Article

Microbiome management by biological and chemical treatments in maize is linked to plant health

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Abstract: The targeted application of plant growth promoting rhizobacteria (PGPR) provides the key for a future sustainable agriculture with reduced pesticide application. PGPR interaction with the indigenous microbiota is poorly understood but essential to develop reliable applications. Therefore, *Stenotrophomonas rhizophila* SPA-P69 was applied as seed coating and in combination with a fungicide based on the active ingredients fludioxonil, metalaxyl-M, captan and ziram. Plant performance and rhizosphere composition of treated and non-treated maize plants of two field trials were analyzed. Plant health was significantly increased by treatment; however overall corn yield was not changed. By applying high-throughput amplicon sequencing of the 16S rRNA and the ITS genes, the bacterial and fungal changes in the rhizosphere due to different treatments were determined. Despite treatments had a significant impact on the rhizosphere microbiota (9- 12%), the field site was identified as main driver (27- 37%). Soil microbiota composition from each site was significantly different, which explains the site-specific effects. In this study we were able to show first indications how PGPR treatments increase plant health via microbiome shifts in a site-specific manner. This way first steps towards a detailed understanding of PGPRs and developments of consistently efficient applications in diverse environments are set.

Keywords: *Zea mays;* maize; corn; rhizosphere; 16S rRNA gene; ITS; fungicide; plant growth promoting rhizobacteria

1. Introduction

Plants live in close interaction with microorganisms and are therefore recognized as holobionts [1]. This close coevolution developed mainly mutualistic, commensal and neutral, but also pathogenic interactions [2–4]. Especially beneficial plant-microbe interactions in the rhizosphere were found to be very important throughout the plants lifecycle [5]. The rhizosphere harbors high abundances of plant growth promoting rhizobacteria (PGPR); their modes of action are nutrient uptake, stress protection, induced resistance and plant growth promotion by production of

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phytohormones [6–8]. However, in all agricultural production systems, pathogens originating from soil are a major factor limiting significantly yield and quality in crops. World-wide, farmers are increasingly specialized on production of distinct crops based on intensive management practice. This has consequences for soil-borne pathogens because they accumulate in soil and enhance disease pressure on crops over time [9]. *Fusarium*, *Rhizoctonia* and *Verticillium* are the most important fungal pathogens in soil, which are difficult to suppress because: i) they form highly persistent survival structures, ii) they have a very broad host range, and iii) specific resistance genes in crops are often not known [10]. Loss in global and local biodiversity is linked to an increase of soil-borne pathogens [11–13]. Annually, they cause loss of income of 4 billion € and of yield up to 80% in single farms but the causes of the world-wide spread are not yet fully understood and effective methods of control are currently lacking [14].

Missing crop rotations lead to reduced microbial diversity because of the missing plant-specific food prints in soil, so-called plant-soil feedbacks were identified as one reason [15]. Plant-mediated changes in the soil microbiome can influence the growth of other plants that grow later in the same soil but they definitively enrich crop-specific pathogens in soil [16]. This demands again more fungicide applications and intense management. A number of synthetic fungicides are applied currently to the field to target soil-borne pathogens and threaten global biodiversity [13,17]. Therefore, application of microorganisms antagonistic towards soil-borne pathogens are a promising alternative to suppress them and avoid outbreaks and yield losses [18]. Recently it was shown that pathogens are able to induce disease-suppressive functions in the endophytic root microbiome [19]. Although antagonistic microorganisms against crop pathogens and PGPR were used for decades to improve plant health and increase field yields in agricultural applications, their inconsistent effect in the field is one reason for missing success. The plant-specific composition of the microbiota is one reason for inconsistency [20]. The interaction of PGPR with the indigenous soil microbiota is poorly understood, and is, according to our hypothesis, another reason for that. To understand the interaction between PGPRs and the indigenous microbiota under field conditions, we studied the interaction of the maize rhizosphere microbiome and the PGPR *Stenotrophomonas rhizophila* SPA-P69 [21]. Domesticated forms of *Zea mays* L. (maize or corn) with a total production of 1.1 billion metric tons (2018) is one of the most grown plants for animal feed and human consumption globally. The biggest production areas are America (52.5%), Asia (29.1%) and Europe (11.2%) [22]. Interestingly, the rhizosphere of maize harbors even a cultivar-specific microbiota [23,24]. Soil-borne pathogens, such as *Phythium sp.*, *Rhizoctonia sp.* and *Fusarium sp.* are a major threat for the plant especially in the early growing period [13,25]. Therefore, reliable treatments in changing environmental conditions are needed.

The PGPR strain *Stenotrophomonas rhizophila* SPA-P69 (=DSM14405^T) was previously studied as a plant growth promoting bacteria in cotton, tomato, oilseed rape and sweet pepper. It was shown to colonize the roots, grow endophytically and, moreover, increase plant health [26]. This PGPR acts as Stress Protecting Agent (SPA) by producing osmolytes and spermidine [21]. As treatment field performance of SPA-P69 varied in previous trials and different locations, we decided to investigate this further. Therefore, we conducted field experiments on maize, involving combined treatments of fungicide and SPA-P69 and studied their impact on plant health, growth promotion, and plant colonizing microorganisms. We evaluated strain establishment in the rhizosphere and, looked at the

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global impact of the treatments using different field sites in the experiment. In our experiment we investigated the effect of seed application of *S. rhizophila* SPA-P69 in combination with a fungicide, based on the active ingredients fludioxonil, metalaxyl-M, captan and ziram. Fungicide treatments were previously shown, apart from their effect on plant health, to also influence the rhizosphere and phyllosphere of plants [27]. We expect that also the SPA-P69 PGPR treatment induced a microbiome shift. By studying the interactions of biocontrol and PGPR strains with the plant and other control products valuable information for the reduction of pesticide use can be gained and first steps for a movement towards more sustainable and integrated agricultural approaches can be set [18].

2. Materials and Methods

Seed treatments and sampling strategy

Untreated single hybrid maize (*Zea mays* L.) seeds (cultivar LG3258, Limagrain, Edemissen, Germany) were inoculated with the stress protecting agent *Stenotrophomonas rhizophila* SPA-P69 (syn. DSM 14405), further mentioned as SPA-P69 in the entire text, obtained from the Strain Collection for Antagonistic Microorganisms (SCAM, Institute of Environmental Biotechnology, Graz University of Technology, Austria). After cultivation in Nutrient Broth (NBII; Sifin, Berlin, Germany) for 24 h at 30°C, cells were harvested by centrifuging at 4000 x g at 4°C and adjusted to 10^8 CFU/ml by resuspending in 0.9% NaCl solution. Seeds were submerged in bacterial suspension for four hours at 22°C under agitation, followed by two washing steps with sterile distilled water and drying for 24 h in a laminar flow cabinet. For the control treatment, bacterial suspension was replaced by a sterile 0.9% NaCl solution. Half of the seeds with or without bacterial coating treatment were additionally coated with a fungicide mix based on the active ingredients fludioxonil, metalaxyl-M, captan and ziram (product names: MAXIM® XL, Syngenta; Korit®, Kwizda Agro GmbH) using sacrust as a carrier (Table 1). The used mixture was MAXIM® XL:Captan:Korit in a 1:1:0.6 ratio and 490 g seeds were coated with a total of 3.5 mL.

