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Effect of biochar and *Trichoderma* application on fungal diversity and growth of *Zea mays* in a sandy loam soil

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Abstract

Abstract. Application of biochar (BC) to agricultural soils has raised global interest. BC could serve as a carrier for immobilization of beneficial microorganisms. Effect of straw-derived BC (50 t ha^{-1}) with and without immobilized *Trichoderma viride* on the growth of maize in a sandy loam soil in Zemgale region (Latvia) was studied in a 128-day mini-field trial. Four treatment types were compared, i.e., without amendments [C], biochar [BC], biochar with *T. viride* [BCT], and T. viride [T]. The highest amount of *Trichoderma* spp. DNA was detected by qPCR in [BCT]. The obtained results demonstrated high heterogenity of soil samples. Addition of straw-derived BC with immobilized *T. viride* to sandy loam soil promoted survival of *Trichoderma* spp. and significantly ($p < 0.05$) increased maize growth. The highest germination index, i.e., 87.5%, after 14 days was observed in the [BCT] treatment. These results provide new methods for the optimization of cultivation conditions for maize in Northern latitudes.

Key words: fungal abundance, maize, nutrient profile of biomass, qPCR, straw-derived biochar, *Trichoderma viride*.
Abbreviations: BC, biochar; CFU, colony forming units; qPCR, quantitative polymerase chain reaction.

Introduction

Improvements in plant breeding and in agronomic practices have made maize (*Zea mays* L.) an economically important culture in the Northern latitudes. Latvia can be considered as a marginal area for maize growing, as maize response to adverse meteorological and crop management conditions is more expressed than that in the case of other crops, e.g., small grain cereals (Gaile 2012; Gaile, Arhipova 2015; Bartusevics, Gaile 2009).

Optimization of growth conditions for maize embraces a broad spectrum of agrotechnological approaches. In particular, recent studies have shown a positive effect of biochar (BC) on yield of maize (Rehman et al. 2016; Arif et al. 2017; Faloye et al. 2017; Gonzaga et al. 2017; Kerré et al. 2017; Naeem et al. 2017; Sarfraz et al. 2017). Addition of BC to soil affects its compaction, porosity, water content, permeability, as well as biochemical processes, which promote element cycles and energy flow (Chan 2007; Tan et al. 2017).

In field experiments on nutrient poor alkaline calcareous soil under a maize-wheat cropping system it has been shown that BC positively influences plant nutrition, crop productivity and soil quality (Arif et al. 2017). Sarfraz et al. (2017) tested the effect of BC (1 to 2%) and nitrogen on the growth of maize in an alkaline calcareous soil, and found an increase of the water-holding capacity, total organic carbon, maize yield, stomatal conductance and nitrogen uptake in plants.

Germination rate, plant growth, N and P concentrations in maize biomass depended on the type of BC and its application rate (Gonzaga et al. 2017). In greenhouse experiments with maize, combination of BC with Fe fertilizer and elemental sulfur significantly increased root and shoot dry weight, grain weight, photosynthetic and transpiration rates, and stomatal conductance (Ramzani et al. 2016). The effect of BC composed of maize-cob residue and inorganic fertilizer on the yield of maize under a drip irrigation system was found to be synergistic (Faloye et al. 2017). Experiments with maize performed in arable soils under historical charcoal kilns (black spots, > 150 years enrichment, 2.2%) in southern Belgium showed a higher maize yield by 23%, compared with adjacent soils without chracoal; these results were attributed to soil physical effects, rather than to nutritional effects (Kerré et al. 2017).

BC in soil leads to changes in microbial community

composition, accompanied by increased microbial biomass and enhanced enzyme activity (Lehmann et al. 2011). This indicates that BC can serve as a carrier for immobilization of beneficial microorganisms in biofertilizers (Głodowska et al. 2016).

