biochar could serve as a fertilizer (Altland and Locke, 2013). In both lettuce and strawberry bio-assays, an increase of nutrients, e.g. P, K, Ca and Mg, was observed when biochar was added to soil or peat, respectively. In nutrient-rich conditions, the soil or substrate already stored a relevant concentration of nutrients, and nutrients are not expected to be limiting for plant growth. Adding more nutrients will increase the nutrient stock but will not directly enhance plant growth. In nutrient-limiting conditions, these changes in nutrients will supply necessary nutrients for the plant and the microbial community, e.g. P, resulting in plant growth promotion. (2) Biochar could have an effect on the plant growth by changing the rhizosphere microbiome. First a higher number of bacteria involved in the microbial N cycling were observed in the rhizosphere of strawberry plants grown in unfertilized peat. This can lead to a higher amount of plant-available N, which subsequently can promote plant growth (Brewin, 2010). Second a higher number of PGPR were observed, which can ease the uptake of nutrients for the plants and enhance the plant availability of phosphorus (Egamberdiyeva, 2007; Krey et al., 2013). Additionally, it has been shown that these stimulatory effects are higher in nutrient deficient soil than in nutrient rich soil (Egamberdiyeva, 2007). Finally, the increased diversity of bacteria in the strawberry rhizosphere might enhance the N mineralization and therefore improve plant nutrition and plant growth (Weidner et al., 2015). These effects of biochar on the rhizosphere microbiome were only observed in the strawberry assay where peat was

not fertilized and limed. The hypothesis stated above might explain partly why this is the only assay where we observed improvement of plant growth by biochar addition.

Within the lettuce bio-assay no effect of leaf infection with *Rhizoctonia* was observed. However, here we used a detached leaf assay, instead of an attached leaf assay as in the strawberry bio-assays. Nonetheless these detached leaf assays are common in phytopathological research, this could have affected our results (Liu et al., 2007). In the strawberry bio-assay, where we used an attached leaf assay, addition of biochar had an effect on plant disease resistance against *B. cinerea* for both the leaves and fruits. We suggest following explanations: (1) Biochar could have an effect on the plant's resistance towards pathogens as fertilizer. Plants that are deficient in potassium are less resistant to pests, diseases and nematode attacks. Addition of K to K-deficient soils can therefore reduce the incidence of plant diseases (Römheld and Kirkby, 2010). In the strawberry assay we observed a significant increase of the K concentration in the substrate when no fertilizer or lime was added. The higher amount of nutrients, especially K, could thus partly explain the higher disease resistance measured of the strawberry plants to *B. cinerea* in nutrient limiting conditions, as indicated by the concentrations being lower than the reference values. In the lettuce bio-assay, K concentrations also increased significantly. However, concentrations of K were already higher than the reference values before biochar was added to the soil, which could explain why the higher amount of K did not have an effect on the disease resistance. (2) The effect of biochar on the rhizobiome could have an effect on the plant's resistance to pathogens. First, we identified species previously described as potential biocontrol agents that increased significantly in relative abundance after biochar addition to the peat. Second, we also detected a higher number of PGPR, which can promote the ISR of the plant. Our observations thus confirm previously published suggestions that biochar can promote the ISR of the plant, resulting in increased disease resistance (Mehari et al., 2015).

In this study, we used two techniques to study the microbial composition of the rhizosphere: PLFA and next-generation sequencing (NGS). This combination is quite new in this research area and provides several benefits. NGS is known to give reliable information for the taxonomy of the sequences, given as OTUs, especially for higher order identification (Poretsky et al., 2014). Also information on species richness and diversity can be calculated. While 16S rDNA amplicon sequencing yields estimates of the relative abundances, it does not provide absolute measures of the real microbial biomass. PLFA analysis, on the other hand, provides complementary data on the total biomass and the biomass per microbial group (based on fatty acid composition). To make a comparison between the two techniques possible, relative abundances of the PLFA biomarkers should be calculated. However, when comparing the two techniques, one should also take into account that the rhizosphere is sampled differently for each technique. Specifically, for 16S rDNA amplicon sequencing, 250 mg rhizosphere soil was taken as defined by Lundberg et al. (2012) and we followed the convention of defining rhizosphere soil as extending up to 1 mm from the root surface. Because of the amount of soil needed for PLFA analysis (6 g), it is impossible to do same soil sampling as for the amplicon sequencing. For this technique, 6 g of soil was taken from the pots. These pots were fully colonized by the roots of the lettuce or strawberry plants, so soil very close to the roots was taken and we believe that this can still be defined as rhizosphere soil.

The extensive amount of literature on biochar application as a soil improver or fertilizer in agriculture shows the high expectations for this product in our society. However, next to the frequently described benefits of the use of biochar mainly in poor tropical soils (Alling et al., 2014), also neutral or in a few cases negative effects are observed, most often in more rich soils (Nelissen et al., 2015). This raises the question if biochar can really improve soil quality and crop production in general. These dissimilarities across studies could be due to the absence of a standardised protocol for biochar production, differences in feedstock and pyrolysis process in the biochar production process, differences in biochar application rate, or as we showed, could even be crop or soil/substrate dependent. From our study we can conclude that the effect of biochar on the plant-soil system is not singular and multiple factors are involved. We show that biochar can alter physicochemical properties of the soil/substrate, plant growth, disease resistance and microbial communities in the plant-soil/substrate system and that these changes are related to each other. Therefore we suggest that future studies should focus on the effect of biochar on the plant, the soil and the microbial system simultaneously. Depending on the crop-soil/substrate system used, biochar can thus be valuable in agriculture. Analysing two different plant-soil systems revealed that biochar is useful under nutrient-limiting conditions, and we hypothesize that it could serve as a fertilizer, affecting plant growth, disease resistance and the microbial composition of the rhizosphere. To study the microbial community, we showed that PLFA and NGS techniques are complementary and give information on the community composition, richness and diversity as well as the microbial abundance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2016.05.001.

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