Wheat varietal diversity affects arbuscular mycorrhizal symbiosis and soil enzymatic activities in the root zone

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Abstract

High-input agriculture has been associated with a drastic reduction of within-field crop genetic diversity, while plant (mostly functional) diversity in natural ecosystems has been shown to promote ecosystem functioning. Increasing intra-specific diversity in agroecosystems is a promising strategy to stabilize crop productivity and promote the associated diversity of fauna and microbiota. We investigated the effect of the within-field diversity of bread wheat varieties on arbuscular mycorrhizal fungi (AMF) and two enzymatic activities involved in organic nitrogen and phosphorus mineralization. A field experiment was designed to test whether the number of mixed wheat varieties in a plot, or their functional diversity (previously assessed), influence the abundance and diversity of AMF and the activity of leucine aminopeptidases and phosphatases in the root zone. The AMF abundance was measured by quantitative polymerase chain reaction, community composition was analyzed by Illumina metabarcoding on two AMF specific markers, and potential microbial activities were quantified by biochemical assays. Wheat traits related to root morphology and susceptibility to fungal diseases previously quantified for each variety were also used. Number of varieties significantly increased AMF abundance in roots, whereas functional cluster number did not, with no impact of root morphology. Functional cluster number influenced AMF diversity, though weakly and not linearly, responding most to binary mixtures. Both wheat variety and functional group number increased the potential leucine amino-peptidase activities in the root zone, while no effect was observed for phosphatase activities. Our results highlight that increasing crop intra-specific diversity triggered changes in key processes involved in nutrient acquisition.

Introduction

Agroecosystems need to be managed more sustainably, in particular by reducing chemical inputs and their environmental impacts. Increasing the diversity of agricultural systems at both field and landscape scales is a key principle of ecological intensification proposed to improve agroecosystem performance and resilience and minimize the need for external inputs (Gaba et al., 2015). Studies in natural and manipulated ecosystems have shown that increasing plant species richness enhances ecosystem functioning, mainly in terms of productivity and stability (Tilman et al., 1997; Weisser et al., 2017; Hong et al., 2022) but also in terms of soil conditions and functions (El Moujahid et al., 2017; Le Roux et al 2013). This type of positive relationship relies on two non-exclusive mechanisms, namely the selection/sampling effect and the complementarity effect (Loreau 1998). Functional trait diversity among plant species was shown to be a good predictor of the ecosystem functioning (Tilman et al., 1997). Although this diversity might be lower at the intra-specific level, intra-specific diversity is not negligible (for wheat, see Cantarel et al. 2021) and variety mixture can lead to substantial overyielding (Litrico & Violle 2015, Wuest et al., 2021). In their meta-analyses including 91 studies on cereals and legumes, Reiss & Drinkwater (2018) found an overall yield increase of 2.2% for varietal mixtures relative to their single-variety components. A meta-analysis focusing on bread wheat varietal mixtures showed a mean overyielding of 3.9%, reaching 6.2% in conditions of high disease pressure (Borg et al., 2018). In the case of intraspecific mixtures of durum wheat, Montazeaud et al (2020) found that root traits such as the root tissue density and root angle were among variables explaining the best the productivity (plant biomass and yield) and grain quality of durum wheat grown in binary mixtures. This makes sense as belowground traits related to nutrient acquisition strategies have been shown to be of major importance for the positive outcomes of crop species or variety mixtures (Barot et al. 2017; Hinsinger et al. 2011; Dubs et al. 2023).

To cope with varying or limiting nutrient resources in soil, plants have evolved different nutrient acquisition strategies, and related traits (e.g. Erel et al. 2017). These strategies involve (i) root morphology (specific root length, branching) to optimize soil exploration (foraging strategy) and (ii) root physiology (release of protons, carboxylates and extracellular enzymes in particular) to mobilize inorganic nutrients or mineralize organic resources (mining strategy). Tight interactions with rhizospheric microorganisms (especially bacteria and fungi) are recognized for their contribution to both foraging and mining mechanisms. Amongst these, external hyphae of arbuscular mycorrhizal fungi (AMF) considerably expand the rhizosphere volume (extending up to several centimeters away from the root surface; Thonar et al., 2011) and thus make a major contribution to the foraging strategy of mycorrhizal plants. The AMF may also enhance nutrient mining from less

available pools through stimulating phosphorus (P) solubilizing bacteria (Wang et al. 2017) and bacterial communities involved in organic P and nitrogen (N) mineralization (Wang et al., 2022, Nuccio et al. 2012) as related to specific enzymatic activities (Ezawa & Saito 2018). The benefit of the mycorrhizal symbiosis is however variable and highly dependent on the environment (e.g., nutrient availability; Ingraffia et al., 2020) but also on the involved plant and fungal partners. Despite the lack of host specificity, AMF display host preferences, and AMF community varies between plant species among a same genus (Pivato et al., 2007) or between genotypes of the same species (Ercoli et al 2017, Ellouze et al., 2018). If different genotypes harbor different AMF communities, increasing genotypic diversity within a field should enrich the total AMF diversity. This vertical effect of plant diversity on AMF communities has been shown at the inter-specific level (van der Heijden et al. 1998; Neuenkamp et al. 2018). However, the role of genetic and phenotypic diversity within the population of host plant species on AMF communities and functioning has received little attention so far, while being a critical leverage for a more sustainable agriculture.

