Effects of Intercropping and Plant Variety on Root Fungal Community

Ming-Hui (Maggie) Hsung

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Supervised by

Pierre Hohmann, FiBL

Martin Hartmann, ETH Zürich

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ETH zürich FiBL ReMIX Species mixtures for redesigning European cropping systems

Ming-Hui (Maggie) Hsung

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Eidgenössische Technische Hochschule Zürich (ETHZ)
Department of Environmental Systems Science
Institute of Agricultural Science
Rämistrasse 101, 8092 Zürich

Research Institute of Organic Agriculture (FiBL)
Department of Crop Science
Plant Breeding Group
Ackerstrasse 113, 5070 Frick

Abstract

Increasing environmental awareness has put new attentions on cereal-legume intercropping system as a possible way to diversify agricultural fields and to efficiently produce food and feed under increasingly adverse conditions caused by climate change. The choice of mixing partners drives plant functional complementarities. In this context, elucidating the effects of this pairing on the plant-associated microbial community can help to better understand and, thus, optimize intercropping systems. This MSc project seeks to understand belowground microbial interactions in intercropping through examining organically-managed cereal-legume on-farm experiments, including a pea (*Pisum sativum* L.)-barley (*Hordeum vulgare* L.) experiment in Switzerland (CH) and a pea-wheat (*Triticum aestivum* L.) experiment in Hungary (HU). Root fungal communities of different varieties in pure and intercropping systems were investigated based on Illumina MiSeq sequencing of the ITS1 gene. In the Swiss pea-barley experiment, five pea varieties (Alvesta, Karpate, Mytic, Respect, Vitra) each mixed with one barley variety (Atrika) were examined; in the Hungarian wheat-pea experiment, three wheat varieties (Kolompos, Kompozit, Nador) each mixed with one pea variety (Aviron) were examined.

Across all varieties, intercropping did not have an effect on alpha fungal diversity. However, there were first indications for variety by cropping system effects on alpha diversity. Fungal richness of roots of particular pea varieties was influenced by the cropping partner either on a species level (CH experiment) or on a genotype level (HU experiment). Pielou's evenness and Shannon diversity in pea roots were influenced by pea variety. Similarly, the analysis of beta diversity also showed intercropping, across all varieties, did not have an effect on root fungal community, for both experiments. A significant crop variety effect was observed for the CH experiment showing distinct fungal community compositions for the pea varieties Respect, Alvesta and Vitra. This study also identified fungal taxa, including putative pea pathogens and beneficials (e.g. Glomeromycota), associated with certain crop varieties and their change in abundances by intercropping. Lastly, the relation between variety fungal community characteristics and their agronomic traits could provide further insights in understanding this complex plant-plant-microbiome interactions.

This thesis project is a pilot study examining intercropping and crop variety effects on fungal communities with results that suggests to consider belowground interactions for the optimization of intercropping systems.

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1 Introduction

1.1 Intercropping

Conventional agriculture and the practice of monoculture (or sole cropping), has become the main agricultural practice over the past decades especially in developed countries. Such practice has known negative impacts on soil, water, and biodiversity. Increased interest in agricultural production systems for higher productivity while promoting sustainability has led intercropping (or mixed cropping), the simultaneous cultivation of more than one crop species, to become the focus for better agricultural practices [1].

Intercropping (**Fig. 1.1**), also known as mixed cropping, takes advantage of differentiated functions of coexisting species to improve system stability, for example, via enhanced resilience against pests and diseases [1, 2]. Intercrops can also more

Fig. 1.1.: Mixed pea-barley intercropping stand (left) and pure barley stand (right) from the Swiss pea-barley experiment. Mixed intercropping is the growing of two or more crops simultaneously with no distinct row arrangement.

efficiently utilize plant growth resources such as light, water and nutrients and

has the potential of yield increases compared to monocultures. Cereal-legume intercropping is a particular focus for organic agriculture due to the functional advantages of legumes. Some of the common types of cereal-legume intercropping include wheat-soybean, wheat-faba bean, and pea-barley. In the absence of synthetic fertiliser inputs, organic farming systems can rely on the legume's symbiotic bacteria to provide the plantation with nitrogen. In addition to higher yields, studies show the potential for cereal quality improvement. For instance, Jensen et al. showed increased grain protein content in wheat and barley when intercropped with faba bean [3]. Barley-pea intercropping was shown to improve the uptake efficiency of limiting nutrients such as phosphorus and potassium [4]. It is well known that such nutrient recycling processes are governed by soil microbial processes, which naturally points to possible changes in soil microbial communities induced by intercropping. Indeed, studies have shown cereal-legume intercrops to induced changes in the bacterial and fungal community in the rhizosphere [5, 6].

1.2 Plant-soil microbiome interaction

Microbial community diversity decreases from bulk soil to plant rhizosphere to root, which is associated with specific groups of microbes. One of the major influences of soil microbiome is soil properties including pH, soil moisture, soil texture [7, 8]. Besides soil types, plant species is a main factor shaping rhizosphere microbial community composition, and different plant species can harbor distinct root-associated microbiomes due to differential root exudation patterns [9, 10, 6]. Root exudate is a major source of nitrogen, carbon and other nutrients for the soil microbiota [11]. Plant root exudation processes could preferentially select for certain groups of microbes, for example, legumes have been described to be able to stimulate certain groups of bacteria [12] or allow colonization of specific arbuscular mycorrhizal fungi by secretion of specific flavonoids [13]. Several studies have also demonstrated changes in microbial composition to be driven by plant genotypes (as reviewed by Wille et al. [14]). Although the selection mechanisms of microorganisms remain poorly understood, there is growing understanding that plants can actively recruit microbial communities in order to combat abiotic and biotic stresses with an impact on plant performance [15]. Root exudation is one form of such mediation between plant host and microbiota assembly. In an intercropping system where different plant roots are in close contact with each other, the plant-specific root-associated microbial communities could also be involved in complex interactions with each other [10]. This interaction could have an effect on plant performance.

There is still little known about the effects of intercropping on belowground biotic interactions. Root-associated fungal communities are of particular interest in legume-based cropping systems due to their roles in recycling and transfer of nutrients, promotion of plant growth and plant defenses, and also in causing harm, as many causal agents of legume root diseases belong to this kingdom. Different studies have shown intercropping to change fungal dominance and result in an increase or decrease in fungal diversities compared to monocropping system [5, 16]. Lian et al. also showed in a soybean-sugarcane intercrop pot experiment that intercropping increased the relative abundances of *Trichoderma* spp., with potentially plant beneficial properties, and *Fusarium* spp., including various species known to attack soybean roots [5]. These results suggest that, while there are numerous recognized benefits for agricultural systems from intercropping, the complex plant-soil interactions might also contribute to potential undesired effects in cropping system that should be minimized.

A particular focus of this thesis is taken on Glomeromycota phylum, which contains large members of arbuscular mycorrhizal fungi (AMF). The beneficial effects of AMF on plants including improving nutrient uptake by enlarging absorbing zone of plant roots and protective abilities against root pathogenic fungi are widely known [17]. But studies on mycorrhizal fungi colonization in intercropping and their contributions to mediating competitive abilities in terms of growth and nutrient uptake of intercrop performance have contradictory results [18].

In Europe, it is an emerging practice for organic farms to intercrop pea with cereal, but it seems yet unexplored how this pairing, including genotype-level differences, shapes soil fungal community in such systems and how plant performance might be affected. Usually, crop varieties used in intercropping system are based on elite monoculture varieties, and their interactions in intercropping has not been carefully evaluated [19]. The selection of crop genotypes to be paired in intercropping system can be important to improve beneficials and reduce pathogenic microbes. So far, the abundance, role and function of microbial communities has appeared to be a missing link to understand plant performance [12]. Plants are subjected to complex microbial community interactions in the field and effects on crop performance may not be caused by individual microbes but on a community-level scale [20]. Recent developments in high-throughput sequencing technologies greatly improved the ability to study microbial community.

1.3 Next generation sequencing for microbiome profiling

Next generation sequencing (NGS) have revolutionized genomic research and provides a culture-independent method to study the diversity of microbial communities. Illumina MiSeq is becoming the most utilized platforms due to high-throughput and low sequencing costs and applications such as in targeted gene sequencing or metagenomics [21].

Illumina is based on DNA polymerase catalyzing the incorportaiong of fluorescently labeled deoxyribonucleotide triphosphates (dNTPs: A,C,G,T) into DNA template strand during sequential cycles of DNA synthesis on a flow cell. Adapters are first ligated to the DNA fragment so that they could be hybridized to flow cell. DNA fragments are clonally amplified in a clonal cluster around original strand, which helps to amplify fluorescence signal and the basis for identification of incorporated base. One base is added at a time and incorporation of the nucleotides are identified by fluorophore excitation. The cycle is repeated 'n' times to create a read of 'n' bases. The process can repeat in the reverse DNA strand, called paired-end sequencing [22].

After sequencing, the nucleotides are identified and subsequent bioinformatics processing could generate reads that can then be clustered into operational taxonomic units (OTUs) or zero-radius taxonomic units (ZOTUs). The latter, also called a sequence variant, are units such that distinct sequence defines a separate OTU. Since this tends to overestimate diversity due to intra-species variations, but ZOTUs could be subsequently clustered based on percentage of sequence similarity as for OTUs, commonly at 97%, 98%, or 99% to lump closely related strains into same cluster [23].

1.4 Research objectives

This study employed Illumina MiSeq 2x300 bp reads sequencing to analyze fungal communities. This work comprised of a pea-barley trial from Switzerland with five pea varieties, and wheat-pea cropping system from Hungary with three wheat varieties. Agronomic data from Switzerland trial was collected.

The overall goal of this study is to understand how cereal-legume cropping system and plant genotypes (crop varieties) influence root fungal community diversity and

how changes might be related to agronomic traits such as root disease expression. In this thesis, we will:

1. Explore fungal communities as affected by intercropping system of pea and with barley/or wheat and different crop varieties by comparing alpha (local) and beta (between environment) diversity and identify fungal taxa that responded to these environments.

2. Relate fungal community diversity and characteristics to below- and aboveground agronomic data.

The main hypotheses are:

1. Fungal community differs between pure and intercropping system (cropping system effect).

2. Fungal community differs between between different crop varieties (variety effect).

3. There is an interaction effect between cropping system and plant variety such that the differences in fungal community between pure and mixed stands are not equal for each plant variety (interaction effect).

Unravelling these plant-microbe interactions will help to gain insights into and make use of efficient plant facilitations in support of sustainable agriculture.

1.5 Organization

This Master thesis is divided into six chapters. Chapter 1 introduces the background and rationale for conducting this research and research gap in understanding fungal communities in intercropping system and between crop varieties. Chapter 2 describes the sampling procedure, including the methods used to study root fungal communities. Results are separated into two major sections to differentiate the field trials from the two countries and reported in Chapter 3 . This is followed by a discussion in Chapter 4 including future outlooks. Chapter 5 summarizes the main findings and their implications for this study. Appendices (A) are included to complement the text, but also additional materials are added to provide a broader view of interesting preliminary results that have been found but not discussed in the main text.

2 Materials and Methods

The data used in this thesis were collected from two different cereal-legume cropping trials located in two different countries, Switzerland and Hungary. These were pea-barley trial from Switzerland and pea-wheat trial from Hungary.

2.1 Design of the field experiments in Switzerland and Hungary

In the Switzerland pea-barley, plant samples were taken from the experimental site located at Niederrohrdorf. The field consisted of pure and intercropping stands of pea and barley with two replicated blocks and incomplete randomization of the cropping stands. The samples investigated in this study consisted of five selected varieties of pea (*Pisum sativa* L.) varieties, Alvesta, Karpate, Mytic, Respect, Vitra, and one selected barley (*Hordeum vulgare* L.) variety, Atrika.

In the Hungary wheat-pea trial, the plant samples used for this study consisted of three wheat (*Triticum aestivum* L.) varieties, Kolompos, Kompozit, and Nador , and one selected pea (*Pisum sativa* L.) variety, Aviron. The field consisted of three replicated blocks and did not have pure stands for pea.

2.2 Sample collection and plant traits assessment - pea-barley - Switzerland

Plant traits covered by this thesis belonged to the pea-barley trial in Switzerland. Root samples collection was performed upon at 50% flowering. Plants were dug up, keeping the roots intact, from opposite subplot spots. These were taken from 1 m into the length of the plot (stand) and second row into the plot to avoid border plants (**Fig. 2.1**, page 8). At pure stands, three plants were sampled making six plants in total per stand. At intercropping stands, three of each plant species (barley and pea) were harvested from each of the two indicated subplot spots in the stand.

The roots coming from the same plot were homogenized, separating plant species in the case of mixed stands, cut and washed, with the last rinse in fresh batch of water to decrease cross-contamination of microbes from different plots. The same procedure was repeated in second replicated block. Plant root samples were frozen at -20°C immediately after harvest and lyophilized a day after. Samples were stored at -20°C until DNA extraction.

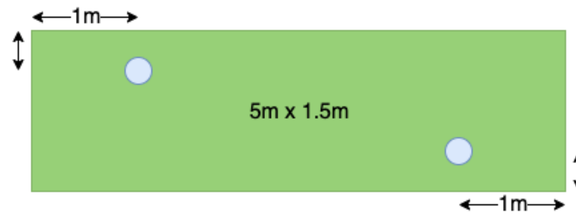


Fig. 2.1.: Sketch of a field plot, which could be either barley or pea pure stand, or pea-barley mixed stand (5 x 1.5 m). Blue circle represents subplots samples were taken from (3 plants per subplot), second row in from the border. These samples could either be barley, pea, or both barley and pea (in the case of intercropping stand).

Traits assessed on the day of root sample collection included root disease score, total plant biomass of the plots. Biomass was scaled to percentage of plant and barley plot cover for intercropping stands. Briefly, total plant biomass was based on scoring of 1 (very low) to 9 (very high biomass). Shoot length were measured as the stands were without artificially errecting the plants. For pea, nodule number and diameter were assessed. Post-harvest traits investigated in this study includes total grain yield and corresponding component yield from pea and barley in intercropping stands, and thousand kernel mass (TKM) by weighing 500 kernels of barley and 300 kernels of pea to calculate the thousand kernel weight.

The sampling from Hungary trial was performed by project partners in Hungary and root samples were sent to Switzerland to be processed together.

2.3 DNA extraction and sequencing

Freeze dried roots were grinded with a 20 mm steel bead at 30 Hz for 5-10 s until fully grinded (TissueLyser II, Qiagen). DNA was extracted from 19-21 mg root powder with the Omega Mag-Bind Plant DNA DS Extraction kit (Omega Bio-tek, United States) according to manufactures instructions. DNA quality check was performed using a Nanodrop2000 Spectrophotometer (Thermo scientific, United States) and electrophoresis (1% agarose, TAE buffer). Roots and DNA were stored at -20°C. This sampling method does not differentiate between firmly attached

rhizoplane and root internal fungi. DNA was normalized to 10 ng/ul for sequencing. Subplot DNA were pooled together prior to Illumina sequencing.

The libraries were sequenced on the Illumina MiSeq Instructument using 2x300 bp cycling mode. Samples were sequenced at Genome Quebec in Canada and bioinformatics were conducted at the Genetic Diversity Centre of ETH Zurich. Briefly, for sequencing, the primers ITS1f and ITS2 with unique barcodes were used to generate the ITS amplicon library. The following thermal programme was used for PCR amplification: 96°C for 15 min, followed by 33 cycles at 96°C , for 30 s, 52°C for 30 s, 72°C for 60 s, followed by an extension at 72°C for 10 min.

2.4 Bioinformatics

Raw paired-end 300nt Illumina MiSeq reads were quality checked using FastQC. In a first processing step, paired-end reads were merged with FLASH. Prior to merging, read ends were trimmed. In the next step, primers were trimmed using USEARCH and the amplicons subsequently quality filtered using PRINSEQ. The UNOISE3 was used to generate amplicon sequence variant (ASV). All sequences identified as unique were unique zero-radius operational taxanomic units (ZOTUs) with additional cluster of ZOTUs at 97% similarity. This workflow was implemented in USEARCH. A count table using the ZOTUs was subsequently generated. Sintax in combination with a UNITE ITS reference database was used for the taxonomic prediction of the ZOTUs. Taxonomic information of unassigned sequences for selected ZOTUs were further explored using BLAST analysis of the Nucleotide collection database. BLAST taxonomic information was considered at query cover 94% and sequence identity of 100%.

2.5 Data analysis

All data analyses were conducted in R v3.6.0 [24]. A more detailed explanation of the data analysis workflow is presented below. As an initial step, all ZOTUs classified as Protista and Plantae were excluded from the dataset. The main packages used for processing the dataset were *phyloseq* [25], *vegan* [26], and *ggplot2* [27], *BiodiversityR* [28], *multcomp* [29]. A flowchart for the workflow for data analysis is provided in **Supplementary Fig. A.1**.

2.5.1 Alpha diversity

Prior to comparing diversity indices, rarefication was performed to remove biases due to uneven sampling depth. Alpha diversity is a measure of local diversity, which can give us insights to an understanding of numerical quantification of the number of species (observed richness), and distribution pattern of the fungal species (evenness and Shannon diversity) on plant roots. Switzerland (CH) and Hungarian (HU) samples were rarefied separately to respective minimum sampling depth with a step size of 100 with ggrare [30]. Package *vegan* function diversity was used to compute Shannon diversity, Pielou's evenness, and observed richness. Higher values of Shannon diversity indicates greater diversity. Observed richness is equivalent to number of distinguishable fungal ZOTUs. Pielou's evenness index ranges from 0 to 1, the closer the value is to 1, the more evenly distributed the fungal species are.

Hypothesis testing were based on linear mixed models, replicated blocks were taken as fixed effects, and the plots (stands) were taken as random effects. In the case of singlular-fit problem, linear models were applied without considering random effects. Statistical tests were performed using general linear hypothesis tests with self-defined contrasts (*multcomp*; [29]). Family-wise error rate (FWER) were used to correct the p-values for the contrasts. The contrasts included between cropping system tests for each species, pair-wise tests for all CH pea or HU wheat varieties, variety-cropping system interaction effects for CH pea or HU wheat, between intercropping and pure stands for CH barley, and between intercropping stands for HU pea. For HU pea, comparison of intercropping stands was only between Kolompos and Kompozit due to having only one replicate in Nador (contrasts can be viewed in **Supplementary A.1.1** and **Supplementary A.2.1**).

2.5.2 Beta diversity

For beta diversity, ZOTUs were filtered to at least four sequences in the whole dataset. Read counts of the samples were normalized by proportion to give relative abundances (%). Bray-Curtis and Jaccard dissimilarities were used to measure fungal community composition. The former dissimilarity takes account of abundance and presence-absence, the latter only takes account of presence-absence and thus weighs more on rare species. To visualize the result, unconstrained ordination principal component analysis (PCoA) was first used to explore the data (*phyloseq*). Analysis was followed by constrained ordination canonical analysis of principal coordinates (CAP) based on discriminant analysis (*BiodiversityR*; [31]), which allows better visualization of patterns based on *a priori* hypothesis (permutations 2999).

Permutational multivariate analysis of variance (PERMANOVA) [32] was used to for hypothesis testing (permutations 9999), complemented by a test for homogeneity of multivariate dispersions (PERMDISP).

2.5.3 Differentially abundant ZOTUs

Microbiome data is typically zero-inflated so it does not meet analysis of variance assumption for hypothesis testing at ZOTU-level. For ZOTUs-level response, R package *edgeR* [33] was employed, originally intended for gene expression, but also used in microbial data analysis (for example, in the study by Hartman et al. [34]). The data from the two countries were split into respective crop species (CH pea, CH barley, HU pea, HU wheat) for this analysis.

The counts were first pruned to at least five sequences in two samples (lowest number of replicates) for a given crop species. This removes low abundance ZOTUs which provide little evidence for differential abundance and present at a cost of lower statistical power after false-discover rate adjustments [33]. Samples were normalized by trimmed mean of M-values (TMM) method, which is the recommended method assuming the majority of ZOTUs are not differentially abundant. Normalized counts were fit with quasi-likelihood negative binomial generalized log-linear model (glmQLFit) and tested in quasi-likelihood test (glmQLFTest) with self-defined contrasts. Contrasts were the same as for alpha diversity mentioned above. ZOTUs were identified based on significance greater than 0.05 in FDR-adjusted p-values.

2.5.4 Glomeromycota abundance testing

To test for Glomeromycota differences, filtered (at least four sequences) and proportion-transformed data was used and hypothesis testing was again based on linear mixed models or otherwise linear models. Statistical test and self-defined contrasts were the same as done for alpha diversity. Linear model fitting and testing on Hungary Glomeromycota data was based on arcsine transformed-data for normal distribution of residuals.

3 Results

This section begins with an overview of the dataset showing differences in fungal community diversity between samples collected at different sites and breaks down to two major sections, presenting the Swiss (CH) pea-barley experiment and the Hungarian (HU) pea-wheat experiment separately. Briefly, each section begins with taxonomic profiling. This is followed by alpha diversity, to evaluate the amount of distinguishable taxa and dominance and evenness patterns of fungi on root samples. Variety effects and indications for interaction effects were observed. This is followed by beta diversity to evaluate fungal community composition between root samples. Fungal composition differences between crop species and crop varieties were observed. At the end of the result section, certain fungal community characteristics are presented in relation to plant agronomic data from the Switzerland pea-barley experiment.

3.1 Global view of fungal community diversity from Swiss pea-barley and Hungarian wheat-pea cropping experiments

After removal of two low-quality samples, a total of 3,582,184 sequences were clustered into 801 fungal operational taxonomic units (OTUs), which were identified from the combined 57 samples. Rarefaction curves (**Fig. 3.1**, page 14) show that the majority of samples have reached an asymptote, so the sampling effort is close to maximizing the number of new distinguishable taxa to be discovered for these samples.

To give an impression of fungal composition of the different plant species collected from different sites, fungal communities were visualized using unconstrained principal coordinate analysis (PCoA) on Bray-Curtis dissimilarity as well as Jaccard dissimilarity on the global data (**Fig. 3.2**, page 14). The pattern shows a clear fungal communities separation between countries and between species, that is between Switzerland experiment (CH) barley, CH pea, and Hungary experiment (HU). Separation of HU pea and wheat were not very obvious on the PCoA plot.

