RESEARCH

Fungal Microbiome Indicators Are Associated with Genotypic Variation in Pea Root Rot Susceptibility when Intercropped with Barley

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ABSTRACT

Intercropping of legume and cereal crop species shows potential to reduce root disease pressures by changing root-associated microbiomes and improving nitrogen (N) use via soil N-dependent fixation of atmospheric N_2 by symbiotic rhizobia. A two-year field study was conducted to evaluate the effect of pea–barley association on crop performance and on the root fungal community. Five pea cultivars (Alvesta, Karpate, Mytic, Respect, and Vitra) were grown either in pure stands or mixed with one variety of barley (Atrika). We measured crop grain yield and root rot incidence and analyzed root fungal communities. In mixed stands, total grain yield was more stable compared with that in each pure stand, but pea root disease incidence was higher except for cultivars Vitra and Karpate. The effect of cropping system on fungal alpha diversity depended on the cultivar, with Vitra showing higher Shannon diversity and Alvesta showing lower richness in mixed compared with pure stands. All four operational taxonomic units (OTUs) belonging to the Didymellaceae family were positively associated with pea root rot, and another disease-associated OTU in pea, *Neoascochyta exitialis*, was found to be also part of the barley core microbiome. Eleven of twelve OTUs belonging to the Glomeraceae family were associated with healthy roots and abundant in cultivar Vitra. This study shows how the phenotype and fungal microbiome of different pea cultivars respond distinctly to intercropping. Furthermore, the identification of disease- and health-associated taxa in the pea root fungal community refines the characterization of different cultivar candidates for intercropping.

Keywords: AMF, disease resistance, fungal diversity, legumes, mixed cropping, on-farm experiment, pea root rot complex, pea–barley intercropping, phytopathology, plant breeding, root mycobiome

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The green revolution increased agricultural homogeneity and relied on inputs to ensure yield and protection against pests and diseases, but this is not sustainable because of pollution, energy use, climate change, and increasing stress vulnerability (Tilman et al. 2001). Increasing plant diversity in agriculture promotes stability and resilience by utilizing inherent resources and functions (Bender et al. 2016; Wagg et al. 2021). Temporal crop diversification in the form of rotations is already widely adopted in many countries to improve soil properties and organic matter and resource efficiency, as well as to break pest lifecycles (Bullock 1992). On the other hand, spatial crop diversification, such as the use of intercropping, still faces biological and technical limitations (Dierauer et al. 2017).

Legume–cereal intercropping, a common practice, effectively reduces nitrogen (N) competition between crops. Legumes have the ability to switch their N-acquisition strategy from soil uptake to atmospheric N fixation through symbiotic rhizobia (Bedoussac et al. 2015; Duchene et al. 2017; Wendling et al. 2017). Additionally, intercropping allows for a more efficient use of light owing to different growth dynamics, which can lead to increased overall crop yields. Furthermore, intercropping helps protect legumes from lodging and reduces weed pressure by occupying space. Below ground, legume–cereal intercropping exhibits complementarity as the roots of different crops explore different soil horizons, owing to either their inherent growth strategies (e.g., shallow maize root system) or their ability to adapt to neighboring crops (e.g., faba bean roots growing deeper in the presence of wheat; Bargaz et al. 2015). Ultimately, successful coexistence and complementarity between legumes and cereals in intercropping systems depend on traits associated with competition for light during flowering and early-stage nitrogen availability. These traits include stem length, early vigor, and leaf size (Haug et al. 2023).

Repeated legume cropping in the same field causes a phenomenon called "soil fatigue," which is a combination of pathogen buildup, microbial dysbiosis, plant disease, and ultimately yield reduction (Wille et al. 2019). Pea root rot is caused by various fungal and oomycetal pathogens, making control measures difficult and requiring long breaks in crop rotations (Katan 2017). Plant-associated microbes can play a significant role in protection against pathogens through niche competition, direct antagonisms, and induced systemic resistance (Berendsen et al. 2012; Pieterse et al. 2014; Vannier et al. 2019). Root exudation plays a vital role in controlling the plant-associated microbiome (Sasse et al. 2018). To develop disease-resistant plants, selection strategies should consider their interactions with entire microbiomes (Hohmann et al. 2020; Wille et al. 2021).

Barley is commonly used in intercropping systems with pea because of their similar maturation timings, typically early in the year. This combination is advantageous as it allows for the two crops to be used together as animal feed, simplifying the harvest and facilitating market valorization of the mixture. Furthermore, barley was shown to reduce the incidence of soil-borne disease (Hauggaard-Nielsen et al. 2008), possibly by altering the root-associated microbiota. This study aimed at assessing the effect of intercropping pea with barley on (i) agronomic performance as well as (ii) the structure and diversity of the fungal community of pea and barley. We further characterized the pea and barley core communities. Finally, (iii) we explored the associations between the agronomic performance of pea and barley and the root fungal microbiome indicators. We hypothesized that both agronomic performance and fungal diversity would be higher in intercropped plots compared with that in pure plots. We further hypothesize that different pea genotypes will react differently to intercropping. To test these hypotheses, we performed a 2-year field experiment with five pea cultivars and one barley variety in a Swiss on-farm field trial.

MATERIALS AND METHODS

Field trial. The pea–barley cropping trials from which a subset was analyzed in this study were two directly neighboring fields located in Fislisbach, Switzerland, respectively used in the 2018 (47.425641N, 8.292059E) and 2019 (47.425233N, 8.293311E) growing seasons. The fields consisted of pure and intercropped stands of pea and barley with two replicated blocks and partial randomization of the cropping stands (Supplementary Table S1). The samples investigated in this study consisted of five pea (*Pisum sativa* L.) cultivars, Alvesta, Karpate, Mytic, Respect, and Vitra, and one

barley (*Hordeum vulgare* L.) cultivar, Atrika. Cultivars differed in several phenotypical traits. Stem length was in the "short" category and semi-leaves were absent for Karpate, Mytic, Alvesta, and Respect; the stem was "long" and semi-leaves were present for Vitra. Early vigor was medium for Karpate and Alvesta, low for Mytic, and high for Vitra. Karpate and Mytic were produced in France, Alvesta in Germany, and Vitra in Latvia. Barley cultivar Atrika, produced in Germany, was chosen for this experiment because its phenotypical traits (stem length, flag leaf size, early height, plancophile/erectophile, tillering capacity) identified it as an average competitor compared with other European two-row barley (Haug et al. 2021). Cumulative air temperatures for the growing season between 1 March and 18 July (140 days) were collected from the nearby meteorological stations at Lupfig and Künten and averaged across both stations, amounting to 1,929°C for 2018 and 1,799°C for 2019. Cumulative precipitations were collected from the Agrometeo database (agrometeo.ch) and amounted to 315 liters m^{-2} in 2018 and 333 liters m^{-2} in 2019. The field soil of the 2018 trial was a sandy loam with brown earth (14.1% clay, 31.3% silt, 52.2% sand, and 2.4% humus), whereas the field soil of the 2019 trial was a loam with brown earth (14.6% clay, 44.5% silt, 38.2% sand, and 2.7% humus). The mineral N and Mn contents of the two soils, measured at sowing time, were 41.9 kg/ha and 294 mg/ha, respectively, in 2018 and 35.0 kg ha^{-1} and 241 mg ha^{-1}, respectively, in 2019 (Haug et al. 2023). The preceding crop was lucerne (alfalfa) as a cover mixture in the 2018 field and winter wheat in the 2019 field.