It has been shown that wheat genotypes varied in their colonization rates, carbon investment into AMF and growth or nutrient uptake response (Garcia de Leon et al., 2020; Elliott et al., 2021). Wheat has long been recognized as a crop with divergent responses to the AMF symbiosis, from negative, neutral to positive effects. Plant dependency to AMF association was shown to be linked to root morphology, with lower dependency for plant species with coarser roots, and higher specific root length (ratio of root length to dry mass of roots; SRL) (Bergmann et al., 2020). At the plant intra-specific species level, this hypothesis (Hetrick et al., 1991) has been poorly studied so far. A side effect of modern breeding might comes from the selection of fungal disease resistant varieties. Because of similarities of the plant immune system responses to infection/colonization processes for AMF and biotrophic pathogens, a trade-off might exist as discussed by Jacott et al (2017). Thus, breeding crops for high resistance against fungal diseases, such as yellow rust and septoriose for wheat, might have reduced AMF colonization potential. Moreover, there is an unclosed debate on the impact of breeding crops under high fertilization inputs on the mycorrhizal responsiveness and dependency of cereals such as wheat and maize (Hetrick et al., 1993, Lehmann et al., 2012; Ellouze et al., 2016; Zhang et al., 2019).

In this study, we used a field trial set up by the Wheatamix consortium using 16 bread wheat varieties characterized for 27 below- and aboveground functional traits (Cantarel et al., 2021). These varieties were divided into four functional clusters according to previously acquired traits' database. The field experiment was designed with 88 wheat plots harbouring a single variety or mixtures of 2, 4 or 8 varieties differing in the number of functional groups (Dubs et al., 2018; Dubs et al., 2023). We aimed to understand how the levels of genetic and functional diversity of bread wheat are affecting AMF communities and two enzymatic activities involved in organic N and P mineralization in the root zone (leucine-amino-peptidase (LAP) and phosphatases, respectively). We hypothesized that wheat diversity (variety number and functional diversity) could enhance AMF diversity and stimulate the potential capacities of wheat mixtures for foraging (AMF colonization) and mining (enzymatic activities) soil nutrients such as N and P.

Material And Methods

Field experiment

The experiment is related to the large Wheatamix project investigating the effect of intraspecific diversity of bread wheat on ecosystem functioning and services (Dubs et al., 2018; Dubs et al., 2023). Our study was conducted on a field experiment carried out in 2016 at the INRAE Experimental Station in Versailles, France (48°48'26"N, 02°05'13"E, elevation 114 m). The full experimental design is detailed in Dubs et al (2018). Soil texture was silty (62.96% silt, 19.36% sand, 17.67% clay) with 12.00 (standard deviation SD 1.05) mg. kg^{-1} of total organic C, 0.93 (SD 0.05) mg. kg^{-1} of total N, and 34.43 (SD 10.62) mg. kg^{-1} of available (Olsen) P. In a previous step, 58 bread wheat varieties (*Triticum aestivum* L.) of diverse origins (elite varieties, modern varieties bred for organic farming, MAGIC recombinant lines and few landraces) were phenotyped for 27 above- and below-ground traits related to agronomic and ecological functions (Cantarel et al., 2021). This database was used for a multi-trait classification of varieties into four functional groups (Dubs et al., 2018), hereafter referred to as clusters. In our

field experiment, a subset of 16 varieties was selected with four varieties of each of the four functional clusters (**Table S1**). Wheat varieties (Altigo, Trémie, F426 and A22) of the cluster 1 (cl1) represent a functional group characterized by highest susceptibility to fungal diseases, low SRL and weak flag leaf N content. The functional group cl2 was composed of wheat varieties (Renan, Skerzzo, Midas, Alauda) less susceptible to fungal diseases (only one susceptible variety) but also with low SRL. The cl3 functional group was composed of tall wheat varieties used in organic agriculture (landrace : "Blé Autrichien", varieties bred for organic agriculture: Hermès, Maxi, Ritter), with high SRL and mean susceptibility to fungal diseases (two out of 4 being rather sensitive). Finally, the cl4 functional group contained elite varieties (Grapeli, Soissons, Arezzo, Boregar) with high SRL and lowest susceptibility to fungal diseases (**Table S1**). Preferential uptake of ammonium or nitrate was also a distinctive trait between wheat clusters, with high level of NO_3^- uptake capacity for cl2 and high levels of NH_4^+ uptake capacity of cl3 (Cantarel et al., 2021).

Varieties were grown alone or as mixtures of two, four or eight varieties. To explore a gradient of functional diversity among varietal diversity, mixtures were composed of wheat varieties belonging to the same or different clusters (Fig. 1). In other words, for a given varietal richness level, the number of functional groups varied from one to the highest possible number. In total, 88 diversity modalities (16 mono varietal and 72 mixtures) were sown on a randomized design, using plots of 10.5 m x 8 m, sown at a density of 180 seeds m^{-2}. Since the objective was to quantify the effects of varietal richness and functional diversity and not to assess significant differences between pairs of mixtures, there was no replicate of each varietal mixture but true replicates (i.e. different variety compositions) for each variety number x functional group number combination, as done in current experimental designs exploring biodiversity-ecosystem functioning relationships (e.g. Weisser et al., 2017). Wheat was sown in October (2015) after a preceding maize crop. The crop received 170 kg N ha^{-1} in two doses, which is matching with a wheat grain yield objective of 8.5 t ha^{-1}. Note that the climatic conditions in France in 2016 were characterized by abnormally warm temperatures in late autumn followed by abnormally wet conditions in spring, leading to extreme yield losses on wheat (Ben-Ari et al., 2018). Seeds were coated with a pesticide mix (CELEST, 2 cm^3 kg^{-1} – Fludioxonil 25 g dm^{-3} and SIGNAM 600 cm^3 kg^{-1} – Cypermethryne 300 g dm^{-3}). No additional fungicide or insecticide treatment was applied afterwards. In March 2016, one herbicide treatment was applied at growth stage 31 (first node detectable; 50 g ha^{-1} Harmony extra, 250 g ha^{-1} Archipel, and 1 dm^3 ha^{-1} adjuvant Actirob 842 g dm^{-3} esterified rapeseed oil base).