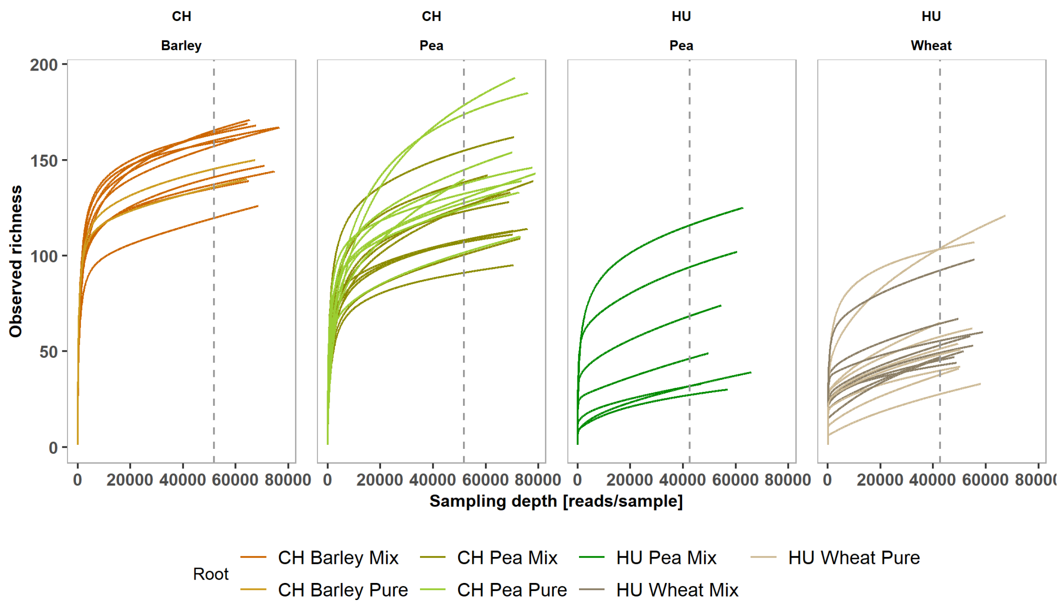


Fig. 3.1.: Rarefaction curves of zero-radius OTUs (ZOTUs)obtained from root samples of intercropping and pure stands of the Swiss (CH) and Hungarian (HU) experiments, showing change in observed richness as a function of sampling depth. Grey dashed lines indicate the minimum sampling depth per country that rareficaction was performed for alpha diversity analysis.

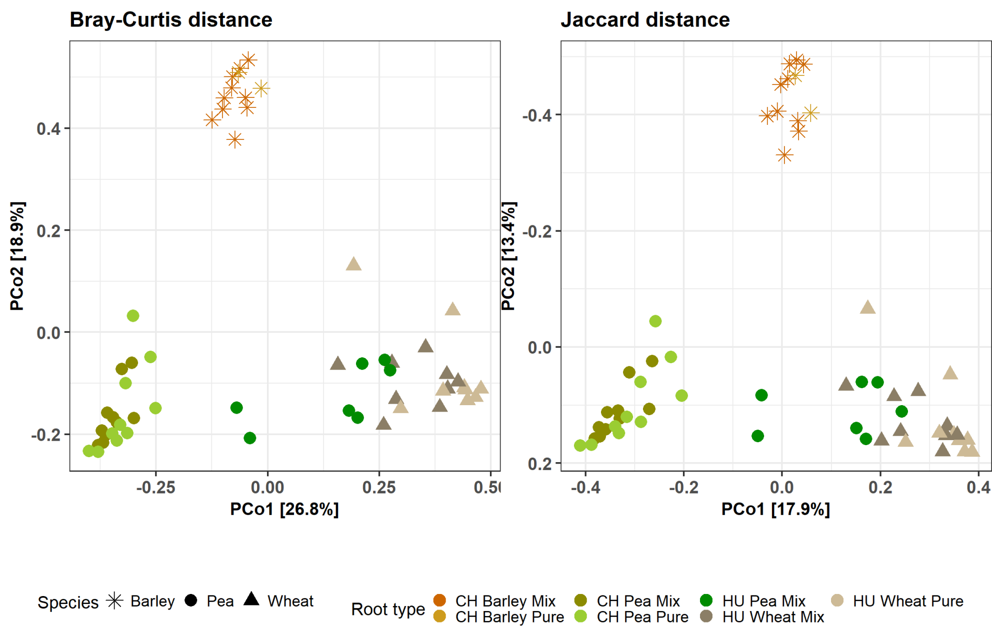


Fig. 3.2.: Principle coordinate analysis (PCoA) by Bray-Curtis (left) and Jaccard (right) dissimilarities on whole fungal dataset of Switzerland (CH) pea-barley and Hungary (HU) pea-wheat experiments. The plot visualizes separation patterns for crop species and cropping systems, intercropping stands (mix) and pure stands (pure).

By Bray-Curtis distance, axis 1 explains 26.8% of the variation in the data and separates the fungal communities by countries and to some extent crop species. 18.9% of the variation is explained by axis 2 and the separation of barley root fungal communities from pea and wheat species. The same pattern was shown when analyzing the data by Jaccard distance, the percentage of the variation in the data explained on both first and second axes were lower, 17.9% and 13.4% respectively.

3.2 Pea-barley — Switzerland

3.2.1 Taxonomic profiling

An overview of the relative abundances of fungal phyla for pea and barley roots are shown in **Fig. 3.3**. The top ten most abundant ZOTUs of pea and barley roots were also explored, provided in **Supplementary Fig. A.8-A.9, Table A.2-A.3**. For

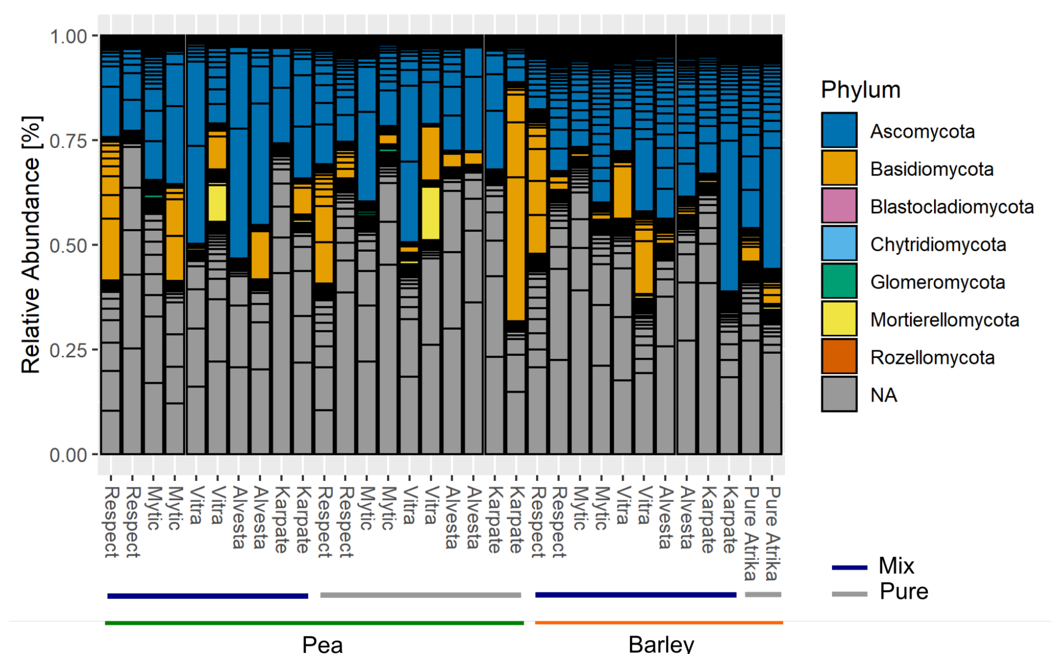


Fig. 3.3.: Relative abundances of fungal ZOTUs grouped at phylum level for pea and barley samples, in intercropping stands (mix) and pure stands (pure). Individual ZOTU abundances were stacked in order from greatest to least.

pea roots, the majority of zero-radius operational taxonomic units (ZOTUs) (54.6%) could not be classified at phylum level. This is followed by the phyla Ascomycota (31.6%), Basidiomycota (11.0%), Mortierellomycota (1.7%), Glomeromycota (0.7%), Chytridiomycota (0.02%), Rozellomycota (0.01%), and Blastocladiomycota

(<0.001%). For barley roots, the phylum abundance ranking was similar to pea roots, the majority of ZOTUs were unclassified (51.3%), followed by Ascomycota (38.7%), Basidiomycota (9.4%), Mortierellomycota (0.5%), Glomeromycota (0.1%), and Chytridiomycota (0.04%).

3.2.2 Alpha diversity

The communities were rarefied to the minimum sampling depth from the Switzerland sample that was 51,801 sequences per sample, which covered most of the observed ZOTU richness (see **Fig. 3.1**, page 14). The alpha diversity results are shown in **Fig. 3.4**, page 17) and the detailed supporting statistical test results in **Supplementary Fig. A.2-A.4**.

Mean fungal richness for pea pure and intercropping stands were 140 and 118, respectively. Linear hypotheses tests showed fungal richness on pea roots was overall significantly higher in pure cropping system (p=0.017). Fungal richness variety means did not differ significantly, ranging from 116 (Mytic) to 138 (Vitra) (p-values ranging from p=0.35 to p=1). The cropping system effect was due to an interaction effect from pea variety Alvesta, with fungal richness in pure stands with a mean of 182 being significantly higher compared with 97 for intercropping stands (p<0.001). For the other four pea varieties, Karpate, Mytic, Vitra, and Respect, no interaction effect on fungal richness was observed (p>0.9). Mean fungal evenness of pea pure and intercropping stands were 0.51 and 0.54, respectively, and did not differ significantly (p=0.997). A significant variety effect was observed for Pielou's evenness of the pea fungal community. Evenness for Alvesta with 0.44 was significantly lower compared with 0.58 and 0.57 for Respect (p<0.01) and Vitra (p=0.016), respectively. There was no significant interaction effect between cropping system and variety (p>0.20 to p=1). Similar to results of evenness, mean fungal Shannon diversity for pea pure and intercropping stands did not differ significantly (2.53 and 2.56, respectively, p=1). Alvesta roots were with 2.16 and significantly lower in fungal Shannon diversity compared with 2.82 and 2.79 for Respect (p=0.011) and Vitra (p=0.019). There was no interaction effect between cropping system and variety (p=0.39 to p=1).

Barley root mean fungal richness between pure and intercropping stands were 141 and 152, respectively, and did not differ significantly (p=1). Barley root fungal richness did not differ significantly between neighbors, ranging from 132 (Respect) to 166 (Vitra) (p>0.9). This means that the identity of neighbor, whether it was barley or different pea varieties, had no effect on barley root fungal richness.

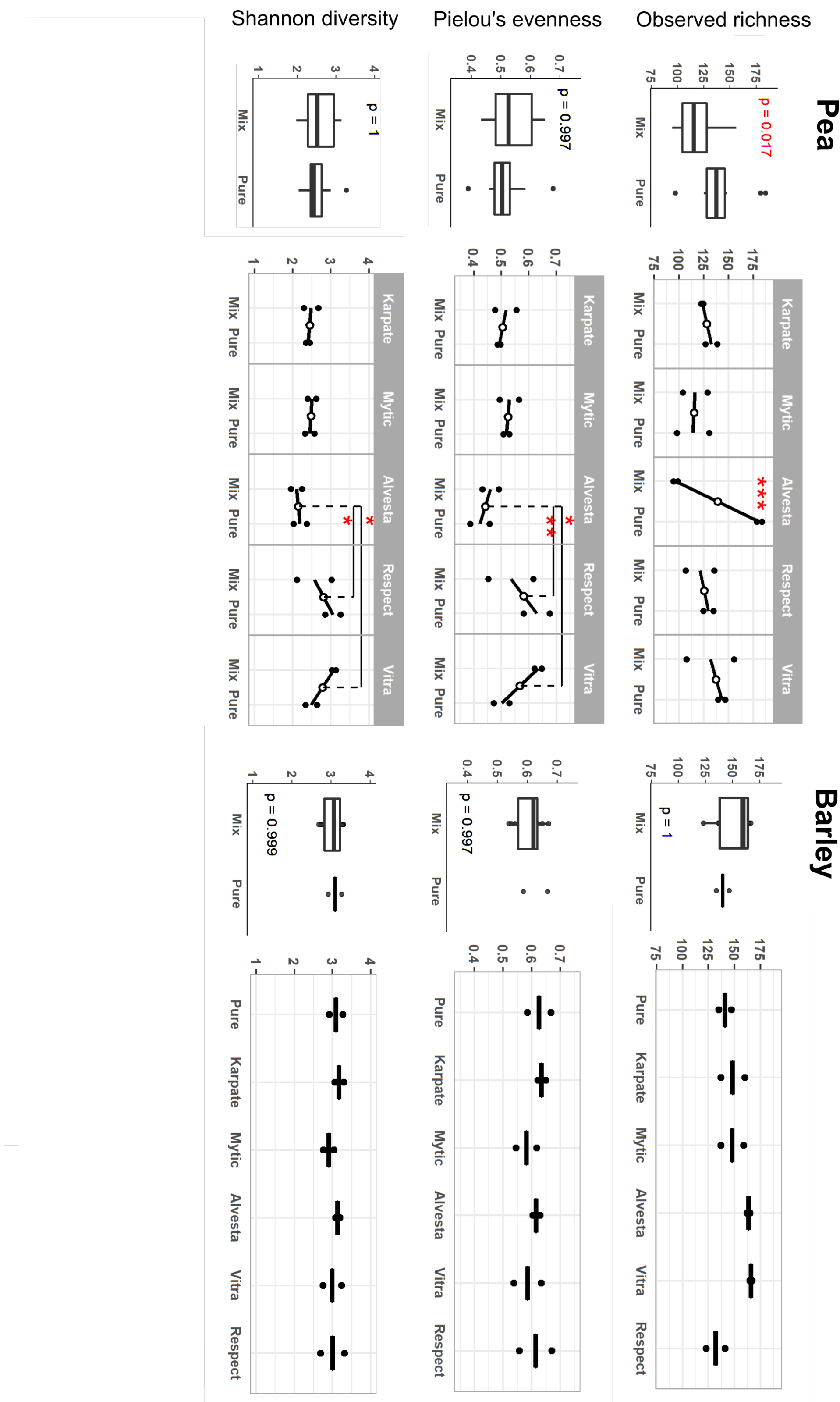


Fig. 3.4.: Fungal observed richness, Pielou's evenness, and Shannon diversity of pea and barley root samples. The left plots (Pea) show the factors cropping system, variety, and their interaction for pea. The right plots (Barley) compare the factors cropping system and neighbor for barley. P-values shown were FWER-adjusted. '***' denotes $p<0.001$, '**' denotes $p<0.01$, and '*' denotes $p<0.05$.

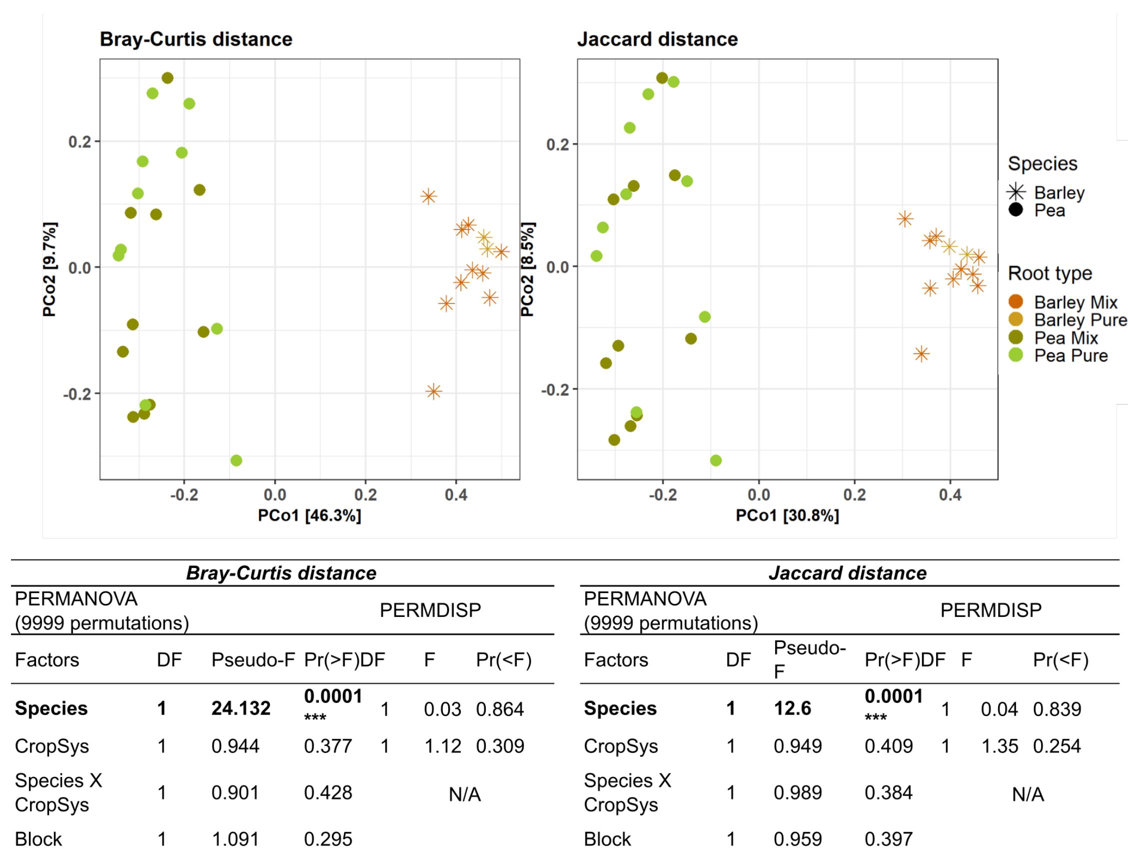
Mean evenness between pure and intercropping stands were 0.63 and 0.61, respectively, and did not differ significantly ($p=0.997$). Root fungal evenness did not differ significantly between neighbors, ranging from 0.58 (Mytic) to 0.64 (Karpate) ($p>0.9$). Barley root Shannon diversity in pure and intercropping stands were 3.10 and 3.04, respectively, and did not differ significantly ($p=0.999$). Shannon diversity ranged from 2.9 (Mytic) to 3.18 (Karpate) and no significant difference was observed between neighbors ($p>0.9$).

3.2.3 Beta diversity

Differences between barley and pea root fungal communities (beta diversity) was visualized with principal coordinate analysis (PCoA) by Bray-Curtis and Jaccard dissimilarities in **Fig. 3.5** (page 19). With both dissimilarities, the results indicated fungal communities between barley and pea were distinct and separated along the first axis. By Bray-Curtis dissimilarity, axis 1 explained 46.3% of total variation and separated cropping species. Jaccard dissimilarity axis 1 explained 30.8% variation. For Bray-Curtis and Jaccard dissimilarity, the first two axes accounted for 56% and 39.3% of total variation, respectively.

The fungal communities of intercropping of each species were clustering with those of the pure stands. Fungal communities difference between species observed in PCoA was confirmed by a significant species effect ($p<0.0001$) by permutational multivariate analysis of variance (PERMANOVA), provided in **Fig. 3.5 (bottom)**. A check for homogeneity of variance and the insignificance of group dispersions ($p>0.8$) (PERMDISP) confirmed that the fungal microbiome differences between species were due to biological variations. No cropping system ($p>0.3$) or interaction effect between species and cropping system was found ($p>0.3$) using Bray-Curtis and Jaccard dissimilarity indices. Replicated block effect was also not significant ($p>0.2$).

In order to have a clearer look at fungal communities associated to different pea varieties and if they might be influenced differently in intercropping system, pea and barley microbial data were further divided into subsets for analysis. PCoA results for pea and barley root fungal communities are shown in **Fig. 3.6** (page 20), accompanied by PERMANOVA and PERMDISP results. PCoA for pea root fungal communities measured by Bray-Curtis dissimilarity showed the first two axes accounted for 40.6% of total variation; for Jaccard, the two axes accounted for 31.0%. It revealed subtle clustering of communities by pea varieties, especially by Vitra, but not by cropping system. This was confirmed by PERMANOVA showing a significant

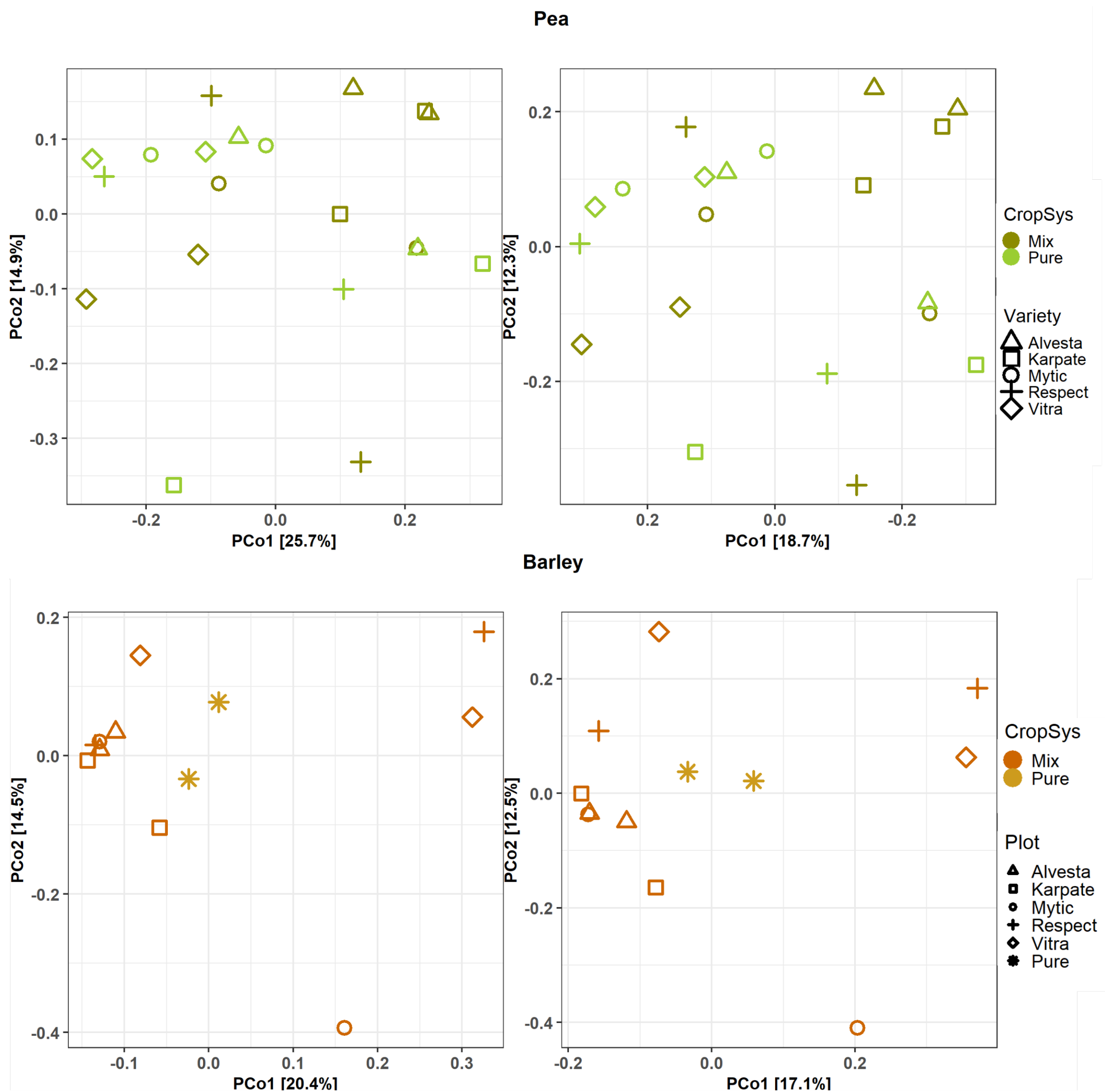


	Bray-Curtis distance						Jaccard distance					
	PERMANOVA (9999 permutations)			PERMDISP			PERMANOVA (9999 permutations)			PERMDISP		
Factors	DF	Pseudo-F	Pr(>F)	DF	F	Pr(<F)	DF	Pseudo-F	Pr(>F)	DF	F	Pr(<F)
Species	**1**	**24.132**	**0.0001 *****	1	0.03	0.864	**1**	**12.6**	**0.0001 *****	1	0.04	0.839
CropSys	1	0.944	0.377	1	1.12	0.309	1	0.949	0.409	1	1.35	0.254
Species X CropSys	1	0.901	0.428		N/A		1	0.989	0.384		N/A	
Block	1	1.091	0.295				1	0.959	0.397			

Fig. 3.5.: (Top) Principal coordinate analysis (PCoA) by Bray-Curtis and Jaccard dissimilarity indices on pea and barley root fungal ZOTUs. (Bottom) The table shows permutational multivariate analysis of variance (PERMANOVA) and permutational dispersion analysis (PERMDISP) results. N/A is given when it is not relevant or statistically reasonable for statistics computations. Bold-face fonts indicated significant effect of the factor.

variety effect measured by Bray-Curtis distance (p=0.023). Variety effect measured by Jaccard distance was also significant (p=0.011). A check for homogeneity of variance and the insignificance of group dispersions (p>0.4) confirmed that the fungal microbiome differences between varieties were due to biological variations. For both Bray-Curtis and Jaccard dissimilarity measurements, no cropping system effect (p>0.5) or variety by cropping system interaction effect (p>0.5) was observed. The effect of the replicated blocks were also not significant (p>0.5).