Fungi influence soil fertility, decomposition, cycling of minerals and organic matter, plant health and nutrition. The ability of *Trichoderma* sp. to colonize plant roots suggests a potential for protection of plant health (Romão-Dumaresq et al. 2016). Biocontrol properties of *Trichoderma* (teleomorph *Hypocrea, Ascomycota*) have been intensively studied in different aspects with the aim to find means to avoid extensive use of agrochemicals. Immobilized and coimmobilized formulations with *Trichoderma* could be applied as biofertilizers and biopesticides (El-Katatny, Idres 2014; Rangel et al. 2015). Many *Trichoderma* bioinoculants are now commercially available (Stewart, Hill 2014).

Trichoderma is a well-known mycoparasite, which secretes cell-wall degrading enzymes and other bioactive compounds with antibiosis effect towards the target pathogen. *Trichoderma atroviride* SG3403 has shown high biocontrol activity against southern maize leaf blight pathogen *Cochliobolus heterostrophus*, by inducing maize defence gene expression against pathogen infection (Wang et al. 2015). Interation of *Trichoderma virens* with maize roots was observed to induce a systemic resistance, which reduced disease in above-ground parts of plants (Lamdan et al. 2015). A positive effect of *Trichoderma harzianum* on maize growth and root development, as well as increased protein levels and activities of β-1,3 glucanase, exochitinase, and endochitinase in both roots and shoots, were reported by Harman et al. (2004). was detected (Harman et al. 2004). Treatment of maize seeds with *Trichoderma asperellum* increased plasma membrane H^+-ATPase activity in roots and shoots (Ĺopez-Coria et al. 2016).

It was hypothesized that application of BC enriched by *Trichoderma* in soils for maize cultivation can be a potential agrotechnological approach. The aim of this study was to test effect of straw-derived BC (50 t ha^{-1}) with and without immobilized *Trichoderma viride* on growth of maize in a sandy loam soil in a 128-day mini-field trial. Changes in the fungal community and in abundance of *Trichoderma* spp. DNA were evaluated using culture-dependent methods and molecular tools.

Materials and methods

Materials

The straw BC was produced at BlackCarbon A/S (Denmark, http://blackcarbon.dk/unit/) from pelletized wheat straw at maximum pyrolysis temperature of 725 °C with a residence time of 1 h, at continuous flow with constant heating. The generated producer-gas had temperature 460 °C. The bulk density of BC was 0.39 g cm^{-3}. BC was divided into two fractions by sieving with a mesh size of 2 mm diameter. The physicochemical characteristics of the BC were as follows: C_{tot} 84.47%; N_{tot} 0.5%; ash 9.5%; S_{tot} 279.4 mg kg^{-1}; BET (Brunauer–Emmett–Teller) surface 2.90 m^2 g^{-1}; pH_{KCl} 8.96 (Muter et al. 2014).

Mini-field trial

Soil was amended by BC and *T. viride* alone or in a mixture, according to the experimental setup. The following treatment types were tested: (1) control [C]; (2) BC [BC]; (3) BC + *T. viride* [BCT]; and (4) *T. viride* [T]. The experimental field was established in Dobele region, Latvia. Eight plots with a two 100-cm-long rows in each were established with a buffer zone of 100 cm between the rows. Four treatment types were randomly applied on the plots in duplicate. For treatment, 250 g BC was applied (alone or with *T. viride*) in the strips (5 × 100 cm), mixing with soil in a 10-cm depth. Maize cv. 'Ambrozja' F1 seeds (16 seeds per one plot) were sown along the BC strip in two parallel rows, eight seeds from each side (distance between seeds 11 cm), with a 5 cm distance from BC.

T. viride (obtained from the Culture Collection of Microorganisms of the Institute of Microbiology and Biotechnology, University of Latvia) was cultivated (25 ± 2 °C, 230 rpm, 72 h) in a liquid medium with the following composition (g L^{-1}): $(NH_4)_2SO_4$ 5.0; KH_2PO_4 15.0; $MgSO_4$ 0.6; $CaCl_2$ 0.6; $FeSO_4$ $7H_2O$ 0.005; $MnSO_4$ H_2O 0.002; $ZnSO_4$ $7H_2O$ 0.002; $CoCl_2$ 0.002; molasses 5.0. The number of colony forming units (CFU) in culture was determined by cultivation on Rose Bengal Agar with chloramphenicol (Biolife, Italy). CFUs were counted after plate incubation for 72 h at 25 ± 2 °C. One litre culture (1.4×10^7 CFU L^{-1}) was mixed with 250 g autoclaved (121 °C, 15 min) BC and incubated overnight at 24 °C. In the case of soil treatment by *T. viride* alone, 1 L per plot of *T. viride* culture was added to soil.