Plant and soil sampling

Plants were sampled in May 2016 at the heading stage (variable according to varieties). On each plot, 50 plants were sampled along two 3-m rows, to ensure that plot heterogeneity was accounted for and that sampling encompassed all the wheat varieties present in the plot considered. Ten soil cores were sampled in the topsoil root zone at the same locations in the plot as for plant sampling, and pooled into a single soil sample per plot. Roots were washed, weighted and three segments of two centimeters were sampled from each plant. The vials containing either soil or root samples were directly covered by liquid N_2 and stored at -80°C. For yield data, the central section of each plot (1.75 × 8 m) was harvested in early August 2016, with a cut-width of 1.75 m (MB Hege 140 combine harvester; Hege Maschinen GmbH). Grain yield are expressed as mean grain weight measured at 15% humidity in t ha^{-1}.

Quantification of soil chemical properties and enzymatic activities

Soil organic C (SOC) and total N (Ntot) were determined by dry combustion (NF ISO 10694 and NF ISO 13878). Available P content was extracted using the Olsen method and assayed colorimetrically (NF ISO 11263). Two enzymatic activities (leucine aminopeptidases (LAP) and phosphatases) were measured after three hours incubation at soil pH 6.55 ± 0.07 (pH in water extract) on 1 g of soil (after 12 hours thawing at 4°C) by fluorometric method using methylumbelliferyl (MUB)-substrates (details in Bell et al. 2013). Enzymatic activities in the soil are expressed in nanomoles of substrate mineralized per g of soil per minute.

Measurements of AM fungal abundance and diversity

Wheat roots were ground in a mortar with liquid N_2, and a volume of 250 mm^3 was used for DNA extraction. DNA quality and concentrations were measured using Invitrogen™ Quant-iT™ PicoGreen™ dsDNA Assay Kit.

Abundance of Glomeromycotina in wheat roots was assessed by qPCR using FLR3-FLR4 primers (approx. 380 pb; Golotte et al. 2004). Final nucleotide "T" was removed from the FLR3 original primer to enclose more AMF species and reduce positive bias toward the Glomeraceae family (personal communication from D. van Tuinen). The qPCR reaction was carried out on 5 ng of root DNA in a final volume of 10 mm^3 comprising 5 mm^3 Mix Sso advanced SYBR green Biorad, 0.5 mm^3 of each primer at 10 µM, 2 mm^3 of DNA extract, and 2 mm^3 of ultrapure water. The PCR cycle was as follows: 2 min at 98°C, (5 sec at 98°C, 30 sec at 60°C, 30 sec at 72°C) for 39 cycles, plus melting curve measurement. Each plate included duplicate reactions per DNA sample and triplicate for standard set. If variation coefficient exceeded 20% between duplicates, the result was confirmed by a third measurement. Standard curves were obtained by serial dilution (10^{-8}–10^{-3}) of linearized plasmids containing a cloned FLR3-FLR4 gene (certified as Glomeromycotina by Sanger sequencing). For conciseness, the number of gene copies per ng of root DNA in wheat roots will hereafter be referred in the text as AMF abundance.

For metabarcoding, amplicons were constructed following a two-step PCR protocol as described in Battie-Laclau et al (2020). Two Glomeromycota specific primer-pairs were used, FLR3/FLR4 (modified on the final nucleotide "T" as previously; approximatively 380 pb; Golotte et al. 2004) and NS31/AML2 (approximatively 480 pb; Simon et al., 1992, Lee et al., 2008), targeting respectively the Large Sub-Unit (LSU) region and the Small Sub-Unit (SSU) region of the ribosomal DNA (rDNA). For the first round of PCR (PCR1), reactions were carried on two replicated dilutions of 15 $ng.mm^{-3}$ of DNA per sample. PCR conditions are presented in Table S5. For each marker, the two PCR1 amplicons were pooled and purified by magnetic beads (Clean PCR, Proteigene, France). The second PCR was performed using a Nextera® XT Index Kit (Illumina, San Diego, USA) following the manufacturer's instructions. After a purification with magnetic beads, these final PCR products were multiplexed and sequenced on a MiSeq Illumina sequencer using MiSeq Reagent Kit v3 (600-cycle; Illumina).

Bioinformatic analyses

We analyzed DNA sequence through the bioinformatics pipeline described in https://adrientaudiere.gitlab.io/solfami_bioinfo_ssu/and/solfami_bioinfo_lsu/. In short, sequences were quality filtered using the filterAndTrim function from the dada2 package (Callahan et al. 2016a), by first truncating reads of a quality score inferior to 10 and second, discarding sequences with less than 50 bp. Then we followed dada2 classic pipeline (Callahan et al. 2016b) to obtain chimera-free amplicon sequence variants (called ASV) using paired-end. Each ASV longer than 200 pb was then taxonomically assigned to MaarjAM database (Öpik et al 2010) with the assignTaxonomy function from dada2 (Callahan et al. 2016a), which implements the RDP classifier of Wang et al. (2007).

