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Response of soil microbial communities to the application of a formulated *Metarhizium brunneum* biocontrol strain

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ABSTRACT

Entomopathogenic fungi are used for biological control of insect pests. *Metarhizium brunneum* Petch (Hypocreales) has potential to control *Diabrotica virgifera virgifera* LeConte (Chrysomelidae), which is a major pest of maize in North America and has recently invaded Europe. The inundative application of an entomopathogenic fungal strain in biological control results in high densities of fungal propagules in the soil which can potentially affect soil microbial communities and their multiple functions in soil. The objective of the present study was to assess potential effects of *M. brunneum* on soil fungal and prokaryotic communities in a pot experiment over a time course of 4 months using high-throughput sequencing (HTS) of ribosomal markers. The application of *M. brunneum* formulated as fungus colonised barley kernels (FCBK) led to a significant increase of the applied strain in soil, as assessed by cultivation-dependent (plating on selective medium followed by genotyping of *Metarhizium* isolates) and cultivation-independent (HTS of ribosomal markers) approaches. Data revealed that soil fungal and prokaryotic community structures did not change after the application of *M. brunneum*. Temporal changes of the fungal and prokaryotic communities were observed and the prokaryotic communities showed minor changes to barley kernels (BK), the matrix of the formulation. Results of this study are in accordance with other investigations lacking any evidence for adverse effects on microbial communities caused by applied entomopathogenic fungi.

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Introduction

Diabrotica virgifera virgifera LeCont (Chrysomelidae) is a devastating univoltine pest in maize (*Zea mays* L.) in North America. Since the late 1990s, it has also established in south-eastern Europe and northern Italy with potential to invade parts of Asia (Bača, 1994; Ciosi et al., 2008;

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Krysan & Miller, 1986), which is expected to cause large damage costs (Wessler & Fall, 2010). The larvae damage maize plants by feeding on the roots, which leads to plant lodging, difficulties during plant harvest and decrease in yield. Adults feed on pollen, silk, kernels, and foliage and as a result, maize ears lack kernels and are more prone to fungal infection (Chiang, 1973). Available control options comprise crop rotation, chemical and biological insecticides, and resistant plants including trans-genetically modified varieties. These measures have been implemented with different success (Toepfer et al., 2009; van Rozen & Ester, 2010). The most frequently used methods are crop rotations and insecticide applications, however, approaches suffer from the development of adapted or resistant *D. v. virgifera* populations (Ciosi et al., 2009; Levine, Spencer, Isard, Onstad, & Gray, 2002). Maize breeding has yielded tolerant varieties, which are characterised by massive and regenerating root systems tolerating larval feeding (Branson, Sutter, & Fisher, 1982). Trans-genetically modified maize plants expressing toxins of *Bacillus thuringiensis* against *D. v. virgifera* are available but resistance to most available toxins have been observed (Jakka, Shrestha, & Gassmann, 2016). Biological control agents such as entomopathogenic nematodes, e.g. *Heterorhabditis bacteriophora*, and fungi, e.g. *Metarhizium* spp. and *Beauveria* spp., have shown promising results for control of *D. v. virgifera* (Toepfer et al., 2009). They bear potential for application in integrated pest management as recommended by European authorities (Directive 2009/128/EC, L309/71). The entomopathogenic fungus *M. brunneum*, formerly known as *M. anisopliae*, reduced 31% of emerging *D. v. virgifera* adults in a field study (Pilz, Keller, Kuhlmann, & Toepfer, 2009). In another study, it has not been successful, probably because of the high pest pressure in the field (Rauch et al., 2017).

The development of a fungal biological control agent (BCA) not only requires identification of an effective and suitable strain, but it also includes the establishment of large-scale production, efficacy-enhancing formulation, and sustainable application, as well as an assessment of quality control and monitoring tools for potential effects on non-target macro- and microorganisms. The latter issue has attracted increasing attention, particularly as it has become a major requirement in the product registration process (e.g. Commission Regulation (EU) No 544/2011, L155/66). Investigation of possible effects on non-target macroorganisms is frequently performed in parallel with studies on host-specificity, which are typically addressed early in the development of a BCA. In contrast, assessment of effects on microorganisms naturally present in soil, such as changes in soil microbial community structures and the functions they fulfil, has received much less attention. However, considering the importance of microorganisms and their functions in ecosystems, knowledge on possible adverse effects on the native soil microbial community is important for safety but also for economic reasons, as it may have implications on benefit-cost analyses (Benjamin & Wessler, 2016).

For a long time, investigations of microorganisms, particularly in soil, were limited to cultivation based approaches like plating of samples on nutrient media or community profiling based on nutrient utilisation. These techniques suffer from a cultivation bias by favouring certain microbial taxa and neglecting the viable but not culturable soil microorganisms (Hugenholtz, 2002). With the development of cultivation-independent biochemical and molecular approaches, it has become possible to expand analyses to the unculturable microbiota and assess communities in much greater detail (Rastogi & Sani, 2011). Amplification of a DNA marker region for instance located on the ribosomal operon (ITS2 for fungi and 16S rRNA V3–V4 for prokaryota) and subsequent sequencing

using high-throughput sequencing (HTS) technology is one of the cultivation-independent approaches that has successfully been applied to study the diversity of microbial communities and the effects certain factors may have on them (e.g. Hartmann, Frey, Mayer, Mader, & Widmer, 2015). This approach allows the parallel assessment of many samples and results in tens of thousands of sequences per sample that can be compared to existing databases (Rastogi & Sani, 2011). Cultivation-independent methods have been used to assess potential effects of applied soil fungi used for control of insects, weeds, or nematodes and these studies have revealed no or only transient effects of the applied strains (Hirsch, Galidevara, Strohmeier, Devi, & Reineke, 2013; Mayerhofer et al., 2017; Rousidou et al., 2013; Zimmermann, Musyoki, Cadisch, & Rasche, 2016).