The experiment lasted for 128 days, in the period from June 9, 2014 until October 15, 2014. Seed germination and plant growth were monitored during the first 14 days and 55 days, respectively. Monthly average temperatures of air and soil surface are summarized in Fig. 1. The average rainfall in June, July, August, September and October was 4.0 mm (14 rainy days); 5.2 mm (9 rainy days); 5.9 mm (19 rainy days); 5.5 mm (8 rainy days) and 21.5 mm (6 rainy days), respectively (www.meteo.lv).

Analytical testing

Soil textural class was determined as sandy loam, according to the Guidelines for Soil Description (FAO 2006).

Dry weight of soil was determined after drying at 105 °C till constant weight. Soil pH was measured with a glass electrode in 1M $BaCl_2$ extract (1:2.5 mass to volume ratio). Exchangeable elements (Ca^{2+}, Mg^{2+}, K+, Na^+, Al^{3+}, Fe^{3+}, Mn^{4+}) were determined by an atom-absorption spectrophotometer (PerkinElmer AAnalyst 200), subsequently calculating element concentrations in

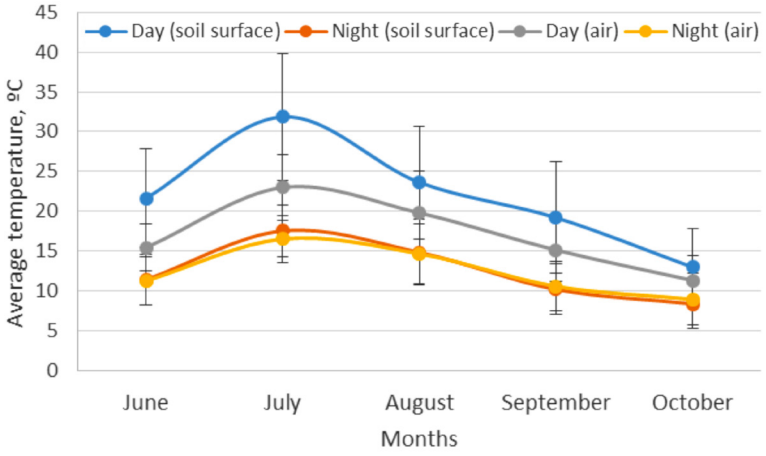

Fig. 1. Monthly average temperatures of air and soil surface in the period from June 9 2014 till October 15 2014 (www.meteo.lv, "Latvian Environment, Geology and Meteorology Centre).

mg kg^{-1}. Total nitrogen and total carbon concentration (%) were determined using a CHNS-O Elemental Analyzer EA3000 Series (EuroVector). Chemical analysis of elements was carried out in three replicates. The laboratory results were considered acceptable when the difference between the values obtained was less than ± 5%.

Soil was characterized by the following parameters: pH_{BaCl_2} 7.27 ± 0.03; C_{tot} 4.94 ± 0.80 %; N_{tot} 0.39 ± 0.04; exchangeable cations (mg kg^{-1}) Na^+ 28.4 ± 2.50; Mg^{2+} 428.9 ± 26.2; K^+ 312.1 ± 12.3; Ca^{2+} 6537.0 ± 434.6; Al^{3+} 0.9 ± 0.1; Fe^{3+} 0.08 ± 0.00; Mn^{4+} 0.24 ± 0.04.

The aboveground biomass of maize after 128 days cultivation was tested at the Analytical Laboratory, Latvia University of Agriculture. The samples were prepared according to standard methods (ISO 6496: 1999; LVS EN ISO 6498: 2012). The dry weight of the aboveground part of harvested plants was determined by sample drying at 60 °C followed by tissue crushing and subsequent drying at 105 °C till constant weight. The total carbon (CS-500) concentration was determined by the Analyzer ELTRA method, acid detergent fibre was measured according to LVS EN ISO 13906: 2008, and crude protein concentration was calculated using nitrogen analysis performed by block digestion and steam distillation (LVS EN ISO 5983-2: 2009). Two plants were sampled from each plot (overall four plants per one treatment type).