PCoA for barley root fungal communities showed By bray-Curtis dissimilarity the first two axes accounted for 34.9% of total variation; for Jaccard, the two axes accounted for 29.6% (**Fig. 3.6**). Microbial communities in pure stands were clustering closely with those of the intercropping stands.



Bray-Curtis distance

	PERMANOVA (9999 permutations)				PERMDISP		
	Factors	DF	Pseudo F	Pr(>F)	DF	F	Pr(<F)
Pea	CropSys	1	0.767	0.698	1	0.998	0.331
	Variety	**4**	**1.608**	**0.023** *	4	1.073	0.404
	CropSys X Variety	4	0.895	0.655	N/A		
	Block	1	0.929	0.515			
Barley	Neighbor	5	1.035	0.371	N/A		
	Block	1	0.954	0.549			

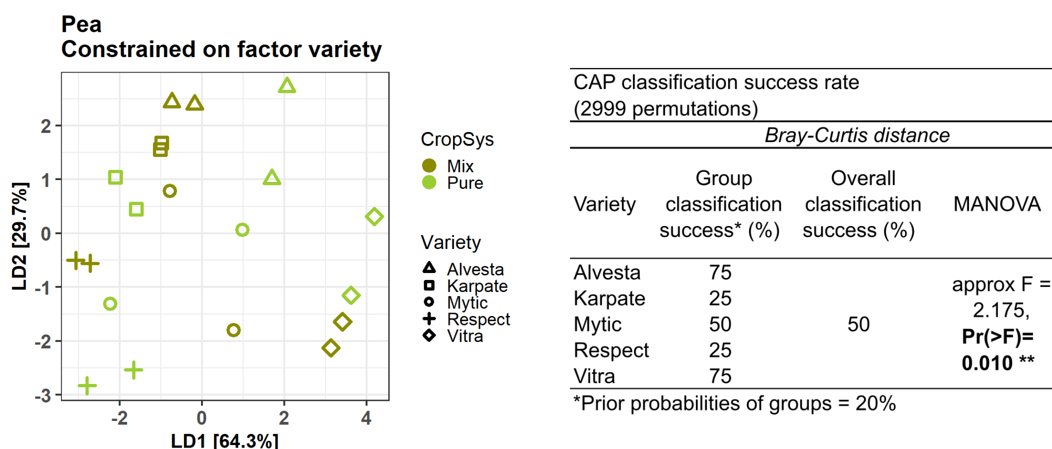
Jaccard distance

	PERMANOVA (9999 permutations)				PERMDISP		
	Factors	DF	Pseudo F	Pr(>F)	DF	F	Pr(<F)
Pea	CropSys	1	0.912	0.576	1	1.05	0.319
	Variety	**4**	**1.476**	**0.011** *	4	1.046	0.417
	CropSys X Variety	4	0.979	0.524	N/A		
	Block	1	0.921	0.565			
Barley	Neighbor	5	1.031	0.35	N/A		
	Block	1	0.947	0.6			

Fig. 3.6.: (Top) Separate PCoA by Bray-Curtis and Jaccard distances for root fungal ZOTUs of different pea varieties and barley with different neighbors. For barley plots, only the pea variety names are given for the stands intercropped with pea. (Bottom) PERMANOVA and PERMDISP results. Factors included are cropping system (CropSys), variety, interaction (CropSysXVariety), replicated blocks (Block), and neighbors (_neighbor). N/A is given when it is not relevant or statistically reasonable for statistics computations. Bold-face fonts indicated significant effect of the factor.

Fungal communities were scattered across the PCoA plane with no clear pattern and no separation between different stands. PERMANOVA confirmed that there was no neighbor effect by both Bray-Curtis dissimilarity and Jaccard dissimilarity ($p>0.3$), so barley root fungal community was not significantly influenced by the identity of the neighbors.

The differences in fungal microbiomes between pea varieties were not immediately clear based on PCoA plot. A subsequent canonical analysis of principal coordinates (CAP) ordination based on discriminant analysis was performed constraining on the factor variety as shown in **Fig. 3.7**. Bray-Curtis distance was used for the analysis.



CAP classification success rate (2999 permutations)			
	Bray-Curtis distance		
Variety	Group classification success* (%)	Overall classification success (%)	MANOVA
Alvesta	75		approx F = 2.175, **Pr(>F)= 0.010 ****
Karpate	25		
Mytic	50	50	
Respect	25		
Vitra	75		

*Prior probabilities of groups = 20%

Fig. 3.7.: Effects of pea varieties on root fungi revealed by constrained ordination. (Left) Canonical analysis of principal coordinates (CAP) ordination based on discriminant analysis using Bray-Curtis distance constraining on the factor variety. (Right) CAP analysis output showing percentage success rate of correct classification of varieties, during permutation tests, in their respective groups. Overall classification success rate and multivariate analysis of variance (MANOVA) were also shown.

PCoA showed the four Vitra samples were clearly clustered separately from the four other varieties on LD axis 1 (64%). Alvesta root fungal microbiomes also formed a cluster, separated from other other varieties on LD axis 2 (29.7%). Vitra, Alvesta, and Respect separated the furthest from each other on the ordination plane. Prior probabilities to successful classification of varieties were 20% for the five varieties. Results showed that Vitra had a high classification success rate of 75% during classification permutation tests with 2999 permutations (**Fig. 3.7 (right)**). Alvesta also had a high 75% classification success rate. For Mytic, success rate was at 50% and Karpate and Respect at 25%. Lower classification success rate for Karpate and Respect is likely due to higher similarities of fungal communities between two Mytic samples, as could also be observed from CAP (**Fig. 3.7 (left)**). Multivariate analysis

of variance (MANOVA) showed overall classification based on variety was 50% and was significant (p=0.01). As a double check, CAP analysis was also performed constraining on the factor cropping system, and MANOVA output showed there was no obvious separation between pure and cropping stands (p=0.320).

Fungal communities of pea were further analyzed in intercropping and pure stands in separate PCoA followed by PERMANOVA and PERMDISP. This revealed slightly different clustering patterns of replicates between the PCoA plots and the presence of crop variety effects in intercropping but not in pure stands. These results are in **Supplementary Fig. A.7** and not extensively presented here because the analyses were based on two replicates and might not be robust.

3.2.4 Differentially abundant ZOTUs

A summary of the number and identities of the differentially abundant ZOTUs identified upon comparisons between cropping systems, varieties, and interaction effect, is shown in **Table 3.1**. The result showed that for all pea root ZOTUs,

Tab. 3.1.: Summary of the identities and number of all differentially abundant ZOTUs obtained upon comparisons between between cropping systems (CropSys), varieties (Variety), and interaction effect (CropSysXVar), for pea and barley. The ZOTUs were identified based on FDR-adjusted p-values to greater than 0.05.

Comparison	Pea		Barley	
	# differentially abundant ZOTUs	ZOTU	# differentially abundant ZOTUs	ZOTU
CropSys	1	*ZOTU175*	2	*ZOTU105, ZOTU405*
Variety†	9	*ZOTU7, ZOTU6, ZOTU59, ZOTU302, ZOTU1148, ZOTU265, ZOTU175, ZOTU1706, ZOTU1132*	0	N/A
CropSys X Var ‡	3	*ZOTU1148, ZOTU265, ZOTU175*	0	N/A

† In the case of barley, pairwise comparison between intercropping stands was made. Number of comparisons were 10 for both pea and barley.

‡ In the case of barley, comparisons between pure stands with each of the intercropping stands were made. Number of comparisons were 5 for both pea and barley.

there were a total of nine ZOTUs that were differentially abundant between pairwise comparisons of pea varieties, and three of which changed in abundance in intercropping for particular pea varieties (interaction effect). For all barley root ZOTUs, two were differentially abundant between cropping systems. Comparisons between pure stands with each of the intercropping stands showed no differentially

abundant ZOTUs. Pairwise comparisons between barley roots in only intercropping stands also showed no differentially abundant ZOTUs.

For barley, the two ZOTUs found to be differentially abundant between cropping systems and their relative abundances in each cropping systems were summarized in **Table 3.2**. These were ZOTU105 and ZOTU408 (both Ascomycota) with pure stand

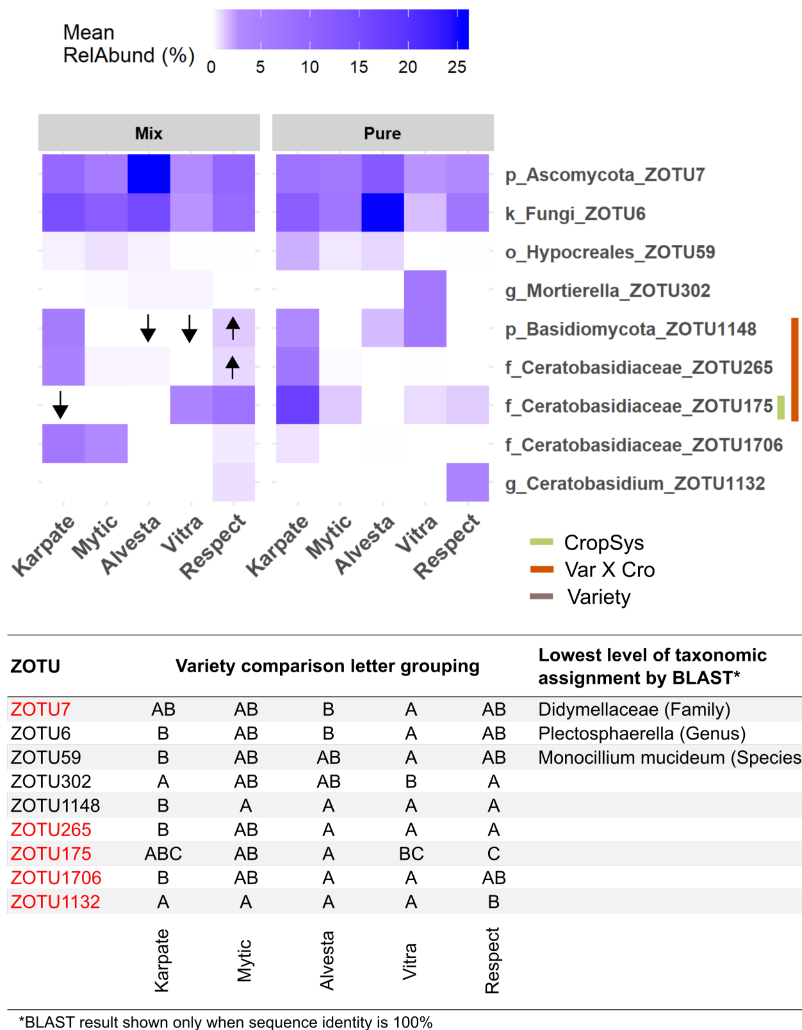
Tab. 3.2.: ZOTUs and their relative abundances in barley roots that were differentially abundant between cropping systems.

ZOTU	Kingdom	Lowest level of taxonomic assignment	Mix RelAbund (%)		Pure RelAbund (%)	
			Mean	SE	Mean	SE
ZOTU105	Fungi	Ascomycota	0.83	0.78	4.55	4.55
ZOTU408	Fungi	Ascomycota	0.03	0.03	1.06	0.86

mean of 4.6% and intercropping stand mean of 0.83% for ZOTU105; and mean of 1% in pure stand and 0.03% in intercropping for ZOTU408. The relative abundances were both significantly higher in pure stands than intercropping stands. Further BLAST search did not identify these ZOTUs at a lower taxonomic rank. There were no ZOTUs found differentially abundant comparing pure stands with each of the five intercropping stands with pea.

Fig. 3.8 (page 24) shows the relative abundances of the nine identified differentially abundant pea ZOTUs in each cropping system. The majority of the discovered differentially abundant ZOTUs between varieties were in the *Ceratobasidiaceae* family (ZOTU1132, ZOTU1706, ZOTU175, ZOTU265). These ZOTUs, along with ZOTU7 (Ascomycota) that was further identified by BLAST search to be a *Didymellaceae*, were fungal groups containing putative pathogens for this experiment. Mean relative abundance of ZOTU7 for Alvesta with 19% was significantly higher compared with 3% for Vitra. ZOTU7 abundance in other varieties ranged from 5% to 10%. For *Ceratobasidiaceae* members, Alvesta consistently had significantly lower abundance when compared to at least one other varieties (**Fig. 3.8 (bottom)**). Mean relative abundance of ZOTU265 was significantly higher in Karpate (6%) compared to Alvesta, Vitra, and Respect, below 1%. ZOTU175 was significantly lower in Mytic and Alvesta (below 0.01%) compared to Vitra (2%) and Respect (4%). ZOTU1706 was significantly lower in Alvesta, Vitra (below 0.01%), compared to Karpate (4%). ZOTU1132 was only present in Respect and thus significantly higher compared to all other varieties.

ZOTU6 (assigned to Kingdom Fungi) was assigned to a *Plectosphaerella* by BLAST search. ZOTU6 for Alvesta at 10% and Karpate at 13% was significantly higher compared to Vitra (1.9%). For Karpate and Respect, the relative abundance for ZOTU6



ZOTU	Variety comparison letter grouping					Lowest level of taxonomic assignment by BLAST*
	Karpate	Mytic	Alvesta	Vitra	Respect	
ZOTU7	AB	AB	B	A	AB	Didymellaceae (Family)
ZOTU6	B	AB	B	A	AB	Plectosphaerella (Genus)
ZOTU59	B	AB	AB	A	AB	Monocillium mucideum (Species)
ZOTU302	A	AB	AB	B	A	
ZOTU1148	B	A	A	A	A	
ZOTU265	B	AB	A	A	A	
ZOTU175	ABC	AB	A	BC	C	
ZOTU1706	B	AB	A	A	AB	
ZOTU1132	A	A	A	A	B	

*BLAST result shown only when sequence identity is 100%

Fig. 3.8.: (Top) Heatmap summary of relative abundances of all nine identified differentially abundant ZOTUs in pea roots in hypotheses testing. Bars next to the taxonomic names indicate which ZOTUs were found differentially abundant between variety (Variety), cropping systems (CropSys), and interaction effect (VarXCrop). Arrows indicate an increase or decrease compared to pure stands. (Bottom) Table showing letter grouping based on variety comparison results. Shared letters indicate no significant differences in mean. ZOTUs highlighted in red were identified as putative pathogens for this trial. BLAST results were provided only when meeting parameters (refer to Chapter 2) or otherwise left blank.

were around 8%. Mean relative abundance of ZOTU59 (*Hypocreales*) was further assigned as *Monocillium mucidum*. In general, the mean abundance was below 0.6% for all varieties, and it was significantly higher in Karpate compared to Vitra, 1% and 0.01% respectively. Mean relative abundance of ZOTU1148 (Basidiomycota) for Karpate was 4% and significantly higher compared to the other four varieties, with means below 3%. *Mortierella* (ZOTU302) was significantly higher for Vitra

compared to Karpate and Respect, with mean relative abundances of 3% compared to complete absence in the Karpate and Respect. Alvesta and Mytic abundance of ZOTU302 were around 0.1%.

ZOTU175 was found to be differentially abundant between cropping systems, and also involved in a significant interaction effect. ZOTU175 was 17% in pure stands for Karpate but was absent in intercropping stands. Another *Ceratobasidiaceae* member involved in interaction effect was ZOTU265, which was absent for Respect in pure stands and increased to 0.8% in intercropping stands. There was no common direction for the change of these *Ceratobasidiaceae* members observed. ZOTU1148 was absent in Respect pure stands and increased to 1% for Respect. For Vitra and Alvesta, ZOTU1148 was 1% and 6% in pure stands but absent in intercropping stands. Although not significantly, it was also noted that ZOTU7 increased from 12% in pure stands to 25% in intercropping stands.

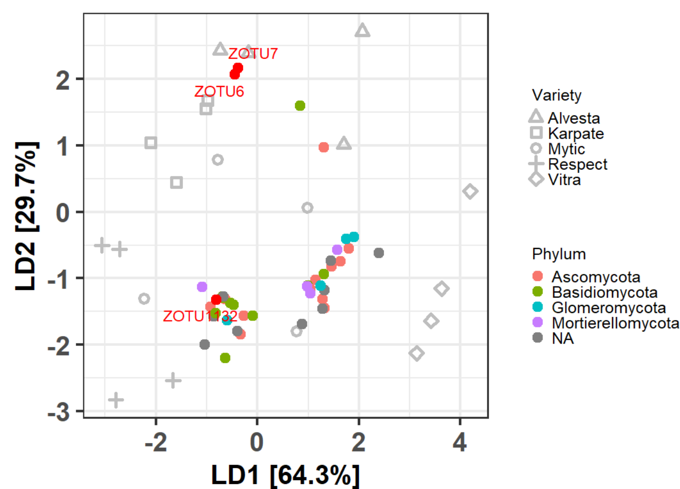


Fig. 3.9.: Biplot of CAP ordination for pea contrained by factor variety with significant fitted ZOTU vectors projected with envfit (prior to FDR adjustments) designated with points. ZOTUs highlighted in red was found to be differentially abundant in *edgeR* pair-wise comparisons of varieties.

In an attempt to see whether the differentially abundant ZOTUs were also explaining the separation of the pea varieties on constrained ordination, ZOTU vectors were fitted on to CAP ordination. There were a total of 44 significant fitted ZOTU vectors prior to p-adjustment for false discovery rate (FDR) (**Supplementary Fig. A.1**). After FDR adjustment, no significant ZOTU vectors were retrieved and thus not extensively presented here. Of the nine ZOTUs, three ZOTUs were observed to be

also responsible for driving the separation of the varieties observed in constrained ordination, visualized in **Fig. 3.9**.

ZOTU vectors ZOTU6 and ZOTU7 were also found to be associated to Alvesta. ZOTU1132 was correlated to Respect. It was additionally noted that Glomeromycota members correlated highly with Vitra. Basidiomycota members were highly correlating with Respect (**Fig. 3.9**).

3.2.5 Glomeromycota occurrences

Fig. 3.10 summarizes the relative abundance of Glomeromycota for barley and

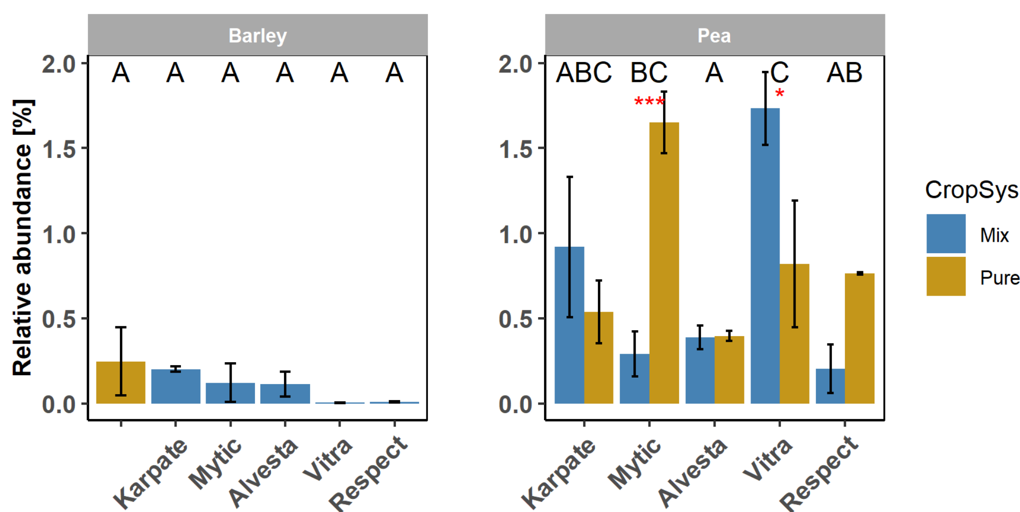


Fig. 3.10.: Mean Glomeromycota abundance in barley and pea roots in different intercropping and pure stands. Error bars show the standard error of the means (n=2). Red asterisks indicate a difference between cropping systems (CropSys) for a variety, '***' denotes p<0.001, '**' denotes p<0.01, and '*' denotes p<0.05. P-values shown were FWER-adjusted. Shared letters indicate no significant differences in mean.

pea. Detailed statistical test results are in **Supplementary Fig. A.5**. There was no significant effect of cropping systems on Glomeromycota relative abundance (p=0.992). Variety effect and interaction effects were observed. The relative abundance of Glomeromycota in Vitra roots with 1.2% was significantly higher in Vitra, compared to with 0.4% and 0.5% of Alvesta (p<0.001) and Respect (p<0.001). Relative abundance of Glomeromycota in Vitra roots grown in intercropping (1.72%) increased by almost double compared to when grown in pure stands (0.82%) (p=0.01). For Mytic it was the opposite, Glomeromycota relative abundance in the

root was much higher when it was in pure compared to intercropping (p<0.001) and lowered from 1.6% to 0.2%. For barley roots, there was no cropping system effect on Glomeromycota abundance (p=1). Glomeromycota relative abundances were under 0.5%, and no significant effect of neighbors was observed (p>0.9).