Sample collection and plant trait assessment. Root sample collection was performed at 50% flowering. Plants were dug up from opposite subplots, keeping the roots intact. These subplots were situated 1 m into the length of the plot and in the second row to avoid border effect. For pure stands, three plants were sampled per subplot, resulting in six plants in total per plot. For intercropping stands, three of each plant species (barley and pea) were harvested from each of the two indicated subplot spots in the plot. The roots coming from the same plot were homogenized, separating plant species in the case of mixed stands, cut, and washed. Plant root samples were frozen at –20°C immediately after harvest and lyophilized 1 day later. Samples were stored at –20°C until DNA extraction.

Pea traits were assessed on the day of root sample collection and included root disease score, shoot length, and nodule number. The assessment of the root disease score was conducted as described in detail. Shoot length was measured without artificially erecting the plants. After the roots were carefully washed, nodules were counted. The assessment of these three parameters was conducted as described in detail by Wille et al. (2020). Postharvest traits included total grain yield and fraction yield from pea and barley in intercropping stands. Grain protein content was measured by near-infrared transmittance technology (FOSS Infratec Grain Analyzer 1241, Denmark) according to manufacturer's instructions at a wavelength range of 570 to 1,050 nm. Grain yield from cultivar Vitra could not be assessed because the grains were not yet at maturity at harvest date, and grain yield from cultivar Respect was not measured in 2019.

DNA extraction and sequencing. Lyophilized roots from pea and barley were ground with a 20-mm steel bead at 30 Hz for 5 to 10 s or until fully ground (TissueLyser II, Qiagen). DNA was extracted from 19 to 21 mg of root powder with the Omega Mag-Bind Plant DNA DS Extraction kit (Omega Bio-tek, U.S.A.) according to the manufacturer's instructions. DNA quality check was performed using a Nanodrop2000 Spectrophotometer (Thermo Scientific, U.S.A.) and electrophoresis (1% agarose, TAE [Tris-acetate-EDTA] buffer). Roots and DNA were stored at –20°C. This sam-

pling method does not differentiate between firmly attached rhizoplane and endophyte fungi.

DNA extracts were sent for PCR and amplicon sequencing to the Genome Quebec Innovation Center in Montréal, Canada. We chose primers ITS1F and ITS2 (Gardes and Bruns 1993; White et al. 1990) to amplify the first internal transcribed spacer region (ITS1) based on our previous work (Bodenhausen et al. 2019). The libraries were sequenced on the Illumina MiSeq platform (Illumina, Inc., San Diego, CA, U.S.A.) using the v3 chemistry (PE300). The raw sequencing data were deposited at the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) database (https://www.ncbi.nlm.nih.gov/sra) with accession number PRJNA894835.

Bioinformatics. Bioinformatic analyses were performed by the Genetic Diversity Centre at ETH Zurich, similar to the workflow described in Bodenhausen et al. (2019). Briefly, raw reads were quality checked and filtered, read ends were trimmed, and paired-end reads were merged using usearch v11.0.667 (Edgar 2010). In the next step, primer sites were trimmed using usearch and the amplicons subsequently quality filtered for sequence size range between 150 and 500, GC range between 30 and 70, and maximum number of Ns = 0 using PRINSEQ-lite 0.20.4 (Schmieder and Edwards 2011). UPARSE (usearch v11.0.667 i86linux64, Edgar 2016) was used to generate 97% cluster operational taxonomic units (OTUs), followed by a denoising step with UNOISe3 of amplicon sequence variants (zero-radius zOTUs). A count table using the 97% cluster OTUs was generated with usearch. Taxonomy assignment of these OTUs was performed with the UNITE reference database (version 8.3) (Nilsson et al. 2019) and using a Naïve Bayes classifier implemented by QIIME2 (version qiime2-2021.2) (Bolyen et al. 2019).

Statistical analysis. All data analyses were conducted in R v3.6.3 (Nilsson et al. 2019) and using Rstudio v1.1.463 (RStudio Team 2016). Before using analysis of variance (ANOVA), homogeneity and normal distribution of residuals were confirmed visually and, if needed, the tested variables were log-transformed prior to analysis. All plant parameters (grain yield, shoot length, protein content, disease score index, and nodule number) were statistically analyzed with ANOVA. For microbiome data, all OTUs classified as Protista and Plantae were excluded from the data set as an initial step. Because the number of sequences was significantly different between the 2018 and 2019 sequencing runs, data were rarefied for subsequent analysis, using as rarefaction threshold the number of sequences of the sample with the lowest number (19,767 sequences). Function "diversity'" from the package *vegan* (Oksanen 2015) was used to compute the Shannon index ("H"), from which Shannon's diversity was calculated as D = exp(H). Richness ("R") was calculated as raw OTU count data, and Sheldon's evenness was calculated as D/R, following Bodenhausen et al. (2013). Differences between groups were tested with ANOVA. Because observed richness is affected by sequence number per sample (data not shown), a generalized linear model (glm) was used to confirm differences between treatment groups in richness, using a Poisson model and including the log number of sequences as offset (Mittelstrass et al. 2021). Association between alpha diversity indicators and disease score index was assessed with linear regression following the formula "alpha diversity indices ~ year × cropping system." For beta diversity analysis, *vegan* functions wrapped by the package *phyloseq* (McMurdie and Holmes 2014) was used to visualize community composition. Multidimensional scaling (MDS) of Bray-Curtis dissimilarities was first used to explore the data with the "metaNMDS" function, followed by distance-based redundancy analysis (dbRDA) (Legendre and Anderson 1999) with "capscale" function, with constraining factor "cultivar" for 2018 and "cropping system" for 2019 pea data and "neighboring plant" for both years

of barley data. Permutational multivariate analysis of variance (PERMANOVA, Anderson 2017) was used for hypothesis testing (999 permutation) with functions "vegdist" and "adonis2," complemented by a test for homogeneity of multivariate dispersions (PERMDISP) (Anderson et al. 2006) with function "betadisper." To test the association between individual OTU relative abundance and root rot disease, cropping system and plant species R package *edgeR* (Robinson et al. 2010) was employed. Trimmed mean of M-values (TMM) normalized counts (Robinson and Oshlack 2010) were fit with a quasi-likelihood negative binomial generalized log-linear model (glmQLFit) and tested in a quasi-likelihood test (glmQLFTest) with self-defined contrasts. Significance of OTU identification was based on false discovery rate (FDR)-adjusted *P* values lower than 0.05 (Benjamini and Hochberg 1995). We defined the core pea community as the OTUs that were present in at least 90% of the pea samples and barley core community as OTUs that were present in 100% of samples).