Microbiological testing

For microbiological testing, 1 g soil from the rhizosphere was taken after the 128 days vegetation experiment in triplicate. The soil-water suspensions were diluted serially and spread on agar media. Fungi were cultivated on Rose Bengal Agar with chloramphenicol (Biolife, Italy). CFUs were counted after plate incubation for 120 h at 25 ± 2 °C. Genera of cultivable filamentous fungi were determined after incubation for 10 days according to morphological characteristics and light microscopy results using identification keys (Watanabe 2002).

Microscopy study

BC particles were sampled from the maize rhizosphere after the experiment and analyzed for the presence of biofilm using a confocal laser scanning microscope (Leica DM RA-2, Germany) equipped with a TCS-SL confocal scanning head. Samples were fixed with 70% ethanol and stained with propidium iodide (20 µM). Propidium iodide is a red-fluorescent nuclear and chromosome counterstain. Sample fixation with alcohol permeabilized the cells thus allow entry of the dye and access to the nucleic acids (Chu et al.,1999). Propidium iodide was excited at 488 nm, and fluorescence was detected at 600 – 640 nm. The reflected light of degraded organic matter was induced with a 488 nm band, and reflection was detected between 490 and 500 nm.

Identification of Trichoderma *spp.*

Soil was sampled at the end of the vegetation experiment from the plant rhizosphere (four plants per each treatment type) and stored at –20 °C until testing. Total DNA was extracted from each soil sample (0.25 ± 0.01 g) in duplicate using the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Soil samples were homogenized using a horizontal Mixer Mill Type MM 301 (Retsch, Germany) at a maximum speed of 30 Hz for 10 minutes. The purity and concentrations of the extracted DNA were determined spectrophotometrically with a NanoDrop 2000 (ThermoScientific, USA) and by 0.8% agarose gel electrophoresis (Yeates et al. 1998). The DNA extracts were stored at –20 °C until use. Quantitative PCR (qPCR) was performed using a 7300 Fast real-time Applied Biosystems PCR system and data analysis was carried out using the software supplied.

Molecular identification of *Trichoderma* spp. was performed with primer set uTf/uTr (Hagn et al. 2007). The qPCR reaction contained 1 × Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific), 0.2 µM of each