The present study was conducted to assess potential effects of the application of *M. brunneum* formulated as fungus colonised barley kernels (FCBK) to control *D. v. virgifera* in maize production on soil fungal and prokaryotic community structures determined by HTS of ribosomal markers. Results were compared to a similar study which was performed in another crop-pest system with a different soil to assess if different soil microbial communities react differently to the same stress, i.e. the application of *M. brunneum*.

Material and methods

Set-up of the pot experiment

The pot experiment consisted of four treatments with six pots per treatment. Pots with a diameter of 32 cm and a height of 33 cm were filled with mixed sandy loam soil with a pH of 6.6 and 3.5% humus content. Soil was obtained from an agricultural field close to the research centre Laimburg (Auer, Italy). Pots were placed in an open greenhouse with a glass roof at the research centre and left undisturbed for 13 days in order to allow equilibration of the soil microbial communities. During the entire experiment, 300 ml of water was applied per day to each pot by a drip irrigation system. Each pot was fertilised with 5 g of Nitramoncal (13.5% ammonium and 13.5% nitrate; Borealis L. A. T, Austria) and 0.66 g of monoammonium phosphate (NP 12-61; Arpa speciali, Mantova, Italy) once at a depth of 10 cm before placing one kernel of *Zea mays* L. cultivar Mas 47. P (Maisadour, Austria) in the centre of each pot at a depth of 5 cm. Soil temperature was monitored hourly at a depth of 5 and 20 cm using an Escort Junior data logger (Escort Messtechnik, Zurich, Switzerland). The daily mean soil temperature at 5 cm depth was 23.2°C on average (ranging between 12.2°C and 41.5°C), and at 20 cm depth, it was 22.7°C (ranging between 13°C and 37.1°C).

Treatments

The entomopathogenic fungus *M. brunneum* strain EAMa01/58-Su (CECT 20764 Spanish Type Culture Collection; E. Quesada Moraga, Universidad de Córdoba, Spain), whose virulence against *D. v. virgifera* has been demonstrated (M. Schumann, personal communication), was applied in form of fungus colonised barley kernels (FCBK). FCBKs were produced as described by Aregger (1992) and Strasser, Abendstein, Stuppner, and Butt (2000). Four g of FCBKs, which correspond to an application dose of about 1×10^7 colony forming units (CFU) per g were applied. The same number of sterile

(autoclaved) barley kernels (BK), which resulted in 2.9 g of BK were used as control for possible effects of non-fungal compounds of the formulations. The insecticides PonchoTM 600 FS (clothianidin, Bayer CropScience AG, Germany) and MesuroTM (methiocarb, Bayer CropScience AG, Germany) were applied in form of dressed maize seeds (Insec). Six untreated pots served as controls (Untreated). All treatments were mixed into the upper 5 cm layer of the soil of each pot by hand immediately after the addition of the maize seed.

Application and assessment of *D. v. virgifera* and root damage of maize

Each pot was inoculated with 150 (± 47) eggs of *D. v. virgifera* 38 days after application of the treatments. The eggs were obtained as an egg/sand mixture from a rearing facility (Austrian Agency for Health and Food Safety, Vienna, Austria). The number of eggs in the egg/sand matrix was assessed by extracting and counting the eggs in 5 g of the egg/sand mixture (10 replicates). Successful hatching was tested by incubating 100- eggs in the laboratory at 25°C for three weeks. In order to assess the number of emerging *D. v. virgifera* adults, individual pots were covered with traps (Rauch, Zelger, & Strasser, 2016). Traps were installed 26 days after the addition of the eggs and checked three times a week. *D. v. virgifera* beetles were collected with an aspirator and counted. Root damage caused by larval feeding was evaluated according to the node-injury scale at the end of the experiment (Oleson, Park, Nowatzki, & Tollefson, 2005).

Soil sampling

Soil samples were collected right before maize sowing and application of treatment (week 0) on 19 May 2014, and 9 and 18 weeks later on 22 July and 24 September 2014. Samples consisted of four soil cores (diameter of 1 cm and a depth of 15 cm) per pot, which were pooled and thoroughly mixed. At each sampling time point 24 soil samples were collected (4 treatments and 6 replicates), sieved through a 5 mm mesh and split into three subsamples, which were used for: (1) the determination of soil dry weight, (2) for the isolation of *Metarhizium* spp. and subsequent genotyping and (3) for the assessment of effects on indigenous soil microorganisms.

Monitoring of the applied strain

Metarhizium spp. colonies were isolated from soil samples using plating on the selective medium according to the protocol described by Laengle, Pernfuss, Seger, and Strasser (2005). Soil suspensions (0.25 g ml^{-1}) of each sample were plated on four selective medium agar plates, *Metarhizium* spp. colony forming units (CFU) were counted and median CFU per sample were calculated. One single colony was randomly selected per soil sample for genotyping. DNA extraction of fungal mycelium was performed according to the protocol described by Kepler, Humber, Bischoff, and Rehner (2014). Isolates were grown on sterile filter paper placed on PDA agar plates until a firm layer of mycelium was observed. Mycelium of 2–4 mm² was scraped off the filter paper and transferred to a 2 ml Eppendorf tube containing a mix of 1 mm (Vitaris, Braun Biotech, Germany) and 3 mm glass beads (Merck KGaA, Germany) and 350 μl of Prepman extraction buffer (Applied