RESULTS

Plant agronomic parameters. *Grain yield, grain protein content, and shoot length.* Pea and barley showed reverse trends across both years, with pea showing higher yield in 2018 than in 2019, and barley the opposite. This led to a higher total yield in pure stands (47.6 t ha^{-1} 4 standard deviation [sd]) than in mixed stands (19 t ha^{-1} 6 sd) in 2018. The opposite pattern was observed in 2019, with a total yield of 15 t ha^{-1} (16 sd) in pure stand and 19.6 (3.5 sd) in mixed stand. Yield and gross income were thus more stable in mixed stands throughout both years, while temporal variability was higher in pure stands (Supplementary Fig. S1). Protein content of pea grains varied between years and cultivars and was systematically higher in 2019 than in 2018 for cultivars that were assessed in both years (Alvesta, Karpate, and Mytic) (Supplementary Fig. S2A). The highest protein content values were observed in Vitra grains (2018), with a mean value of 21.2 against 17.7 for all other cultivars. For the three cultivars measured both years, an effect of cropping system was clear in 2019, showing lower protein content in mixed than in pure stands; the response was much less pronounced or nonexistent in 2018 (Supplementary Table S2). Shoot length varied between cultivars mainly due to Vitra, which showed a higher average shoot length (94 cm) than all other cultivars (82.7 cm) (Supplementary Fig. S2B). Further, a difference could be seen between the two cropping systems, but this difference depended strongly on the year of measurement (Supplementary Table S2). Indeed, the drop between cropping systems was most dramatic in 2018, where pea shoot length in mixture was lower than the cultivar's baseline.

Roots: Disease score and nodule number. The disease score index showed overall low values for all pea cultivars, on average 1.9 (0.78 sd) on a scale from 0 to 9. Globally, cultivar Vitra showed lower disease incidence (on average 1.1) than all other cultivars (on average 2.1). In addition, Alvesta, Respect, and Mytic showed a significantly higher disease incidence in mixture than in pure stand. On the other hand, Vitra and Karpate showed no difference of disease incidence between the two cropping systems (Supplementary Fig. S3A). Nodule number in pea roots was evaluated only in year 2018 and showed mainly a very high number of nodules in cultivar Vitra, with an average of 13.1, compared with all four of the other cultivars, with an average of 2.5 (Supplementary Fig. S3B).

Fungal microbiome diversity. The number of sequences resulting from pea and barley root-associated fungal community profiling was higher for the 2018 sequencing run, ranging from 50,989 to 77,154 with a median at 69,128.5, than for the 2019 sequencing run, ranging from 19,797 to 35,664 with a median at 28,108.5. The

rarefaction curve (Supplementary Fig. S4) shows, however, that the coverage was sufficient to capture the true OTU richness in our samples. Sequencing libraries yielded 613 OTUs for 2018 and 414 for 2019, from which 304 were common to both experiments. After rarefaction, there were 600 OTUs left in 2018 and 404 in 2019, of which 283 were in common.

Alpha diversity: Shannon index, richness, and evenness. Pea cultivars differed in OTU richness and evenness, but not in the Shannon index. When inspecting in detail the response of each cultivar, we found cultivar Alvesta having a higher richness and Vitra a higher evenness than all other cultivars (Fig. 1). Cropping system did not have a systematic effect on alpha diversity (Supplementary Table S3), but significant interactions between cropping system and cultivar were observed for richness ($P < 0.001$) and close to significant interaction for the Shannon index ($P = 0.05$) (Supplementary Table S3). Indeed, richness in roots of Alvesta responded negatively to mixture compared with those of pure stands (Fig. 1), with a mean richness of 127 in pure and 72 in mixed stands. On the other hand, the Shannon index of cultivar Vitra responded positively to mixture compared with pure stands, with mean values of 9.6 in pure and 17.3 in mixed stands (Fig. 1). There is no evidence for an effect of the neighboring plant (pea cultivars or barley) on diversity indicators of barley root fungal communities (Supplementary Fig. S5).

Beta diversity. MDS ordination on Figure 2 shows how root fungal communities are separated along the *x*-axis according to the factor "crop species," while the *y*-axis separates the samples according to the factor "year," with a stronger differentiation between species in 2018 data compared with the 2019 data. These observations were confirmed by PERMANOVA analysis including all factors, which shows that 19, 13, and 7% of the total variation were explained by species, year, and their interaction, respectively. In comparison, variations explained by cropping system and cultivar were 1 and 6%, respectively (Supplementary Table S4).

For further analysis, we separated the pea and barley data set and observed that besides the highly significant temporal component, the effects of cropping system, cultivar, and the interaction between year and cropping system on pea fungal communities were close to significant (Table 1). Given the strong temporal effect (Fig. 2), we further split the data by year. Communities from the 2018 data set showed a significant response to cultivar ($R^2 = 0.32$, $P = 0.02$), but not to cropping system (Table 1). The cultivar mainly responsible for the cultivar effect was Vitra, clustering away from all other cultivars, but most strongly from Alvesta (Fig. 3A). Contrast between cultivar Respect, on one end of the *y*-axis, and both Vitra and Alvesta on the other end, also drove an important part of the variation. On the contrary, communities from the 2019 data set did not cluster according to cultivar but differed significantly according to cropping system ($R^2 = 0.1$, $P < 0.05$) (Table 1; Fig. 3B). Of note, heterogeneity of variance between groups was significant as well and may contribute to beta-diversity differences detected by the PERMANOVA, along with the biological factors.

Barley beta diversity was strongly influenced by year ($R^2 = 0.2$, $P < 0.01$), but neither the effect of neighboring plant nor interaction between both factors was found. Looking at the MDS, we observe that both years show a similar sample clustering pattern where the *x*-axis captures differences between neighboring pea cultivar Vitra and pea cultivars Karpate and Mytic (14.5 and 17.8% of the total variability for both years, respectively), and the *y*-axis capturing differences between neighboring plant barley and neighboring pea cultivars Vitra and Mytic (11 and 10.5% of the total variability for both "years") (Fig. 3C and D). Thus, in this study, differences between barley fungal communities are not higher between pure and mixed stand than between mixed stand with different pea cultivars.

Pea and barley specific and common fungal communities. The pea "core community," representing the OTUs that were present in at least 90% of pure pea root samples (i.e., in nine samples), comprised 15 OTUs, and the barley core community, present in 100% of pure barley samples (i.e., in four samples) comprised 20 OTUs (Table 2). Nine OTUs overlapped between the two core communities. Log fold-change (logFC) pea/barley in Table 2 indicates whether these core OTUs have significant higher abundance in pea or in barley, to evaluate whether these OTUs characterize the pea or barley communities in terms of abundance as well as frequency. Most of the OTUs that are core of one specie also show significant higher abundance in the same specie (10 out of 11 for barley, 3 out of 6 for pea). Some of the ubiquitous OTUs ("core pea + barley") show significant "preference" for one or the other specie (*Fusarium waltergamsii, F. acutatum*, and the unassigned 62 OTU *Didymella* species were enriched in pea, *Cladosporium delicatulum, Mycosphaerella tassiana*, and an unassigned OTU in barley). Of these core OTUs, only one, *N. exitialis,* exhibited differential abundance according to cropping system. This OTU was characteristic of the barley core community and showed higher abundance in pea roots in mixed compared with pure stand, suggesting a potential influence of barley on the pea community (Table 2).

Link between agronomic parameters and fungal community. *Microbiome diversity and root disease.* Among the alpha diversity metrics, Shannon index (eta$^2 = 0.21$, $P < 0.01$) and Sheldon's evenness (eta$^2 = 0.09$, $P = 0.07$) but not observed richness showed a significant or close to significant negative relationship with disease score when taking in account the highly significant effect of year (Fig. 4), and Shannon index showed a close to significant interaction between year and disease score (eta$^2 = 0.24$, $P = 0.08$). Further, when testing root disease score index as an explanatory factor, we find a small but significant association with pea fungal community composition (adjusted $R^2 = 0.08$, $P < 0.01$) (Supplementary Table S5).