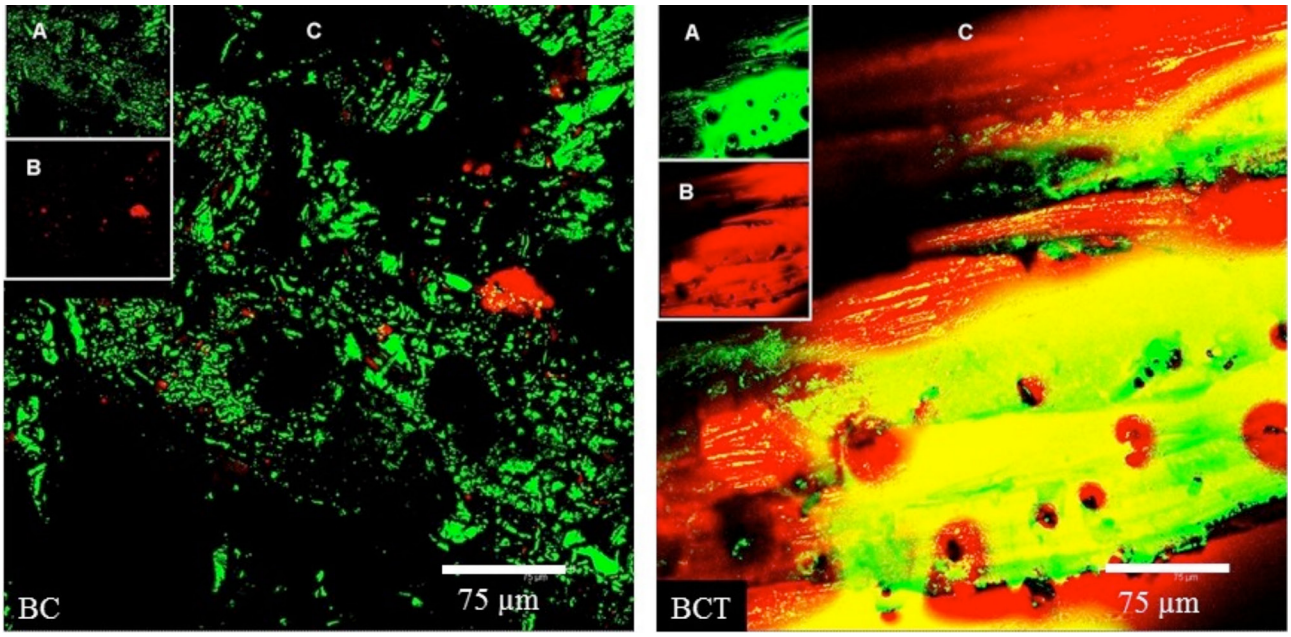

Fig. 2. Confocal laser scanning micrographs of the straw-derived biochar surface with biofilm. Biochar was sampled from soil after the vegetation experiment with maize. BC, biochar; BCT, BC + *T. viride*. Green color corresponds to zones without nucleic acids (A), while red and yellow to zones with nucleic acids (B). C shows summary visualization of distribution of both zones. Black zones correspond to non-scanned surfaces situated either above or below the scanning zone.

primer, and 1 µL of template DNA in a total volume of 25 µL reaction. The cycler conditions were as follows: initial 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s and detection. *Trichoderma asperellum* MSCL 309 DNA (1 to 103 pg µL^{-1}) was used to make a standard curve for quantification. All reactions were done in triplicate. After quantification the samples were subjected to melting curve analysis and agarose gel electrophoresis.

Statistical analysis

The statistical data analysis was performed with single factor ANOVA using Microsoft Excel software at a significance level $p = 0.05$.

Results

Microscopy of biochar particles after the experiment

Effect of BC with immobilized *T. viride* on maize growth was expected to differ from the effects of BC and fungi, which were applied separately. After the 128 days experiment, BC particles were sampled from the rhizosphere soil and tested under confocal laser microscopy in order to compare the pre-treatment of BC by *Trichoderma* [BCT] with the non-treated BC [BC]. As shown in Fig. 2, BC particles from [BCT] were covered by a dense continuous biofilm, while BC particles from [BC] contained only separate sites with viable organic matter. This indicated the suitability of the tested BC as a carrier for immobilization of *T. viride*.