Table 1. Overview of treatments used in two field trials. The used fungicide mixture was MAXIM® XL:Captan:Korit in a 1:1:0.6 ratio.

Treatment	PGPR strain	Fungicide
CB	No	No
CF	No	YES
P69B	*Stenotrophomonas rhizophila* SPA-P69	No
P69F	*Stenotrophomonas rhizophila* SPA-P69	YES

Field trials were carried out at two field sites located in Lower Austria, Austria (Melk, Austria [48°9'20.28'N; 15°30'45.10'E] with neutral (pH 6.6-7.2) soil consisted of lime-free loamy sand with average humus content and Mitterdorf a.d. Raab, Styria, Austria [47°10'42.29'N; 15°36'45.16'E] with a slightly acidic to acidic (pH 4.6- 6.5) soil type predominated by lime-free sandy loam with average humus content). The randomized plot design included three replicates per treatment. In late April 220 seeds per plot in Mitterdorf and 180 seeds in Melk were planted in four rows. Four weeks post-planting, emerged plants were counted and the number of plants per row equalized to 40 or 30

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individuals (160 or 120 plants per plot/replicate) in Mitterdorf and Melk respectively. The fresh weight of the shoot was assessed from six out of the extracted plants. For microbiome analysis one composite sample per plot (three samples per treatment), consisting of roots with adhering soil from four randomly chosen plants, was collected from both locations. Additionally, four soil samples (10-15 cm depth) were taken from random locations on the field. At the end of October, corn yield was recorded from the two inner rows of a plot after mechanical harvesting.

DNA extraction and amplicon library construction

After arrival in the lab (cooled and within 24h), total community DNA was extracted from the samples. Therefore, 5 g of the soil and root samples were homogenized using a stomacher (Bagmixer; Intersciences, St. Nom, France) for 3 min using 50 mL of sodium chloride (0.85%). A total of 4 mL of homogenized solution was pelleted and further used for DNA extraction. The total community DNA was extracted using the FastDNA SPIN Kit for Soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's protocol. DNA was quality checked using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and stored at -20°C for PCR reactions.

Isolated DNA from two locations, four treatments and bulk soil samples were used for amplification of the 16S rRNA gene V4 hypervariable region and ITS1 region. The 515f/806r primer pair (515f: 5'-GTGYCAGCMGCCGCGGTAA-3'; 806r: 5'-GGACTACNVGGGTWTCTAAT-3') for bacteria and the ITS1f/ITS2r (ITS1f: 5'-CTTGGTCATTTAGAGGAAGTAA-3'; ITS2r: 5'-GCTGCGTTCTTCATCGATGC-3') for fungi were used [28–30]. All PCR reactions were performed in triplicates. The PCR mix was amplified in 35 cycles at 94°C denaturation for 45 s, 50°C annealing for 60 s and 72°C elongation for 90 s. The amplicons were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and pooled in equimolar concentrations. The paired-end Illumina MiSeq sequencing (2×300bp) of the barcoded Illumina library was performed by GATC Biotech (Berlin, Germany).

Bioinformatic analysis

Paired end reads were quality checked and demultiplexed using cutadapt [31]. Bioinformatic analysis for amplicon sequencing analysis was performed using the open-source QIIME2 version 2018.4.0 pipeline (https://qiime2.org, [32]). Primer sequences were removed using cutadapt [31], forward and reversed reads were joined and feature table and representative sequences (amplicon sequences variants (ASVs)) were generated using the DADA2 algorithm using quality filtering, denoising and chimeric filtering steps in QIIME2 [33]. The generated ASVs were classified using the vsearch algorithm and the SILVA v132 and UNITE v8.0 as bacterial and fungal reference databases, respectively [34–37].

Statistical analysis

The R version (R Core Team, 2017) was used to perform statistical analysis and create NMDS plots. Differences in agronomic data were tested using a pairwise t-test. Resulted ASV tables and taxonomy from QIIME2 were imported into R via phyloseq [38]. Bacterial and fungal analysis were performed by rarefying the dataset to the lowest amount of sequences in each dataset by randomly selecting subsets of sequences to account uneven sequencing depth. Alpha diversity significance ($p<$

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0.05) was tested using Kruskal Wallis tests, followed by pairwise comparisons and Tukey HSD post-hoc test. Beta diversity based on Bray-Curtis dissimilarities were tested using permutational analysis of variance (PERMANOVA, 999 permutations). The distance matrices were visualized by using a non-metric multidimensional scaling (NMDS) plot. Differences in taxa abundance between treatments was determined by DESeq2 analysis [39]. Prior DESeq2 analysis, ASVs with relative abundance less than 0.1% were removed. Shown significant differences below 0.05 were P value adjusted using the Benjamini-Hochberg method for multiple testing.

qPCR analysis of SPA-P69 gene copy numbers

Total DNA extracts from the samples were further used to specifically quantify *S. rhizophila* SPA-P69 by real-time PCR using TaqMan® and the primers Strh_for (5′-CACCTGAAAGAATGTAGGAGTGG-3′) and Strh_rev (5′-CTCGCTCTTTTCCCTAGTGC-3′) in combination with the probe Strh_pr (5′-CAGGGAAGCAAGCGCACCGT-3′). The quantification was performed on a Corbett Research TM thermocycler (Rotor-Gene 6000, Corbett Research, United Kingdom) The following approach was used to perform the qPCR (total volume 10 µl): 5µL TaqMan® Environmental Master Mix 2.0; 0.05µL of Primers (fwd/rev); 0.2µL Probe; 0.5µL Sample; 4.2µL nuclease-free water. The temperature program for the qPCR was composed of denaturation at 95°C for 10 min, 45 cycles of annealing at 95°C for 0.4 min and extension at 60°C for 1 min. The standard regression curve was obtained using the 160 bp long target fragment with known concentration and further 1:10 dilutions. Three replicates of each standard dilution were prepared to generate a mean value. The standard regression curve was employed to determine the gene copy numbers in the analyzed samples. All PCR reactions were performed in triplicates.

3. Results

Plant growth performance and SPA-P69 colonization in the rhizosphere

To investigate treatment effect on maize plants, different plant parameters were observed during the field trial. While no effect of the treatment on corn yield was observed between treatments, a significant higher emergence rate and shoot fresh weight was observed for the combined treatment of fungicide and SPA-P69 in Mitterdorf. Moreover, shoot fresh weight was significantly increased by fungicide treatment, especially with a combined treatment using stress protectant strain SPA-P69 (Figure 1A-C). When copy numbers of SPA-P69 were analyzed for all samples via qPCR a significantly ($p< 0.01$) higher abundance was found in SPA-treated samples (1000-fold increase). Therefore, the colonization of the rhizosphere by the SPA-P69 strain was successful. Interestingly, while in Melk *Stenotrophomonas* was also detected in control plants, in Mitterdorf only the treated samples showed detectable copies of SPA-P69 (Figure 1D).