3.3 Wheat-pea — Hungary

3.3.1 Taxonomic profiling

An overview of the relative abundances of fungal phyla for wheat and pea roots are shown in **Fig. 3.11**. The top ten most abundant ZOTUs of wheat and pea roots were also explored, provided in **Supplementary Fig. A.13-A.14, Table A.5-A.6**. For

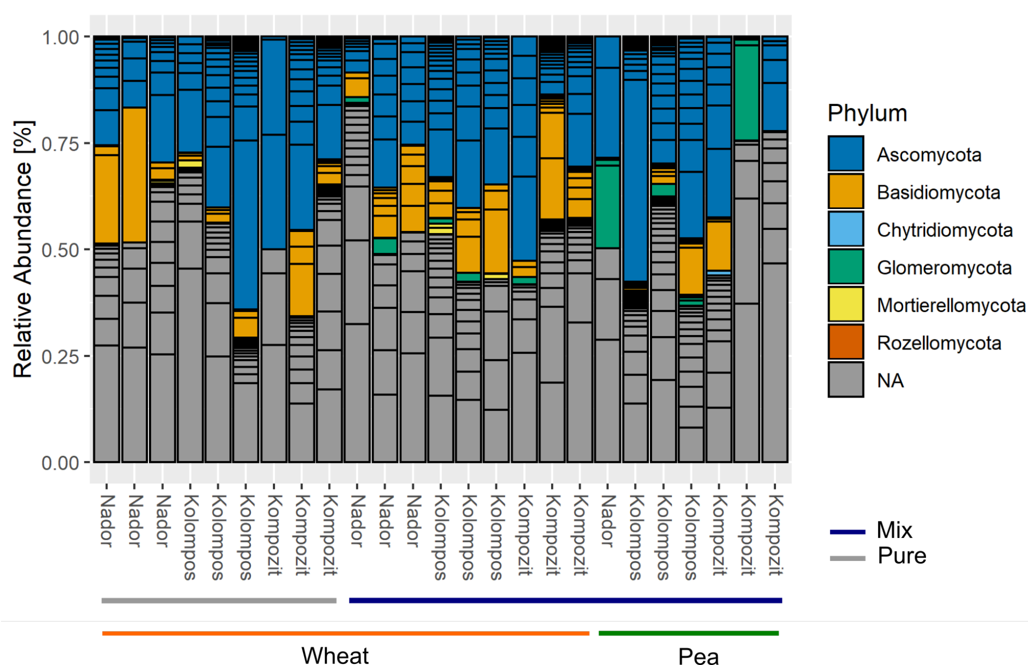


Fig. 3.11.: Relative abundances of fungal ZOTUs grouped at phylum level for wheat and pea samples, in intercropping stands (mix) and pure stands (pure). Individual ZOTU abundances were stacked in order from greatest to least.

wheat roots, the the majority of ZOTUs (53.0%) could not be classified at phylum level. This is followed by Ascomycota (33.4%), Basidiomycota (12.6%), Glomeromycota (0.6%), Mortierellomycota (0.4%), and Chytridiomycota (0.01%). In pea roots, the majority of ZOTUs (55.1%) were also not classified, followed by Ascomycota (32.6%), Glomeromycota (7.4%), Basidiomycota (4.7%), Chytridiomycota (0.2%), Mortierellomycota (0.05%), and Rozellomycota (<0.01%).

3.3.2 Alpha diversity

The communities were rarefied to the minimum sampling depth from the Hungary sample that was 42,601 sequences per sample, which covered most of the observed ZOTU richness (see **Fig. 3.1**, page 14). Alpha diversity result is shown in **Fig. 3.12** and the detailed statistical test results in **Supplementary Fig. A.10-A.11**. Mean

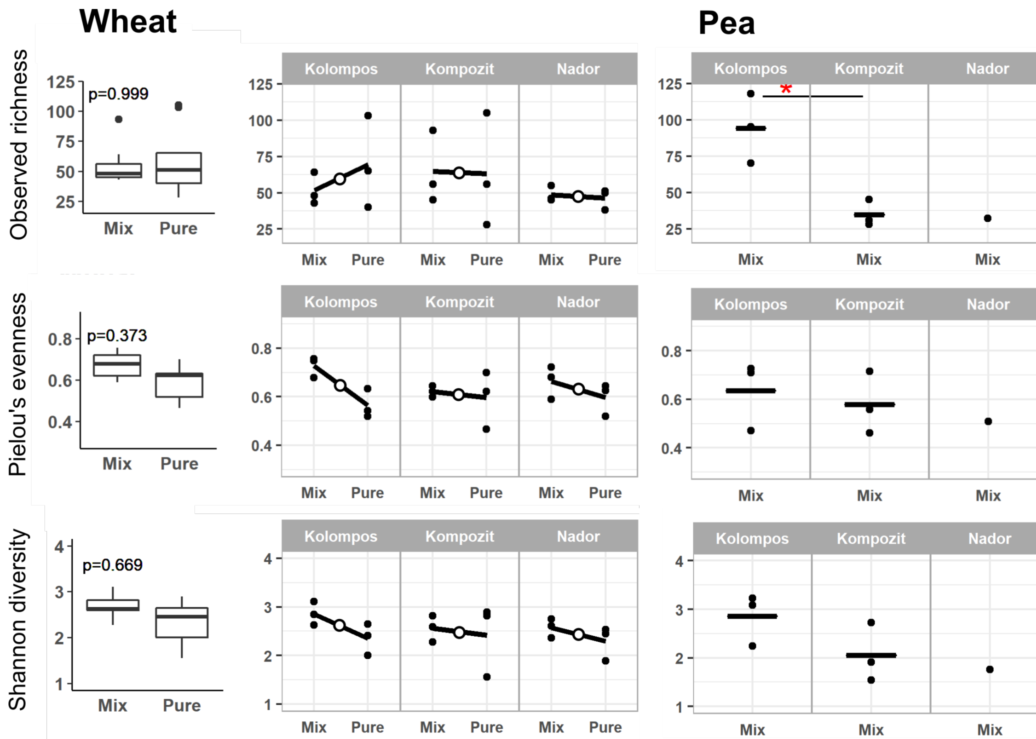


Fig. 3.12.: Fungal observed richness, Pielou's evenness, and Shannon diversity of wheat and pea root samples. The left plots (Wheat) show the factors cropping system, variety, and their interaction for wheat. The right plots (Pea) compare the factor neighbor for pea. P-values shown were FWER-adjusted. '***' denotes $p<0.001$, '**' denotes $p<0.01$, and '*' denotes $p<0.05$.

fungal richness for wheat pure and intercropping stands were 60 and 55 respectively. Linear hypotheses tests showed that fungal richness on wheat roots did not differ between cropping systems (p=0.999). Fungal richness variety means ranging from 48 (Nador) to 64 (Kompozit) did not differ significantly (p>0.9). There was no significant interaction effect between cropping system and variety (p>0.9). Mean fungal evenness for wheat pure and intercropping stands were 0.59 and 0.67, respectively. There was no cropping system effect although intercropping tended to be higher (p=0.373). Variety means ranging from 0.61 (Kompozit) to 0.65 (Kolompos) were not significantly different (p>0.9). There was no significant interaction effect observed (p-values ranging from p=0.278 to p=1). Mean fungal Shannon diversity

for pea pure and intercropping stands with 2.35 and 2.66, respectively, did not significantly differ between cropping systems (p=0.669). Variety means ranging from 2.43 (Nador) to 2.60 (Kolompos) were not significantly different (p>0.9). There was no interaction effect between cropping system and variety (p=0.716 to p=0.999).

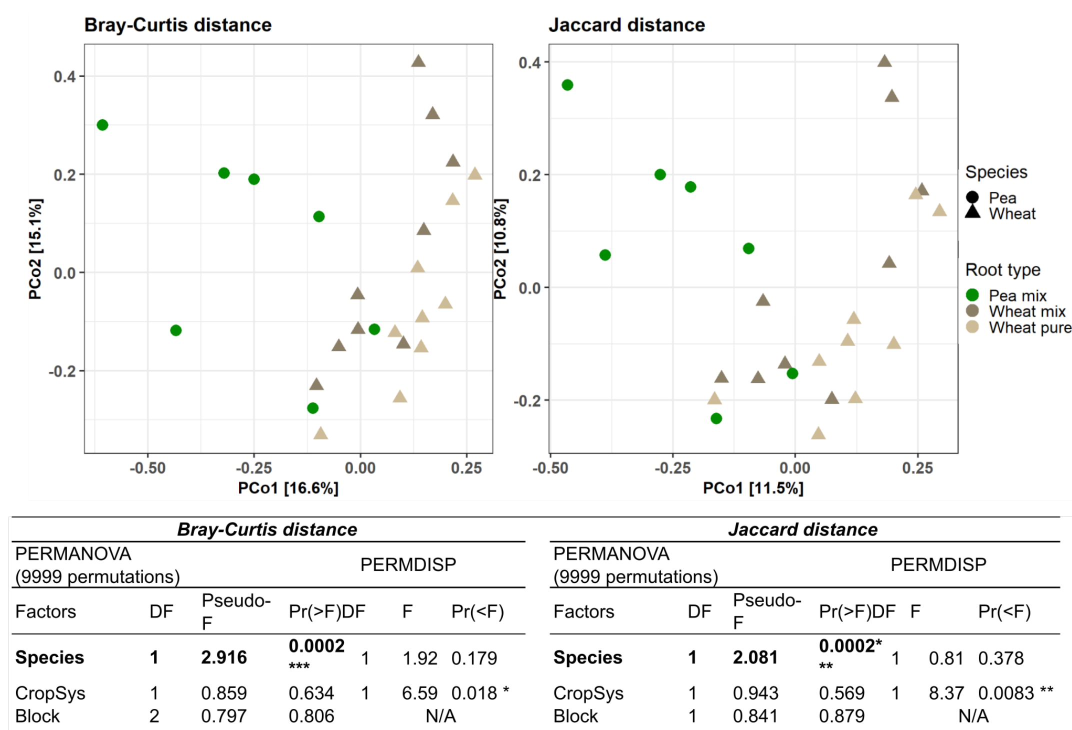
The effect of neighbor on pea fungal richness was observed. Pea fungal richness intercropped with Kolompos with 94 was significantly higher compared with 34 for intercropped with Kompozit (p=0.0168). Fungal evenness for pea roots did not differ significantly between Kolompos and Kompozit with mean of 0.64 and 0.58, respectively (p=0.973). Pea Shannon diversity intercropped with Kolompos was 2.85 and with Kompozit was 2.06 were also not significantly different (p=0.973). The one replicate for pea intercropped with Nador had root fungal richness of 32, evenness of 0.51, and Shannon diversity 1.76, and similar to with Kompozit.

3.3.3 Beta diversity

Differences between wheat and pea root fungal communities (beta diversity) was visualized with PCoA by Bray-Curtis and Jaccard dissimilarities in **Fig. 3.13** (page 30). With both dissimilarities, PCoA showed separation of wheat and pea fungal communities. By Bray-Curtis dissimilarity, axis 1 separated cropping species and explained 16.6% of total variation. Axis 2 explained 15.1% of total variation. Similar picture was observed with Jaccard dissimilarity, axis 1 explained 11.5% total variation and axis 2 explained 10.8% of total variation.

The fungal communities of wheat intercropping stands of each species were clustering with those of the pure stands. Fungal communities difference between species observed in PCoA was confirmed by a significant species effect (p=0.0002) by PERMANOVA, provided in **Fig. 3.13 (bottom)**. A check for homogeneity of variance showed group dispersions were not significant (p>0.2), which confirmed that the fungal microbiome differences between species were due to biological variations. No cropping system effect was observed for both dissimilarities (p>0.5). Effect of replicated blocks was not significant (p>0.8).

To have a closer look at fungal communities associated to different wheat varieties and the influences of cropping system, wheat and pea microbial data were further divided into subsets for analysis. PCoA results for wheat and pea root fungal communities are shown in **Fig. 3.14** (page 31), accompanied by PERMANOVA and PERMDISP results. PCoA for wheat fungal communities measured by Bray-Curtis



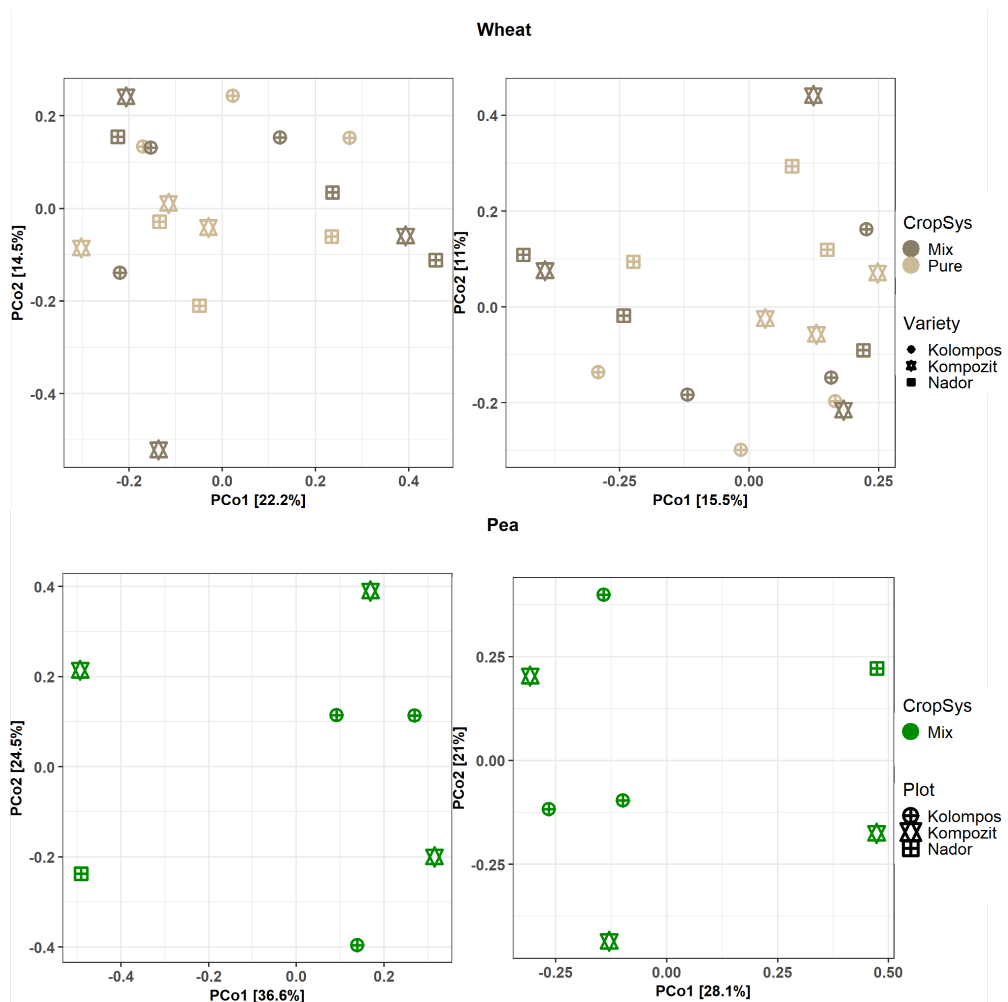
Bray-Curtis distance						
PERMANOVA (9999 permutations)				PERMDISP		
Factors	DF	Pseudo-F	Pr(>F)	DF	F	Pr(<F)
Species	**1**	**2.916**	**0.0002 *** **	1	1.92	0.179
CropSys	1	0.859	0.634	1	6.59	0.018 *
Block	2	0.797	0.806	N/A		

Jaccard distance						
PERMANOVA (9999 permutations)				PERMDISP		
Factors	DF	Pseudo-F	Pr(>F)	DF	F	Pr(<F)
Species	**1**	**2.081**	**0.0002** **	1	0.81	0.378
CropSys	1	0.943	0.569	1	8.37	0.0083 **
Block	1	0.841	0.879	N/A		

Fig. 3.13.: (Top) PCoA by Bray-Curtis and Jaccard dissimilarity indices on wheat and pea root fungal ZOTUs. (Bottom) The table shows permutational multivariate analysis of variance (PERMANOVA) and permutational dispersion analysis (PERMDISP) results. N/A is given when it is not relevant or statistically reasonable for statistics computations. Bold-face fonts indicated significant effect of the factor.

dissimilarity showed the first two axes accounted for 36.7% of total variation; for Jaccard, the two axes accounted for 26.5%. No immediate patterns were separating between wheat varieties or cropping systems. This was also shown in PERMANOVA result (**Fig. 3.14 (bottom)**). It showed that for both dissimilarities, cropping system effect was not significant ($p>0.4$). Variety effect on fungal communities measured by Bray-Curtis tended towards significance ($p=0.061$); similarly, Jaccard measured variety effect tended towards significance ($p=0.053$). There was no replicated block effect ($p>0.9$).

PCoA for pea root fungal communities by Bray-Curtis dissimilarity showed the first two axes accounted for 61.1% of total variation; for Jaccard, the two axes accounted for 49.1%. No immediate patterns were separating pea fungal communities between stands.

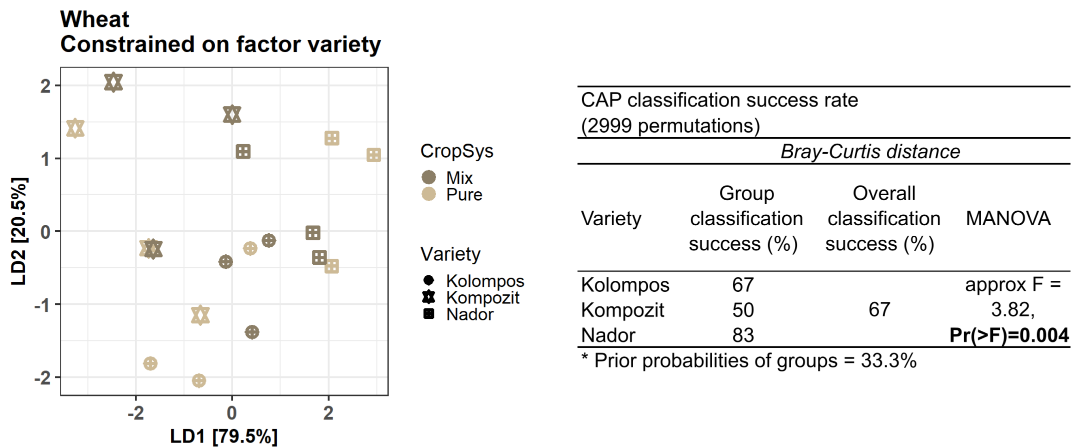


	Bray-Curtis distance							***Jaccard distance***						
	PERMANOVA (9999 permutations)				PERMDISP			PERMANOVA (9999 permutations)				PERMDISP		
	Factors	DF	Pseudo F	Pr(>F)	DF	F	Pr(<F)	Factors	DF	Pseudo F	Pr(>F)	DF	F	Pr(<F)
Wheat	CropSys	1	0.948	0.514	1	1.603	0.224	CropSys	1	1.008	0.448	1	0.507	0.612
	Variety	2	1.442	0.061 .	2	2.898	0.086 .	Variety	2	1.286	0.053 .	2	2.911	0.085 .
	CropSys X Variety	2	0.883	0.671		N/A		CropSys X Variety	2	0.969	0.558		N/A	
	Block	2	0.603	0.979				Block	1	0.76	0.972			
Pea	Neighbor	2	1.393	0.216		N/A		Neighbor	2	1.203	0.219		N/A	
	Block	2	1.811	0.1				Block	2	1.383	0.107			

Fig. 3.14.: (Top) Separate PCoA by Bray-Curtis and Jaccard distances for root fungal ZOTUs of different wheat varieties and pea with different neighbors. For pea plots, only the wheat variety names are given for the stands intercropped with wheat. (Bottom) PERMANOVA and PERMDISP results. Factors included are cropping system (CropSys), variety, interaction (CropSysXVariety), replicated blocks (Block), and neighbors (_neighbor). N/A is given when it is not relevant or statistically reasonable for statistics computations. Bold-face fonts indicated significant effect of the factor.

The replicates were scattered across the PCoA plane, with slightly closer clustering of communities in intercropped stands with Kolompos on one side of the plane compared to in Kompozit. PERMANOVA confirmed that there was no neighbor effect by both Bray-Curtis dissimilarity and Jaccard dissimilarity ($p>0.2$), so pea root fungal community was not significantly influenced by the identity of the neighbors.

A subsequent CAP ordination based on discriminant analysis was performed constraining on the factor variety as shown in **Fig. 3.15**. Bray-Curtis was used for the analysis. The figure showed that Nador and Kompozit formed clusters subtly



CAP classification success rate (2999 permutations)			
Bray-Curtis distance			
Variety	Group classification success (%)	Overall classification success (%)	MANOVA
Kolompos	67		approx F =
Kompozit	50	67	3.82,
Nador	83		**Pr(>F)=0.004**

* Prior probabilities of groups = 33.3%

Fig. 3.15.: Effects of wheat varieties on root fungi revealed by constrained ordination. (Left) CAP ordination based on discriminant analysis using Bray-Curtis distance constraining on the factor variety. (Right) CAP analysis output showing percentage success rate of correct classification of varieties, during permutation tests, in their respective groups. Overall classification success rate and multivariate analysis of variance (MANOVA) were also shown.

separated on LD axis 1 (79.5%), and Kolompos clustering between the two varieties and separated slightly on LD axis 2 (20.5%). Prior probabilities for successful classification of varieties were 33.3% for the three varities. Results showed that Nador had the highest classification success rate of 83% during classification permutation tests with 2999 permutations (**Fig. 3.15 (right)**). This was followed by Kolompos with 67% success rate and Kompozit with 50% success rate. Multivariate analysis of variance (MANOVA) showed overall variety classification was significant ($p=0.004$).

Fungal communities of wheat were further analyzed in intercropping and pure stands in separate PCoA followed by PERMANOVA and PERMDISP. This revealed different clustering patterns of replicates between the PCoA plots and the presence of crop variety effects in pure but not in intercropping stands. These results are in **Supplementary Fig. A.12** and not extensively presented here.

3.3.4 Differentially abundant ZOTUs

A summary of the number and identities of the differentially abundant ZOTU identified upon comparisons between cropping systems, varieties, and interaction effect, is shown in **Table 3.3**. The result showed that of all the ZOTUs found in this trial, there were in total four ZOTUs differentially abundant due to wheat variety differences.

Tab. 3.3.: Summary of the identities and number of all differentially abundant ZOTUs obtained upon comparisons between between cropping systems (CropSys), varieties (Variety), and interaction effect (CropSysXVar), for wheat and pea. The ZOTUs were identified based on FDR-adjusted p-values to greater than 0.05. It should also be noted that the value for Nador was based on one replicate.

Comparison	Wheat		Pea	
	# differentially abundant ZOTUs	ZOTU	# differentially abundant ZOTUs	ZOTU
CropSys	0	N/A	N/A	N/A
Variety[†]	1	*ZOTU191*[‡]	3	*ZOTU122, ZOTU69, ZOTU128, ZOTU490*
CropSys X Var	0	N/A	N/A	N/A

[†] In the case of pea, pairwise comparison between intercropping stands was made. Number of comparisons were 3 for both wheat and pea

[‡] Unclassified Fungi without further identification by BLAST. With mean abundance of 2.9% in Kompozit and absent in Kolompos.

Comparing wheat fungal ZOTUs between cropping system, variety, and interaction effect showed there were no differentially abundant ZOTUs discovered between cropping system or involved in interaction effect. One ZOTU was to be found differentially abundant between Kolompos and Kompozit. ZOTU191 with a relative abundance of 2.9% in Kompozit and absent in Kolompos was significantly higher in the former. BLAST search did not further identify this fungus to a lower taxonomic rank.