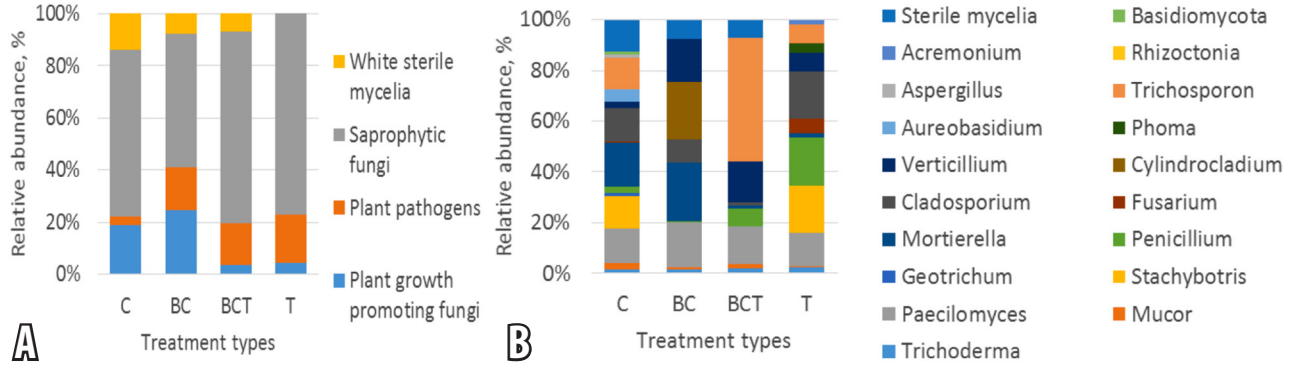

Fig. 3. Relative abundance of cultivable fungal genera in the maize rhizosphere after growth in a sandy loam soil for 128 days. A, three ecological groups of filamentous fungi and non sporulating white sterile mycelia; B, all fungal genera detected in soil. C, control; BC, biochar; BCT, BC + *T. viride*; T, *T. viride*. Data represent the average of 4 replicates.

Characteristics of cultivable fungi

All identified cultivable sporulating fungi in the present investigation were divided into three ecological groups: plant pathogens, saprophytes and plant growth promoting fungi (Fig. 3). Genera belonging to the plant pathogens were: *Fusarium, Verticillium, Phoma, Rhizoctonia*, and *Acremonium*. Plant growth promoting genera were *Trichoderma* and *Mortierella*. Members of the genus *Mortierella* produce arachidonic acid, a suppressive agent against plant pathogenic fungi (Eroshin, Dedyukhina 2002; Fakas et al. 2009). Among saprophytic fungi the following genera were represented: *Mucor, Stachybotrys, Geotrichum, Penicillium, Cladosporium, Aureobasidium, Trichosporon, Aspergillus, Cylindrocladium*, and *Paecilomyces*.

Non-sporulating fungi were grouped in a separate group. BC treatment increased the relative abundance of plant growth promoting fungi, while other two treatments (BCT and T) reduced the relative abundance of these fungi and increased the proportion of saprophytic fungi (Fig. 3A). BC treatment increased the relative abundance of *Mortierella* from 17.55 to 22.87%, as previously observed in a study conducted in an apple orchard in Australia (Abujabhah et al. 2016).

Treatments [BC] and [BCT] increased the CFU count of *Paecilomyces*. Similar effect of BC on this fungal genus has been described also in another study on the effect of BC influence on soil microbial populations (Hu et al. 2014). All treatments increased the relative abundance of plant pathogenic fungi (Fig. 3).

Abundance of Trichoderma *in soil*

qPCR was used to quantify *Trichoderma* DNA in soil samples. A set of uTf/uTr primers used was developed by Hagn (2007) for the detection, identification and quantification of common *Trichoderma* species in soil samples .

The amount of the total soil DNA ranged from 11.9 up to 19.3 μg g^{-1} of dry soil. *Trichoderma* spp. DNA was detected in 5 samples among 16, which represented four treatment types performed in four replicates (Fig. 4). In particular, for treatments [C]; [BC] and [T], *Trichoderma* DNA was detected in one sample of four replications, while for [BCT] in two samples. Moreover, the observed amount of *Trihoderma* DNA was highest in the [BCT] treatment (Fig. 4).

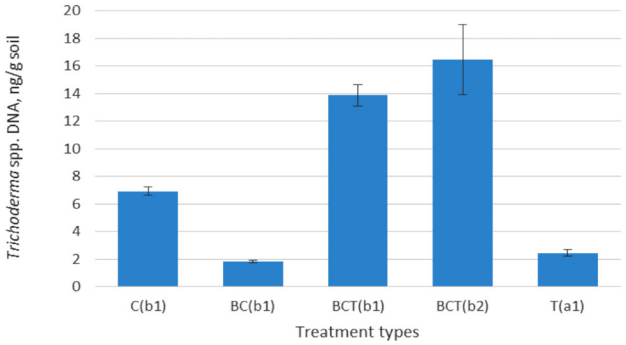

Fig. 4. Abundance of *Trichoderma* DNA in maize rhizosphere soil after 128 days. C, control; BC, biochar; BCT, BC + *T. viride*; T, *T. viride*. (a1); (b1); (b2), replications of treatment, soil was taken from different plants.