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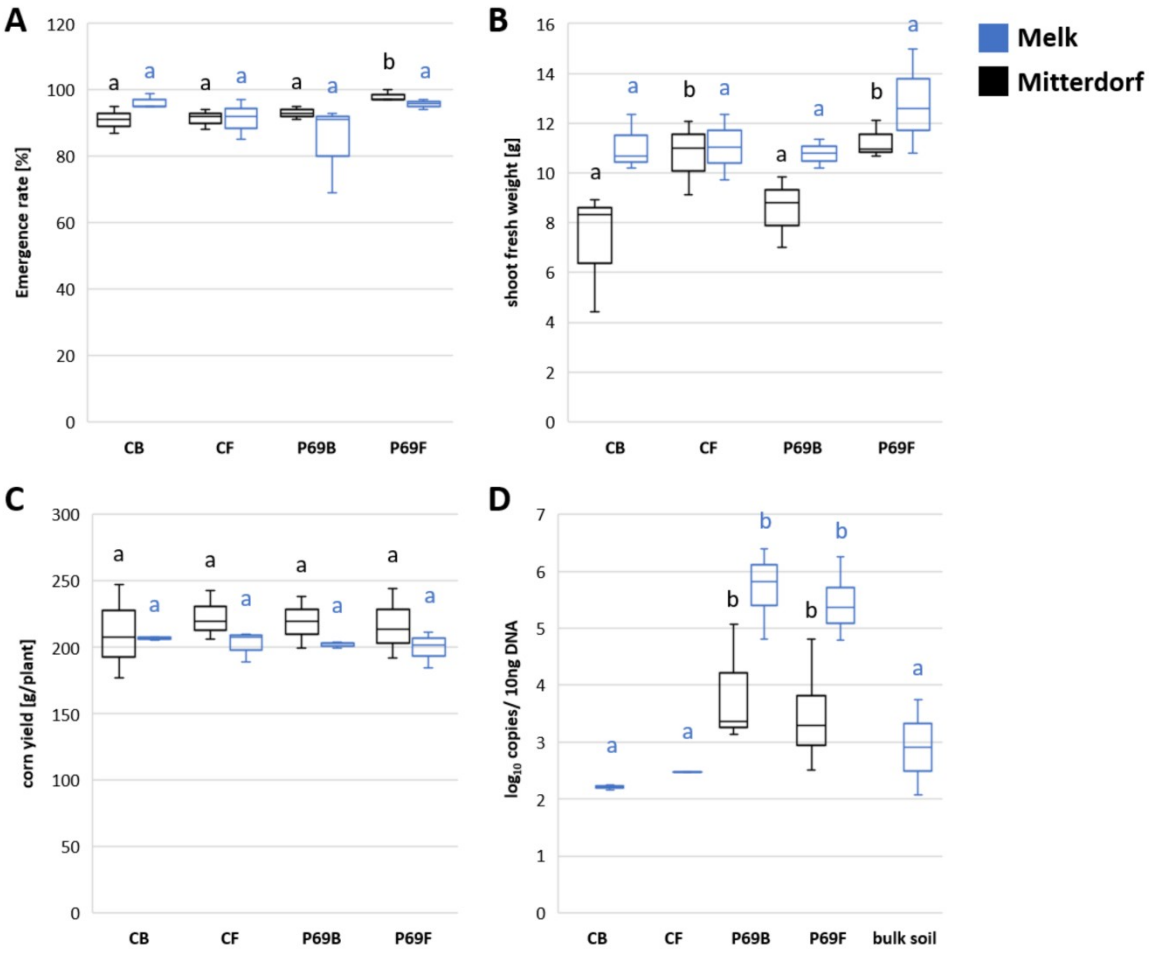

Figure 1. Plant and microbiota data (rating, qPCR). Emergence rate (A), shoot fresh weight (B), corn yield (C) and SPA-P69 copy numbers observed for different maize treatments (D). Treatments CB (control), CF (fungicide), P69B (SPA-P69) and P69F (SPA-P69 and fungicide) are labeled on the x-axis. Significance was tested using pairwise t-test within locations (indicated by letters). Detailed statistic report can be found in Table S1.

Microbial diversity analysis of bulk soil from the two sites

A total of 927,021 and 166,333 high quality reads were recovered after filtering and removing of non-target taxa for 16S rRNA and ITS gene amplicon datasets, representing 7012 and 2395 bacterial and fungal ASVs respectively (Table S2 and Table S3). Taxonomic assignment of ASVs indicated that the bulk soil was dominated by six bacterial phyla (>80% of total reads) i.e *Actinobacteria*, *Firmicutes*, *Acidobacteria*, *Proteobacteria*, *Verrucomicrobia* and *Chloroflexi* (Figure 2A). A relative abundance of *Acidobacteria* and *Chloroflexi* were relatively higher at Mitterdorf site (23.2% and 7.6%, respectively) when compared to the Melk site (18.8% and 6.8%, respectively). In contrast, *Proteobacteria* and *Verrucomicrobia* relative abundances were higher in Melk (28% and 5.8%) in comparison to Mitterdorf (22.1% and 4.2%, respectively). In deeper taxonomic resolution, five bacterial classes namely *Alphaproteobacteria, Gammaproteobacteria, Subgroup 6, Actinobacteria* and *Thermoleophilia* dominated both soils which accounted 50.7% and 42.7% of total reads at Mitterdorf and Melk site, respectively. Relative abundances of *Gammaproteobacteria* and *Subgroup* 6 were relatively higher in Melk (11.9% and 11.8%, respectively) in comparison to the Mitterdorf site (8.7% and 8.6%, respectively). Three

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fungal phyla i.e. *Mortierellomycota*, *Basidiomycota* and *Ascomycota* were prevalent in bulk soil from both sites (Figure 2B). These phyla accounted up to 98.4 and 99.1% of total reads from bulk soil sampled from Mitterdorf and Melk sites. Overall, a higher relative abundance of *Mortierellomycota* was observed in Mitterdorf (88.2%), while *Mortierellomycota* and *Basidiomycota* were similarly abundant in Melk (44.2% and 42.8%, respectively). At fungal class level, *Mortierellomycotina* cls Incertae sedis relative abundance was higher in Mitterdorf site (88.2%) in comparison to Melk (44.2%), whereas *Tremellomycetes* showed the opposite pattern (2.9% and 38.4%, respectively).