Four pea root ZOTUs were differentially abundant between intercropping stands, which were all significantly higher in relative abundance intercropped with Nador compared to with Kompozit as shown in **Fig. 3.16** (page 34). Two of four ZOTUs were identified to be Glomeromycota. ZOTU69 (Glomeromycota) was further assigned as *Glomeraceae*. Relative abundance of ZOTU69 in pea roots intercropped with Nador was 19.4% and with Kompozit was 7.4%. ZOTU69 for pea roots intercropped with Kolompos was 1.5% and not detected to be differentially abundant to the former two stands. ZOTU490 (*Glomeraceae*), ZOTU128 (Fungi), and ZOTU122 (Ascomycota) relative abundances in pea roots intercropped with Nador were 1.3%, 28.8%, and 7.4%, respectively, and all were absent in intercropping stands with Kompozit.

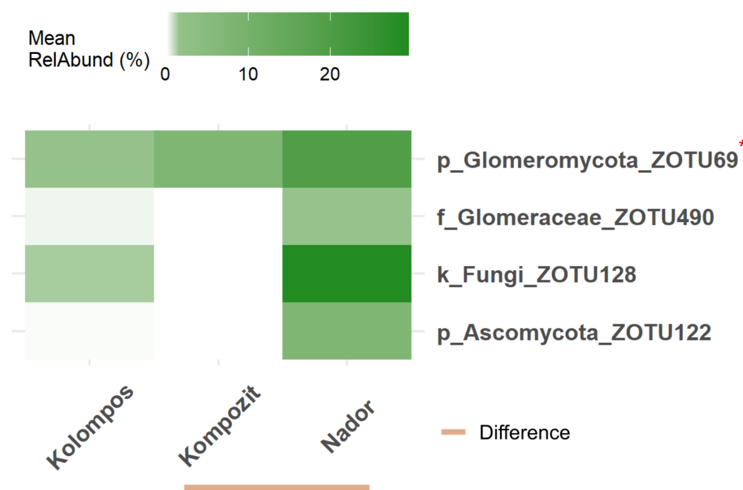


Fig. 3.16.: Heatmap summary of relative abundances of all four identified differentially abundant ZOTUs in pea roots in hypotheses testing. Differentially abundant ZOTUs were found between intercropping stands of Kompozit and Nador, denoted by a bar below the variety names. BLAST results were provided (in footnote) only when meeting parameters (refer to Chapter 2).

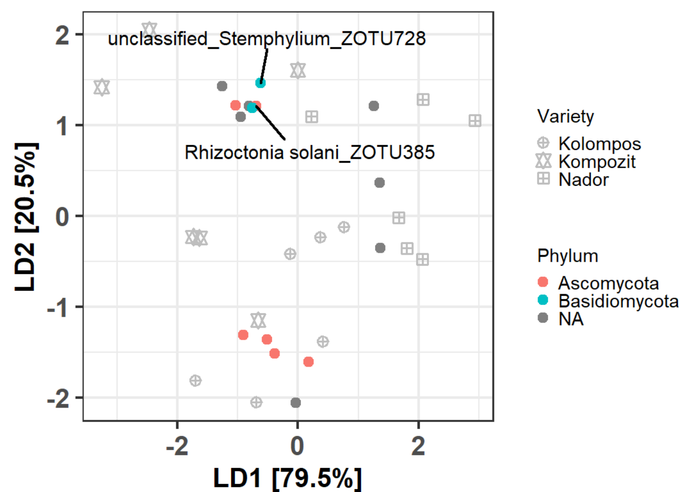


Fig. 3.17.: Biplot of CAP ordination for wheat contrained by factor variety with significant fitted ZOTU vectors projected by envfit (prior to FDR adjustments) designated with points. ZOTUs were differentially abundant between Kompozit and Nador.

In an attempt to see whether the differentially abundant ZOTUs were also explaining the separation of the pea varieties on constrained ordination, ZOTU vectors were fitted on to CAP ordination. There were a total of 23 significant fitted ZOTU vectors prior to p-adjustment for FDR (**Supplementary Table A.4**), which were no longer significant after the adjustment and thus not extensively presented here. ZOTU191 was not identified to be responsible for driving the separation of the varieties observed in constrained ordination, visualized in **Fig. 3.17**. Among these ZOTUs, it was noticed that two ZOTUs correlating with Kompozit were identified by BLAST as a *Stemphylium* and the other *Rhizoctonia solani*, which were both important plant pathogens.

3.3.5 Glomeromycota occurrences

Fig. 3.18 summarizes the relative abundance of Glomeromycota for wheat and

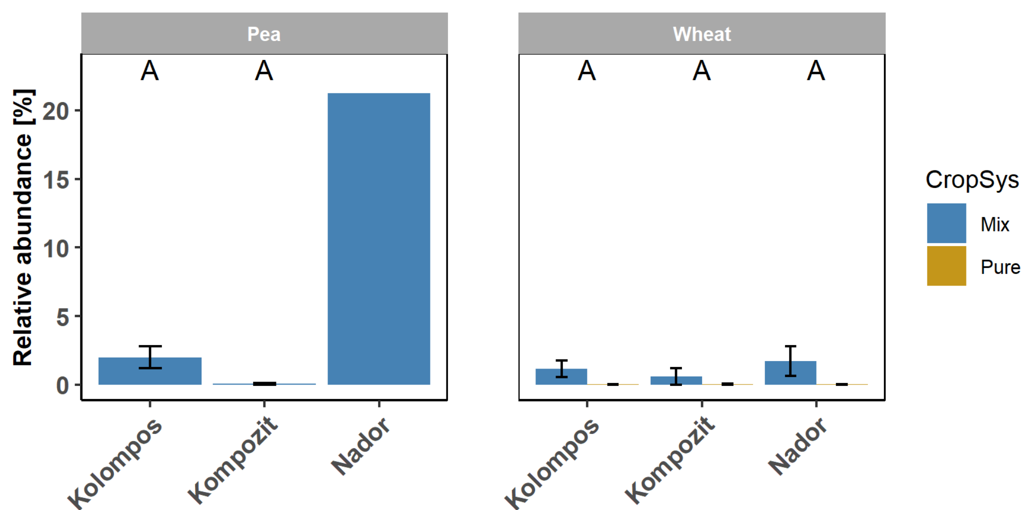


Fig. 3.18.: Glomeromycota abundance in wheat and pea roots in different intercropping and pure stands. Standard errors are shown with n=3 for wheat, n=1 for pea in Nador, n=2 for pea in Kompozit after the elimination of an outlier, n=3 for Kolompos. Means sharing a letter are not significantly different (User-defined contrasts, FWER-adjusted comparisons). For pea, no statistical test was made for Nador due to low sample size.

pea. Detailed statistical test results are in **Supplementary Fig. A.11**. Mean relative abundance of Glomeromycota on wheat root in intercropping stand with 1.2% compared to less than 0.01% in pure stands tended towards a significant cropping system effect ($p=0.09$). Variety means for Kolompos, Kompozit, and Nador were 0.6%, 0.3%, and 0.9%, and did not differ significantly ($p>0.8$). There was no

interaction effect between cropping system and variety ($p=0.22$ to $p=0.95$). For pea roots, Glomeromycota relative abundance in pea roots intercropped with Kolompos was 2.0% and not significantly higher than with Kompozit, 0.06% ($P=0.313$).

3.4 Fungal community diversity and plant agronomic traits

3.4.1 Intercropping and pure stands

Pea and barley agronomic traits and selected microbial variables were analyzed by principle component analysis and visualization of the plot is shown in **Fig. 3.19** (page 37). PCA axes 1 and 2 explained a total of 43.4% variations in the dataset. Axis 1 explained 29% and axis 2 explained 14.4%, respectively. In general, pea pure stands formed a distinct cluster on positive values of PC2, and intercropping stand spanned across PC1, which explained 29% of the total variation. PC1 also separated the intercropping stand of barley-Vitra from other intercropping stands. Vitra pure and intercropping stands, two Respect pure stands and Mytic stands were associated with positve PC1 values. The two replicates from barley pure stands were not distinctly different than ones in intercropping stands and clustered with the intercropping stands (**Fig. 3.19 (top)**).

Fig. 3.19 (bottom) shows barley kernel mass ($cos2=0.63$), barley total mass ($cos2=0.51$), pea kernel mass ($cos2=0.52$), pea disease score ($cos2=0.57$) were well-represented by PC1. These were associated to negative values of PC1. Other variables well-represented by PC1 included pea Glomeromycota abundance ($cos2=0.54$), nodule number ($cos2=0.51$) and were associated with positive PC1 values. Overall grain yield and pea grain component yield was associated to positive PC2 values and pea pure stands ($cos2=0.64$ and $cos2=0.67$, respectively). Pea vegetative growth parameters, shoot length and total biomass were associated with the first quadrant (upper-right). Barley grain yield was more associated to negative values of PC1, in the direction of intercropping stands, negatively correlated with pea biomass and grain yield. Pea fungal diversity and evenness were quite equally represented by PC1 and PC2, positively correlated to the direction of the fourth quadrant (lower-right) ($cos2>0.2$). In general, fungal community variables, including barley root biodiversity indices, barley Glomeromycota abundance, and pea fungal richness, were not very well-represented by the first two PCs ($cos2<0.35$).

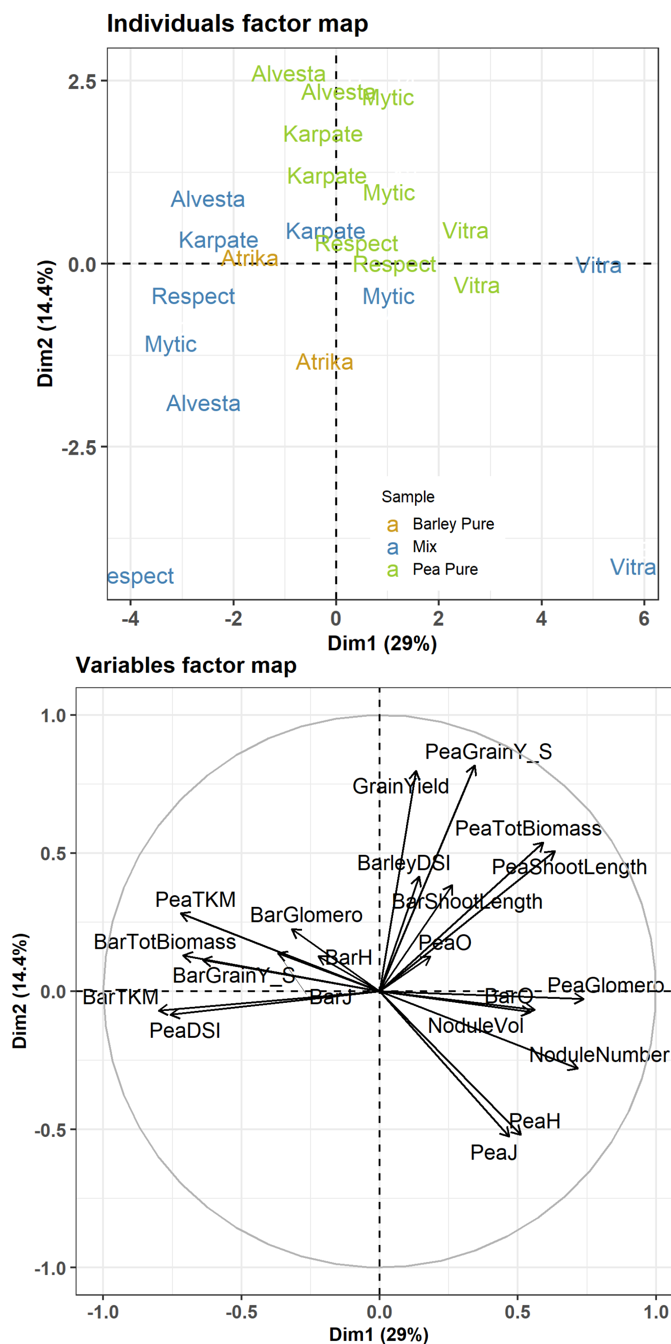


Fig. 3.19.: (Top) Individual factor map for pea and barley. (Bottom) Variables factor map. Variables were subset into to pea and barley, except for grain yield, which included total grain yield in intercropping stand. The variables abbreviations include 'H' for Shannon diversity, 'J' for evenness, 'O' for observed richness, 'GrainY_S' for scaled component grain yield. 'Glom' for Glomeromycota abundance, 'TKM' for thousand kernel mass. 'TotBiomass' for total biomass. 'NodulesVol' for pea nodule volume. Missing values of Vitra grain yield were imputed by the mean. Where 'Bar' was indicated, the variable was a barley trait.

3.4.2 Pea fungal community diversity and agronomic traits

Closer examination of how pea root fungal community characteristics might be reflected on agronomic traits was investigated. Pea fungal community data, including biodiversity indices and the nine differentially abundant ZOTUs, were analyzed with with pea and barley (intercropping) agronomic traits, visualized in **Fig. 3.20** (page 39). An additional PCA without Vitra is constructed and supplied in **Supplementary Fig. A.6**.

PC1 explained 26.3% of the total variation in the dataset, and PC2 explained 14.6% of the total variation in the dataset. PCA showed separate clusters of pure and intercropping stands, where the pure stands were associated with positive values of PC1 and included Vitra intercropping stands. Alvesta and Respect were on the far end in negative values of PC1. Variables pea disease score (cos2=0.65), pea kernel mass (cos2=0.57), barley total biomass (cos2=0.49), ZOTU7 (cos2=0.5), were well-represented by PC1 and associated to negative PC1 values. These variables were associated to Alvesta and Respect intercropping stands. In the opposite direction to these variables were pea Glomeromycota abundance (cos2=0.47), pea shoot length (cos2=0.49), pea total biomass (cos2=0.44), pea nodule number (cos2=0.49), nodule volume (cos2=0.39) in positive PC1 values. These were associated to Vitra pure and intercropping stands. ZOTU6 was also correlated to negative values of PC1 (cos2=0.39). Besides the ones mentioned, the other ZOTUs were not well-represented by the first two PCs and were also not by PC3 in a subsequent check (cos2<0.35). Exceptions were ZOTU1148 and ZOTU302 well-represented by PC3 (cos2=0.56 and cos2=0.47, respectively). The ZOTU variables were generally pointing in the direction of the pea varieties that they were found to be differentially abundant in. Fungal diversity and evenness were as observed in **Fig. 3.19** positively correlated with pea Glomeromycota abundance with Vitra in intercropping stands. Fungal richness was not well-represented in the first two PCs (cos2<0.1).

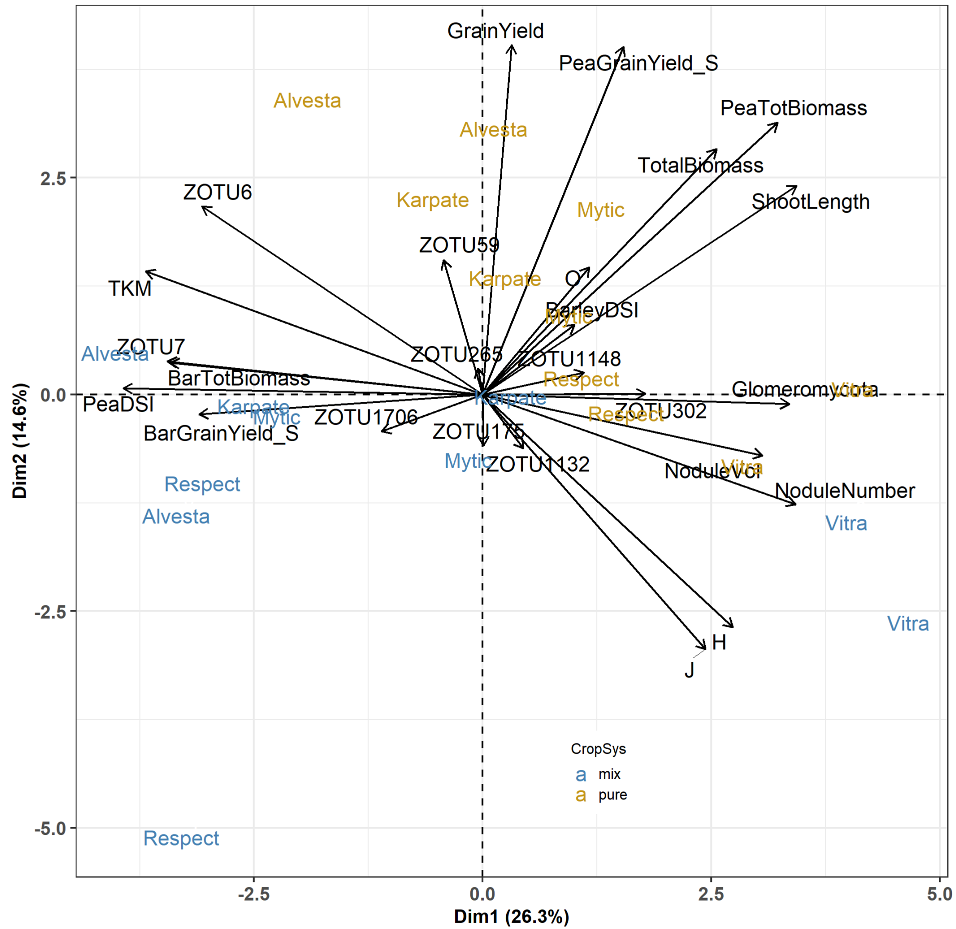


Fig. 3.20.: PCA visualizing pea fungal community data, with differentially abundant ZOTUs, and selected pea and barley agronomic traits. The barley traits included only intercropping data. Where 'Bar' was indicated, the variable was a barley trait, where absent, it is a pea trait. The variables abbreviations include 'H' for Shannon diversity, 'J' for evenness, 'O' for observed richness, 'GrainY_S' for scaled component grain yield. 'Glom' for Glomeromycota abundance, 'TKM' for thousand kernel mass. 'TotBiomass' for total biomass. 'NoduleVol' for pea nodule volume. Missing values of Vitra grain yield were imputed by the mean.

4 Discussion

It is now no longer a question to consider plant and its root-associated microbiome as an entity to understand plant performance [15]. Through the utilization of high-throughput sequencing technology to examine plant root-associated fungal communities, this study was able to gain a better understanding of intercropping and plant variety influences on root fungal communities. This study demonstrated that the strongest drivers of root fungal community diversity in the two cereal-legume trials were geographical location and crop species. Previous work observed rhizosphere community shift due to intercropping [5]. The results from this study showed root fungal composition was overall not impacted by intercropping, but alpha diversity results from CH and HU indicated genotype-dependent influences. Thus, the hypothesis that "intercropping has an effect on root fungal community" is rejected, but the hypothesis that "there is an interaction effect between cropping system and plant variety on fungal community" is not rejected. So far, limited studies have looked at genotype effect on root fungal community but it was observed before in a study on *Arabidopsis* [35]. In this study, ordination (CH and HU) and alpha diversity (CH) results showed significant variety effects on fungal community diversity, confirming the hypothesis that "crop variety has an effect on root fungal community". By employing differential abundance analysis, fungal taxa characteristic of crop varieties were identified and further related to agronomic data in pure and intercropping systems.

4.1 Crop species and geographical location are main drivers of root fungal diversity

The strongest drivers of root fungal community diversity in the two cereal-legume trials were geographical location and crop species. The global ordination result showed a clear separation between barley, CH pea, and HU intercrops root fungal communities. Strong crop species effect could be observed by the separation between CH barley with CH pea on the global ordination. Although the separation of HU wheat with CH barley and CH pea is confounded by crop species, a location effect

could be determined by comparing CH and HU pea communities. Dissimilarities from the two pea root fungal communities of the two trial sites could be due to difference in pea variety or geographical location. The latter conjecture is more plausible because the global ordination also did not reveal separations in CH pea varieties. The reason that plant root microbial community composition could differ between geographical locations is because plants can only recruit from the pool of indigeneous microorganisms available, and this pool is determined by soil properties that could greatly vary between sites [10]. Taxonomic profiles also showed a difference in fungal community composition between HU pea and CH pea roots, with higher Glomeromycota relative abundance in the former compared to the latter. Numerous previous studies already show the root and rhizosphere microbiome compositions are dominantly driven by abiotic soil properties [5, 8, 36, 37]. Separate ordinations for Switzerland and Hungary experiments showed fungal communities separate based on intercrop species. Previous work also showed based on UniFrac distances bacterial profiles differ between cereal and legume [36]. This is in line with the numerous studies showing that different plant species have distinct microbiomes [9, 10, 36].

Taxonomic profiling showed the majority of the ZOTUs could not be classified at phylum level, followed by taxa classified as members of Ascomycota and Basidiomycota, or Glomeromycota in the case of HU pea roots. The high proportion of unclassified ZOTUs was not expected as other studies only have a small proportion of fungal taxa not classified at phylum level [5, 38]. Not considering the unclassified ZOTUs, Ascomycota and Basidiomycota are indeed found to be dominating in the roots of other plant species such as rice [39], wheat [40], grasses [38], and soybean or sugarcane [5]. A likely explanation for a high proportion of unclassified ZOTUs could be attributed to differences in bioinformatics processing, since there is no clear rules of the algorithms (e.g. Sintax or BLAST), database (e.g. UNITE or NCBI), or confidence level setting to give taxonomic predictions to the (Z)OTUs.

Fungal communities were analyzed using both Bray-Curtis and Jaccard dissimilarities in this study because they don't measure fungal composition the same way. While Bray-Curtis takes account of fungal abundance pattern and presence-absence, Jaccard shows presence-absence. In general, a high similarity between ordination results using both Bray-Curtis and Jaccard dissimilarities was observed. The latter always explained slightly less variation compared to Bray-Curtis on both linear discriminant axes. The same pattern in both ordinations indicates that the main driving force for differences in crop species were due to fungal presence-absence, but that abundances patterns provided added value to the separation.

4.2 Crop variety drives fungal diversity

Diversity indices, albeit keeping fungal taxonomic identity anonymous, gives us insights to an understanding of numerical quantification of the number (i.e. observed richness), and dominance pattern of fungal taxa (i.e. evenness and Shannon diversity) on plant roots. On the other hand, multivariate analysis with dissimilarity indices provided insights to differences in fungal community composition. Based on these indices crop variety effects on fungal diversity was observed.

A significantly lower fungal evenness and Shannon diversity was observed on Alvesta roots in comparison with Respect and Vitra in the Switzerland pea-barley trial. Shannon diversity accounts for both richness or evenness, but since no significant difference for fungal richness was observed, lower evenness better explains for lower Shannon diversity in Alvesta roots. This indicated different fungal colonization patterns on Alvesta roots whereby there are certain dominating fungi. Wheat varieties Kompozit and Nador ordination result showed a separation of these varieties based on fungal composition, but not alpha diversity. Changes in fungal community composition but not fungal diversity had been observed before in other studies [34]. One explanation is that an increase and decrease of certain taxa is compensated by the changes in other taxa. It was observed that PERMANOVA showed variety effect tended to be significant, whereas CAP uncovered significant variety effect. This was due to confounding effects of block and interaction factors, and taking them out of the model PERMANOVA would also have shown significant variety effects.