Seed germination and plant growth

The effect of BC and *T. viride* on maize was evaluated by seed germination and plant height. The highest seed germination among the tested treatments after 14 days was found in the [BCT] treatment (87.5 ± 0.0%), which was significantly ($p < 0.05$) different from that in the [T] treatment (71.9 ± 4.4%). Seed germination in treatment [C] and [BC] was 75.0 ± 8.8% and 78.1 ± 4.4%, respectively.

Effect of soil treatments on the maize growth after 18 days and 55 days was determined. As shown in Fig. 5, average height of plants grown in the presence of [BCT] exceeded other treatment types after 18 days and 55 days.

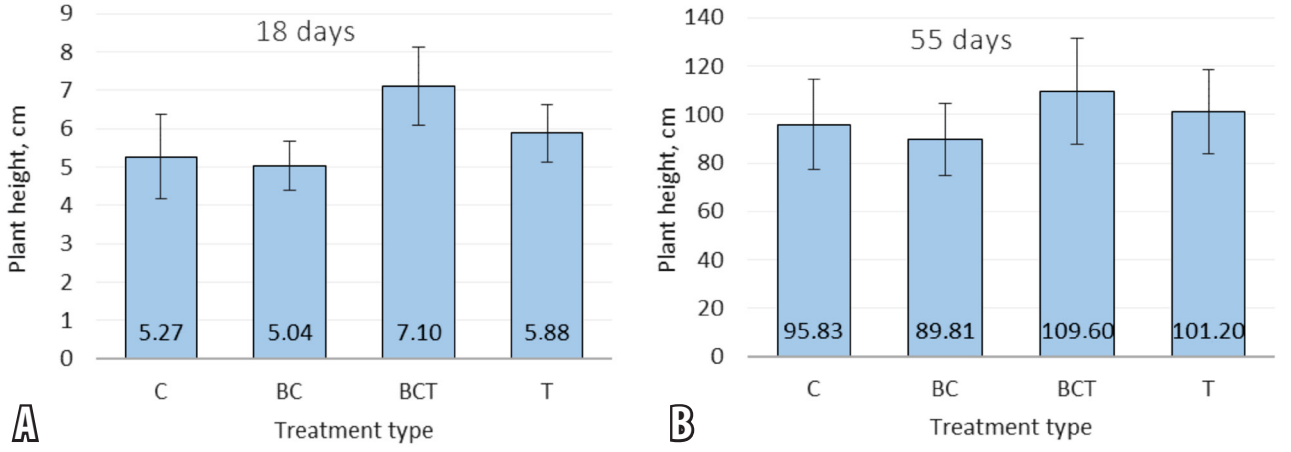

Fig. 5. Height of maize *Zea mays* growth under various treatment types after 18 days and 55 days from the beginning of the mini-field vegetation experiment. C, control; BC, biochar; BCT, BC + *T. viride*; T, *T. viride.* The values represent averages from two plots for each treatment type.

Table 1. Yield components and nutritional parameters of *Zea mays* aboveground biomass after 128 days. C, control; BC, biochar; BCT, BC + *T. viride*; T, *T. viride*. Data represent the average of 4 plants. *, average levels in ten maize hybrids cultivated in the Zemgale region, Latvia and harvested in October 3rd, 2009. **, not determined

Parameter	C	BC	BCT	T	REF*
Dry weight (%)	21.4 ± 1.5	19.9 ± 1.8	19.7 ± 1.6	21.3 ± 2.3	24.2
Carbon (% DW)	44.9 ± 1.9	46.7 ± 0.3	45.3 ±1.6	45.0 ± 1.5	n.d.**
Crude protein (% DW)	7.8 ± 0.4	7.9 ± 0.9	8.9 ± 0.8	8.5 ± 0.7	6.9
Acid detergent fiber (% DW)	28.2 ± 2.8	28.3 ± 3.0	27.0 ± 2.1	28.0 ± 1.5	23.3

The difference between [C] and [BC] was not statistically significant, while treatment pairs [BC] vs [BCT]; [C] vs [BCT]; [BC] vs [T] showed significant ($p < 0.05$) differences in plant height for both measurement periods (Fig. 5).