AMF diversity

Alpha-diversity was evaluated by Hill numbers (using the 'vegan' R package, Oksanen et al 2022) without previous normalization following McMurdie & Holes (2014). Hill diversity indices (Hill 0 = Richness; Hill 1 = Hill-Shannon; Hill 2 = Hill-Simpson) consider both the number and the relative abundance of species, with decreasing sensitivity to rare species and to sample size (Roswell et al., 2021). Differences in terms of AMF community composition (beta-diversity) between functional clusters was analyzed by PERMANOVA on Bray-Curtis distances using the 'adonis' function from the 'vegan' R package (Oksanen et al 2022), after normalization by standardizing abundances to the median sequencing depth.

Statistical analyses

All statistical analyses were realized using R (version 4.0.4). Linear mixed models were performed with the package 'nlme' to determine whether the number of wheat varieties and the number of functional wheat clusters influenced variables related to wheat plant characteristics involved in nutrient acquisition, focusing on AMF abundance in roots as well as phosphatases and LAP enzymatic activities in the root zone. The same analyses were done on the alpha-diversity of AMF in roots.

The full linear mixed models also included soil proprieties as co-variables that could have an impact on the variables of interest (C, N, Olsen P) and the spatial y-coordinates was set as random effect as first exploration with linear models detected some spatial heterogeneity along the y-axis of the field experimental site (Fig. 1). Co-variables of the models were standardized (centered on the matrix and scaled on each variable's standard deviation; *scale* function in R) to discard spurious effects of various units of covariates included models. The final model was selected step by step by keeping variables that significantly contributed to the model using the Akaike information criterion. Main factors of interest (number of wheat genotypes and of functional clusters) were always kept in the final model. All models were tested for normality and AMF abundance and Hill indexes had to be log-transformed. Analyses of Deviance (Anova Type II) were performed on the final models, with the *Anova* function of the 'car' R package. The full model is detailed in **Table S2**. Direct correlations between enzymatic activities and AMF abundance were analyzed by Pearson coefficient and plotted with *ggscatter* (package 'ggpubr').

Comparisons of the measured variables between the four functional wheat clusters were realized on a data subset including only functionally homogeneous mixtures (plots composed of varieties belonging to the same cluster). This dataset included seven plots for each cluster type (four with one variety, two with two varieties, and one with four varieties).

Results

Wheat shoot mass, nitrogen content and grain yield

Neither the number of wheat genotypes nor the number of functional clusters in mixtures did explain shoot biomass and total N uptake (p-values >0.1; **Table S2**) at the heading stage or the final yield (p-value >0.1; **Table S2**). However, yield was significantly different between the four functional clusters (wheat grown as functionally homogeneous mixtures), with a significantly higher grain yield for Cluster 4 composed of disease-resistant elite varieties (**Fig. 2. A**.). Such difference between the functional clusters in mixtures was not visible on shoot biomass at the heading stage in May(**Fig 2. B.**), and no significant difference between total N content in shoot biomass was found (**Fig. 2. C**).

Effect of wheat diversity on enzymatic activities in the root zone and AMF abundance in roots

There were no significant differences in enzymatic activities in the root zone between the four functional clusters (**Fig. 2 D.** and **Fig. 2 E.**). The number of wheat varieties and of functional clusters did not affect phosphatase activities either (**Table 1**). However, both varietal and functional diversity had a significant and positive effect on the LAP activities in the root zone (**Table 1**), with significantly higher LAP activities measured on soil from the root zone of mixtures with four or eight varieties compared to lower numbers of varieties (two or one varieties) (**Fig. 3 A.)**. Nevertheless, there was no significant effect of functional group number on LAP activities, based on pairwise comparisons (**Fig. 3 B.)**. Total N in shoot biomass was significantly impacted by LAP activities (p-val= 0.0407) with a slight but positive relation (slope= 0.36; **Table S1 B**).

There were no significant differences in AMF abundance in roots between the four functional clusters (**Fig. 2 F.**). The number of wheat varieties however significantly impacted AMF abundance in roots (**Table 1**), with higher abundance (2.3-fold increase on average) in plots mixing eight wheat varieties compared to pure cultures (**Fig. 3 E**.). There was no effect of functional cluster number on AMF abundance in roots (**Fig. 3 F**.).

As co-variable in linear mixed models, total N in soil had a positive correlation with LAP activities slope=0.019; **Table 1**) but it was negatively correlated with phosphatases activities (slope=-0.22; **Table 1**)). The AMF abundance in roots was also impacted by total N in soil, with a positive correlation (slope=0.179; **Table 1**), but negatively affected by available (Olsen) P in soil (slope=-0.208; **Table 1**). Phosphatase activities were negatively and linearly related with AMF abundance in roots (Pearson, R=-0,37, p-val = 0.002). On the contrary, LAP activities exhibited a positive linear relationship with the abundance of AMF in roots (**Fig. 4;** Pearson,R=0.49, p-val < 0.001).

AMF community composition

For the LSU and SSU regions respectively, a total of 240 and 409 Glomeromycota ASVs (Amplicon Sequence Variants) were obtained from 8 753 361 and 18 228 610 sequences. For the LSU, mean sequencing depth per sample was 99 661, grouped into 277 ASVs after discarding ASVs with less than five reads. For the SSU, mean sequencing depth per sample was 209 016 after discarding one outlining sample with less than 50 000 sequences, and 392 ASVs (> 5 sequences).

In the same order of abundance, the number of ASVs from the LSU and SSU regions belonged to the orders of Glomerales (LSU: 153; SSU: 206), Diversisporales (LSU: 3; SSU: 44), Archeosporales (LSU: 1; SSU: 41), Paraglomerales (LSU: none; SSU: 3), and non-identified (LSU: 83; SSU: 115). The ten most abundant ASVs represented 92.32 % of all LSU sequences, among which *Funneliformis* was the dominant genus (8 out of 10 ASVs; **Table 2**). For the SSU region, the ten most abundant ASVs represented 96.45 % of all sequences, with ASVs from the genera of *Funneliformis*, *Scutelospora*, *Archeospora*, *Diversispora* and two uncultured Glomeromycota. For both LSU and SSU regions, the most abundant ASV blasted on *Funneliformis caledonium* (**Table 2**).