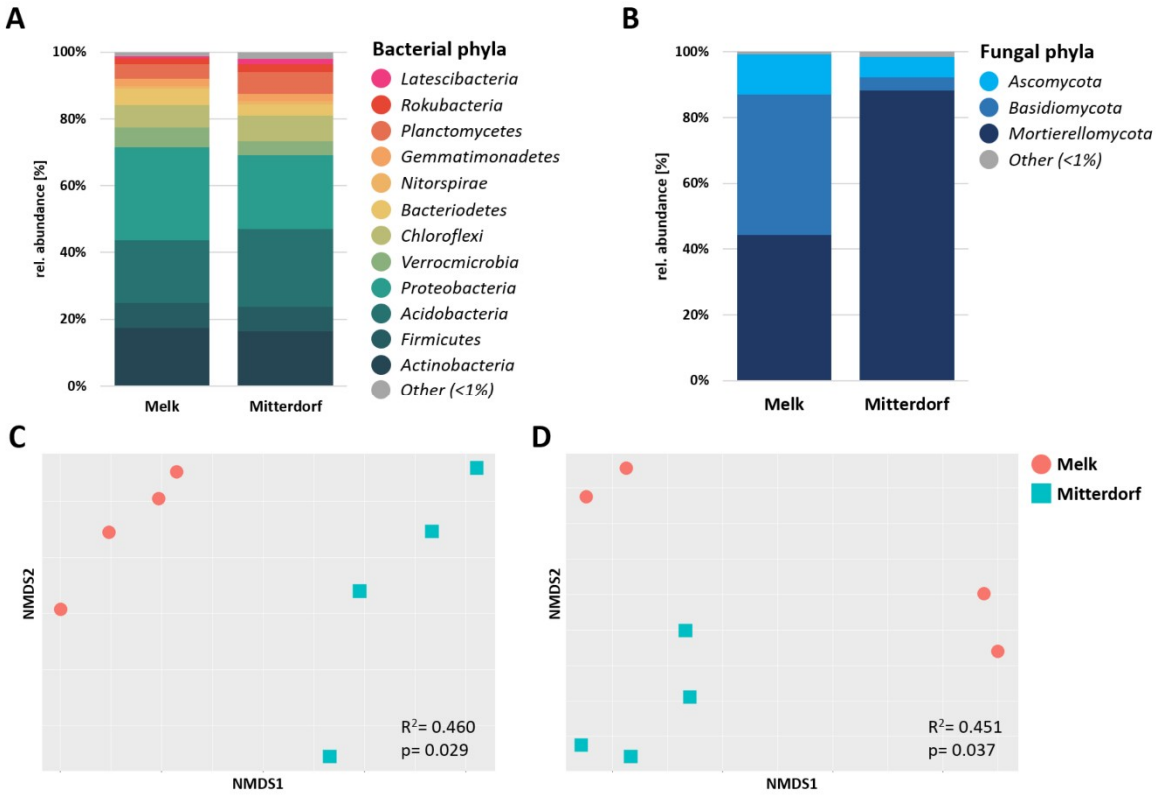

Figure 2. Taxonomic composition (A, B) and observed beta diversity (C, D) of bulk soil in two different locations. Beta diversity is shown as non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity measures. Significance was tested using PERMANOVA

The bacterial and fungal richness in the bulk soil of both locations was assessed using Shannon diversity metrics (H'). Alpha diversity between the locations did not differ significantly (p= 0.205) for bacteria (H'= 6.0 in Melk and H'= 6.4 in Mitterdorf, Table S2), however the fungal diversity was significantly (p= 0.003) higher in Melk (H'= 2.7, Table S3) compared to Mitterdorf (H'= 1.9, Table S3). Looking at non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity measures on the bulk soil from two different experimental locations, an overall clear separation between both locations was found (Figure 2C,D). The calculated beta diversity showed significant difference in the composition of both bacterial (p= 0.029) and fungal (p= 0.037) microbiomes exhibiting variations of 46.0% and 45.1%, respectively. Overall, sites used in this study harbored different indigenous microbial communities.

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Influence of seed treatments on the alpha and beta microbial diversity

The microbial composition of rhizospheres from four differently treated maize samples were analyzed. Regarding the beta diversity, field site was found to explain 27.5% bacterial variation, while treatment only explained 11.9%. Field site was also the major factor that shaped the fungal community structure by contributing 37.3% of fungal community variation while seed treatment only explained 8.7% of the variation. Adonis test indicated sampling site significantly affected the bacterial and fungal community structure (p= 0.001 and p= 0.001, respectively). In contrast seed treatment did not significantly affected the community (p= 0.098 and p= 0.385, respectively) (Figure 3A,B). Due to the major influence of field site on the community the rhizospheres of the two locations were further analyzed separately. After separation, the variation within the treatment groups in Melk and Mitterdorf showed clear segregation in NMDS plots (Figure 3C-F).

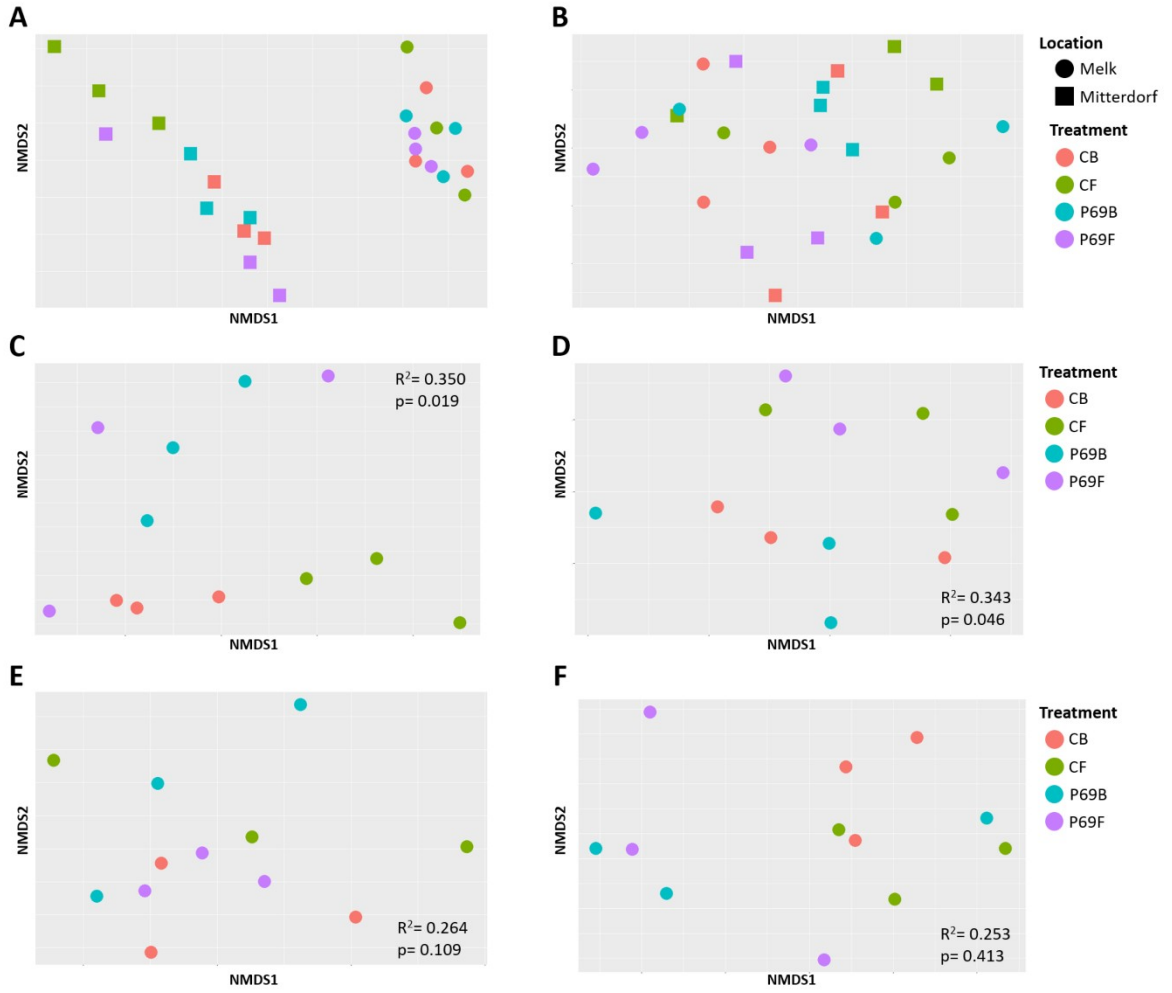

Figure 3. Beta diversity observed for bacterial (A, C, E) and fungal (B, D, F) communities in treated rhizospheres overall (A, B) and for the locations Mitterdorf (C, D) and Melk (E, F). Beta diversity is shown as non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarity measures. Significance was tested using PERMANOVA.