Biochemical characteristics of plant biomass after the vegetation experiment

The aboveground biomass of maize was characterized in treatment effect on yield and forage quality potential of the crop. Table 1 summarizes the yield components and nutritional parameters of maize aboveground biomass after 128 days. No significant differences in the tested parameters among treatment types were found.

Discussion

In this study effects of the wheat straw derived BC and *T. viride* alone and in combination on fungal diversity and maize growth in a sandy loam soil were determined. Soil was characterized by a high heterogenity in composition, which influenced the reproducibility of data obtained by culture-dependent methods and molecular tools. It should be noted that because of the adsorption of DNA to BC, microbial DNA extraction and purification from BC-amended soil can be difficult. Leite et al. (2014) compared different DNA extraction kits and showed that the PowerSoil kit was the most efficient for extracting microbial genomic DNA from soil treated with different types of biochar (Leite et al. 2014). Some studies have shown that the incorporation of BC into soil may influence soil microbial communities in different ways (Lehmann et al. 2011). Distinction must be made between BCs derived from manure- and crop residue-based feedstocks versus BCs derived from ligno-cellulosic feedstock, as well as BCs produced at a lower production temperature (< 400 °C) versus BCs generated at a higher production temperature (≥ 600 °C) (Gul, Whalen 2016).

Addition of BC and *T. viride* to soil caused changes in the fungal community, i.e., relative abundance of three ecological groups: plant pathogens, saprophytes and plant growth promoting fungi. Several fungal ecological groups have been described, such as litter decomposing fungi, wood-attacking fungi, chitinolytic fungi, keratinophilic fungi, fungicolous fungi, coprophilous fungi, pyrophilous fungi, ammonia fungi (Suzuki 2009), plant pathogens, plant endophytes and saprophytes (Rodriguez, Redman 1997). Such divisions can be imprecise, as, for example, some pathogenic fungi can be also saprophytic, depending on environmental conditions and available food sources. For example, *Fusarium* species are well known plant pathogens in agriculture but they can act as decomposers and carbon sequestrators as well (LeBlanc et al. 2015).

Addition of BC with immobilized *T. viride* to soil significantly increased seed germination and growth of maize. In this respect, it could be hypothesized that the tested BC is suitable for *Trichoderma* immobilization and further survival in soil. This was shown by culture-dependent methods, qPCR and microscopy. *Trichoderma* itself could play a positive role for maize growth. *Trichoderma* may also promote leaching of nutrients from BC, which should be tested in future experiments.

The treatment with BC and *T. viride* alone did not significantly influence seed germination and plant height, as compared to the control. It is important to emphasize the role of fertilizers in the case of BC addition to soil. Zhu et al. (2017) described the effect of maize-straw-derived BC at an application rate up to 30 t ha^{-1} on soil properties and maize growth. No significant effect of BC on nitrogen uptake by seedlings, plant height, biomass dry weight was found. Conversely, cultivation of maize in a glasshouse for 45 days in a sandy podzol soil with addition of BC (30 t ha^{-1}) resulted in increased dry matter and plant height. The authors found that BC alone is not able to supply enough nutrients for maize growth, and that additional fertilizers are required (Syuhada et al. 2016). In particular, Fe fertilization with BC might be beneficial for maize growth and Fe bioavailability, resulting in increasing starch, protein and fat concentrations in the plants (Ramzani et al. 2016).

Our vegetation experiment with maize did not include additional fertilizer treatment. According to recommendations (Karklins, Ruza 2013), additions of different amounts of N, P_2O_5 and K_2O result in a range of maize yield from 30 up to 80 t ha^{-1} in Latvia. In particular, the application rate of N in the case of maize cultivation should be from 100 to 180 kg ha^{-1} and it is recommended to divide this amount into three applications, i.e., at the beginning of cultivation, in the 5 to 6 leaf stage, and in the 8 to 10 leaf stage (Karklins, Ruza 2013). The concentration of N in the tested soil was 0.39 ± 0.04%, which was not sufficient for obtaining the maximum maize yield. Furthemore, maize yield depends on climatic conditions, particularly active temperature sum and precipitation (Gaile, Arhipova 2015).

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