Similar to the observations of plant genotype effects on root bacterial microbiota by Bulgarelli et al. [8], this study observed crop varieties as a driver of fungal community. This was observed in ordination analyses. In the other studies showing a significant plant genotype effect on root microbiome, the plants were originated from very different origins such as *Arabidopsis* obtained from different countries [35] or wild and different domestication accessions of barley [41]. The results from this thesis found even differences in root microbiota within genetically similar commercial varieties. Fungal community composition of Vitra was outstanding from the rest of the varieties. One possible explanation for this would be that unlike the other varieties, Vitra is a fodder pea cultivar. In conjunction with previous studies' results, it could be hypothesized that plant varieties, depending on how closely related in the plant's phylogenetic distance, could have an effect on the plant's relationship with its root-associated microbial community. On the other hand, Alvesta, Respect are more similar to each other compared to Vitra because they are both edible pea, but still fungal composition differed between the two varieties. One

way to explain is they recruit different microbiota through distinct root exudation patterns [42], as such root traits can be variable among crop genotypes [43].

On the other hand, ordination analyses also showed root fungal communities between certain crop varieties to be less differentiable. One explanation for this is that the active exudation of a range of compounds from plants to establish plant-microbial relationship are modulated by stress [42]. For example, a recent study on different rice genotypes showed that rice endospheric fungal microbiota composition shifted under drought and with a more extensive genotype effect [39]. It could be hypothesized that strong differentiation in root-associated microbial communities between crop varieties would be observed under more stressful conditions.

4.3 Small, genotype-dependent effects of intercropping on fungal microbiome

If host-specificity of root microbiome is strong for crop species as numerous studies have indicated [38, 36], one might expect to find that root microbial diversity be increased in an intercropping system when different plants exert influence in the soil through differentiated root exudation processes, accumulating different fungal species. Yet, the result from alpha and beta diversity showed that fungal diversity was not impacted by intercropping. In the case of Alvesta, richness was lower in intercropping stands compared to pure stands. In a study by Mommer et al. comparing different levels of plant species richness on fungal diversity, they similarly demonstrated that when plant species are grown together it does not lead to an increase in diversity of root-associated fungal communities [38]. They observed that there was a negative density dependence effect, the idea that an accumulation of host-specific fungal species decreases with declining relative abundances of their host plants [38] because root fungi could have trouble locating their hosts in diverse plant communities.

While overall fungal community was not influenced by intercropping, there were small and genotype-dependent effects of intercropping on alpha diversity and on fungal abundances at different taxonomic levels. Alpha diversity was influenced by intercropping partner in two cases. First, Alvesta root fungal richness decreased in intercropping with barley; and second, fungal richness was lower in Aviron (HU pea) roots when intercropped with Kompozit compared to with Kolompos. Differential abundance analysis showed two unclassified ZOTUs, ZOTU105 and ZOTU408, were significantly different in barley roots between intercropping and

pure stands. This is consistent with earlier studies that also observed intecropping influencing fungal abundances at different taxonomic levels [5, 16]. Intercropping also altered Glomeromycota abundances incongruously for crop varieties. While a prior study observed greater mycorrhizal fungi colonization in intercropped faba beans compared with faba bean monoculture [18], here, it was shown that the response is dependent on variety. For Mytic, intercropping decreased Glomeromycota relative abundance and increased for Vitra. Because it is known that mycorrhizal fungi symbiotic relationships generate benefits for the plant hosts [44], it could be suggested that intercropping negatively affected Mytic. Lastly, it was also observed that a member of *Basidiomycota* (ZOTU1148), and members of *Ceratobasidiaceae* (ZOTU265, ZOTU175) abundances changed in different directions depending on crop variety.

Such gradual effects of intercropping on fungal community has been observed in an intercropping silviculture study on soil fungal community by Rachid et al. (2015). They showed that rather than having distinctly different fungal diversity between monocultures and intercropping cultures, alpha diversity and different fungal phyla abundance in the soil of intercropping stand was observed to be in an intermediate state of two monocultures [16]. On the other hand, Lian et al. showed a decrease in fungal diversity and significant change in fungal composition of sugarcane and soybean in intercropping system. They owed the explanation to an indirect effect from lowering of pH in intercropping soil compared to monoculture [5]. From the study it wasn't clear why pH was impacted in intercropping system, but it could be suspected that the rhizosphere soil was acidified during legume nitrogen fixation process, during which protons are excreted to balance internal pH [11]. It had been observed that legume relied more on nitrogen fixation in intercropping system [4] so acidification could be more pronounced in intercropping system. Such significant effect of intercropping on fungal community was not observed in this study, but the results did show that the effects of intercropping on alpha diversity depends on crop variety.

4.4 Fungal taxa associated with crop varieties

A combination of results from differential abundance analysis, Glomeromycota analysis, and fitted ZOTUs on constrained ordination enabled us to identify characteristic fungal taxa that differentiate between crop varieties. Many of these fungal taxa were putative pathogens. Fungal taxa that distinguish Alvesta from Vitra included

higher association with a member of *Didymellaceae* (ZOTU7) and a member of *Plectosphaerella* (ZOTU6). The *Didymellaceae* family included economically important pea pathogens *D. pinodes* and *D. pinodella* [14]. *Plectosphaerella cucumerina* is also a crop pathogen, associated to root rots of horticultural crops [**Carlucci2012**]. Glomeromycota abundance was significantly lower in Alvesta compared to Vitra. It could also be observed from fitted ZOTUs on constrained ordination showed one of the driving force for separation of Vitra from the other varieties is the association with members of Glomeromycota. Differential abundance analysis showed Vitra could be distinguished from at least one other pea variety based on higher relative abundance of ZOTU302, a member of *Mortierella*. *Mortierella* species are saprophytes found to assist crops and mycorrhizal fungi in phosphorus acquisition and litter decomposition [45]. Earlier study by Xu et al. on comparing fungal communities in bulk soil, rhizosphere soil, and roots of pea in the fields, found that *Plectosphaerella* spp., members of *Didymellaceae*, *Mortierella* spp. were almost exclusively found in bulk soil and/or rhizosphere [46]. This study found them to be colonizers of pea roots. Lastly, *Monocillium mucidum* (ZOTU59), found significantly more abundant in Karpate compared to Vitra, has function as degraders of organic compounds [47].

Ceratobasidiaceae (ZOTU1132) was only present in Respect, and in general, Alvesta was less abundant in four members of *Ceratobasidiaceae* (ZOTU175, ZOTU265, ZOTU1706, ZOTU1132) compared to at least one other pea varieties. *Ceratobasidium* are binucleate telemorphs of *Rhizoctonia* spp. [48]. While *R.* solani is a major cereal and pea pathogen [48, 49], the binucleate telemorph has been shown to be either non-virulent on pea or causing root rot without adversely affecting pea emergence and growth [48]. On the other hand, *Ceratobasidium cereale* has been reported to be the cause of sharp eyespot of cereal causing lesions on stems and adversely affecting cereal growth [50]. Pathogenicity on cereals might also depend on the specific anastomosis groups of the binucleate *Rhizoctonia*, as another study shows the binucleate telemorph are nonpathogenic on cereals [49].

In the Hungarian wheat-pea trial, Kompozit was found more associated to *Stemphylium* and *Rhizoctonia solani* compared to Nador. The former include species that are known plant pathogens causing leaf blight with a wide host range including wheat and pea [51]. The latter, *Rhizoctonia* spp., was as mentioned earlier also pathogens of cereals and pea [49, 48]. Differential abundance analysis identified ZOTU191 (kingdom Fungi) to be lower in Kolompos compared to Kompozit.

Differential abundance analysis and projection of ZOTUs onto constrained ordination are different methods to understand ccrop-associated fungal taxa. Differential abundance analysis finds which ZOTUs were significantly different between varieties,

and differentiates between crop varieties based on ZOTU abundance differences. Fitted ZOTUs onto constrained ordination in another way differentiates between crop varieties by showing ZOTUs that drives the separations of crop varieties on constrained ordination. These two methods are not exactly comparable due to different normalization and hypotheses testing method; in the former, community data was normalized by TMM with hypotheses testing based on quasi-likelihood test, the latter was based on proportion-normalized data followed by permutation test. However, the overlaps in ZOTUs between these two analyses together give stronger indication for the association of fungal taxa with crop variety, as was the case for the Swiss pea-barley experiment.

4.5 Relation between fungal communities and agronomic traits

Fungal richness was not very well represented by the first two PCs. Fungal evenness and Shannon diversity were observed to be equally well-represented in quadrant 4, but it appeared to be driven by Vitra. It has been reviewed that diverse and even microbial communities increased competition between microorganisms and reduces the niche spaces available for potential pathogen invaders [7], it could explain for a negative correlation of Shannon diversity and evenness with disease expression. This pattern also persisted when Vitra was taken out of the PCA analysis (**Supplementary Fig. A.6**).

Among the identified putative pathogens of cereals and pea, including ZOTU175, ZOTU265, ZOTU1706, ZOTU1132 (Ceratobasidiaceae members), ZOTU7 (Didymellaceae), PCA showed only a strong positive correlation between abundances of putative pathogens ZOTU7 with pea root disease score. Although care should be taken not to see correlation and causation, the correlation between ZOTU7 with disease score might be reasonable as it was identified to be a putative pea pathogen. To identify whether it is a causal factor for root disease expression requires a test of the pathogenicity of this fungus in a re-inoculation study (Koch's postulate). Even though disease pressure was generally low in the field in this experiment, sometimes pathogens may be present but become aggressive only when the environment becomes conducive [52]. While ZOTU6 (Plectosphaerella), a putative horticultural crop pathogen, was also positively correlated with disease score, it might be that this correlation was due to its high abundance in Alvesta. Alvesta appeared to be a key driver of disease score.

In terms of belowground traits, nodulation was positively correlated with Glomeromycota abundance. This correlation could be observed in light of nitrogen fixation being an energy intensive process requiring phosphorus. Mycorrhizal fungi symbiosis could improve phosphorus uptake by plants and stimulate nitrogen fixation [53]. Vitra appeared to be key driver of nodulation and Glomeromycota abundance. However, the positive correlation between nodulation and Glomeromycota abundance still persisted when Vitra was taken out of the PCA analysis (**Supplementary Fig. A.6**).

PCA showed that Vitra clearly separated from the other varieties, especially from Alvesta, based on fungal community composition but also plant performance. Vitra separated on PCA from the other intercropping stands by positive correlation with Glomeromycota abundance, better vegetative growths, and negative correlation with disease score, and putative crop pathogens (ZOTU6 and ZOTU7). The positive correlation between Glomeromycota abundance and better plant performance is in line with the knowledge of benefits of symbiotic relationship with mycorrhizal fungi [44]. In addition, earlier study comparing healthy and diseased pea roots found that Glomeromycota were almost exclusively found on healthy pea roots [46]. The combination of observations on PCA suggested Vitra has in general healthier roots.

4.6 Limitations

This study aims to understand differences between pure and cereal-legume cropping system fungal communities and plant variety effects. There were certain limitations while exploring the aim of the study including potential rooms for exploration. These points should also hopefully be guide future researches on this topic.

First, low replication makes it hard to detect interaction effect with multivariate analysis. Separate PCoA plots for pea or wheat fungal communities in intercropping and pure stands appears to suggest that there might be some interactions, but was not detected by PERMANOVA (**Supplementary A.7, Supplementary A.12**). This study drew inferences on taxon abundance mainly based on the results from *edgeR* analysis, which had been used and published in studies related to microbial community (e.g. by Hartman et al. [34]), but were initially intended for gene expression use. This study rarefied dataset to analyze alpha diversity to account for library size differences in microbial dataset. While rarefying could lower the false

discovery rate in differential abundance testing [54], it could potentially eliminate valid data, including rare taxa [55].

In addition to biases against underrepresented fungal groups with quite different ITS1 region length, error profiles for the Illumina sequencing technology are still poorly understood [21]. Because each DNA clonal clusters produce a signal during synthesis, when synthesis of a molecule lags behind or advances too quickly, it creates noise for detection of the nucleotide fluorophore signal. The number of affected sequences also tends to increase with each cycle [21]. Unless this is resolved, we would not know the true biological variations of the microbial community analyzed by such high-throughput sequencing platform. There is no one set way to handle microbial community data and analyzing such a data requires numerous decision steps involved from bioinformatics processing of sequences to downstream analysis of the (Z)OTU data. The various filtering and trimming strategies were aimed to decrease error rates, however, the choices were not well-defined, and possibly remain to be so if the exact error profiles in high-throughput sequencing platform could not be known.

As mentioned in introduction, there are no clear rules to taxonomic predictions. Taxonomic assignments of the ZOTUs are an approximate but not full certainty of the true identity of the ZOTU. More than half of the ZOTUs remained unclassified at phyla level. This could mean that what we were observing in this study only comprised half of the total community, and being informed about their identity could potentially change the picture. It might be useful to try a combination of methods to get lower level taxonomic predictions and improve efficiency when examining fungal identity.

In this study, when discussing abundances, we do not know the absolute abundances of fungal community, but refer to the abundance of the ZOTU relative to the sum of the ZOTUs found in the roots. The positive side is that we could look at fungal community based on fungal 'replacement'. Lastly, by focusing on root microbiome we might be missing out on the interaction in rhizosphere, a highly dynamic environment [9] with higher levels of microbial diversity compared to roots [42]. Similarly, studying bacterial community such as plant growth-promoting bacteria, could also be informative on plant performance. And while PCA is a good method to combine microbial data with plant traits, the interpretation of the data is still difficult because correlation is not necessarily causation.

5 Conclusion

This work advances the current efforts to gain a better understanding of plant-soil microbiome interaction behavior in the context of intercropping system. In this study, microbiome data was generated based on Illumina Miseq sequencing on ITS1 region of root samples from pea-barley and wheat-pea intercropping experiments. The goal of this thesis is to understand how cereal-legume cropping system and plant genotypes influence fungal community diversity by comparing root fungi between pure and intercropping and between crop varieties. To do this, we began with a view of root fungal diversity with alpha diversity indices, and then we compared fungal composition differences between roots with beta diversity. We proceeded to explore differences in certain fungal abundances between these environments, crop varieties and cropping systems, and identified taxa that were influenced by these environments. Finally, this study took an additional step to put root fungal community data in context with plant agronomic traits in pure and intercropping systems.

Intercropping requires selecting crop varieties to be paired. Yet, there has been limited studies examining what might be the consequences of the pairing in below-ground interactions. Understanding belowground interactions in intercropping is all the more important, because of a growing body of literature showing host-specificity of root microbiome. This specificity is observed in this work, and we saw that even genetically similar commercial varieties can have distinct fungal community. We further observed certain characteristics in fungal community between crop varieties, including association with putative pathogens versus beneficials, that were significantly different between the crop varieties.

It was put forward in introduction the idea that the plant-specific root microbiome could potentially influence each other in intercropping. Here, the results suggest root fungal composition was overall not impacted by intercropping. However, plant genotype-dependent effects was observed, and abundances of putative pathogens and beneficials changed in different directions in intercropping depending on crop varieties. In two instances, intercrop neighbor influenced fungal richness. In one case, by another crop species and in the other, by partner variety. The combined results from this study suggest that crop pairings matter for the changes in root

microbial community, which also suggests to consider crop selections more carefully in intercropping.

This study also showed analyzing fungal community data, in conjunction with PCA with agronomic traits, allowed us to distinguish between crop varieties and hint at which varieties might be better or suboptimal for intercropping system. For example, Vitra with higher Glomeromycota abundance in general and in intercropping in particular could make it a better choice as an intercrop due to known benefits of mycorrhizal fungi. Positive correlations between more rigorous plant growth, lower root disease expression and higher Glomeromycota abundance were also shown in PCA. While it is understood that PCA displays only correlations but not causations, this study shows combining agronomic traits with microbial data in PCA is a good methodology to reveal what could potentially be the microbial community characteristics to explain plant performance.

Overall, this study demonstrated that there are influences of intercropping system on root-associated fungal microbiota. The extent of the influence can be different between varieties. This thesis's results also expanded evidences for variety influences on root fungal community composition and demonstrates that much information could be gained by studying plant root-associated fungal community. To the best of my knowledge, this is the first study comparing different varieties in intercropping. We saw indications that plant varieties are influenced by cropping systems differently. Future studies should be conducted to substantiate its results.

Future work should aim to increase the number of replicates to enhance resolution to better observe interaction effects that was limited in this study, especially for multivariate analysis. For example, using the same trial and resources as this project, one way to circumvent the lack of replicated blocks could be to create technical replicates within replicated blocks. Certainly, the fungal community characteristics associated to the intercrops observed in this study could be affirmed by a repetition of this study. As an extension to this study, we could look at how soil properties or abiotic disturbances, such as drought, changes the results from this study. Both are important for farming purposes because while the former informs whether crop influences on fungal community is consistent across sites, the latter is relevant due to increasingly variable climatic conditions. Perhaps there are certain environments that would become conducive to diseases and alter fungal community composition and performances of the crops. Such research would allow a more comprehensive understanding of the potentials of the cereal-legume pairings studied in this thesis. Research work is still required to understand the plant root traits mediating microorganism recruitment process. This could allow breeders to combine sets

of favourable plant aboveground and belowground traits to obtain crop varieties targeted for intercropping. Lastly, identifying systematic errors from Illumina sequencing data will be necessary for more accurate taxonomic profiling of microbial community, which will allow us to draw out more accurate conclusions on microbial community. These efforts combined could contribute to the effort in building a more sustainable agricultural practice, and in an increasingly adverse environmental conditions caused by climate change, to efficiently and sustainably to meet feed and food demand.

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Appendix A

A.1 Swiss pea-barley experiment dataset

A.1.1 Supplementary materials to main results

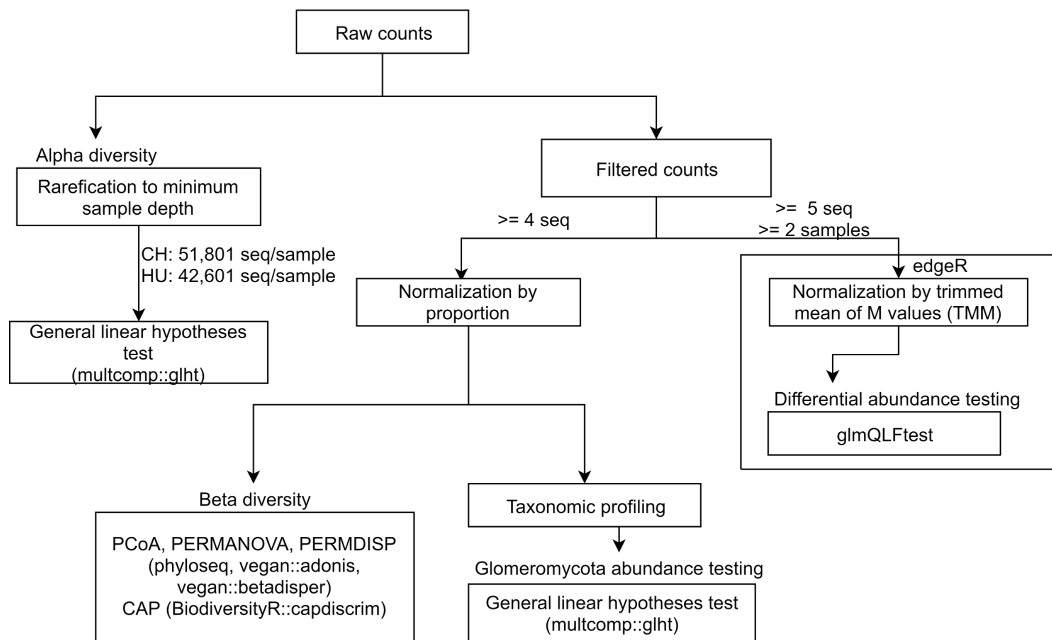

Fig. A.1.: Flowchart outlining data analysis steps.

CH Observed richness

```
         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: User-defined Contrasts


Fit: lmer(formula = O ~ spf + rep + (1 | new_plotID), data = index.dat)

Linear Hypotheses:
                              Estimate Std. Error z value Pr(>|z|)
mixPea - purePea == 0          -22.300      6.678  -3.339   0.0169 *
mixBar - pureBar == 0            4.600      7.901   0.582   1.0000
P13 - P11 == 0                 -13.000     10.559  -1.231   0.9742
P13 - P18 == 0                 -23.750     10.559  -2.249   0.3525
P13 - P23 == 0                 -22.250     10.559  -2.107   0.4559
P13 - Respect == 0             -10.250     10.559  -0.971   0.9971
P11 - P18 == 0                 -10.750     10.559  -1.018   0.9953
P11 - P23 == 0                  -9.250     10.559  -0.876   0.9990
P11 - Respect == 0               2.750     10.559   0.260   1.0000
P18 - P23 == 0                   1.500     10.559   0.142   1.0000
P18 - Respect == 0              13.500     10.559   1.279   0.9650
P23 - Respect == 0              12.000     10.559   1.137   0.9870
mixP11 - pureP11 == 0           -9.000     14.932  -0.603   1.0000
mixP13 - pureP13 == 0            2.000     14.932   0.134   1.0000
mixP18 - pureP18 == 0          -84.500     14.932  -5.659   <0.001 ***
mixP23 - pureP23 == 0          -11.500     14.932  -0.770   0.9998
mixRespect - pureRespect == 0   -8.500     14.932  -0.569   1.0000
mixBarP11 - pureBar == 0         7.500     14.932   0.502   1.0000
mixBarP13 - pureBar == 0         7.000     14.932   0.469   1.0000
mixBarP18 - pureBar == 0        22.500     14.932   1.507   0.8869
mixBarP23 - pureBar == 0        25.000     14.932   1.674   0.7905
mixBarRespect - pureBar == 0    -9.000     14.932  -0.603   1.0000
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Adjusted p values reported -- single-step method)
```

Fig. A.2.: Linear hypothesis test output for observed richness.

Evenness

```
         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: User-defined Contrasts


Fit: lmer(formula = J ~ spf + rep + (1 | new_plotID), data = index.dat)

Linear Hypotheses:
                               Estimate Std. Error z value Pr(>|z|)
mixPea - purePea == 0          0.022632   0.023796   0.951   0.9972
mixBar - pureBar == 0         -0.026919   0.028156  -0.956   0.9970
P13 - P11 == 0                 0.019734   0.037625   0.524   1.0000
P13 - P18 == 0                 0.082408   0.037625   2.190   0.3893
P13 - P23 == 0                -0.043584   0.037625  -1.158   0.9826
P13 - Respect == 0            -0.057735   0.037625  -1.534   0.8662
P11 - P18 == 0                 0.062675   0.037625   1.666   0.7881
P11 - P23 == 0                -0.063318   0.037625  -1.683   0.7765
P11 - Respect == 0            -0.077469   0.037625  -2.059   0.4875
P18 - P23 == 0                -0.125992   0.037625  -3.349   0.0163 *
P18 - Respect == 0            -0.140143   0.037625  -3.725    <0.01 **
P23 - Respect == 0            -0.014151   0.037625  -0.376   1.0000
mixP11 - pureP11 == 0          0.024727   0.053210   0.465   1.0000
mixP13 - pureP13 == 0          0.010710   0.053210   0.201   1.0000
mixP18 - pureP18 == 0          0.038528   0.053210   0.724   0.9998
mixP23 - pureP23 == 0          0.132984   0.053210   2.499   0.2020
mixRespect - pureRespect == 0 -0.093788   0.053210  -1.763   0.7200
mixBarP11 - pureBar == 0       0.009071   0.053210   0.170   1.0000
mixBarP13 - pureBar == 0      -0.044881   0.053210  -0.843   0.9992
mixBarP18 - pureBar == 0      -0.009977   0.053210  -0.187   1.0000
mixBarP23 - pureBar == 0      -0.040358   0.053210  -0.758   0.9997
mixBarRespect - pureBar == 0  -0.012168   0.053210  -0.229   1.0000
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Adjusted p values reported -- single-step method)
```

Fig. A.3.: Linear hypothesis test output for evenness.