Biosystems, USA). Fungal mycelium was homogenised with a ball-mill (MM301, Retsch, Germany) at maximum speed for 15–30 s. Following homogenisation, tubes were incubated in a heating block at 99°C for 5 min, mixed by inverting, and incubated again for 5 min. After incubation, the tubes were centrifuged at 14,000 g for 5 min, rotated by 180°, and centrifuged again for 5 min. The supernatant (175 µl) was transferred to a new tube and stored at –20°C. DNA extracts were diluted by 1:10 or 1:100 for PCR analyses. Genotyping of isolates was performed with simple sequence repeat (SSR) marker analysis according to the protocol of Mayerhofer et al. (2015). SSR marker set I (Ma2049, Ma2054, Ma2063) and set V (Ma195, Ma327, Ma2287) were amplified in multiplex reactions and amplicon sizes were determined with an ABI 3130XL (Applied Biosystems, USA) using 36 cm capillaries, POP-7™ and GENESCAN™ 400HD ROX™ as an internal size standard. *M. brunneum* ARSEF 7524 served as reference for allele sizes. PCR fragment sizes were calculated with GeneMarker® v. 2.4.0 software (SoftGenetics®, USA).

Determination of the ITS2 sequence of the applied strain

The sequence of internal transcribed spacer region 2 (ITS2) of strain *M. brunneum* EAMa01/58-Su was determined as described previously (Mayerhofer et al., 2017) using Sanger sequencing with primer pair ITS3 (5' CAHCGATGAAGAACGYRG 3')/ITS4 (5' TCCTSCGCTTATTGATATGC 3') (Tedersoo et al., 2014). The sequence was submitted to NCBI GenBank database (Accession No KY786030; Benson et al., 2015).

Analyses of microbial community composition

Soil DNA extraction was performed as described by Mayerhofer et al. (2017) which included three consecutive DNA extractions from 0.5 g soil of each sample. Soil DNA was dissolved in TE buffer (at 1 ml per 1 g soil dry weight) and subsequently subjected to a clean-up procedure using NucleoSpin® gDNA clean-up kit (Machery-Nagel, Germany). Soil DNA concentration was determined with a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Australia) and the extract subsequently diluted to a concentration of 2 ng/µl. The fungal ITS2 was amplified using primer pair ITS3/ITS4 (Tedersoo et al., 2014) and the prokaryotic (bacterial and archaeal) variable region (V3–V4) of the small subunit of the ribosomal RNA (16S rRNA) was amplified using a modified version of primer pair 341F (5' CCTAYGGGDBGWCWSCAG 3')/806R (5' GGAC-TACNVGGGTHCTAAT 3') as published by Frey et al. (2016). The 5' end of the forward and reverse primers were tagged with CS1 and CS2 adapters, respectively, in order to allow multiplexing with the Fluidigm Access Array System (Fluidigm, USA). PCR amplifications were performed as described by Mayerhofer et al. (2017). PCRs were conducted in four replicates for each soil sample. Replicates were pooled and sent to the Génome Québec Innovation Center at the McGill University (Montréal, Canada) for barcoding (Fluidigm Access Array technology), purification with AMPure XP beads (Beckman Coulter, USA) and paired-end sequencing using the Illumina MiSeq v3 platform (Illumina Inc., USA). Raw sequence data and metadata were submitted to NCBI SRA (PRJNA386177; Leinonen, Sugawara, Shumway, & on behalf of the International Nucleotide Sequence Database, 2011).

Sequence processing and taxonomic classification

The sequences were processed as described by Mayerhofer et al. (2017) and according to the customised pipeline reported by Frey et al. (2016) using UPARSE implemented in USEARCH v8.0.1623 (Edgar, 2010, 2013) if not stated differently. In short, quality control included merging of overlapping paired-end reads using `fastq_mergepairs` (Edgar & Flyvbjerg, 2015), correction of substitution errors using BayesHammer algorithm implemented in SPAdes 3.5 (Nikolenko, Korobeynikov, & Alekseyev, 2013; Nurk et al., 2013), removing PCR primer sequences with `Cutadapt 1.8.1` (Martin, 2011) and filtering reads with a maximum expected total error of 1 using `fastq_filter` in USEARCH (Edgar & Flyvbjerg, 2015). After exact dereplication using `derep_fulllength` in USEARCH, singletons were removed and reads identified as being chimeric were discarded during clustering at 97% sequence identity using `cluster_otus` in USEARCH (Edgar, 2013). The presence of ribosomal signatures was verified with ITSx (Bengtsson-Palme et al., 2013) or Metaxa2 (Bengtsson-Palme et al., 2015) for eukaryotic or prokaryotic centroids, respectively, and all centroids lacking ribosomal signatures were discarded. Taxonomic classification was performed with the naïve Bayesian classifier implemented in MOTHUR v.1.35.1 (Schloss et al., 2009) using a custom-made database extracted from NCBI GenBank (Benson et al., 2015) and the UNITE (Abarenkov et al., 2010) database for eukaryotes. Prokaryotic V3–V4 sequences were queried against the GREENGENES database (DeSantis et al., 2006; McDonald et al., 2012). Non-fungal sequences were discarded from the eukaryotic dataset. In the prokaryotic dataset only archaeal and bacterial sequences were retained and OTUs assigned as organelles, i.e. chloroplasts and mitochondria were excluded. In order to avoid influences of the abundance of the OTU of the applied strain (OTU 3) on statistical tests, it was excluded from the dataset prior to comparison of microbial diversities among treatments.