OTUs associated with root disease. The most abundant OTU of the common core community of pea and barley was a disease-associated *Didymella* species (62 OTUs) (Table 2). The Didymellaceae *N. exitialis* (159 OTUs) from the barley core community was associated with disease as well. Interestingly, the *Didymella* species (62 OTUs) was significantly more abundant in pea roots, while *N. exitialis* was significantly more abundant in barley roots (Table 2). When inspecting the abundance among cultivars, we observe that the disease-associated 62 OTU *Didymella* species was more abundant in Alvesta, with an average relative abundance of 12.9

TABLE 1
Effect of year, cropping system, cultivar, and their interactions on beta diversity of pea root fungal communities, on both years and for each year separately[a]

Variable	Both years	2018	2019
Cropping system	0.03	0.04	0.1*
Cultivar	0.1	0.32*	0.19
Cultivar−cropping system	0.07	0.17	0.23
Year	0.19***	−	−
Year−cropping system	0.03	−	−
Year−cultivar	0.09	−	−
Year−cultivar−cropping system	0.09	−	−
Residual	0.39	0.47	0.48

[a] R^2 values are presented, and asterisks mark significant effect of variables ($P < 0.05$, *; $P < 0.01$, **; and $P < 0.001$, ***), based on permutational multivariate analysis of variance.
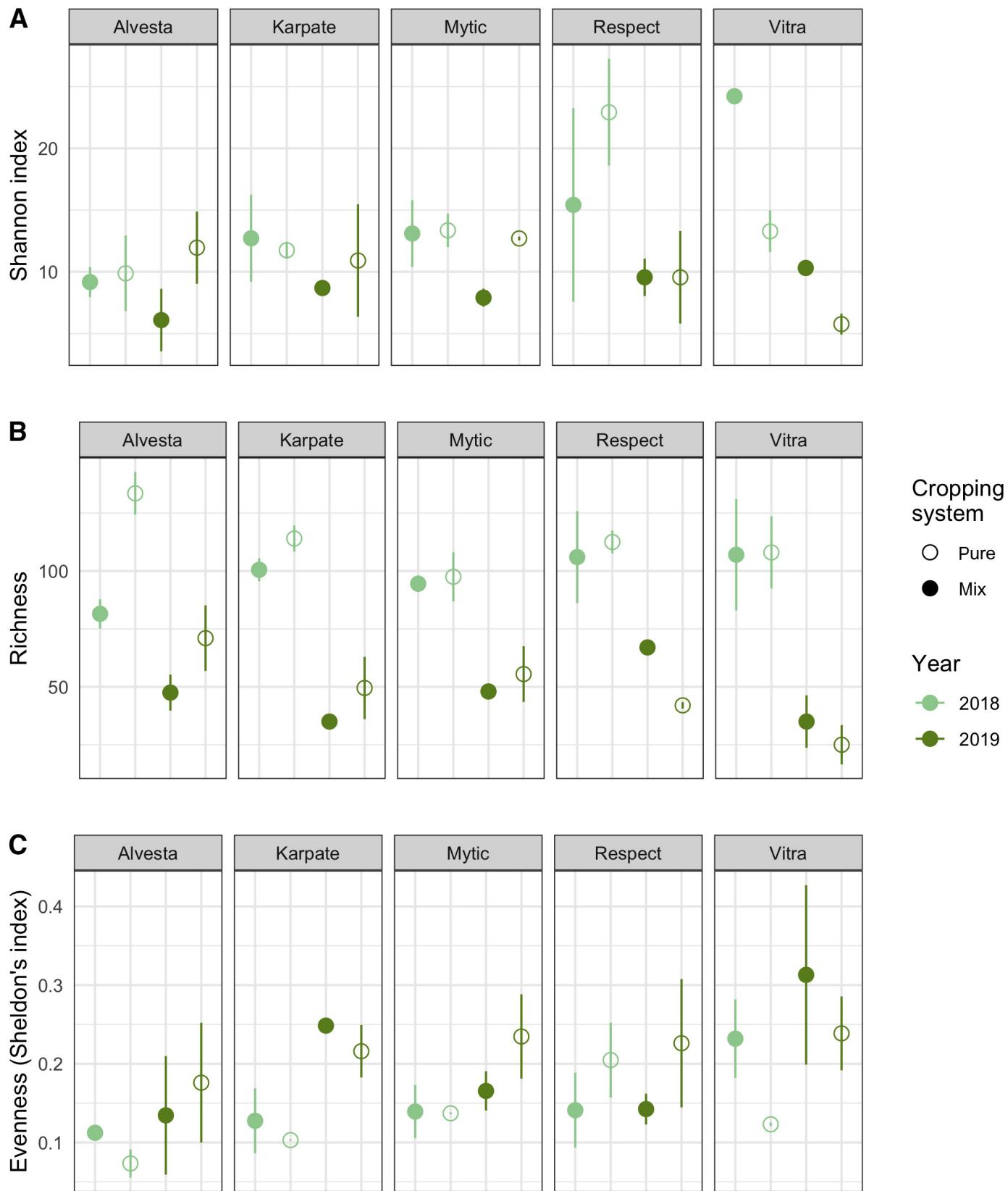
Fig. 1. Alpha diversity estimates of pea root fungal communities. **A,** Shannon index, **B,** observed richness, and **C,** Pielou's evenness based on operational taxonomic unit (OTU) counts. Means and standard deviations ($n = 2$) are shown, with colors showing year of experiment, filled or empty circles showing cropping systems, and facets showing different pea cultivars.

and 16.9% in 2018 and 2019, respectively (Supplementary Fig. S6A). This *Didymella* species (62 OTUs) was overall more abundant in mixed than in pure stand—except in Vitra roots—with an average relative abundance of 6 and 9.5% in pure stand, in 2018 and 2019, respectively, and 6.5 and 17.3% in mixtures. While the abundance of this OTU was higher in pea when mixed with barley, it was high in pure pea as well and close to zero in barley roots in 2018 (Supplementary Fig. S6A). *N. exitialis* (159 OTUs) showed a high abundance in barley roots in association with Alvesta, Karpate, Respect, and Vitra in 2019 (Supplementary Fig. S6B) and a relatively higher abundance in pea roots of Alvesta, Respect, and Vitra in 2019 when mixed with barley in 2019 (0.6% in mixed against 0.002% in pure stand on average for these three cultivars). On the other hand, two barley core community OTUs were associated with

healthy pea roots, one unassigned OTU and *Mortierella fatshederae* (Table 2).