CH Shannon diversity

```
         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: User-defined Contrasts


Fit: lmer(formula = H ~ spf + rep + (1 | new_plotID), data = index.dat)

Linear Hypotheses:
                              Estimate Std. Error z value Pr(>|z|)
mixPea - purePea == 0          0.03264    0.12124   0.269   1.0000
mixBar - pureBar == 0         -0.11755    0.14345  -0.819   0.9993
P13 - P11 == 0                 0.03512    0.19169   0.183   1.0000
P13 - P18 == 0                 0.33058    0.19169   1.725   0.7450
P13 - P23 == 0                -0.30301    0.19169  -1.581   0.8386
P13 - Respect == 0            -0.33224    0.19169  -1.733   0.7390
P11 - P18 == 0                 0.29546    0.19169   1.541   0.8604
P11 - P23 == 0                -0.33813    0.19169  -1.764   0.7166
P11 - Respect == 0            -0.36736    0.19169  -1.916   0.5984
P18 - P23 == 0                -0.63359    0.19169  -3.305   0.0192 *
P18 - Respect == 0            -0.66282    0.19169  -3.458   0.0113 *
P23 - Respect == 0            -0.02923    0.19169  -0.152   1.0000
mixP11 - pureP11 == 0          0.08518    0.27109   0.314   1.0000
mixP13 - pureP13 == 0          0.05696    0.27109   0.210   1.0000
mixP18 - pureP18 == 0         -0.08806    0.27109  -0.325   1.0000
mixP23 - pureP23 == 0          0.59368    0.27109   2.190   0.3875
mixRespect - pureRespect == 0 -0.48459    0.27109  -1.788   0.6990
mixBarP11 - pureBar == 0       0.07928    0.27109   0.292   1.0000
mixBarP13 - pureBar == 0      -0.19592    0.27109  -0.723   0.9998
mixBarP18 - pureBar == 0       0.04408    0.27109   0.163   1.0000
mixBarP23 - pureBar == 0      -0.10204    0.27109  -0.376   1.0000
mixBarRespect - pureBar == 0  -0.09601    0.27109  -0.354   1.0000

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Adjusted p values reported -- single-step method)
```

Fig. A.4.: Linear hypothesis test output for Shannon diversity.

CH Glomeromycota hypotheses

```
         Simultaneous Tests for General Linear Hypotheses
Multiple Comparisons of Means: User-defined Contrasts


Fit: lmer(formula = Glomero.abund ~ spf + rep + (1 | new_plotID),
    data = Glomero.dat)

Linear Hypotheses:
                                Estimate Std. Error z value Pr(>|z|)
mixBar - pureBar == 0         -0.0012035  0.0013978  -0.861   0.9992
P13 - P11 == 0                 0.0024202  0.0018679   1.296   0.9612
P13 - P18 == 0                 0.0057816  0.0018679   3.095   0.0383 *
P13 - P23 == 0                -0.0030527  0.0018679  -1.634   0.8161
P13 - Respect == 0             0.0048709  0.0018679   2.608   0.1568
P11 - P18 == 0                 0.0033614  0.0018679   1.800   0.7005
P11 - P23 == 0                -0.0054729  0.0018679  -2.930   0.0640 .
P11 - Respect == 0             0.0024508  0.0018679   1.312   0.9572
P18 - P23 == 0                -0.0088343  0.0018679  -4.730   <0.001 ***
P18 - Respect == 0            -0.0009107  0.0018679  -0.488   1.0000
P23 - Respect == 0             0.0079236  0.0018679   4.242   <0.001 ***
mixP11 - pureP11 == 0          0.0038076  0.0026415   1.441   0.9155
mixP13 - pureP13 == 0         -0.0135982  0.0026415  -5.148   <0.001 ***
mixP18 - pureP18 == 0         -0.0000832  0.0026415  -0.031   1.0000
mixP23 - pureP23 == 0          0.0091284  0.0026415   3.456   0.0115 *
mixRespect - pureRespect == 0 -0.0055782  0.0026415  -2.112   0.4528
mixBarP11 - pureBar == 0      -0.0004611  0.0026415  -0.175   1.0000
mixBarP13 - pureBar == 0      -0.0012510  0.0026415  -0.474   1.0000
mixBarP18 - pureBar == 0      -0.0013343  0.0026415  -0.505   1.0000
mixBarP23 - pureBar == 0      -0.0024311  0.0026415  -0.920   0.9983
mixBarRespect - pureBar == 0  -0.0023843  0.0026415  -0.903   0.9986

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Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Adjusted p values reported -- single-step method)
```

Fig. A.5.: Linear hypothesis test output for Glomeromycota abundance.

Tab. A.1.: List of significant ZOTU vectors fitted onto pea constrained ordination plot, prior to FDR adjustment.

ZOTUs with significant vectors on pea constrained ordination prior to FDR adjustments

Phylum	Class	Order	Family	Genus	Species	LD1	LD2	ZOTU
Ascomycota	NA	NA	NA	NA	NA	0.435408	0.324887	ZOTU311
Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae	Podospora	NA	-0.21213	-0.43669	ZOTU291
Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriaceae	NA	NA	0.598507	-0.18288	ZOTU241
NA	NA	NA	NA	NA	NA	-0.14896	0.688863	ZOTU6
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	NA	NA	0.485987	-0.27554	ZOTU91
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Ilyonectria	Ilyonectria_robusta	0.44058	-0.48211	ZOTU14
NA	NA	NA	NA	NA	NA	0.798887	-0.2055	ZOTU4
Ascomycota	Sordariomycetes	Hypocreales	NA	NA	NA	0.428365	-0.43887	ZOTU215
NA	NA	NA	NA	NA	NA	-0.22576	-0.42386	ZOTU379
Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	Humicola	Humicola_grisea	0.545945	-0.24707	ZOTU83
NA	NA	NA	NA	NA	NA	0.439278	-0.39282	ZOTU236
NA	NA	NA	NA	NA	NA	-0.28778	-0.52389	ZOTU191
Ascomycota	NA	NA	NA	NA	NA	-0.12777	0.720717	ZOTU7
Basidiomycota	NA	NA	NA	NA	NA	0.436545	-0.31419	ZOTU532
Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	NA	NA	-0.08884	-0.52279	ZOTU161
Basidiomycota	NA	NA	NA	NA	NA	-0.20989	-0.73419	ZOTU113
NA	NA	NA	NA	NA	NA	-0.345	-0.66482	ZOTU439
Ascomycota	NA	NA	NA	NA	NA	-0.11173	-0.61507	ZOTU254
NA	NA	NA	NA	NA	NA	-0.13179	-0.59973	ZOTU70
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Exophiala	NA	-0.30762	-0.4761	ZOTU667
NA	NA	NA	NA	NA	NA	0.48283	-0.24542	ZOTU399
Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Exophiala	NA	0.38346	-0.34317	ZOTU1437
NA	NA	NA	NA	NA	NA	0.293265	-0.56433	ZOTU8
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	NA	NA	-0.03011	-0.5226	ZOTU431
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Psathyrella	NA	0.278906	0.531978	ZOTU78
Basidiomycota	Agaricomycetes	Auriculariales	Exidiaceae	NA	NA	-0.27268	-0.51006	ZOTU155
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	NA	NA	-0.22681	-0.42437	ZOTU1753
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Ceratobasidium	NA	-0.27037	-0.442	ZOTU1132
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	NA	NA	-0.1782	-0.45732	ZOTU1129
Basidiomycota	NA	NA	NA	NA	NA	-0.22585	-0.43278	ZOTU602
Basidiomycota	Tremellomycetes	NA	NA	NA	NA	0.333037	-0.37045	ZOTU804
Basidiomycota	NA	NA	NA	NA	NA	-0.15844	-0.46584	ZOTU898
Ascomycota	Dothideomycetes	Pleosporales	NA	NA	NA	0.347359	-0.38803	ZOTU31
NA	NA	NA	NA	NA	NA	-0.22569	-0.42394	ZOTU384
Mortierellomycota	Mortierellomycetes	NA	NA	NA	NA	-0.36199	-0.37739	ZOTU625
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	Mortierella	NA	0.326881	-0.37243	ZOTU1592
Mortierellomycota	Mortierellomycetes	Mortierellales	NA	NA	NA	0.525329	-0.19155	ZOTU77
Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	Mortierella	NA	0.34762	-0.41022	ZOTU285
Glomeromycota	Paraglomeromycetes	Paraglomerales	NA	NA	NA	0.583863	-0.13599	ZOTU1223
Glomeromycota	Glomeromycetes	NA	NA	NA	NA	-0.19969	-0.54513	ZOTU1853
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	NA	NA	0.631921	-0.12608	ZOTU1463
NA	NA	NA	NA	NA	NA	0.426355	-0.48521	ZOTU128
Glomeromycota	Glomeromycetes	Glomerales	Glomeraceae	NA	NA	0.415419	-0.37128	ZOTU1052

A.1.2 Additional analyses

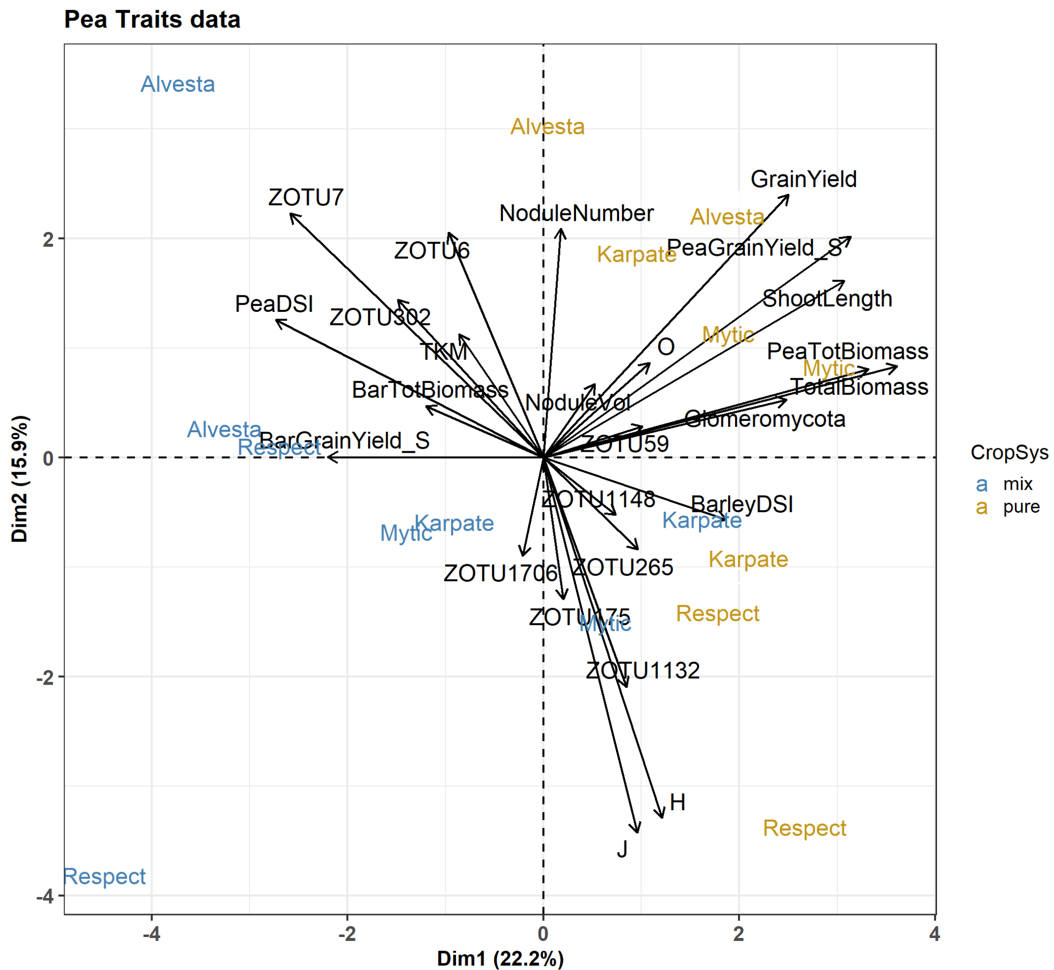
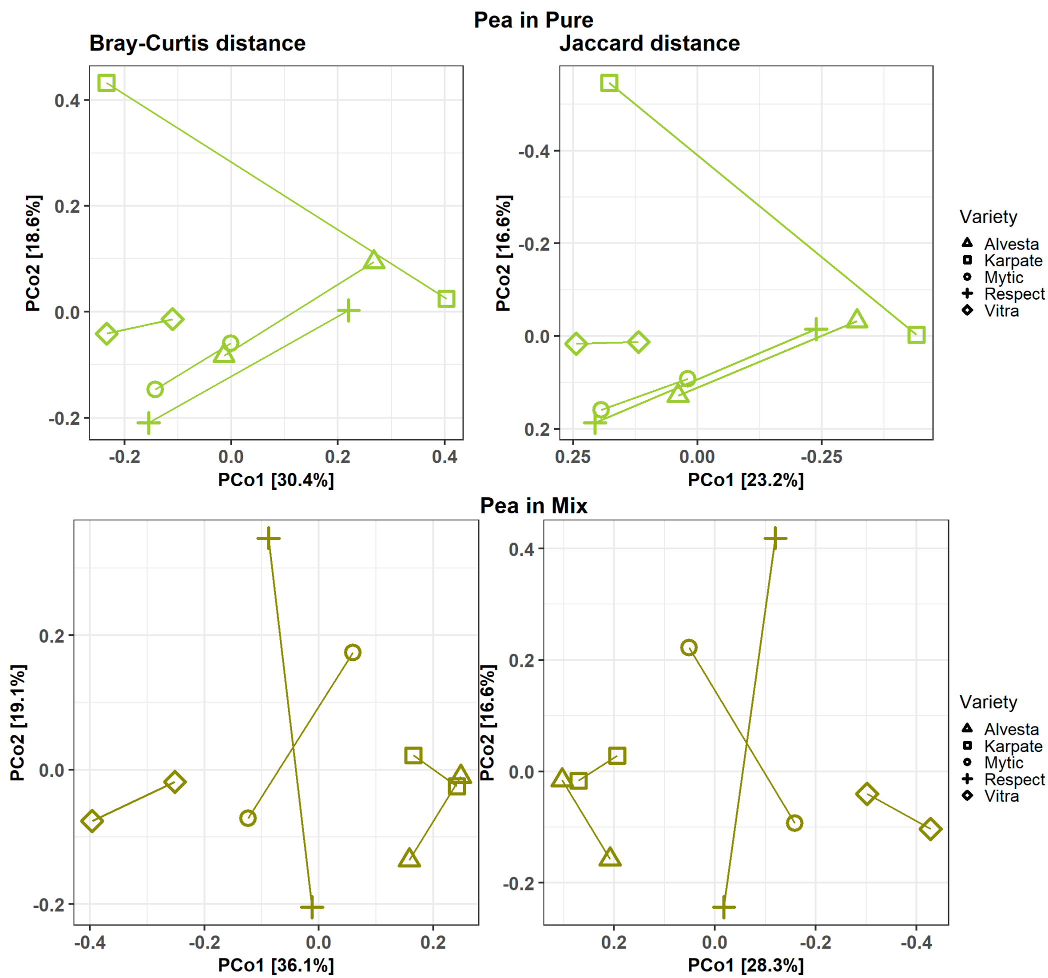


Fig. A.6.: PCA visualizing pea fungal community data without Vitra, with differentially abundant ZOTUs, and selected pea and barley agronomic traits. The barley traits included only intercropping data. Where 'Bar' was indicated, the variable was a barley trait, where absent, it is a pea trait. The variables abbreviations include 'H' for Shannon diversity, 'J' for evenness, 'O' for observed richness, 'GrainY_S' for scaled component grain yield. 'Glom' for Glomeromycota abundance, 'TKM' for thousand kernel mass. 'TotBiomass' for total biomass. 'NoduleVol' for pea nodule volume. Missing values of Vitra grain yield were imputed by the mean.



	Bray-Curtis distance							*Jaccard distance*						
	PERMANOVA (9999 permutations)				PERMDISP			PERMANOVA (9999 permutations)				PERMDISP		
	Factors	DF	Pseudo-F	Pr(>F)	DF	F	Pr(<F)	Factors	DF	Pseudo-F	Pr(>F)	DF	F	Pr(<F)
Pea in pure	Variety	4	0.927	0.595		N/A		Variety	4	0.988	0.49		N/A	
	Block	1	0.816	0.643				Block	1	0.868	0.659			
Pea in mix	Variety	4	2.048	0.003 **		N/A		Variety	4	1.691	0.003 **		N/A	
	Block	1	1.747	0.094				Block	1	1.421	0.117			

Fig. A.7.: Separate PCoA and PERMANOVA , PERMDISP analysis for CH pea by cropping system.

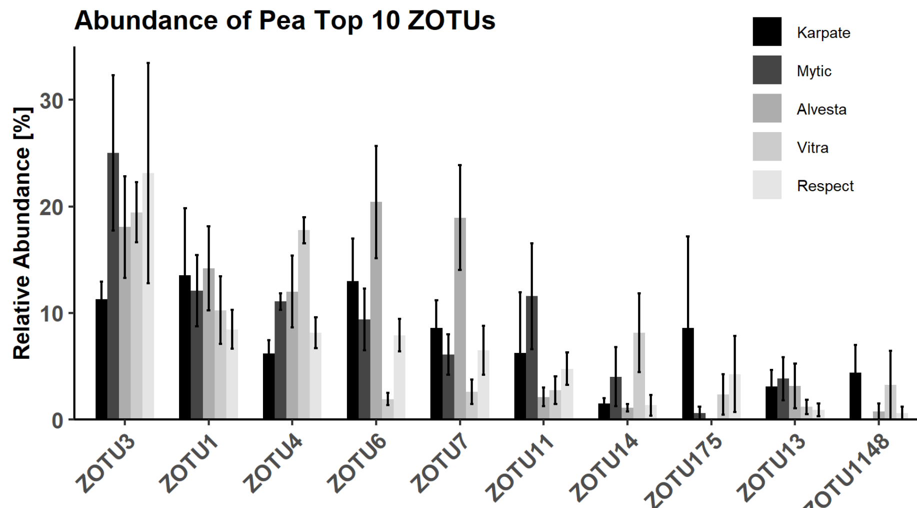


Fig. A.8.: Top 10 ZOTUs in pea roots grouped by variety. Error bars show the standard error of the means (n=2).

Tab. A.2.: Top 10 ZOTUs in pea roots in intercropping and pure stands with mean relative abundance and standard error. *BLAST results were provided only when meeting parameters (refer to Chapter 2).

CH Pea

Phylum	Class	Lowest level of taxonomic assignment by UNITE	Lowest level of taxonomic assignment by BLAST*	ZOTU	Mean RelAbund (%)	SE
			Mix			
NA	NA	Fungi (Kingdom)	Fusarium (Genus)	ZOTU3	17.74	3.32
Ascomycota	Sordariomycetes	Microascales (Order)	Fungi (Kingdom)	ZOTU1	13.74	2.65
Ascomycota	NA	Ascomycota (Phylum)	Didymellaceae (Family)	ZOTU7	10.68	2.80
NA	NA	Fungi (Kingdom)	Plectosphaerella (Genus)	ZOTU6	10.59	1.99
NA	NA	Fungi (Kingdom)	Nectriaceae (Family)	ZOTU4	9.51	1.26
NA	NA	Fungi (Kingdom)	Hypocreales (Order)	ZOTU11	5.75	2.43
NA	NA	Fungi (Kingdom)	Clonostachys rosea (Species)	ZOTU13	3.70	1.19
Ascomycota	Sordariomycetes	Ilyonectria robusta (Species)	Ilyonectria (Genus)	ZOTU14	3.48	1.27
Basidiomycota	Agaricomycetes	Ceratobasidiaceae (Family)	Ceratobasidium (Genus)	ZOTU175	2.27	1.60
Basidiomycota	Agaricomycetes	Ceratobasidiaceae (Family)	Ceratobasidium (Genus)	ZOTU1706	2.00	1.23
			Pure			
NA	NA	Fungi (Kingdom)	Fusarium (Genus)	ZOTU3	21.02	4.39
NA	NA	Fungi (Kingdom)	Nectriaceae (Family)	ZOTU4	12.56	1.90
NA	NA	Fungi (Kingdom)	Plectosphaerella (Genus)	ZOTU6	10.46	3.41
Ascomycota	Sordariomycetes	Microascales (Order)	Fungi (Kingdom)	ZOTU1	9.66	1.93
Ascomycota	NA	Ascomycota (Phylum)	Didymellaceae (Family)	ZOTU7	6.39	1.87
NA	NA	Fungi (Kingdom)	Hypocreales (Order)	ZOTU11	5.21	2.21
Basidiomycota	Agaricomycetes	Ceratobasidiaceae (Family)	Ceratobasidium (Genus)	ZOTU175	4.06	3.38
Ascomycota	Sordariomycetes	Ilyonectria robusta (Species)	Ilyonectria (Genus)	ZOTU14	2.94	1.73
Basidiomycota	NA	Basidiomycota (Phylum)	Ceratobasidium (Genus)	ZOTU1148	2.24	1.37
Ascomycota	Sordariomycetes	Sordariaceae (Family)	Sordariomycetes (Class)	ZOTU53	1.96	0.87

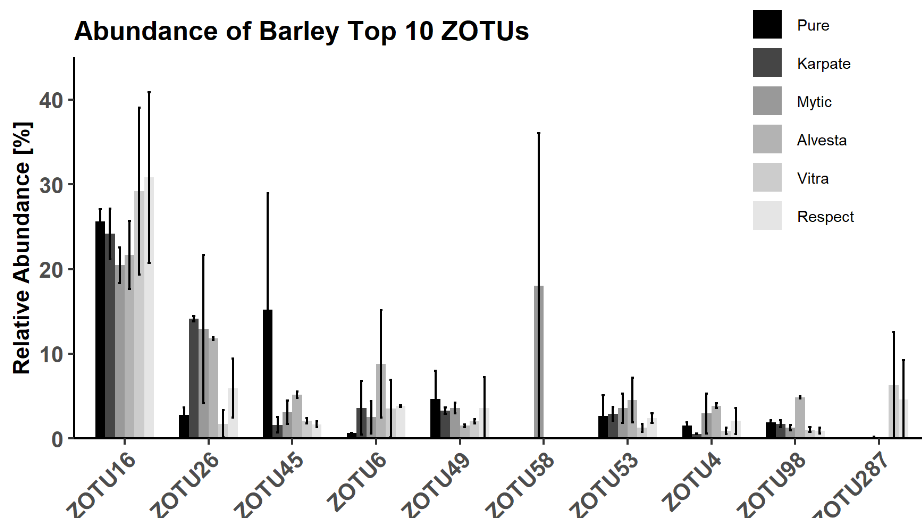


Fig. A.9.: Top 10 ZOTUs in barley roots grouped by plot. Error bars show the standard error of the means (n=2).