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According to the Shannon index, bacterial richness (α diversity) was slightly affected by treatment in Mitterdorf site at the 90% confidence interval (p= 0.052, Table S4). Post-Hoc analysis with Tukey's test indicated that the rhizosphere of control plants (CB) had a higher bacterial richness (H'= 6.1) in comparison to the rhizosphere of fungicide treated plants at the 90% confidence interval (p= 0.063). Moreover, a similar result was observed when the latter was compared to the rhizosphere of SPA-P69 and fungicide treated plants. Despite, in Melk, the control rhizosphere had a higher bacterial richness (H'= 5.9, Table S2) in comparison to other treatments (H'= 5.3- 5.8, Table S2), seed treatment did not significantly affect bacterial richness (p= 0.767). When beta diversity was assessed by PERMANOVA, treatment significantly affected bacterial community structure (p= 0.019) in Mitterdorf and was shown to explain 35% bacterial community variation. Biologically treated and combinatory treated (SPA-P69 and fungicide) samples clustered closer together. Moreover, fungicide treated samples clustered separately (Figure 3C). Contradicting results were obtained from the location Melk as beta diversity analysis suggested that seed treatment did not significantly change the bacterial rhizosphere community structure (p= 0.109, R^2= 0.264) (Figure 3E).

In the location Mitterdorf, fungal richness was higher in the rhizosphere of control samples (H'= 2.7, Table S3) in comparison to other rhizosphere samples (H'= 2.6- 2.1, Table S3). A similar trend was also observed in the Melk site. However, ANOVA analysis suggested there was no significant difference in fungal richness between treatment on both of location (p= 0.703 and p= 0.223, respectively, Table S4). Despite no effect on fungal richness were found, PERMANOVA analysis revealed that seed treatments significantly (p= 0.046) affected the fungal rhizosphere community structure in Mitterdorf. Seed treatment was shown to explained 34% fungal community variation in the rhizosphere. The rhizosphere of samples treated with fungicide and treated with a combination of fungicide and SPA-P69 tend to cluster together (Figure 3D). In contrast to the result in Mitterdorf, seed treatment did not influence the fungal rhizosphere community structure in Melk site (p= 0.413, R^2= 0.213) (Figure 3F). Taken altogether, seed treatment had a site-dependent effect on fungal community structure as previously described above on the bacterial community structure.

Microbial composition in response to seed treatments

Taxonomic assignment of ASVs indicated seven bacterial phyla, including *Actinobacteria*, *Firmicutes*, *Acidobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Bacteroidetes* and *Chloroflexi* dominated the rhizosphere regardless the seed treatment (88.2%- 94.9% of total reads). When taxonomic profiling was performed in each field site, a distinct bacterial composition was observed between seed treatments (Figure 4A). In Mitterdorf, *Proteobacteria* abundances were relatively higher in samples treated with PGPR (without and with fungicide, 33.9% and 33.5%) in comparison to untreated and fungicide treated samples (27.2% and 26.7%). In contrast, *Acidobacteria* relative abundances displayed the opposite pattern (23.4% and 24.5% in untreated and fungicide treated samples, respectively; 17.9% and 16.4% in SPA-P69 treated samples and in samples with a combination of PGPR and fungicide, respectively). Different results were observed in Melk site. Although *Proteobacteria* abundances were higher in samples treated with SPA-P69 (52.8%) in comparison to the control (34.7%), the abundance in samples treated with fungicide (51.6%) was relatively similar to samples treated with SPA-P69. Moreover, relative abundances of *Acidobacteria* in treated samples (9.2%- 13.4%) were relatively lower in comparison to untreated control samples (18.5%).

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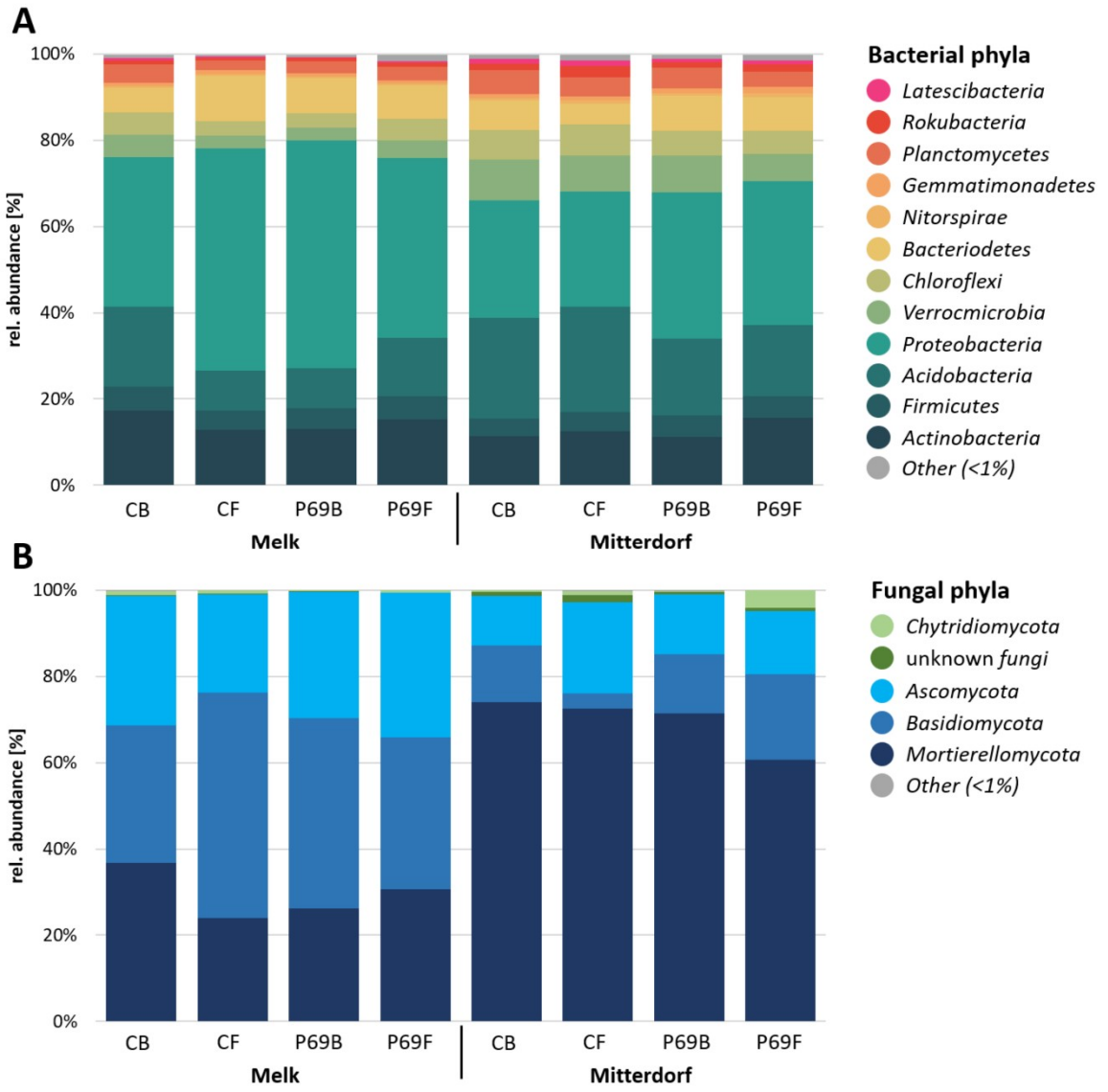

Figure 4. Microbial response in the rhizosphere after seed treatment. Bacterial (A) and fungal (B) relative taxonomic composition on phylum level is shown for individual treatments.