When considering the entire fungal community, we identified a total of 63 OTUs that showed a significantly higher relative abundance in diseased root and 101 OTUs that showed a significantly higher relative abundance in healthy roots (Supplementary Table S6). Among the disease-associated OTUs, we found four OTUs belonging to the Didymellaceae family, which accounted for 6% of all disease-associated OTUs, while no Didymellaceae OTUs were found among the health-associated OTUs. The overall abundance of the Didymellaceae family was lower in cultivar Vitra compared with all other cultivars (Fig. 5). In addition to *N. exitialis*, another OTU from the Didymellaceae family, *Phomatodes aubrietiae*, was significantly more abundant in mixed stands com-

TABLE 2
Details about the core pea and/or barley operational taxonomic units (OTUs)[a]

Zero-radius OTUs	Family	Species	Core (pure)[b]	logCPM[c]	logFC pea/barley[d]	logFC cropping system pea[e]	logFC disease[f]
zOTU370	Phaeosphaeriaceae	*Parastagonospora caricis*	Core barley	10.21	−9.12	–	–
zOTU59	Helotiaceae	*Articulospora proliferata*	Core barley	14.05	−8.28	–	–
zOTU195	Pleosporaceae	*Alternaria* unassigned	Core barley	13.66	−7.05	–	–
zOTU12	NA	NA	Core barley	17.10	−6.58	–	–
zOTU533	Helotiales_fam_Incertae_sedis	*Collembolispora aristata*	Core barley	11.89	−3.93	–	–
zOTU40	Didymellaceae	*Epicoccum dendrobii*	Core barley	12.61	−5.08	–	–
zOTU60	Nectriaceae	*Fusicolla septimanifiniscientiae*	Core barley	12.46	−4.31	–	–
zOTU346	NA	NA	Core barley	10.53	−3.15	–	–
zOTU155	Nectriaceae	*Ilyonectria robusta*	Core pea	14.13	3.66	–	–
zOTU129	Nectriaceae	*Fusarium ornamentatum*	Core pea	11.42	2.82	–	–
zOTU1456	Nectriaceae	*Fusarium acutatum*	Core pea	13.75	2.03	–	–
zOTU174	Mortierellaceae	*Mortierella* unassigned	Core pea	10.75	−1.22	–	–
zOTU10	Nectriaceae	*Fusarium* unassigned	Core pea	14.71	0.15	–	–
zOTU34	Olpidiaceae	*Olpidium brassicae*	Core pea	10.89	0.90	–	–
zOTU2	Nectriaceae	*Fusarium waltergamsii*	Core pea + barley	15.46	4.66	–	–
zOTU18	NA	NA	Core pea + barley	15.75	−3.42	–	–
zOTU49	Mycosphaerellaceae	*Mycosphaerella tassiana*	Core pea + barley	12.50	−1.98	–	–
zOTU3	Nectriaceae	*Fusarium acutatum*	Core pea + barley	16.11	2.13	–	–
zOTU190	Cladosporiaceae	*Cladosporium delicatulum*	Core pea + barley	10.06	−1.37	–	–
zOTU613	Herpotrichiellaceae	*Exophiala equina*	Core pea + barley	9.82	−1.18	–	–
zOTU4	Plectosphaerellaceae	*Plectosphaerella* unassigned	Core pea + barley	15.38	−1.19	–	–
zOTU11	Nectriaceae	*Ilyonectria macrodidyma*	Core pea + barley	15.04	0.69	–	–
zOTU427	NA	NA	Core barley	9.78	−7.72	–	−1.25
zOTU424	Mortierellaceae	*Mortierella fatshederae*	Core barley	8.04	−1.36	–	−1.58
zOTU159	Didymellaceae	*Neoascochyta exitialis*	Core barley	13.69	−10.15	3.75	2.18
zOTU62	Didymellaceae	*Didymella* unassigned	Core pea + barley	15.92	2.03	–	1.57

[a] Taxonomic identification at the levels of family, genus, and species are specified. Only significant values at a false discovery rate (FDR)-corrected *P* value threshold of <0.05 are shown.
[b] Core indicates whether OTUs belong to the pure core pea (present in at least 90% of the samples, $n = 10$), barley (present in 100% of the samples, $n = 4$), or both communities (the overlap between the pure core communities).
[c] logCPM, average log count per million.
[d] logFC pea/barley, log-fold change between differentially abundant OTUs in pea (positive values) and barley (negative).
[e] logFC cropping system pea, log-fold change between differentially abundant OTUs in mixed (positive) or pure (negative) stand in pea roots (barley roots are not represented because none of these core OTUs showed differential abundance between cropping systems in barley roots).
[f] logFC disease, log-fold change between differentially abundant OTUs in diseased (positive) or healthy (negative) pea root.

pared with pure stands. This OTU is a potential plant pathogen (Supplementary Table S6). Among the health-associated OTUs, we identified 12 OTUs belonging to the Glomeraceae family, accounting for 12% of the health-associated OTUs, while only one OTU was associated with disease (2%) (Supplementary Table S6). The Glomeraceae family was predominantly present in the roots of cultivar Vitra, representing 0.31% of the sequences in Vitra in 2018 data and 1.35% in 2019. In cultivar Respect in 2018, it represented 0.13% of the sequences (Fig. 5). In all other cultivars and years, the proportion of Glomeraceae was lower than 0.1%. Additionally, six OTUs belonging to the genus *Mortierella* were associated with healthy roots (6%), while only one of them was associated with diseased roots (2%) (Supplementary Table S6).

DISCUSSION

This study aimed at characterizing the root fungal microbiome of pea cultivars in intercropping systems and explored the relationship between the occurrence of fungal taxa and the agronomic performance of pea. The response to cropping systems of both pea agronomic performance and fungal diversity depended on the year of measurement and on the cultivar. Diversity, community structure, and abundance of particular fungal taxa were related to cropping system and root rot incidence.

Temporal variability is higher for pure stand yield and influences microbiome response to cropping systems. In this 2-year field experiment, we observed contrasting responses between the

Fig. 2. Beta diversity of fungal communities associated with pea and barley roots. Multidimensional scaling (MDS) based on Bray-Curtis dissimilarities calculated from operational taxonomic unit (OTU) counts in 2018 and 2019. Colors correspond to species, hues to years of experiment, and filled or empty circles to cropping system.

2 years, particularly in terms of pea yield and root fungal composition. The intercropped plots showed greater stability in total yield compared with pure stands of pea, which aligns with the observed "insurance effect" resulting from complementarity in intercropping practices (Justes et al. 2021). The differential drought sensitivity of the two crop species may be responsible for this effect (Sun et al. 2021). Both years experienced below-average rainfall, but 2018 was also warmer than the 30-year average, leading to higher evaporation compared with that in 2019 (see field trial for meteorological data in the Materials and Methods section or Haug et al. 2023). Barley suffered more from drought stress, allowing pea to dominate in the intercropped plots. However, in 2019, we observed systematically lower protein content in pea grains from intercropped plots compared to pure stands. This could be attributed to nitrogen limitation resulting from increased competition from barley, possibly exacerbated by lower soil nitrogen levels in the 2019 field compared to the 2018 field (see field trial for soil nitrogen data in the Materials and Methods section or Haug et al. 2021). Pea plants can be particularly sensitive to nitrogen competition during early growth stages when nodules are not yet formed (Bedoussac and Justes 2010).

It is important to consider that although the 2018 and 2019 experiments were conducted in two nearby fields, there may be spatial and historical differences between them. Factors such as microtopography, soil characteristics, and field management history can influence the distribution of microbial communities (Ramirez et al. 2018; Walsh et al. 2021). These differences may also play a role in the response of the microbiome to intercropping or pea cultivar, along with climatic variations between years (Tedersoo et al. 2014) and feedback from crop responses to this combination of factors. Additionally, the sequencing was performed in two different runs, which could introduce some technical variation in fungal diversity measures. Considering these factors, the higher similarity observed between barley and pea fungal communities in the 2019 experiment compared with the 2018 experiment may be attributed to differences in the initial soil fungal pool, including its composition or lower richness, resulting in a more homogeneous root community. Alternatively, the increased drought conditions in 2019 could have influenced differences in exudation or rooting depth, leading to the recruitment of more crop-specialized fungi (following the "gradient of stress" hypothesis, where stress conditions enhance the development of complementary phenotypes in mixed cropping systems; He et al. 2013). Another possibility is that the higher proportion of barley biomass in the mixture in 2019 exerted a stronger influence of the barley microbiome on the pea microbiome. This hypothesis is supported by the fact that a cropping system effect on fungal community composition was detectable only in 2019, whereas compositional differences between cultivars were detected only in 2018. The cropping system effect in 2019 might have masked crop genotypic differences.