Tab. A.3.: Top 10 ZOTUs in barley roots in intercropping and pure stands with mean relative abundance and standard error. *BLAST results were provided only when meeting parameters (refer to Chapter 2).

CH Barley

Phylum	Class	Lowest level of taxonomic assignment by UNITE	Lowest level of taxonomic assignment by BLAST*	ZOTU	Mean RelAbund (%)	SE
			Mix			
NA	NA	Fungi (Kingdom)	Microdochium bolleyi (Species)	ZOTU16	25.26	2.63
NA	NA	Fungi (Kingdom)	Microdochium nivale (Species)	ZOTU26	9.31	2.12
NA	NA	Fungi (Kingdom)	Plectosphaerella (Genus)	ZOTU6	4.46	1.41
Ascomycota	Eurotiomycetes	Eurotiomycetes (Class)	Eurotiales (Order)	ZOTU58	3.61	3.61
Ascomycota	Sordariomycetes	Sordariaceae (Family)	Sordariomycetes (Class)	ZOTU53	2.93	0.62
Ascomycota	Sordariomycetes	Sordariomycetes (Class)	Myrmecridium schulzeri (Species)	ZOTU49	2.80	0.63
Ascomycota	NA	Ascomycota (Phylum)	Sordariales (Order)	ZOTU45	2.72	0.51
Basidiomycota	Agaricomycetes	Trechisporales (Order)	Fungi (Phylum)	ZOTU287	2.20	1.47
NA	NA	Fungi (Kingdom)	Nectriaceae (Family)	ZOTU4	2.09	0.59
Ascomycota	Dothideomycetes	Pleosporales (Order)	Fungi (Phylum)	ZOTU87	2.05	1.72
			Pure			
NA	NA	Fungi (Kingdom)	Microdochium bolleyi (Species)	ZOTU16	25.64	1.42
Ascomycota	NA	Ascomycota (Phylum)	Sordariales (Order)	ZOTU45	15.23	13.72
Ascomycota	Sordariomycetes	Sordariomycetes (Class)	Myrmecridium schulzeri (Species)	ZOTU49	4.64	3.35
Ascomycota	NA	Ascomycota (Phylum)	Fungi (Phylum)	ZOTU105	4.55	4.55
NA	NA	Fungi (Kingdom)	Microdochium nivale (Species)	ZOTU26	2.79	0.85
Ascomycota	Sordariomycetes	Sordariaceae (Family)	Sordariomycetes (Class)	ZOTU53	2.63	2.49
Ascomycota	Sordariomycetes	Chaetomiaceae (Family)	Apodus deciduus (Species)	ZOTU672	2.04	1.92
NA	NA	Fungi (Kingdom)	Fusarium (Genus)	ZOTU3	2.01	0.96
Ascomycota	Dothideomycetes	Alternaria (Genus)	Alternaria infectoria (Species)	ZOTU98	1.93	0.25
Basidiomycota	Agaricomycetes	Agaricomycetes (Class)	Fungi (Phylum)	ZOTU415	1.70	1.70

A.2 Hungary wheat-pea dataset

A.2.1 Supplementary materials to main results

HU Observed richness

```
         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: User-defined Contrasts


Fit: lmer(formula = O ~ spf + rep + (1 | new_plotID), data = index.dat.
hu)

Linear Hypotheses:
                           Estimate Std. Error z value Pr(>|z|)
mixWhe - pureWhe == 0        -4.556     11.271  -0.404   0.9992
Kol - Kom == 0               -3.333     13.804  -0.241   1.0000
Kol - Nad == 0               13.000     13.804   0.942   0.9310
Kom - Nad == 0               16.333     13.804   1.183   0.8221
mixKol - pureKol == 0       -17.667     19.522  -0.905   0.9424
mixKom - pureKom == 0         1.667     19.522   0.085   1.0000
mixNad - pureNad == 0         2.333     19.522   0.120   1.0000
mixPeaKol - mixPeaKom == 0   59.667     19.522   3.056   0.0168 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Adjusted p values reported -- single-step method)
```

HU Evenness

```
         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: User-defined Contrasts


Fit: lm(formula = J ~ spf + rep, data = index.dat.hu)

Linear Hypotheses:
                           Estimate Std. Error t value Pr(>|t|)
mixWhe - pureWhe == 0       0.08497    0.04411   1.926    0.373
Kol - Kom == 0              0.03700    0.05402   0.685    0.982
Kol - Nad == 0              0.01549    0.05402   0.287    1.000
Kom - Nad == 0             -0.02151    0.05402  -0.398    0.999
mixKol - pureKol == 0       0.16235    0.07639   2.125    0.278
mixKom - pureKom == 0       0.02529    0.07639   0.331    1.000
mixNad - pureNad == 0       0.06726    0.07639   0.880    0.944
mixPeaKol - mixPeaKom == 0  0.05745    0.07639   0.752    0.973
(Adjusted p values reported -- single-step method)
```

Fig. A.10.: Linear hypothesis test output for observed richness and evenness.

HU Shannon diversity

```
        Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: User-defined Contrasts


Fit: lm(formula = H ~ spf + rep, data = index.dat.hu)

Linear Hypotheses:
                           Estimate Std. Error t value Pr(>|t|)
mixWhe - pureWhe == 0       0.31025    0.21604   1.436    0.669
Kol - Kom == 0              0.11093    0.26460   0.419    0.999
Kol - Nad == 0              0.17217    0.26460   0.651    0.986
Kom - Nad == 0              0.06124    0.26460   0.231    1.000
mixKol - pureKol == 0       0.50929    0.37419   1.361    0.716
mixKom - pureKom == 0       0.14068    0.37419   0.376    0.999
mixNad - pureNad == 0       0.28078    0.37419   0.750    0.973
mixPeaKol - mixPeaKom == 0  0.79582    0.37419   2.127    0.277
(Adjusted p values reported -- single-step method)
```

HU Glomeromycota hypotheses

```
               Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: User-defined Contrasts


Fit: lm(formula = Glomero.abund ~ spf + rep, data = Glomero.dat)

Linear Hypotheses:
                           Estimate Std. Error t value Pr(>|t|)
mixWhe - pureWhe == 0       0.07258    0.02595   2.797   0.0929 .
Kol - Kom == 0              0.02123    0.03178   0.668   0.9843
Kol - Nad == 0             -0.01109    0.03178  -0.349   0.9995
Kom - Nad == 0             -0.03232    0.03178  -1.017   0.8963
mixKol - pureKol == 0       0.07747    0.04494   1.724   0.4903
mixKom - pureKom == 0       0.03755    0.04494   0.836   0.9548
mixNad - pureNad == 0       0.10272    0.04494   2.285   0.2199
mixPeaKol - mixPeaKom == 0  0.10478    0.05096   2.056   0.3128
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Adjusted p values reported -- single-step method)
```

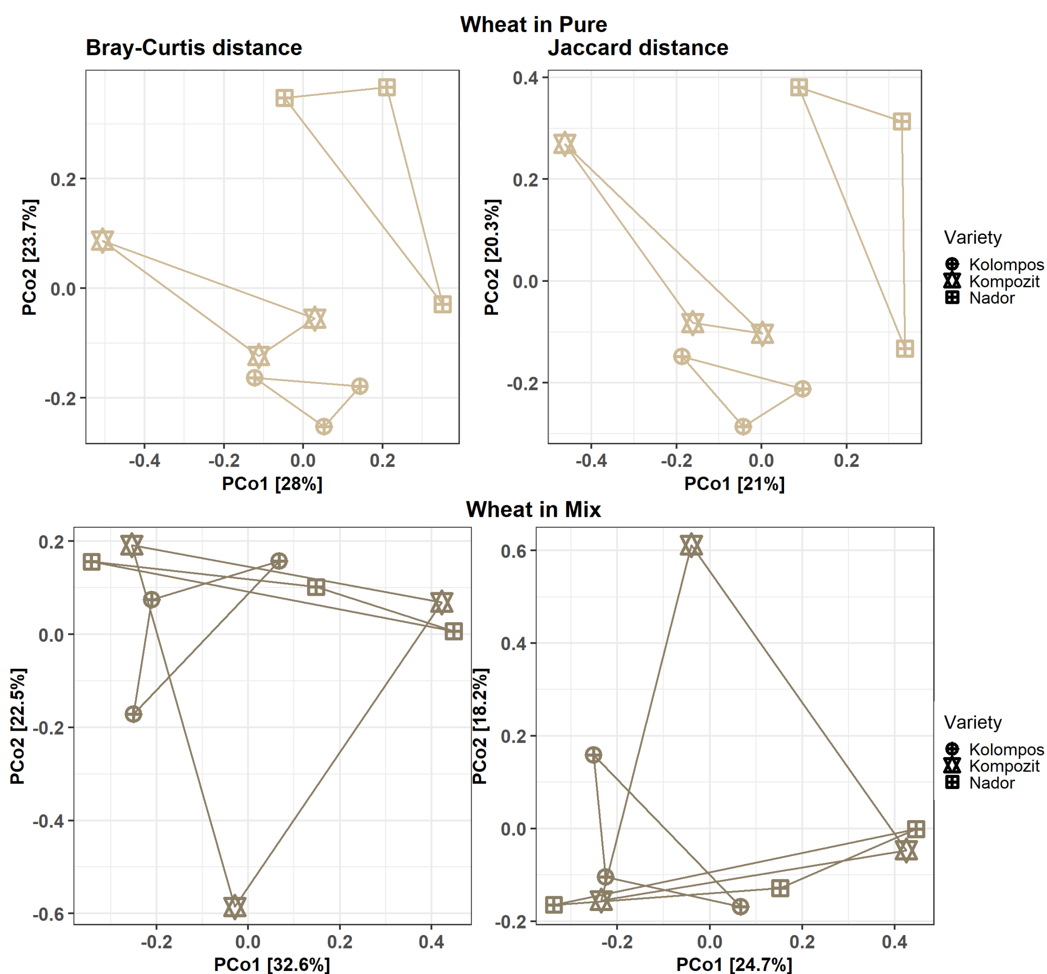
Fig. A.11.: Linear hypothesis test output for Shannon diversity and Glomeromycota abundance.

Tab. A.4.: List of significant ZOTU vectors fitted onto wheat constrained ordination plot, prior to FDR adjustment.

ZOTUs with significant vectors on wheat constrained ordination prior to FDR adjustments

Phylum	Class	Order	Family	Genus	Species	LD1	LD2	BLAST result*	ZOTU
NA	NA	NA	NA	NA	NA	-0.502955549	0.569842812	g_Plectosphaerella	ZOTU6
Ascomycota	Sordariomycetes	Branch06	NA	NA	NA	-0.156215088	-0.60604697	p_Ascomycota	ZOTU816
Ascomycota	Sordariomycetes	Hypocreales	NA	NA	NA	-0.278502958	0.484315663	k_Fungi	ZOTU731
Ascomycota	Sordariomycetes	Hypocreales	Stachybotryaceae	NA	NA	-0.279138318	0.483829192	s_Achroiostachys_humicola	ZOTU808
Ascomycota	Sordariomycetes	NA	NA	NA	NA	-0.205391175	-0.543711922		ZOTU343
Ascomycota	NA	NA	NA	NA	NA	-0.279014446	0.483705161	f_Lasiosphaeriaceae	ZOTU709
NA	NA	NA	NA	NA	NA	-0.380980629	0.434230345	k_Fungi	ZOTU245
NA	NA	NA	NA	NA	NA	0.505349489	0.483937962	p_Ascomycota	ZOTU646
Ascomycota	Sordariomycetes	NA	NA	NA	NA	-0.413494841	0.487085701	f_Lasiosphaeriaceae	ZOTU122
Ascomycota	Sordariomycetes	Sordariales	NA	NA	NA	-0.279138318	0.483829192	g_Chaetomium	ZOTU1442
NA	NA	NA	NA	NA	NA	-0.279138318	0.483829192	s_Chaetomium_globosum	ZOTU223
Ascomycota	Pezizomycetes	Pezizales	Pyronemataceae	NA	NA	-0.279138318	0.483829192	f_Pyronemataceae	ZOTU1117
Ascomycota	Dothideomycetes	Pleosporales	NA	NA	NA	0.06969523	-0.642673407		ZOTU789
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Stemphylium	NA	-0.279806308	0.483927027	g_Stemphylium	ZOTU728
NA	NA	NA	NA	NA	NA	-0.326197411	0.483927065	k_Fungi	ZOTU80
NA	NA	NA	NA	NA	NA	-0.279138318	0.483829192	k_Fungi	ZOTU1680
NA	NA	NA	NA	NA	NA	-0.014556898	-0.823445852	s_Alternaria_longissima	ZOTU39
NA	NA	NA	NA	NA	NA	0.546037016	-0.14060055	p_Ascomycota	ZOTU686
NA	NA	NA	NA	NA	NA	0.540323196	0.144695398	p_Ascomycota	ZOTU1172
Ascomycota	NA	NA	NA	NA	NA	-0.279138318	0.483829192	k_Fungi	ZOTU1024
Ascomycota	NA	NA	NA	NA	NA	-0.361529265	-0.523595844	s_Periconia_macrospinosa	ZOTU10
Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	NA	NA	-0.246742017	0.586125529	s_Rhizoctonia solani	ZOTU385
Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Conocybe	Conocybe_deliquescens	-0.304609613	0.475672009	s_Conocybe_deliquescens	ZOTU152

A.2.2 Additional analyses



	Bray-Curtis distance							*Jaccard distance*						
	PERMANOVA (9999 permutations)				PERMDISP			PERMANOVA (9999 permutations)				PERMDISP		
	Factors	DF	Pseudo-F	Pr(>F)	DF	F	Pr(<F)	Factors	DF	Pseudo-F	Pr(>F)	DF	F	Pr(<F)
Wheat in pure	**Variety**	**2**	**1.654**	**0.043 ***		N/A		**Variety**	**4**	**1.406**	**0.037 ***		N/A	
	Block	2	1.028	0.446				Block	1	1.04	0.4			
Wheat in mix	Variety	2	0.934	0.553		N/A		Variety	4	0.979	0.499		N/A	
	Block	2	0.876	0.63				Block	1	0.926	0.621			

Fig. A.12.: Separate PCoA and PERMANOVA, PERMDISP analysis for HU wheat by cropping system.

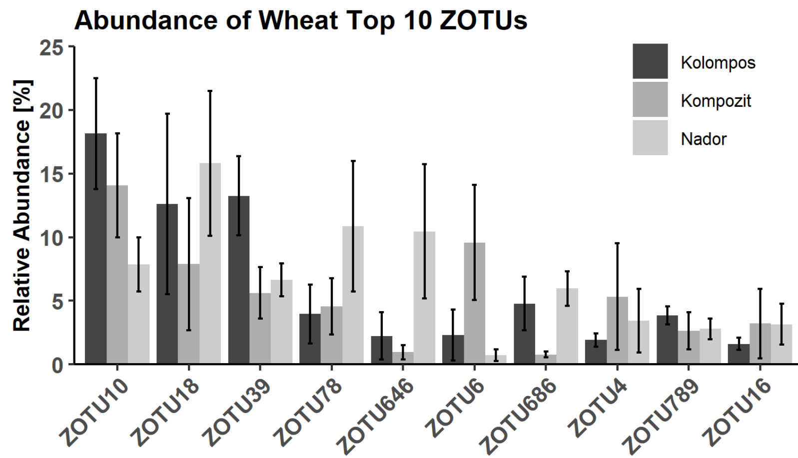


Fig. A.13.: Top 10 ZOTUs in wheat roots grouped by variety. Error bars show the standard error of the means (n=3).

Tab. A.5.: Top 10 ZOTUs in wheat roots in intercropping and pure stands with mean relative abundance and standard error. *BLAST results were provided only when meeting parameters (refer to Chapter 2).

HU Wheat

Phylum	Class	Lowest level of taxonomic assignment by UNITE	Lowest level of taxonomic assignment by BLAST*	ZOTU	Mean RelAbund (%)	SE
			Mix			
NA	NA	Fungi (Kingdom)	Fungi (Kingdom)	ZOTU18	11.88	4.93
Ascomycota	NA	Ascomycota (Phylum)	Periconia macrospinosa (Species)	ZOTU10	9.09	2.24
NA	NA	Fungi (Kingdom)	Alternaria longissima (Species)	ZOTU39	6.01	1.56
NA	NA	Fungi (Kingdom)	Ascomycota (Phylum)	ZOTU686	5.49	1.67
NA	NA	Fungi (Kingdom)	Nectriaceae (Family)	ZOTU4	5.49	3.01
Basidiomycota	Agaricomycetes	Psathyrella (Genus)		ZOTU78	4.64	1.72
NA	NA	Fungi (Kingdom)	Plectosphaerella (Genus)	ZOTU6	3.78	2.20
Ascomycota	Dothideomycetes	Pleosporales (Order)		ZOTU789	3.24	0.98
Ascomycota	Dothideomycetes	Pleosporales (Order)	Paraphoma chrysanthemicola (Species)	ZOTU50	3.15	0.74
NA	NA	Fungi (Kingdom)	Ascomycota (Phylum)	ZOTU646	3.10	1.50
			Pure			
Ascomycota	NA	Ascomycota (Phylum)	Periconia macrospinosa (Species)	ZOTU10	17.63	3.42
NA	NA	Fungi (Kingdom)	Fungi (Kingdom)	ZOTU18	12.33	4.91
NA	NA	Fungi (Kingdom)	Alternaria longissima (Species)	ZOTU39	11.00	2.30
Basidiomycota	Agaricomycetes	Psathyrella (Genus)		ZOTU78	8.29	3.73
NA	NA	Fungi (Kingdom)	Ascomycota (Phylum)	ZOTU646	5.99	3.86
NA	NA	Fungi (Kingdom)	Plectosphaerella (Genus)	ZOTU6	4.63	3.02
NA	NA	Fungi (Kingdom)	Microdochium bolleyi (Species)	ZOTU16	3.42	1.97
Ascomycota	Dothideomycetes	Pleosporales (Order)		ZOTU789	2.94	0.70
Ascomycota	Sordariomycetes	Sordariomycetes (Class)	Lasiosphaeriaceae (Family)	ZOTU122	2.77	2.45
NA	NA	Fungi (Kingdom)	Xylariales (Order)	ZOTU81	2.20	1.86

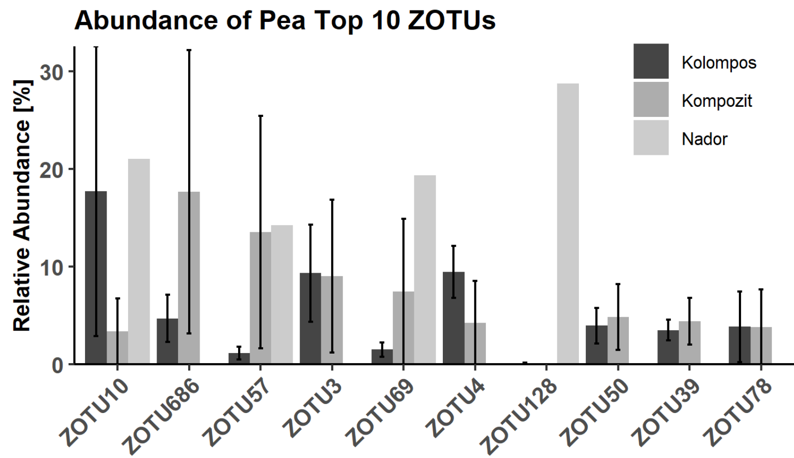


Fig. A.14.: Top 10 ZOTUs in HU pea roots grouped by plot. Error bars show the standard error of the means (n=3 for pea in Kolompos and Kompozit).

Tab. A.6.: Top 10 ZOTUs in HU pea roots in intercropping stands with BLAST result.

HU Pea				
Phylum	**Class**	**Lowest level of taxonomic assignment by UNITE**	**Lowest level of taxonomic assignment by BLAST***	**ZOTU**
Ascomycota	NA	Ascomycota (Phylum)	Periconia macrospinosa (Species)	ZOTU10
NA	NA	Fungi (Kingdom)	Ascomycota (Phylum)	ZOTU686
NA	NA	Fungi (Kingdom)	Glomeromycota (Phylum)	ZOTU57
NA	NA	Fungi (Kingdom)	Fusarium (Genus)	ZOTU3
Glomeromycota	NA	Glomeromycota (Phylum)	Glomeraceae (Family)	ZOTU69
NA	NA	Fungi (Kingdom)	Nectriaceae (Family)	ZOTU4
NA	NA	Fungi (Kingdom)		ZOTU128
Ascomycota	Dothideomycetes	Pleosporales (Order)	Paraphoma chrysanthemicola (Species)	ZOTU50
NA	NA	Fungi (Kingdom)	Alternaria longissima (Species)	ZOTU39
Basidiomycota	Agaricomycetes	Psathyrella (Genus)		ZOTU78

Scope of Tasks: Edited from Master Thesis Research Proposal

Submitted: 27 May 2019
Ming-Hui Hsung, ETHZ MSc student
Collaboration with FiBL (CH), Supervisor: Dr. Pierre Hohmann
ETH Supervisor: Dr. Martin Hartmann

Introduction

(eliminated)

Objectives

The overall goal is to understand how cereal-legume cropping system and plant genotypes effect soil fungal community structure

a) Compare and explore different diversity indices and relative abundances of key OTUs/taxa among different plant varieties in sole and mixed cropping system of pea with barley or wheat.
b) Relate fungal community structure to below- and aboveground agronomic data.

Materials and work plan

<table>
<tr>
<td>Materials
Swiss trial:
1 barley variety: KWS Atrika
5 pea varieties: Karpate, Mytic, Alvesta, Vitra, Respect
Hungarian trial:
3 wheat varieties: Nádor, Kolompos, Kompozit
1 pea variety: (TBA)
Experimental setup
CH: mixed and pure stands of all combinations, 2 replicates, randomised block design
Hungary: mixed stands and wheat pure stands, 3 replicates, randomised block design</td>
<td>Work plan
Harvest, assessments, sample processing
DNA extraction
Clean up OTU data (which to keep/remove)
Data analysis with R</td>
</tr>
</table>

Work schedule

	May	Jun	Jul	Aug	Sep	Oct	Nov
Research proposal and literature review	x						
R script review	x	x					
Harvest work		x					
Lab work		x					
Analysis work		x	x	x	x	x	
Final thesis writing						x	x

References

(eliminated)

Eidgenössische Technische Hochschule Zürich
Swiss Federal Institute of Technology Zurich

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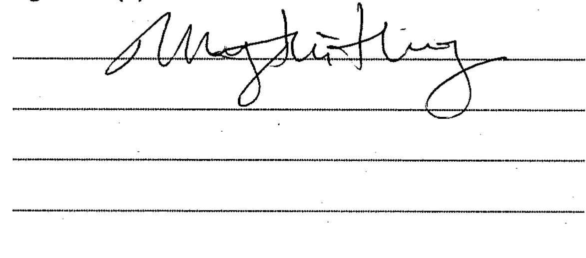

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