Fungal taxonomic assignment indicated that three fungal phyla *Mortierellomycota*, *Basidiomycota* and *Ascomycota* are dominant in the rhizosphere, regardless the seed treatment (95.2%- 99.7% of total reads) (Figure 4B). Relative abundance of *Basidiomycota* was lower in samples treated with fungicide (3.5%) in comparison to untreated samples (13.1%) in Mitterdorf. In contrast, the opposite result was observed in Melk for *Basidiomycota* (52.3% - seed treated with fungicide; 31.9% - untreated seed). Moreover, relative abundance of *Mortierellomycota* were lower in treated samples (23.9%- 30.6%) in comparison to untreated samples (36.8%) in Melk. A congruent result was not observed in Mitterdorf, where relative abundances of *Mortierellomycota* were relatively similar between treatments (71.4%-74%), except for samples treated with a combination of fungicide and SPA-P69 (60.7%).

Identification of responder taxa in response to seed treatments

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Given the observed high impact of the treatments on beta diversities and on the microbial taxonomic composition, DESeq2 analysis was performed to observe in-depth changes triggered by the treatments (Table S5-S8). The highest number of ASVs differentially abundant were found comparing samples treated with fungicide and untreated samples in both of field sites (n=19 and n=7 in Mitterdorf and Melk, respectively). Among them, relative abundance of four (Mitterdorf) and two (Melk) ASVs, belonging to *Subgroup 6*, were significantly reduced in the rhizosphere of fungicide treated samples compared to the rhizosphere of the control. In the location Mitterdorf, relative abundance of an ASVs that belong to *Candidatus Xiphinematobacter* (*Verrucomicrobiae*) consistently decreased after fungicide and combinatory treatment. Additionally, the genus *Stenotrophomonas*, where the stress protectant strain SPA-P69 belongs to, was shown to be decreased by fungicide treatment. Moreover, in Melk, relative abundances of two ASV that belong to *Janthinobacterium* and *Pedobacter* were consistently enriched in in the rhizosphere of treated samples compared to the control. In the fungal dataset, generally a low number of significantly increased and decreased taxa were found. One ASV that belong to *Mortierella* significantly increased in Mitterdorf after fungicide and combinatory treatment compared to the control group. Additionally, another ASV from the genus *Mortierella* significantly increased in Mitterdorf in all treated samples.

4. Discussion

Seed treatment using fungicide and/or PGPR improved plant biomass and emergence rate as well as significantly increased plant health. Corresponding to these phenotypic effects in plants, we observed statistically significant shifts in the plant-associated bacterial and fungal communities. Another interesting observation is that the field site, mainly the indigenous soil microbiome, was identified as main driver of the rhizosphere microbiota as well as the PGPR interaction and effect.

The ability of PGPR to colonize the rhizosphere, the so-called rhizosphere competence, has an effect on plant growth [40]. In this study, seed treatment successfully delivered bacteria to colonize rhizosphere of maize as shown using qPCR. However, successful colonization of the PGPR seems not the only factor to determine the efficacy of PGPR. We observed no analogous effects on the microbial community composition between the two fields on higher taxonomic levels. Rather, the opposite effect was often observed. This could be linked to the contrasting plant growth effect observed on the field. The mode of action of PGPRs was intensively studied [8], while their interaction with the indigenous microbiome was neglected. Using PGPR treatments, the microbial community should be changed towards a healthier and more diverse composition [12,41]. In our experiment the microbial alpha diversity did not significantly increase with different treatments. Nevertheless, treatments were shown to have a high (25- 35%) influence on the beta diversity of bacterial and fungal communities in Mitterdorf, were plant growth promoting effects were observed on the field. The two observed locations differed significantly in their indigenous soil microbiome. Different indigenous microbial communities are likely due to a complex range of environmental factors such as pH, moisture and nutrient availability of each site [42–44]. A previous study indicated that SPA-P69 promotes plant growth by modifying fungal communities in the rhizosphere and by eliminating deleterious microorganisms under greenhouse conditions using standard soil [26]. Our study supports these previous findings, as SPA-P69 was able to change rhizosphere microbial communities in Mitterdorf under field conditions. However, no effect was visible in Melk, where also no growth

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promotion was observed. These findings underline that i) the composition of the indigenous soil microbiota, which depends also on soil parameters, is crucial for the effect *in planta* and, ii) inducing a microbiome shift is an important mode of action of PGPR agents.

In comparison to seed treated with PGPR, fungicide treatment had a higher impact on the microbial community. In alignment with a previous study by Nettes et al. (2016) [45], the significant effect was primarily observed on the fungal community structure but not on the fungal richness. Nevertheless, current studies have also demonstrated off-target effects of fungicide on soil microbial communities [46–48]. In the present study, bacterial richness tends to decrease in response to fungicide treatment. The field trial in Mitterdorf indicated both off-target and site-specific effects of fungicide. Moreover, bacterial community compositions were significantly affected by the fungicide treatment. However, the mechanism by which the fungicide treatment affected the bacterial community structure is not clear. Seed treatment with fungicide may eliminate certain bacteria and fungi during the early colonization then create a new niche or reduce competition for others to colonize [45,49]. Moreover, a higher impact on the bacterial community indicated that bacteria are more responsive toward fungicide treatment during early colonization in the rhizosphere. To confirm this, multiple sampling timepoints may be useful to investigate effect of seed treatment on the microbial richness.

When the impacts on the microbial community composition were assessed in detail, seed treatments were shown to affect various taxa in the rhizosphere which may be a link to plant growth. A consistent decrease of multiple ASVs belonging to *Subgroup 6*, from the phylum *Acidobacteria*, commonly found in soil [50], was observed. A similar effect was found in both locations, however not in a combined treatment with PGPR strain SPA-P69 and fungicide. Moreover, a significant decrease of *Stenotrophomonas*, the same genus SPA-P69 belongs to, was observed after fungicide treatment. While SPA-P69 treatment alone had no significant effect on plant performance, a combinatory treatment with the fungicide, however, significantly increased emergence rate and fresh weight as well as population density of *Stenotrophomonas.* Our data further showed a significant decrease of *Candidatus_Xiphinematobacter*, from the family *Verrucomicrobiae* in seed treated with fungicide with and without PGRP that also improved plant biomass. *Candidatus_Xiphinematobacter* was previously found to be a endosymbiont of plant-parasitic nematodes [51,52]. Moreover, efficient treatments further had significant effects on several *Mortierella* ASVs. The genus *Mortierella* is generally believed to be plant-beneficial and is moreover applied to control plant diseases and root-knot nematodes [53–55]. We speculated that seed treatment may also have an indirect impact on plant parasitic nematodes which lead to the improvement of plant biomass. This could be an interesting target for further investigations to assess off-target impact of fungicide treatment on the nematode community due to shifts of the microbial community.