Effect of wheat diversity on AMF diversity in roots

There was no difference in AMF community composition between wheat functional clusters, with no significant variations in beta-diversity (Permanova, *p-value*=0.165 and 0.098, on respectively the LSU and SSU data; **Table S3**). However, on the SSU, wheat functional clusters significantly differed in alpha-diversity (Hill 1 index; **Fig 2 G**.) with significantly higher AMF diversity in wheats of cluster 4 than for that of cluster 1. No such significant difference between wheat functional clusters was found for the LSU data (Hill 1 index; **Fig 2 H**.). The number of wheat varieties did not influence AMF alpha-diversity neither on the LSU nor on the SSU sequencing data (**Table 1**). However, the number of wheat functional clusters had a significant impact on Hill1 index for the LSU and SSU sequencing data (**Table 1, Fig. 3 H., J.**) but Tukey's post-hoc test revealed significant differences between mixtures treatments only for the SSU marker, with higher diversity for mixtures composed of two than three wheat functional clusters (**Fig. 3 J**.). The Hill 0 index (specific richness) was never significantly impacted by wheat diversity, while Hill 2 followed the trend of Hill 1(**Table 1**).

Discussion

The number of varieties, but not the type or number of variety functional groups, increases AMF abundance

Mixtures with increasing numbers of wheat varieties significantly increased AMF abundance in roots (Table 1, Fig. 3E). Different non-exclusive mechanisms could explain such an effect. First, this could be due to a sampling effect, where increasing the number of varieties in mixtures also increases the chance to include particularly mycotrophic varieties. An *ex situ* experiment showed indeed a high variability in AMF colonization rates between genotypes in durum wheat (*Triticum turgidum* L.), ranging from 7–84% (Ganugi et al., 2021). Such an increase in AMF abundance could also be due to a complementarity effect where host diversity enabled niche differentiation for AMF colonization ; or simpler said: each AMF strain found its "favorite" wheat variety partner. Also, if increasing the number of wheat varieties enabled higher plant photosynthesis (through potential above- and belowground resource sharing, higher disease resistance, and competitive replacement), carbon availability to support AMF symbiosis could have been enhanced, thus increasing AMF abundance. In the experiment, there was no evidence however of improved biomass or grain yield when increasing the number of wheat varieties. This does not necessarily relates with the amount of carbon allocated to AMF, though, which would have been extremely difficult to measure in such a field experiment. In addition, as the ten most abundant ASV represented > 90% of all sequences, with no differences between functional wheat clusters (Permanova), these common AMF strains were certainly present on all wheat, thus creating a dense common mycorrhizal network. Investment into common mycorrhizal networks has been shown to depend on the identity of mixed plant species or genotypes. Engelmoer et al. (2015) found that mixing different host species (*Daucus carota* L., *Cichorium intybus* L. and *Medicago truncatula* Gaertn.) reduced the investment of plants into extraradical hyphae of their common network. At the intra-specific level of host plant diversity, File et al (2012) showed higher hyphal length in an AMF network between sibling plants compared to populations of more distantly related plants of the same species (*Ambrosia artemisiifolia* L.). As extra-radical hyphal length has been shown to be the best

indicator of AMF contribution to plant nutrition in the study of Sawers et al. (2017), it would have been interesting to evaluate the effect of intra-specific diversity on plant investment into the common mycorrhizal network, to verify whether it followed the observed increase of AMF abundance in roots with increasing genetic diversity in wheat variety mixtures. The role of AMF on wheat development was not directly investigated here, but a pot experiment on maize recently showed substantial overyielding of varietal mixtures only occurred when plants were grown in association with the AM fungus *Funneliformis mosseae* (Wang et al., 2020).

In contrast with the effect of wheat variety diversity, there was no effect of the functional diversity of wheat variety mixtures on AMF abundance (Table 1, Fig. 3F.). Since the clusters of wheat varieties differed for criteria such as root morphology and resistance against pathogens (**Table S1**), this offered an interesting opportunity to challenge two common statements and debated hypotheses, concerning trade-offs between AMF root colonization and either root morphology or disease resistance. Actually, the four wheat clusters did not differ in root AMF abundance (Fig. 2F.). We noted no difference between clusters with high SRL (Clusters 3 and 4) and those with low SRL (Clusters 1 and 2) and thus found no visible trade-off between soil foraging strategies by AMF root colonization and high SRL (Fig. 2F.; **Table S2**), as previously found by Hetrick (1991). Ruiz-Lozano et al. (1999) showed that genes involved in resistance to powdery mildew in barley (*Hordeum vulgare* L.) reduced root colonization by the AM fungus *Funneliformis mosseae* (called by then *Glomus mosseae*). However, resistance to fungal-borne disease of wheat did not reduce AMF abundance in roots in our experiment, when comparing wheat plots of Cluster 4, composed of only elite resistant varieties, to those of the highly susceptible varieties of Cluster 1 (Fig. 2F.). When looking at the direct effect of mean susceptibility to yellow rust on the abundance of AMF in roots (regardless of functional cluster, on the total trait matrix), a significant effect was observed (p-value < 0.001; **Table S2**) but with a negative correlation. This result was confirmed when looking at the effect of the observed pressure of yellow rust on wheat plots during the experiment and the AMF abundance (p-value < 0.05; **Table S2**). Disease pressure was very high in France in 2016 due to abundant rainfall in Spring, as occurred at our experimental site, and the effect of wheat variety mixtures in the present field experiment have been further discussed by Vidal et al. (2020). This correlation showed that disease symptoms altering plant development also had a negative impact on AMF colonization, possibly by limiting C allocation or AMF colonization.