Statistical analyses

Statistical analyses were performed as outlined in Mayerhofer et al. (2017). Procedures using iterative subsampling such as the assessment of OTU richness, rarefaction curve analyses, and calculation of Bray Curtis (BC) dissimilarities were performed at the lowest sampling depth of a sample in the dataset, i.e. 6180 and 8942 fungal and prokaryotic sequences, respectively. Differences in microbial community structures across treatments and sampling time points were assessed with overall and pairwise PERMANOVA based on BC dissimilarities using the functions `adonis` within `vegan` and `pairwise.perm.manova` within `RVAideMemoire` (Hervé, 2018; Oksanen et al., 2018) and Benjamini-Hochberg *p*-value correction in R (R-Core-Team, 2016). Multivariate homogeneity of groups' dispersions (variances) of fungal and prokaryotic community structures were assessed among treatments and sampling time points using the function `betadisper` within the package `vegan` in R and were based on BC distances. Non-metric multidimensional scaling (NMDS) was calculated in R with the function `metaMDS` included in the package `vegan` (Oksanen et al., 2018; Shepard, 1962). Overall and pairwise PERMANOVA tests and tests for multivariate homogeneity of groups' dispersions of relative sequence abundance at OTU level among treatments and sampling time points were based on Euclidean distances and were performed as described above. Fungal and prokaryotic community

structures of untreated pots were compared between the present dataset and another dataset derived from a study with a very similar set-up comprising a biocontrol experiment against *Agriotes* spp. in potato (Mayerhofer et al., 2017; SRA accession number PRJNA386024) using OTU richness, overall PERMANOVA, and multivariate homogeneity of groups' dispersions as described above. Principal coordinates analyses (PCO) based on BC distances were calculated using `cmdscale` included in the R core package (Gower, 1966; R-Core-Team, 2016). Venn diagrams displaying shared OTUs were drawn using the function `draw.pairwise.venn` within the package `VennDiagram` in R (Chen & Boutros, 2011).

Results

Abundance of the applied strain, number of adult beetles and damage assessment

The abundance of *Metarhizium* spp. increased significantly from a median of 2,451 CFU g⁻¹ soil dry weight over all pots at week 0 to a median of 64,521 and 23,993 CFU g⁻¹ soil dry weight in pots treated with FCBK at week 9 and 18, respectively (Figure 1(A)). Five and six out of six isolates from FCBK-treated pots had the genotype (SSR marker based) of *M. brunneum* EAMa01/58-Su at week 9 and 18, respectively (Table S1). *M. brunneum* EAMa01/58-Su was not detected at week 0 but one out of six isolates from the treatment Insec and the control at week 9, and two isolates from treatment Insec at week 18 had the genotype of the applied strain (Table S1). The mean number of emerging *D. v. virgifera* adults ranged from 4.7–7.8 per treatment with standard deviations ranging from 3 to 5.3. The number of *D. v. virgifera* adults and damage of maize plants across all treatments correlated weakly but significantly (Spearman $r = 0.53$, $p < 0.01$), and both measures did not differ significantly when comparing individual treatments with untreated pots (data not shown).

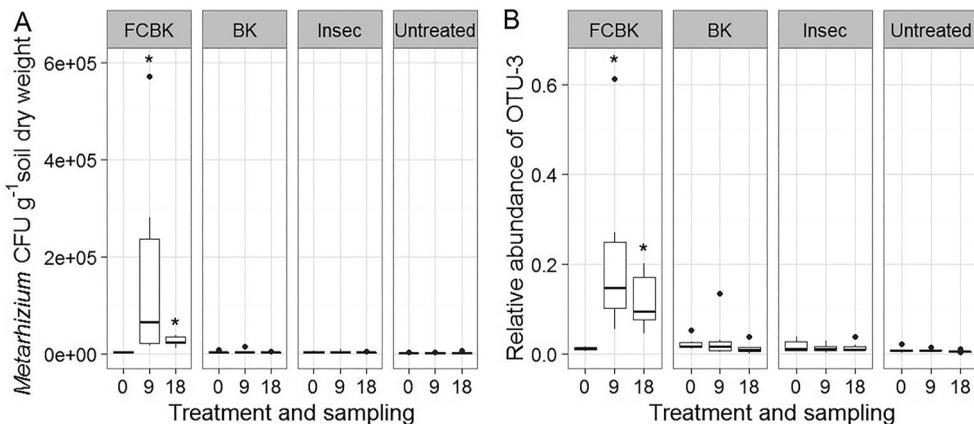


Figure 1. Abundance of *Metarhizium* spp. in CFU g⁻¹ soil dry weight (A) and relative abundance of the OTU (B) including the sequence of the applied strain to total sequence abundance per treatment (fungus colonised barley kernels [FCBK], barley kernels [BK] and clothianidin & methiocarb coated seeds [Insec]) at week 0, 9 and 18. * indicates significant differences to untreated control at the respective sampling time points ($n = 6$; $p < 0.05$).

Soil microbial diversity

After quality filtering the 24 samples contained in total 1,601,688 fungal (22,246 ± 8686 sequences per sample) and 1,233,720 prokaryotic sequences (17,135 ± 2958 sequences per sample), including 21,160 archaeal sequences. The sequences were clustered into 2022 fungal (388 ± 65 OTUs per sample) and 9428 prokaryotic OTUs (including 39 archaeal OTUs; 2670 ± 242 OTUs per sample). Rarefaction analyses indicated that the sampling effort for fungal sequences of most samples was close to saturation (Fig S1C). The variation among samples, however, was higher for fungal as compared to prokaryotic samples (Fig S1C and S1D). Fungal OTUs were assigned to six phyla (Fig S2A). The total prokaryotic community comprised 45 phyla (including three archaeal phyla; Fig S2B). OTU 3 with an abundance of 57,664 sequences was assigned to *M. brunneum*. Within OTU 3 14,889 sequences exactly matched the ITS2 sequence of *M. brunneum* EAMa01/58-Su. The relative abundance of OTU 3 increased significantly only in the FCBK-treated pots from a median of 1% of all sequences per sample prior to the application to 15% and 10% at 9 and 18 weeks after the treatment (Figure 1(B)). CFU counts and relative OTU 3 abundance strongly correlated ($r = 0.7$, $n = 72$, $p < 0.001$). After the exclusion of OTU 3 (to avoid interference with statistical analyses) the total number of fungal sequences decreased to 1,544,024 (21,445 ± 8700 per sample).