The presence of root rot and disease-associated *Didymella* pathogen is higher in intercropping plots for some cultivars. In our study, we found four Didymellaceae OTUs linked to diseased roots. Members of the Didymellaceae family, namely *D. pinodes* and *D. pinodella*, have already been identified as belonging to a pathogen complex responsible for pea root rot (Wille et al. 2020, 2021). We found that most cultivars were more sensitive to root rot when intercropped; however, the most abundant disease-associated OTU, an unassigned *Didymella* species, was also abundant in pea roots in pure stands, suggesting that it may not originate from the barley community. We speculate that the presence of barley could create a microclimate, for example, when a shading effect of barley or a higher plant density would increase soil moisture retention and favor root rot development (Sippell and Hall 1982; Wong et al. 1984). Another hypothesis could be that the presence of
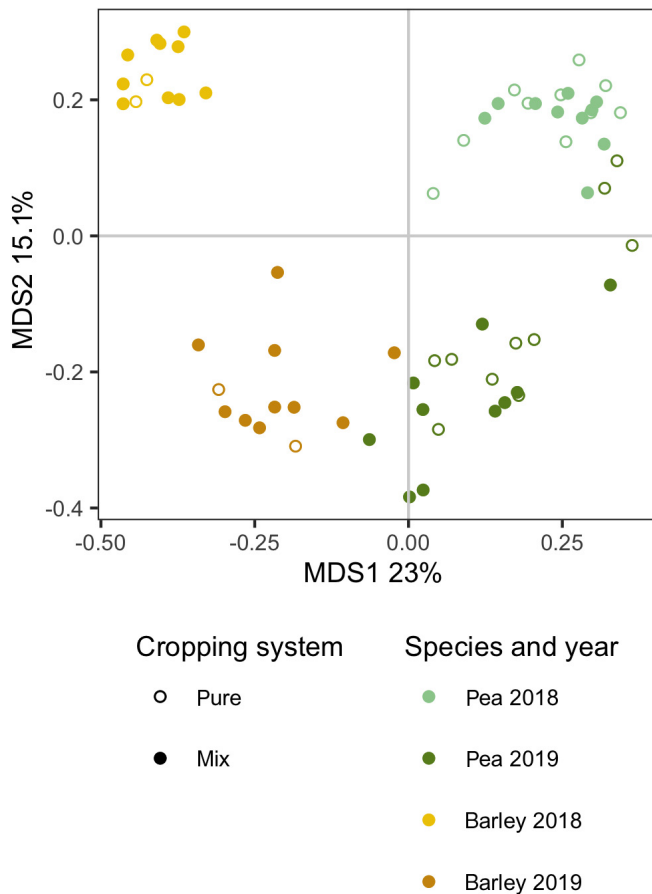
other plant pathogens in the barley community, such as *N. exitialis*, formed a complex with pathogens present in pea roots like the (62 OTU) *Didymella* species and enhanced the global pathogenicity (Wille et al. 2021). Its higher presence in pea in 2019 corroborates the hypothesis of higher microbial exchanges between crops in mixed stands in the 2019 experiment. Globally, the higher disease incidence in mixtures for some cultivars indicates that the intercropping benefits may depend on the context, including climate or genotype, and should be further studied in order to better control pea root disease.

Disease incidence showed negative association with fungal community Shannon index and evenness. Possibly, complementary or redundant activity from beneficials are an advantage of even more microbial communities (Chen and Zhou 2015; van Elsas et al. 2002) and give stability to the system (Yachi and Loreau 1999). This may prevent dominance of one species and reduce disease virulence. The association of healthy pea roots with a high number of taxa belonging to the *Mortierella* genus and to the Glomeraceae family is in line with results found by Hossain et al. (2021). These taxa are known to have beneficial effects on the colonized plants, including pathogen protection (Cameron et al. 2013; Jung et al. 2012; Ozimek and Hanaka 2021; Wang et al. 2022). Arbuscular mycorrhizal fungi (the majority of which belong to the Glomeraceae family) are one of the prime plant symbionts and have a key role in the provision of agricultural ecosystem services (Rillig et al. 2019), including nutrient and water uptake (Finlay 2008), soil stability
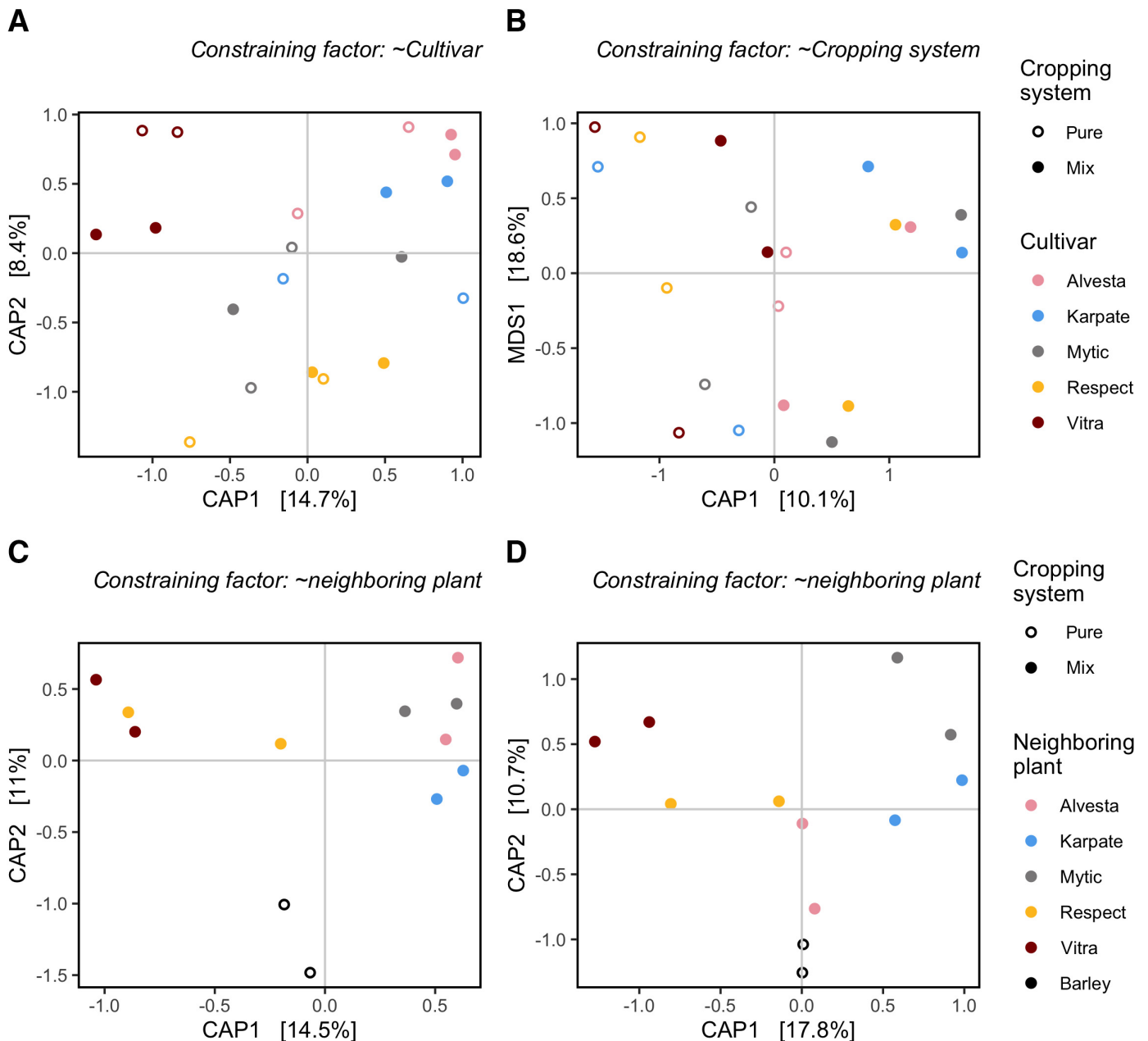