5. Conclusions

Our study highlights how microbial changes in the rhizosphere are linked to plant growth performance and health. Improved plant performance was associated with significant changes in the plant microbial community. We also have demonstrated the off-target effect of pesticide on the bacterial community richness and structure providing an evidence of adverse effect of fungicide use.

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Using microbial inoculant in combination with fungicide treatments an improved plant performance can be achieved and linked to induced microbial shifts. Moreover, the challenges of varying performance of treatments in changed field conditions were shown. Therefore, not only a field experiment to determine optimum inoculum concentration and timing to achieve optimum performance in the field is suggested, but also an analysis of the indigenous soil microbiota is required.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1,

Table S1: Statistical evaluation of agronomic parameters and qPCR measurements tested using pairwise t-Test.

Test set	Category		CB	CF	P69B
complete	Emergence rate	**CF**	0.493	-	-
		P69B	0.15	0.435	-
		P69F	0.355	0.116	0.024
	Fresh weight	**CF**	0.168	-	-
		P69B	0.811	0.269	-
		P69F	0.021	0.339	0.043
	Yield	**CF**	0.74	-	-
		P69B	0.87	0.87	-
		P69F	0.94	0.69	0.82
Melk	Emergence rate	**CF**	0.434	-	-
		P69B	0.084	0.282	-
		P69F	0.915	0.496	0.099
	Fresh weight	**CF**	0.98	-	-
		P69B	0.85	0.88	-
		P69F	0.24	0.28	0.22
	Yield	**CF**	0.55	-	-
		P69B	0.53	0.98	-
		P69F	0.34	0.7	0.72
Mitterdorf	Emergence rate	**CF**	0.889	-	-
		P69B	0.414	0.493	-
		P69F	0.017	0.021	0.063
	Fresh weight	**CF**	0.031	-	-
		P69B	0.353	0.141	-

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		P69F	0.017	0.713	0.078
	Yield	**CF**	0.58	-	-
		P69B	0.70	0.87	-
		P69F	0.78	0.78	0.91

		Bulk_soil	**CB**	**CF**	**P69B**
qPCR	**CB**	1	-	-	-
	CF	1	1	-	-
	P69B	0.00013	5.90E-05	2.00E-05	-
	P69F	6.30E-05	2.90E-05	1.00E-05	1

Table S2: Sequencing overview of 16S amplicons for each sample and observed alpha diversity.

Sample ID	Sample type	Field location	Treatment	Filtered quality sequences	Shannon diversity index
soil_Mitterdorf1_bacteria	soil	Mitterdorf	none	36260	6.3
soil_Mitterdorf2_bacteria	soil	Mitterdorf	none	33070	6.3
soil_Mitterdorf3_bacteria	soil	Mitterdorf	none	16690	5.9
soil_Mitterdorf4_bacteria	soil	Mitterdorf	none	9193	5.6
soil_Melk1_bacteria	soil	Melk	none	21547	6.2
soil_Melk2_bacteria	soil	Melk	none	12472	6.0
soil_Melk3_bacteria	soil	Melk	none	68602	6.8
soil_Melk4_bacteria	soil	Melk	none	37257	6.5
rhizosphere_Mitterdorf_CB1_bacteria	rhizosphere	Mitterdorf	CB	30527	6.2
rhizosphere_Mitterdorf_CB2_bacteria	rhizosphere	Mitterdorf	CB	53169	6.3
rhizosphere_Mitterdorf_CB3_bacteria	rhizosphere	Mitterdorf	CB	14234	5.9
rhizosphere_Melk_CB1_bacteria	rhizosphere	Melk	CB	30068	6.2
rhizosphere_Melk_CB2_bacteria	rhizosphere	Melk	CB	14246	5.1
rhizosphere_Melk_CB3_bacteria	rhizosphere	Melk	CB	40783	6.0
rhizosphere_Mitterdorf_CF1_bacteria	rhizosphere	Mitterdorf	CF	7766	5.5
rhizosphere_Mitterdorf_CF2_bacteria	rhizosphere	Mitterdorf	CF	2790	4.9
rhizosphere_Mitterdorf_CF3_bacteria	rhizosphere	Mitterdorf	CF	5393	5.2
rhizosphere_Melk_CF1_bacteria	rhizosphere	Melk	CF	40360	5.8
rhizosphere_Melk_CF2_bacteria	rhizosphere	Melk	CF	27217	5.2
rhizosphere_Melk_CF3_bacteria	rhizosphere	Melk	CF	11194	5.1
rhizosphere_Mitterdorf_P69B1_bacteria	rhizosphere	Mitterdorf	P69B	11280	5.6
rhizosphere_Mitterdorf_P69B2_bacteria	rhizosphere	Mitterdorf	P69B	32102	6.0
rhizosphere_Mitterdorf_P69B3_bacteria	rhizosphere	Mitterdorf	P69B	19881	5.7
rhizosphere_Melk_P69B1_bacteria	rhizosphere	Melk	P69B	106544	3.8

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rhizosphere_Melk_P69B2_bacteria	rhizosphere	Melk	P69B	13501	5.7
rhizosphere_Melk_P69B3_bacteria	rhizosphere	Melk	P69B	48768	6.0
rhizosphere_Mitterdorf_P69F1_bacteria	rhizosphere	Mitterdorf	P69F	6503	5.5
rhizosphere_Mitterdorf_P69F2_bacteria	rhizosphere	Mitterdorf	P69F	31655	6.3
rhizosphere_Mitterdorf_P69F3_bacteria	rhizosphere	Mitterdorf	P69F	51874	6.6
rhizosphere_Melk_P69F1_bacteria	rhizosphere	Melk	P69F	48190	5.5
rhizosphere_Melk_P69F2_bacteria	rhizosphere	Melk	P69F	20570	5.5
rhizosphere_Melk_P69F3_bacteria	rhizosphere	Melk	P69F	23315	6.0

Table S3: Sequencing overview of ITS amplicons for each sample and observed alpha diversity.