Effects of treatments on microbial diversity

There were no significant differences in fungal and prokaryotic OTU richness among the treatments and the untreated control at any sampling time point (Fig S1A and S1B). Prior to application (week 0) no significant differences in the fungal and soil prokaryotic communities were detected based on BC dissimilarities and assessed with overall PERMANOVA (fungi: pseudo $F = 1.14$, $p = 0.13$; prokaryota: pseudo $F = 1.09$, $p = 0.13$). Overall PERMANOVA including all time points revealed that soil fungal communities were affected slightly by treatments, however, pairwise tests did not reveal differences among treatments and untreated pots (Tables 1 and 2). Multivariate homogeneity of groups' dispersion tests revealed no differences in the dispersion of fungal community structures among treatments. Also, overall PERMANOVA of the relative sequence abundance per fungal OTU revealed no significant changes after the application of any of the treatments. Small overall differences in the soil prokaryotic communities among the treatments were

Table 1. Differences of the community structures of all fungi and prokaryota between treatments at sampling time points assessed with overall PERMANOVA.

Organism	Factor	Degrees of freedom	Sums of squares	Mean of sums of squares	Pseudo F -statistic	R2	p -value
Fungi	Treatment	3	0.5199	0.1733	1.6123	0.0543	0.0131
	Time	2	1.9782	0.9891	9.2026	0.2068	0.0001
	Treatment x time	6	0.6195	0.1033	0.9607	0.0648	0.5758
	Residuals	60	6.4487	0.1075	NA	0.6741	NA
	Total	71	9.5663	NA	NA	1.0000	NA
Prokaryota	Treatment	3	0.2834	0.0945	1.7386	0.0605	0.0002
	Time	2	0.8168	0.4084	7.5162	0.1744	0.0001
	Treatment × time	6	0.3231	0.0539	0.9911	0.0690	0.4928
	Residuals	60	3.2602	0.0543	NA	0.6961	NA
	Total	71	4.6835	NA	NA	1.0000	NA

Table 2. Differences of the community structures of all fungi and prokaryota between treatments at sampling time points assessed with pairwise PERMANOVA.

Organism	Pairwise comparison of each treatment with untreated (<i>p</i> -value)		
	BK	FCBK	Insec
Fungi	0.3515	0.5930	0.5694
Prokaryota	0.0300	0.1962	0.1962

Organism	Pairwise comparison of sampling time points (<i>p</i> -value)		
	Week 0 and 9	Week 0 and 18	Week 9 and 18
Fungi	0.0001	0.0001	0.0001
Prokaryota	0.0001	0.0001	0.0001

detected (Table 1, Figure 2(A) and (B)) and pairwise comparisons among treated and untreated pots revealed significant changes of the soil prokaryotic communities in BK-treated pots (Table 2). Multivariate homogeneity of groups' dispersion tests revealed no differences in the dispersion of prokaryotic community structures among treatments. Also, relative sequence abundances of none of the prokaryotic OTUs was changed significantly after the application of any treatments.

Temporal changes of the microbial diversity

Soil fungal and prokaryotic community structures were assessed at three sampling time points over a period of 18 weeks. OTU richness of fungal and prokaryotic communities in untreated pots did not change significantly during this time period (Fig S1A and S1B). However, a tendency of decreased OTU richness of fungal communities and an increased OTU richness of prokaryotic communities was observed in untreated pots and in all treatments over time (Fig S1A and S1B). Overall PERMANOVA showed that fungal and prokaryotic communities changed over time and pairwise tests revealed that

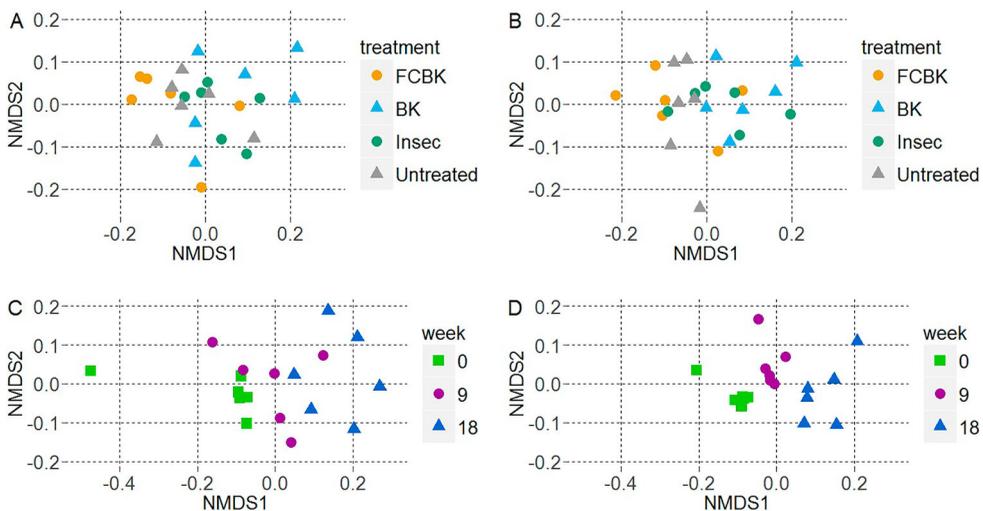


Figure 2. NMDS of prokaryotic samples showing treatment-effects at week 9 (A, stress = 0.13) and week 18 (B, stress = 0.15) and NMDS of fungal (C, stress = 0.12) and prokaryotic communities (D, stress = 0.09) in untreated control samples over time ($n = 6$).