Fig. 3. Beta diversity of fungal communities associated with pea and barley roots. Constrained analysis of principal coordinates (CAP) ordinations for pea roots communities in **A,** 2018 field experiment, constrained by factor cultivar, and **B,** 2019 field experiment, constrained by cropping system factor. Colors show cultivars, and filled or empty circles show cropping systems. CAP ordinations for barley root communities in **C,** 2018 field experiment and **D,** 2019 field experiment, constrained by neighboring plant (pea cultivars or barley) factor. Colors show neighboring plant (pea cultivars or barley), and filled or empty circles show cropping systems.

(Gianinazzi et al. 2010), nutritional quality (Cavagnaro 2008), biotic and abiotic stress tolerance in general (Harrier and Watson 2004; Marulanda et al. 2009), and control of root pathogens in particular, mostly via indirect mechanisms, i.e., regulation of phytohormones (Gutjahr and Paszkowski 2009; Hause et al. 2007; Jung et al. 2012) and defense-related compounds (López-Ráez et al. 2010; Pozo and Azcón-Aguilar 2007; Slezack et al. 2000), but also via direct antagonism (Linderman 1994; Whipps 2004). Thus, Glomeraceae OTUs show high potential to serve as useful indicators to select vigorous crop cultivars, as demonstrated by Wille et al. (2021) for resistance against pea root rot.

Disease incidence was also associated with shifts in beta diversity of fungal communities. It has indeed been demonstrated that the presence of pathogens can modify the microbiome composition, directly or indirectly through modified exudation from plant roots in the presence of disease (Sasse et al. 2018). However, the causality goes also in the reverse direction: the microbiome composition also plays a role in controlling disease presence in plant roots (Berendsen et al. 2018; Mendes et al. 2011). Therefore, owing to the mainly descriptive nature of this study and its focus on correlations, further investigation is necessary to establish the causal role of community structure, alpha-diversity indicators, individual fungal taxa, or pathogen complexes in influencing the presence or control of diseases and the interaction with cropping system. This can be achieved through mechanistic experiments that involve simplified and controlled fungal communities (Gu et al. 2022). In addition,
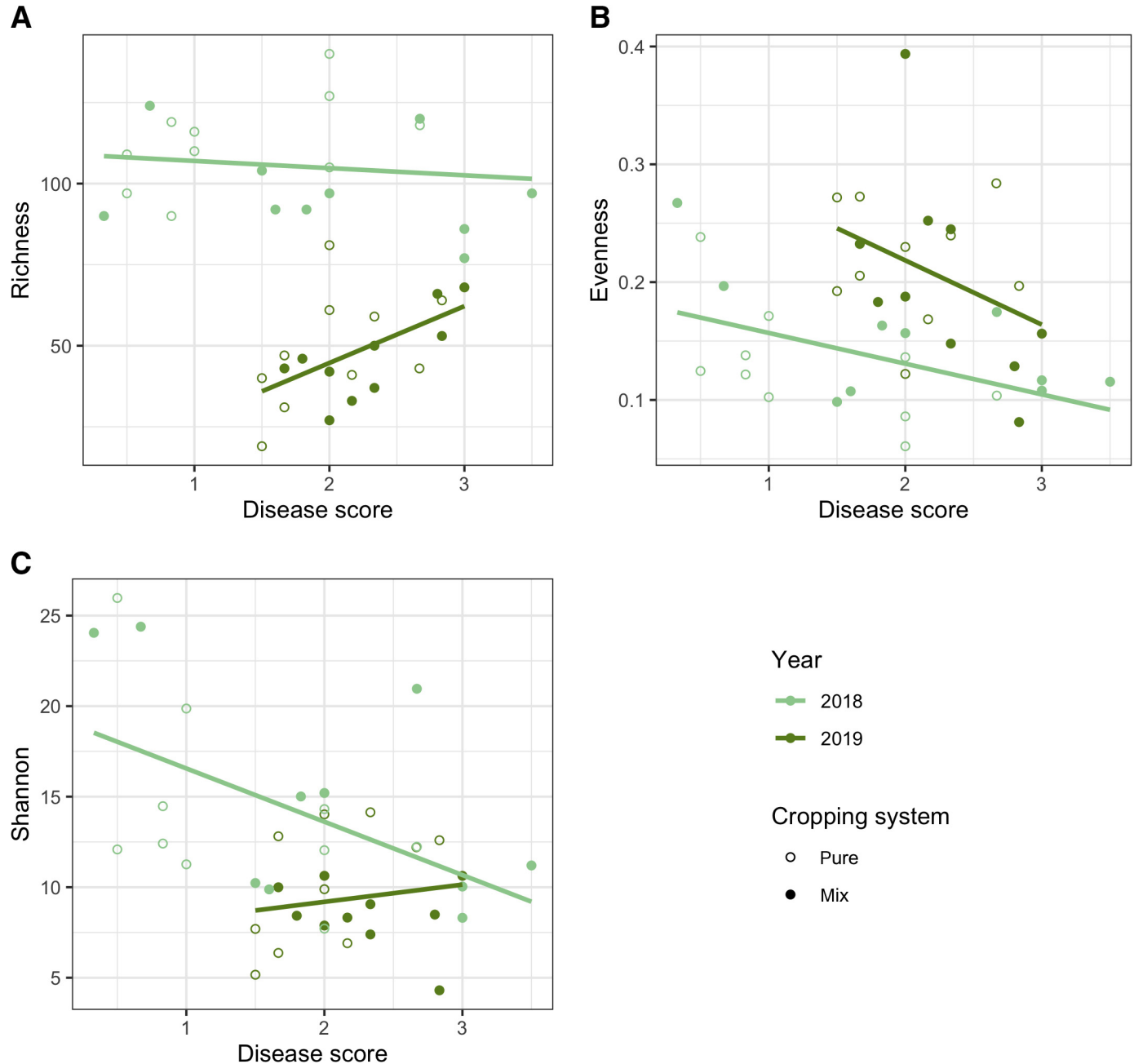

Fig. 4. Relationship between observed richness (A), Sheldon's evenness (B), and Shannon diversity (C) of pea-associated fungal operational taxonomic units (OTUs) and the pea root disease score index. Colors show year of field experiment, and filled or empty circles show cropping systems.