Activities of leucine aminopeptidases but not phosphatases positively correlated to wheat variety diversity and AMF abundance

Neither the number of wheat varieties nor the number of wheat functional clusters did impact phosphatase activities in the root zone (Table 1; Fig. 3C. **and 3 D.**). In contrast, these two components of wheat diversity, i.e. genetic and functional diversity, both had a positive effect on LAP activities in the root zone (Table 1; Fig. 3A.). Leucine aminopeptidases (LAPs) are metallopeptidases that cleave N-terminal residues from proteins and peptides and are expressed by soil bacteria (Loeppmann et al., 2016). Proteins are an important source of N, which can represent 40% of the total soil N (Schulten & Schnitzer et al., 1997). Increasing genetic and functional diversity of wheat might have triggered the demand for uptake of N by plants, stimulating mechanisms to access organic N pools. LAP activities in the root zone were positively correlated to total N content in wheat shoot biomass (**Table S1**), as well as to AMF abundance in roots (Fig. 4). The relation between LAP activities and AMF abundances might not be direct: both variables can be affected by a common external factor or variable, such as wheat diversity. However, enhanced LAP activities concomitant to increased AMF abundance in the root can also be the consequence of increasing root–microorganism competition for soil inorganic N (Kuzyakov & Xu 2013). Liu et al. (2021) showed that N uptake from organic patches via AM fungal hyphae was directly affected by soil LAP activities, possibly due to a stimulation of the microbial activities in the soil. Previous studies demonstrated that AMF can stimulate soil microbial activities related to N cycling by influencing the structure of bacterial communities (Nuccio et al., 2012; Jansa et al., 2019 for a review). Although much less probable, it should be noted that little is known about LAP activities of AMF *per se*. In the genome of *Rhizophagus irregularis*, three genes have been identified, each encoding a protein with a putative LAP function (JGI mycocosm.jgi.doe.gov; Chen et al 2018; **Table S4)**. One of the genes (coding for the 1524872 protein) is highly expressed in the extraradical mycelium and the mycelium in planta (personal com. C. Roux; unpublished data). Additionally, a peptide signal, enabling the release of the protein outside the cell, is predicted only for this protein (not present on the two

other genes), but with low prediction robustness. Hence, this protein could be an interesting candidate to explain patterns of LAP activities variation with the amount of AMF copies measured in the roots. However, its occurrence in the soil remains to be verified. Further studies should also test to which extent protocols used for soil enzymatic activities may extract cytosolic enzyme by breaking hyphae, as they often include steps of soil freezing and thawing, sieving and blending (as was the case in the protocol used here).

The type or number of functional groups, but not the number of varieties, weakly alters AMF diversity

To our knowledge, this is the first study focusing on the effect of plant intra-specific diversity and its vertical effect on AMF diversity. We expected an increased AMF diversity with increasing variety number. However, wheat variety diversity had no significant effect on AMF diversity (Table 1, Fig. 3G. **and 3 I.**), while there was a significant but weak effect of the functional diversity of wheat variety mixtures on some of the indicators of AMF diversity, such as SSU Hill 1, SSU Hill 2 and LSU Hill (Table 1, Fig. 3J.). In addition, the functional clusters of wheat varieties significantly differed in AMF diversity (Fig. 2G.). Indeed, based on the LSU data, wheat plots of Cluster 4 (composed of resistant elite varieties), displayed significantly higher AMF diversity than those of Cluster 1 (composed of less resistant elite varieties and varieties from a highly recombinant inbred MAGIC panel). Again, AMF diversity was possibly linked to host resistance, due to (i) either a functional link between disease resistance mechanisms and AMF selectivity, or (ii) the amount of carbon allocated by the host plants, with stronger competition between AMF when the resource is scarce (i.e. in wheat affected by fungal pathogens). The rather weak effect of wheat functional diversity on AMF diversity observed in the present experiment can be explained by similar AMF communities composition between functional clusters (**Table S3**), and between varieties (not tested here). As we expected that intra-specific diversity of host plants might induced subtle variation in AMF community composition (compared to communities of different plant species), we used two different AMF markers. The SSU region was used for its better coverage of the different AMF families, completed by the LSU marker, for its better taxonomic resolution than the slowly evolving SSU region (Krüger et al., 2012; Hart et al., 2015; Delavaux et al., 2021). On both markers, the genus *Funneliformis* was strongly dominating. On the SSU, a single *Funneliformis* ASV representing 93.3% of all sequences, subdivided into eight dominating ASVs representing 90.7% of all sequences on the LSU. *Funneliformis* was also the dominating genus in two field studies in Canada on large sets of durum wheat genotypes including landraces and commercial varieties (Ellouze et al., 2018; Stefani et al., 2020). Both studies sequenced different regions of the SSU and revealed only weak differences in alpha-diversity and composition of AMF communities between wheat genotypes in roots, rhizosphere and bulk soil. Stefani et al. (2020) found no difference at all, while Ellouze et al. (2018) did not find any difference when looking at roots, but observed that durum wheat genotype differently shaped the composition of AMF communities in their rhizosphere. Jacquiod et al. (2021) found significant differences in the diversity and the composition of rhizosphere microbial communities of wheat elites and landraces, with root-associated fungi being particularly dependent of the interaction between the plant genotype and the environment. Under low fertilizer inputs, OTU richness was higher in ancient wheat varieties than in modern varieties, but it was the opposite upon addition of inorganic fertilizers. This phenotypic plasticity with varying environmental conditions needs to be hold in mind: indeed, the observed responses of the wheat variety functional clusters discussed in our study were obtained under a specific environment, which corresponded to a rather high N and P fertilizer input history. Would an increase of AMF diversity induce a better functioning of agroecosystems is still an open question, which the present work hardly addressed. This would require to explore a broader diversity of environmental conditions, including a range of low fertilizer input agroecosystems. AMF fungi are recognized to have some degree of functional diversity (Van Der Heijden et al., 2018), with a positive effect on some key functions in ecosystems. AMF diversity effect on plant species complementarity as has been investigated in experimental settings (microcosm or macrocosm experiments; Wagg et al., 2015), but remain challenging to demonstrate and thus hardly tangible in field conditions.