fungus and prokaryotic community structures differed at all sampling time points (Tables 1 and 2). Dispersion of fungal and prokaryotic community structures also changed significantly over time (fungi: pseudo $F = 8.49$, $p = 0.0008$; prokaryota: pseudo $F = 10.47$, $p = 0.0002$). NMDS plots showed continuous changes of the fungal and the prokaryotic communities in untreated pots from week 0–9 and 18 (Figure 2(C) and (D)). Overall PERMANOVA of relative sequence abundance of each OTU among sampling time points revealed that 9.7% of the fungal OTUs (197) were significantly affected by time and 64% of these fungal OTUs varied also in dispersion. Most of these significantly affected OTUs belonged to the phylum Ascomycota (108), followed by Glomeromycota (34), Basidiomycota (28), Zygomycota (12), unclassified fungi (12), and Chytridiomycota (3). Of these five fungal phyla, Glomeromycota (pseudo $F = 54.6$, $p < 0.001$) and Chytridiomycota (pseudo $F = 3.9$, $p < 0.05$) were the only phyla for which relative sequence abundances changed at phylum level. Means and dispersions of relative sequence abundances of Chytridiomycota (in total 102 OTUs) and Glomeromycota (in total 126 OTUs) changed significantly over time. Chytridiomycota increased slightly at week 9 followed by a decrease at week 18. Glomeromycota were significantly more abundant in soil of all treated and untreated pots at week 9 and 18 than at week 0 (Figure 3(A)) and the community structure of Glomeromycota based on BC distances changed significantly over time (pseudo $F = 21$; $p = 0.0001$; Figure 3(B)). The 34 OTUs belonging to the Glomeromycota which changed significantly over time belonged to the genera *Ambispora*, *Claroideoglossum*, *Glomus*, *Paraglossum*, *Rhizophagus*, and *Septoglossum* (or unknown). Overall PERMANOVA analyses at the OTU level showed that 13.2% of the prokaryotic OTUs (1249) were affected by time and thereof 78% also changed significantly in dispersion. These OTUs belonged to 31 different phyla and the five most prevalent phyla were

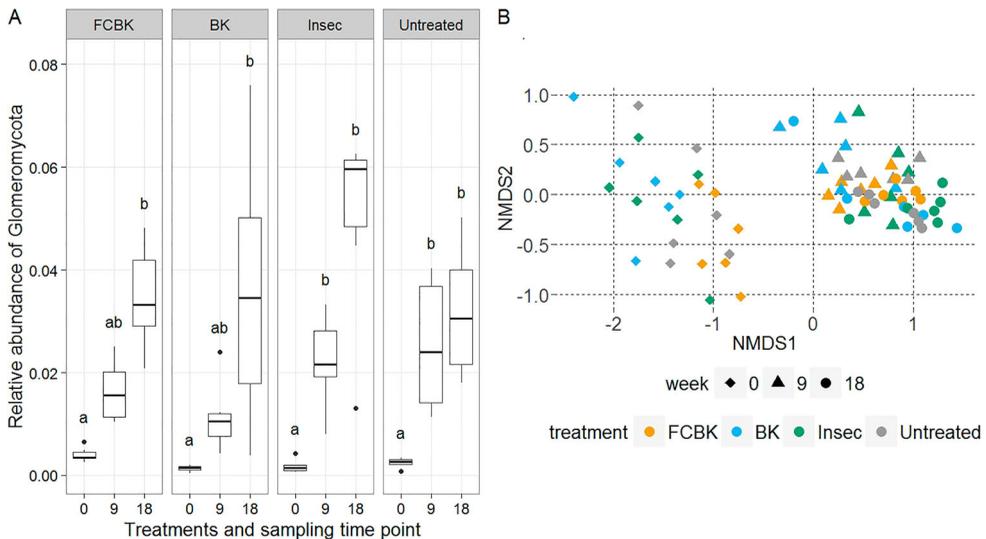


Figure 3. Relative sequence abundance of the phylum Glomeromycota per treatment and sampling time point (A). Letters indicate significant difference ($p < 0.05$) among sampling time points for each treatment individually ($n = 6$). Ordination (NMDS) of community structures of Glomeromycota (B, stress = 0.11) per treatment and sampling time point ($n = 6$).

Proteobacteria (348), Acidobacteria (137), Verrucomicrobia (136), Bacteroidetes (133), and Planctomycetes (108).

Comparison of effects on soil microbial communities to a similar pot experiment

Finally, fungal and prokaryotic OTU richness and community structures in untreated pots were compared to a similar pot experiment performed previously (Mayerhofer et al., 2017). The comparison revealed a significantly lower fungal OTU richness in the present study, but no differences in prokaryotic richness (Figure 4(A) and (B)). Venn diagrams displayed that 30.8% and 46.2% of fungal and prokaryotic OTUs were shared between the untreated pots of both experiments (Figure 4). Furthermore, PCO and overall PERMANOVA analyses showed major and significant differences in fungal and prokaryotic communities between the two experiments (fungi: pseudo $F = 39.04$, $p = 0.0001$, prokaryota: pseudo $F = 53.23$, $p = 0.0001$; Figure 4(C) and (D)). Also, dispersions