the lack of community profiling from other members of the root microbiome, such as bacteria, protists, nematodes, and viruses, is one limit of this study. Notably, rhizosphere bacteria are known to play an important role in disease suppressiveness (Berendsen et al. 2018; Pieterse et al. 2014), are influenced by neighboring plants (Taschen et al. 2017; Ulbrich et al. 2022), and synergistically interact with arbuscular mycorrhizal fungi (Barea and Ascón-Aguilar 1983; Shtark et al. 2012). Thus, besides the increased presence of Glomeraceae OTUs, the lower disease-associated OTUs of cultivar Vitra might also be connected to its increased nodulation status. In
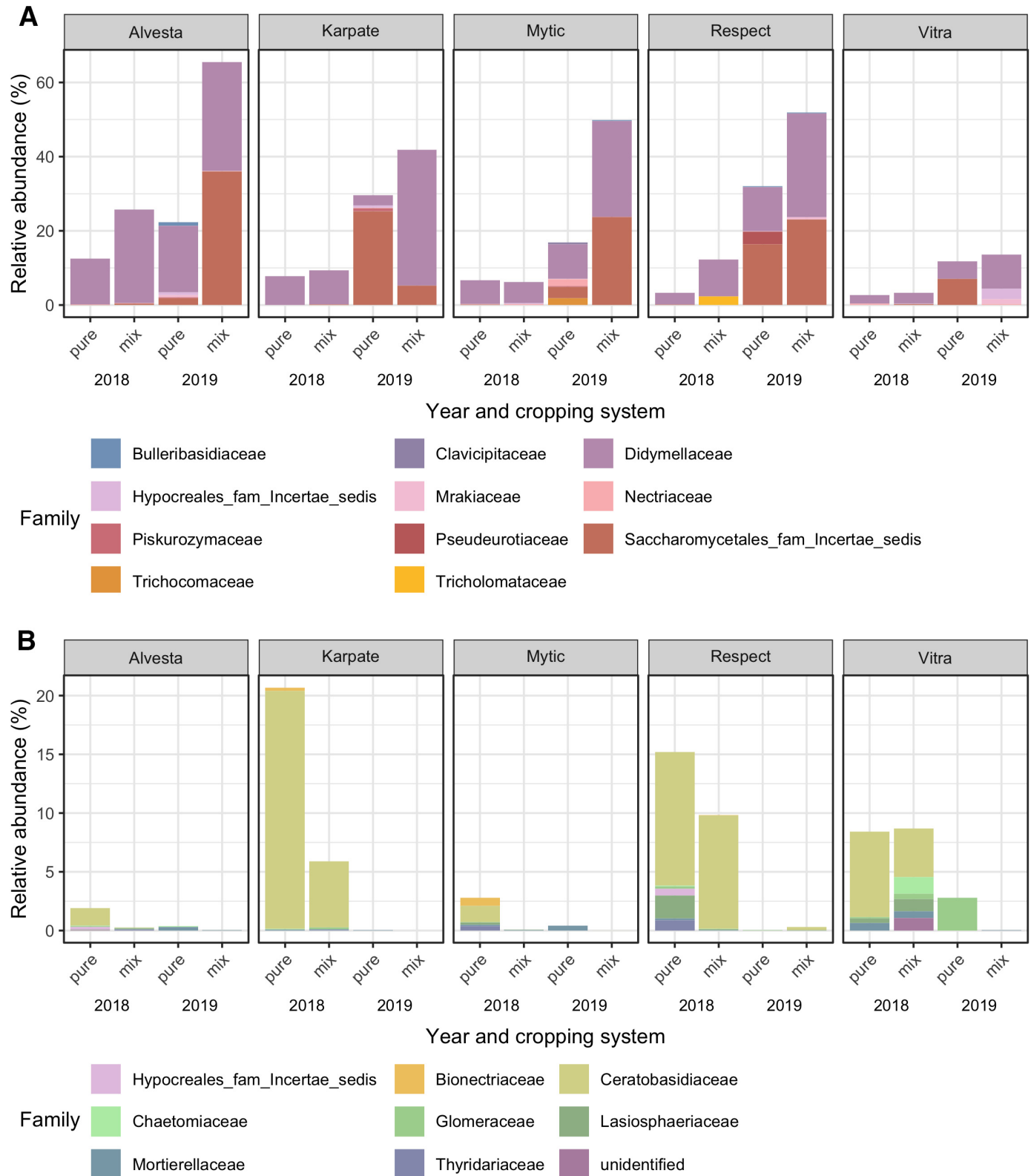

Fig. 5. Families of disease-associated fungal operational taxonomic units (OTUs). Colors show the mean relative abundance of families of **A,** disease-associated OTUs and **B,** health-associated OTUs for each cropping system and year of experiment. Different pea cultivars are shown with facets.

addition, the soil type and its specific microbial pool may interact with a cultivar's response to cropping system. To have a complete picture of the microbial component of cultivar response to cropping systems, a study including such additional factors of variation will be a necessary input toward a more general conclusion.

Vitra stands out as a good intercropping candidate and harbors a contrasting microbiome. Cultivar Vitra differed from the other four genotypes in many microbial indicators. Vitra showed a higher Shannon diversity index and Pielou's evenness of fungal communities in intercropped plots than in pure stands. It also had a lower proportion of *Didymella* fungi, a higher proportion of arbuscular mycorrhizal fungi, and a higher nodule number than all other cultivars. This fungal microbiota composition high in beneficial organisms might have contributed to Vitra's particular disease resistance. Most notably, Vitra did not show, in contrast to the other cultivars, a higher disease score when intercropped with barley compared with pure stands—showing a potentially better adaptation to mixtures with barley.

These contrasting characteristics are consistent with the fact that cultivar Vitra differs in other fundamental morphological and phenological aspects that are relevant criteria to be considered when deciding to use specific pea cultivars for intercropping with cereals. Vitra belongs to the leafy pea type, whereas the other cultivars were semi-leafless (*afila*) types. Leafy types, as well as taller plants, are not favored in pure stands because of a higher lodging risk, but this criterion may be less important in the presence of intercropped cereals that can protect to a certain degree against lodging. However, yield from leafy genotypes was found to be less stable across years (Haug et al. 2023).

The genotype Vitra is a late maturity cultivar from Latvia that is adapted for the photoperiod under those latitudes. In Switzerland, it was not ripe for harvest in mid-July at the same time as the other cultivars, and more importantly, as barley. Thus, it is difficult to compare agronomic performances between cultivars of different maturity stages. A possible way of disentangling the phenology effect and adapting this cultivar to intercropping with barley in Switzerland would require new breeding efforts or finding a late-maturing barley partner.

Conclusion. Altogether, our study showed that effects of intercropping with barley on the performance and root fungal community of pea depended on the yearly climatic and/or edaphic factors and on the pea cultivar. Although the presence of *Didymella* pathogens associated with disease presence, was favored in intercropped pea stands, better total yield stability was found across the 2 years when compared with that in pure pea stands. In addition, cultivar Vitra contrasted with other cultivars by not showing higher disease presence in mixed stands. Vitra showed an overall higher abundance of arbuscular mycorrhizae and nodules in the roots compared with other cultivars and was the only cultivar with high fungal evenness in mixed compared with pure stands. These might be important indicators associated with the roots of healthy pea plants and highlight the potential of this cultivar for legume cultivation in general and mixed cropping in particular. Overall, our results indicate that the sensitivity of intercropping systems to genotypic variation might be related to specific fungal taxa. Such indicators could be a useful tool to support breeding programs in identifying crops suitable for intercropping.

ACKNOWLEDGMENTS

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