Sample ID	Sample type	Field location	Treatment	Filtered quality sequences	Shannon diversity index
soil_Mitterdorf1_fungi	soil	Mitterdorf	none	5541	1.9
soil_Mitterdorf2_fungi	soil	Mitterdorf	none	1293	1.8
soil_Mitterdorf3_fungi	soil	Mitterdorf	none	4181	2.2
soil_Mitterdorf4_fungi	soil	Mitterdorf	none	1944	1.6
soil_Melk1_fungi	soil	Melk	none	1540	2.4
soil_Melk2_fungi	soil	Melk	none	2825	2.8
soil_Melk3_fungi	soil	Melk	none	15208	2.9
soil_Melk4_fungi	soil	Melk	none	5733	2.9
rhizosphere_Mitterdorf_CB1_fungi	rhizosphere	Mitterdorf	CB	3109	2.7
rhizosphere_Mitterdorf_CB2_fungi	rhizosphere	Mitterdorf	CB	1072	3.0
rhizosphere_Mitterdorf_CB3_fungi	rhizosphere	Mitterdorf	CB	9758	2.4
rhizosphere_Melk_CB1_fungi	rhizosphere	Melk	CB	4016	3.1
rhizosphere_Melk_CB2_fungi	rhizosphere	Melk	CB	1936	3.0
rhizosphere_Melk_CB3_fungi	rhizosphere	Melk	CB	7557	2.7
rhizosphere_Mitterdorf_CF1_fungi	rhizosphere	Mitterdorf	CF	6712	3.2
rhizosphere_Mitterdorf_CF2_fungi	rhizosphere	Mitterdorf	CF	3837	2.3
rhizosphere_Mitterdorf_CF3_fungi	rhizosphere	Mitterdorf	CF	5318	2.4
rhizosphere_Melk_CF1_fungi	rhizosphere	Melk	CF	5581	2.9
rhizosphere_Melk_CF2_fungi	rhizosphere	Melk	CF	2948	1.7
rhizosphere_Melk_CF3_fungi	rhizosphere	Melk	CF	4714	2.5
rhizosphere_Mitterdorf_P69B1_fungi	rhizosphere	Mitterdorf	P69B	13190	2.2
rhizosphere_Mitterdorf_P69B2_fungi	rhizosphere	Mitterdorf	P69B	5307	3.0
rhizosphere_Mitterdorf_P69B3_fungi	rhizosphere	Mitterdorf	P69B	5263	1.4
rhizosphere_Melk_P69B1_fungi	rhizosphere	Melk	P69B	4183	2.4
rhizosphere_Melk_P69B2_fungi	rhizosphere	Melk	P69B	6224	2.5
rhizosphere_Melk_P69B3_fungi	rhizosphere	Melk	P69B	10616	2.4
rhizosphere_Mitterdorf_P69F1_fungi	rhizosphere	Mitterdorf	P69F	7158	2.9
rhizosphere_Mitterdorf_P69F2_fungi	rhizosphere	Mitterdorf	P69F	5744	2.7

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rhizosphere_Mitterdorf_P69F3_fungi	rhizosphere	Mitterdorf	P69F	7342	1.7
rhizosphere_Melk_P69F1_fungi	rhizosphere	Melk	P69F	1626	2.9
rhizosphere_Melk_P69F2_fungi	rhizosphere	Melk	P69F	4077	2.5
rhizosphere_Melk_P69F3_fungi	rhizosphere	Melk	P69F	780	3.0

Table S4: Statistical evaluation of explained impact of different factors on bacterial and fungal diversity.

Factor	Bacterial richness analysis		Fungal richness analysis	
	F value	P value	F value	P value
Soil only dataset				
Location	2.019	0.205	23.63	0.003
Rhizosphere Mitterdorf dataset				
Treatment	3.97	0.052	0.483	0.703
Rhizosphere Melk dataset				
Treatment	0.384	0.767	1.812	0.223

Table S5: DESeq2 analysis of bacterial ASVs on the site Mitterdorf. Significantly different ASVs with log-change above 2 are shown.

Comparison	log2FoldChange	p_{adj}	Class	Genus
CB:CF	5.3	0.033	*Holophagae*	*uncultured Subgroup 7*
	6.2	0.045	*Gammaproteobacteria*	*Stenotrophomonas*
	6.2	0.006	*Subgroup_6*	*uncultured Subgroup 6*
	6.2	0.001	*Bacteroidia*	*uncultured env.OPS17*
	6.2	0.048	*Subgroup_6*	*uncultured Subgroup 6*
	6.2	0.048	*Gammaproteobacteria*	*Massilia*
	6.2	0.007	*Gammaproteobacteria*	*Ellin6067*
	6.3	0.008	*Subgroup_6*	*uncultured Subgroup 6*
	6.4	0.045	*Gammaproteobacteria*	*Ellin6067*
	6.4	0.002	*Verrucomicrobiae*	*Candidatus Xiphinematobacter*
	6.6	0.001	*KD4-96*	*uncultured KD4-96*
	6.6	0.030	*Bacilli*	*Bacillus*
	6.8	0.031	*Alphaproteobacteria*	*Nordella*
	7.4	<0.001	*Verrucomicrobiae*	*Candidatus Xiphinematobacter*
	7.4	0.010	*Subgroup_6*	*uncultured Subgroup 6*
	7.4	0.048	*Bacteroidia*	*Flavobacterium*
	7.9	0.030	*Gammaproteobacteria*	*Rhizobacter*
CB:P69B	-9.7	0.045	*Verrucomicrobiae*	*Opitutaceae*
	7.7	0.030	*Anaerolineae*	*uncultured Anaerolineaceae*
	7.7	<0.001	*KD4-96*	*uncultured KD4-96*

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CB:P69F	7.7	<0.001	*Verrucomicrobiae*	*Candidatus Xiphinematobacter*

Table S6: DESeq2 analysis of bacterial ASVs on the site Melk. Significantly different ASVs with log-change above 2 are shown.

Comparison	log2FoldChange	p_{adj}	Class	Genus
CB:CF	-23.0	<0.001	*Gammaproteobacteria*	*Janthinobacterium*
	-22.2	<0.001	*Bacteroidia*	*Pedobacter*
	-10.3	0.005	*Gammaproteobacteria*	*Luteibacter*
	7.0	0.001	*Holophagae*	*uncultured Subgroup 7*
	8.0	<0.001	*Subgroup 6*	*uncultured Subgroup 6*
	11.0	0.002	*Gammaproteobacteria*	*Achromobacter*
CB:P69B	-20.6	<0.001	*Bacteroidia*	*Pedobacter*
	-20.4	<0.001	*Gammaproteobacteria*	*Janthinobacterium*
	-11.6	0.002	*Gammaproteobacteria*	*Luteibacter*
	9.7	0.020	*Gammaproteobacteria*	*Massilia*
CB:P69F	-21.0	<0.001	*Gammaproteobacteria*	*Janthinobacterium*
	-19.3	<0.001	*Bacteroidia*	*Pedobacter*
	11.5	0.001	*Gammaproteobacteria*	*Achromobacter*

Table S7: DESeq2 analysis of fungal ASVs on the site Mitterdorf. Significantly different ASVs with log-change above 2 are shown.

Comparison	log2FoldChange	padj	Class	Genus
CB:CF	22.0	<0.001	*Sordariomycetes*	*Unidentified Nectriaceae*
	-20.2	<0.001	*Mortierellomycotina cls Incertae sedis*	*Mortierella*
CB:P69F	-21.2	<0.001	*Mortierellomycotina cls Incertae sedis*	*Mortierella*

Table S8: DESeq2 analysis of fungal ASVs on the site Melk. Significantly different ASVs with log-change above 2 are shown.

Comparison	log2FoldChange	padj	Class	Genus
CB:CF	-20.5	<0.001	*Mortierellomycotina cls Incertae sedis*	*Mortierella*
	-21.3	<0.001	*Sordariomycetes*	*unidentified Nectriaceae*

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CB:P69B	-22.6	<0.001	*Mortierellomycotina cls Incertae sedis*	*Mortierella*
	21.9	<0.001	*Spizellomycetes*	*Spizellomyces*
	-22.0	<0.001	*Sordariomycetes*	*unidentified Nectriaceae*
CB:P69F	-24.2	<0.001	*Sordariomycetes*	*unidentified Nectriaceae*
	-20.7	<0.001	*Mortierellomycotina cls Incertae sedis*	*Mortierella*

Data Availability: The 16S rRNA gene and ITS gene raw reads obtained from the sequencing company were deposited at the European Nucleotide Archive (ENA) under the project number PRJEB39535.

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