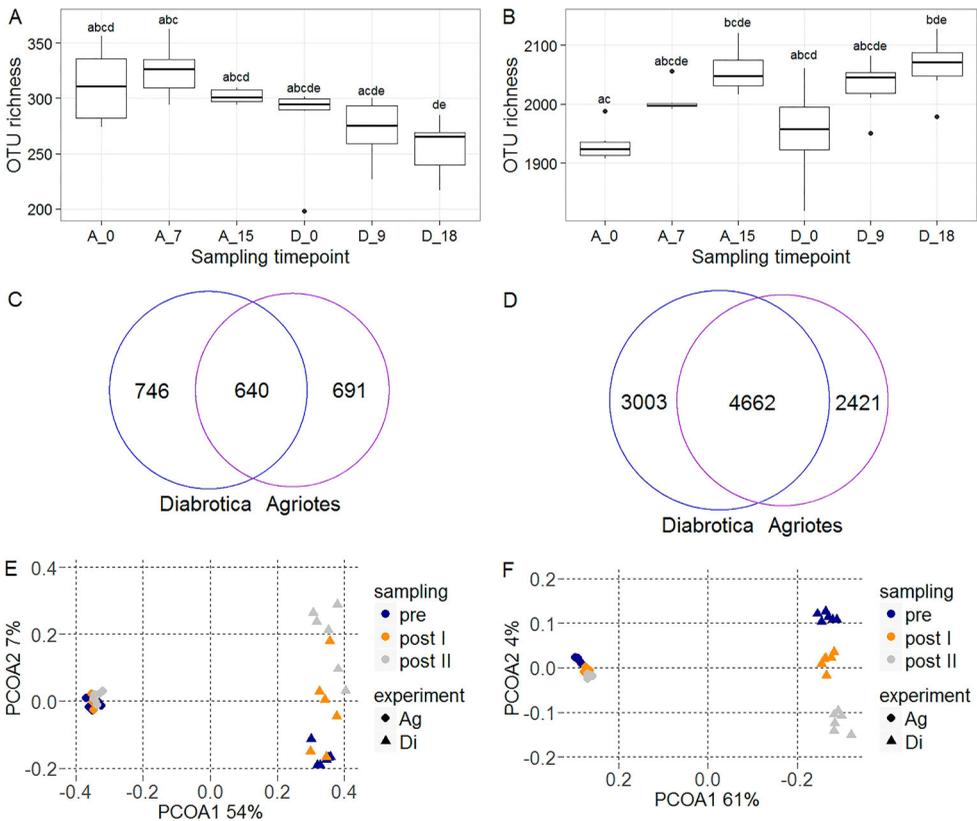


Figure 4. Comparative analysis of soil fungal communities in untreated pots of the biocontrol experiment to control *Agriotes obscurus* (A, Mayerhofer et al. 2017) and *D. v. virgifera* (D, present study): OTU richness of fungal (A) and prokaryotic communities (B) in untreated pots at different sampling time points ($n = 6$). Letters indicate significant differences ($p < 0.05$). Venn diagrams showing the shared number of fungal (C) and prokaryotic (D) OTUs between the untreated pots of both experiments. Ordination revealing fungal (E) and prokaryotic (F) community structures in untreated pots before application (pre) and after application (post I and post II) for each study ($n = 6$).

of fungal and prokaryotic community structures in untreated pots were significantly greater in the present study compared to the Agriotes-pot experiment (Mayerhofer et al., 2017; $p < 0.01$, Figure 4(E) and (F)).

Discussion

Assessment of presence and abundance of an applied BCA strain in soil is important for evaluating efficacy and possible effects on native soil microbial communities. In our study, this was achieved by using a cultivation-dependent as well as a cultivation-independent approach, i.e. assessment of *Metarhizium* spp. CFU g⁻¹ soil dry weight followed by SSR marker based genotyping of selected isolates as well as an assessment of the OTU which included the marker sequence of the applied strain within the HTS dataset. Results of both methods showed significant increases in the pots treated with FCBKs, but not in the other treatments. *M. brunneum* EAMa01/58-Su was also detected in some pots treated with the insecticide and untreated control pots, however, at low prevalence (one to two out of six strains analysed). In these treatments, the applied strain was possibly transferred from treated to untreated pots by insects as has been reported also in other studies (e.g. Baverstock, Baverstock, Clark, & Pell, 2008). Although data indicate that the abundance of the applied strain increased to a median of about 6.5×10^4 CFU g⁻¹ soil dry weight in the FCBK-treated pots, no acute biocontrol effects were observed. The variation of the mean number of *D. v. virgifera* beetles per treatment was high (standard deviation ranging from 3 to 5.3), which was possibly due to variability in inoculum quantities (150 ± 47 eggs per pot) leading to an unequal infestation rate with *D. v. virgifera*. This may have obscured detection of small effects of the different treatments. Varying efficacies of *M. brunneum* (formerly *M. anisopliae*) to infect *D. v. virgifera* have also been found in field experiments, i.e. no biocontrol effect (Rauch et al., 2017) or 31% reduction of *D. v. virgifera* beetles (Pilz et al., 2009). Despite the fact that no biocontrol effects were observed in this study, the 19-fold increase of the density of *M. brunneum* EAMa01/58-Su in the FCBK treatments observed in our study demonstrated that native soil was sufficiently exposed to the applied strain, which allowed assessment of possible effects on microbial communities.

Prior to registration of plant protection products in Europe, the assessment of potential effects of applied microorganism on native soil microbial populations is required (Commission regulation No 544/2011, L155/66). The present study revealed that neither changes in soil fungal nor prokaryotic community structures as well as relative sequence abundance of individual OTUs could be detected upon the application of *M. brunneum* formulated as FCBK. This is in accordance with previous studies that detected no or only transient effects on soil microorganisms involving *Beauveria bassiana* (Hirsch et al., 2013; Rai & Singh, 2002), *B. brongniartii* (Rai & Singh, 2002; Schwarzenbach, Enkerli, & Widmer, 2009), *Metarhizium anisopliae* sensu lato (Hu & St Leger, 2002; Kirchmair, Neuhauser, Huber, & Strasser, 2008), and *M. brunneum* (Mayerhofer et al., 2017). In the latter study, a different *M. brunneum* strain formulated as FCBK and BK alone have been used in a pot experiment and small changes in fungal community structures were observed (Mayerhofer et al., 2017). However, because fungal spores alone did not affect fungal communities it was suggested that effects resulted mainly from the compounds of the formulation. Moreover, the effects of FCBK were only observed in the pot experiment and not in the field.