Conclusion & Perspectives

Although wheat variety diversity did not affect aboveground biomass and yield, the within-field number of wheat varieties grown in mixtures stimulated AMF abundance in roots and enhanced leucine amino-peptidase (but not phosphatase)

activities in the root zone. This possibly impacted the plant nutrient acquisition capacity through improved access to N from organic sources. The effect of functional cluster diversity on these processes was less prominent, and underlying mechanisms (complementarity, sampling effect...) need to be further understood. Concerning AMF diversity, mixing varieties of two different wheat functional clusters enhanced AMF diversity, but the effect was subtle and did not increase with increasing wheat variety diversity. Our results showed the presence of a limited number of dominant AMF strains, suggesting a dense common mycorrhizal network between plants in wheat variety mixtures. The ecological (and economic) costs of the excess use of inorganic N and P fertilizers call for an urgent reduction of those inputs. Our results showed that crop intra-specific diversity is an interesting lever to induce changes in processes involved in nutrient acquisition strategies, although this should be explored under a diversity of environmental conditions. In this respect, our work is calling for additional studies under more limiting conditions (in terms of nutrient availability), where positive interactions could be even more important, according to the stress gradient hypothesis.

Declarations

Acknowledgments

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Authors contribution: Jérôme Enjalbert and Xavier Le Roux contributed to the field study conception and design. Field sampling was performed by Philippe Hinsinger, Didier Arnal, Damien Dezette, Jérôme Enjalbert and Xavier Le Roux. Laboratory analyses were made by Josiane Abadie, Damien Dezette and Elisa Taschen, and bioinformatics by Adrien Taudière. Data collection and analysis were performed by Elisa Taschen, who also wrote the first draft of the manuscript, with helpful discussion with Claude Plassard and Esther Guillot, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Wheatamix consortium regroups many collaborators participating in the field experiment and the elaboration of the trait data set of wheat varieties."

Data Availability

The datasets generated during and/ analyzed during the current study are available in the Data INRAE repository, [PERSISTENT LINK TO DATASETS : Submission in progress].

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Tables

Table 1. ANOVA analyses on linear models searching for the effect of wheat diversity, in terms of numbers (Nb) of varieties or of functional clusters, on two soil enzyme activities (leucine amino-peptidases and phosphatases), AMF abundance in wheat roots and AMF diversity (Hill indices) evaluated using two markers targeting the Large Sub-Unit (LSU) or Small Sub-Unit (SSU) region of the ribosomal DNA.

	Explanatory variables	Chisquare	Df	Pr(>Chisq)	P-value	Intercept	slope
Leucine amino peptidase activities	**Nb varieties**	11.6436	3	0.008709	**		
	Nb clusters	9.1372	3	0.027521	*		
	Total N	9.3264	1	0.002259	**	0.219	0.019
Phosphatase activities	**Nb varieties**	0.3782	3	0.9447			
	Nb clusters	4.4307	3	0.2186			
	Total N	27.1270	1	1.905e-07	***	2.96	-0.22
AMF abundance ¤	**Nb varieties**	11.7872	3	0.008149	**		
	Nb clusters	6.1081	3	0.106467			
	Pi Olsen	8.8856	1	0.002874	**	7.092	-0.208
	Total N	8.9464	1	0.002780	**	7.092	0.179
SSU Hill 0 ¤	**Nb varieties**	3.9492	3	0.2670			
	Nb clusters	3.4090	3	0.3328			
	Total N	16.5858	1	4.65e-05	***	42.31	
SSU Hill 1 ¤	**Nb varieties**	5.1316	3	0.1624113			
	Nb clusters	9.0520	3	0.0286080	*		
	Total N	11.3465	1	0.0007559	***	0.431	0.054
SSU Hill 2 ¤	**Nb varieties**	4.5809	3	0.205185			
	Nb clusters	9.2412	3	0.026250	*		
	Total N	7.5348	1	0.006052	**	0.113	0.018
LSU Hill 0 ¤	**Nb varieties**	3.2251	3	0.3582			
	Nb clusters	2.2177	3	0.5285			
LSU Hill 1 ¤	**Nb varieties**	2.2634	3	0.5195775			
	Nb clusters	9.2822	3	0.0257653	*		
	Total C	11.7779	1	0.0005994	***	1.339	0.113
LSU Hill 2 ¤	**Nb varieties**	1.0462	3	0.7900645			
	Nb clusters	7.3533	3	0.0614488	.		
	Total C	11.0527	1	0.0008856	***	1.339	0.113
¤ log transformed							

Table 2. Blast results (NCBI) of the ten most abundant Amplicon Sequence Variants (ASV), representing 92.2 % of the sequences of the Large Sub-Unit (LSU) dataset and 96.5 % of the Small Sub-unit (SSU) dataset. When possible, the closest blast was selected from reliable taxonomic identified species only (isolates and strains, referenced in collections).