Regarding effects of BK treatment, only in the present study changes on soil prokaryotic communities were observed, but not in the previous study by Mayerhofer et al. (2017). The experimental set-up of the two studies was similar but differed in biotic factors including insect pest species (*Agriotes* spp. vs. *D. v. virgifera*), plant species (potatoes vs. maize), and applied *M. brunneum* strain (ART2835 vs EAMa01/58-Su). Moreover, the soil microbial community structures appeared to be different as reflected in significant differences in fungal OTU richness, low percentages of shared OTUs in untreated pots (30.8% of the fungal and 46.2% of the prokaryotic OTUs), and major differences in community structures (Tables 1 and 2, Figure 4). These differences in microbial communities suggest that the degree of resistance of a microbial community, also referred to as insensitivity to disturbance (e.g. by Shade et al., 2012), to applications of FCBK and BK may depend on soil specific microbial communities. To our knowledge, differences in resistance to massive application of microorganisms in soil between different microbial community structures have not been assessed. However, in a review on the effects of various disturbances, such as heat, addition of heavy metals, organic matter, or pesticides, freeze–thaw or dry-wet cycles, compression, and tillage on soil microorganisms, Griffiths and Philippot (2013) concluded that resistance depends on the type of disturbance, soil physical–chemical parameters, as well as the indigenous soil microbial community composition and soil history. A prerequisite to assess the resistance of a microbial community to disturbance is the assessment of the intrinsic variability of the system, which comprises fluctuations in community composition without perturbation (Shade et al., 2012). Thus, the lacking effects of FCBK on fungal communities in the present study may be explained by the variability of the system, as shown in untreated control pots (Figure 4(E)).

A variety of carrier materials are used to formulate entomopathogenic fungi as BCAs, including kernels (rice or barley), liquids (water or oils), or granules (alginate; Glare & Moran-Diez, 2016). Carrier materials provide a structure for the product and they can protect the fungus from biotic and abiotic stresses and/or serve as a nutrient source to support growth. BK had a small effect on prokaryotic community structures, whereas the corresponding product FCBK did not affect the prokaryotic community structures and none of them had an effect on individual OTUs. Decomposition of the carrier materials may be the reason for the prokaryotic community changes in the BK treatment, however, changes were subtle as no single affected OTU could be identified. Barley kernels, which include compounds such as starch, lipids, proteins, dietary fibre (particularly beta-glucans), vitamins, and minerals (Šterna, Zute, Jansone, & Kantane, 2017), were shown to be degradable by microorganisms such as *Rhizopus oligosporus*, various *Saccharomyces*, and *Lactobacillus* species in the fermentation process of the food product barley tempkeh (Feng, Passoth, Eklund-Jonsson, Alminger, & Schnurer, 2007) or by microorganisms such as *Lactobacillus plantarum*, *Aspergillus niger*, *Trichoderma reesei*, *R. oligosporus* and *Geotrichum candidum* during malting in the beer production process (Hattingh, Alexander, Meijering, van Reenen, & Dicks, 2014). In FCBK the niche (BK) may have been occupied and/or the respective compounds may have been consumed by *M. brunneum* and thereby it may have become inaccessible to the soil microbial communities.

The fungal and prokaryotic communities changed significantly over time. This was reflected in differences in community structures and in differences in relative sequence abundances of 10% of the fungal and 13% of the prokaryotic OTUs. Temporal variations were also observed in the pot experiment conducted by Mayerhofer et al. (2017). Changes

in biotic factors represented by the addition of plants and insects as well as abiotic factors such as temperature or moisture (Dematheis et al., 2012; Guo et al., 2017; Poll, Marhan, Back, Niklaus, & Kandeler, 2013) may have caused these temporal effects. The addition of maize was probably responsible for the major part of the temporal changes in fungal communities. These changes were strongly associated with six genera belonging to Glomeromycota, also known as arbuscular mycorrhiza, forming symbiotic interactions with maize roots (Schüßler, Schwarzott, & Walker, 2001). In a study comparing the diversity of arbuscular mycorrhiza in fields including maize crop rotations or grassland only, one species was exclusively found in the maize field (Oehl et al., 2003), however, the specificity of these interactions is still under debate (Sanders, 2003). Furthermore, temporal effects might have been caused by root exudates released by the maize plant, which differ in quantitative and qualitative composition among growth stages (reviewed in Badri & Vivanco, 2009). The maize plants at the three sampling time points were at different growth stages (week 0 no plants, week 9 approximately 10 leaves unfolded, week 18 mature plant) and thus may have differently affected microbial communities in the rhizosphere as shown in other studies (e.g. Cavaglieri, Orlando, & Etcheverry, 2009). Another biotic factor contributing to temporal changes might have been *D. v. virgifera* larvae feeding on roots, which has been shown to alter soil microbial community structures (Dematheis et al., 2012), and may have affected community structures in our study. Furthermore, effects of changing temperatures on microbial communities are well documented (e.g. Riah-Anglet et al., 2015). As the daily median temperature in our experiment ranged from 16 to 29°C with the minimal temperature of 12°C and with the maximal temperature of 42°C, this factor most likely also contributed to observed variabilities.

Soil microbial communities appeared to be resistant to the massive application of *M. brunneum* in our study. This is consistent with previous studies that have reported no or only minor and transient changes of microbial community structures upon the application of entomopathogenic fungi (*Beauveria* and *Metarhizium*). The growing number of studies revealing resistance of soil microorganisms to *Metarhizium* and *Beauveria* applications suggests that compositional diversity of soil microbial communities is not affected by these applications. Whether stability of soil microbial communities at the taxonomic level transfers into the stability of functions provided by soil microorganism awaits further investigation.

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