rDNA portion	ASV	% of sequences	Genus	Blast species	E value	Per. Ident	Accession
LSU	ASV_1	67.8	*Funneliformis*	*Funneliformis caledonium*	3E-156	97.58%	FN547496.1
	ASV_18	11.6	*Funneliformis*	*Funneliformis caledonium*	3E-156	97.58%	JQ048873.1
	ASV_40	3.21	*Funneliformis*	*Funneliformis caledonium*	3E-156	97.58%	JQ048874.1
	ASV_58	2.93	*Funneliformis*	*Funneliformis mosseae*	1E-169	**100.00%**	AY541909.1
	ASV_49	2.04	*Funneliformis*	*Funneliformis geosporum*	2E-158	97.89%	EU931263.1
	ASV_102	1.27	*Funneliformis*	*Funneliformis geosporum*	3E-160	98.18%	EU931263.1
	ASV_77	1.1	*Funneliformis*	*Funneliformis caledonium*	1E-159	98.18%	JQ048874.1
	ASV_91	0.9	*Rhizophagus*	*Rhizophagus irregularis*	3E-165	99.39%	HF968916.1
	ASV_151	0.77	*Funneliformis*	*Funneliformis caledonium*	1E-154	97.28%	JQ048874.1
	ASV_85	0.7	*Septoglomus*	*Septoglomus constrictum*	2E-163	98.79%	AF304971.1
SSU	ASV_1	93.6	*Funneliformis*	*Funneliformis caledonium*	3E-126	**100.00%**	KU136397.1
	ASV_86	0.7	*Scutellospora*	*Scutellospora calospora*	1E-124	99.60%	KU136427.1
	ASV_105	0.5	*Archaeospora*	*uncultured Archaeospora*	2E-123	99.60%	MH629114.1
	ASV_80	0.4	*Archaeospora*	*Archaeospora trappei*	3E-126	**100.00%**	Y17634.3
	ASV_155	0.3	*Archaeospora*	*Archaeospora trappei*	1E-124	99.60%	Y17634.3
	ASV_95	0.2	*Diversispora*	*Diversispora sp.*	6E-123	99.20%	MF621782.1
	ASV_78	0.2	*Uncult.Glomeromycotina*	*Uncult. Glomeromycotina*	2E-123	99.60%	JN794959.1
	ASV_138	0.2	*Uncult. Glomeromycotina*	*Uncult. Glomeromycotina*	3E-125	100.00%	LT833532.1
	ASV_206	0.1	*Funneliformis*	*uncultured Funneliformis*	6E-118	98.01%	MH629586.1
	ASV_122	0.1	*Diversispora*	*Diversispora aurantia*	9E-126	**100.00%**	EF581880.1

Figures

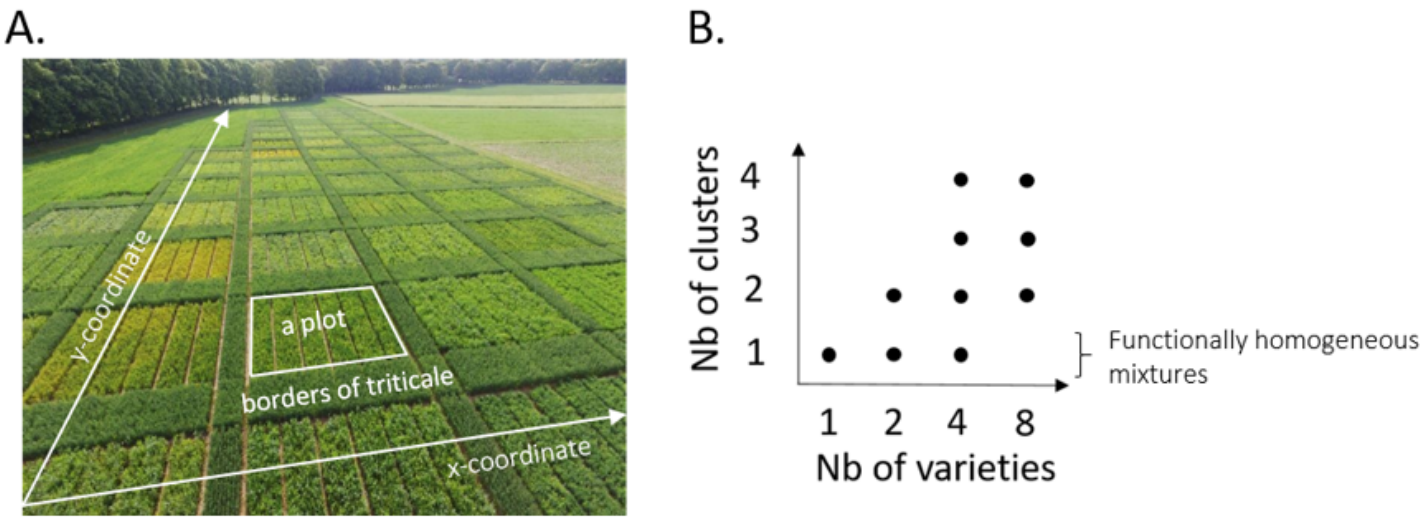

Figure 1

Aerial picture and design of the field experiment. A. Spatial distribution of the wheat plots in the field experiment (with x and y-coordinates), each plot being buffered by rows of triticale. B. Scheme displaying how wheat diversity varied in terms of variety number and number of functional clusters of these varieties. Plots composed of varieties belonging to a single functional cluster are called “functionally homogeneous mixtures”.

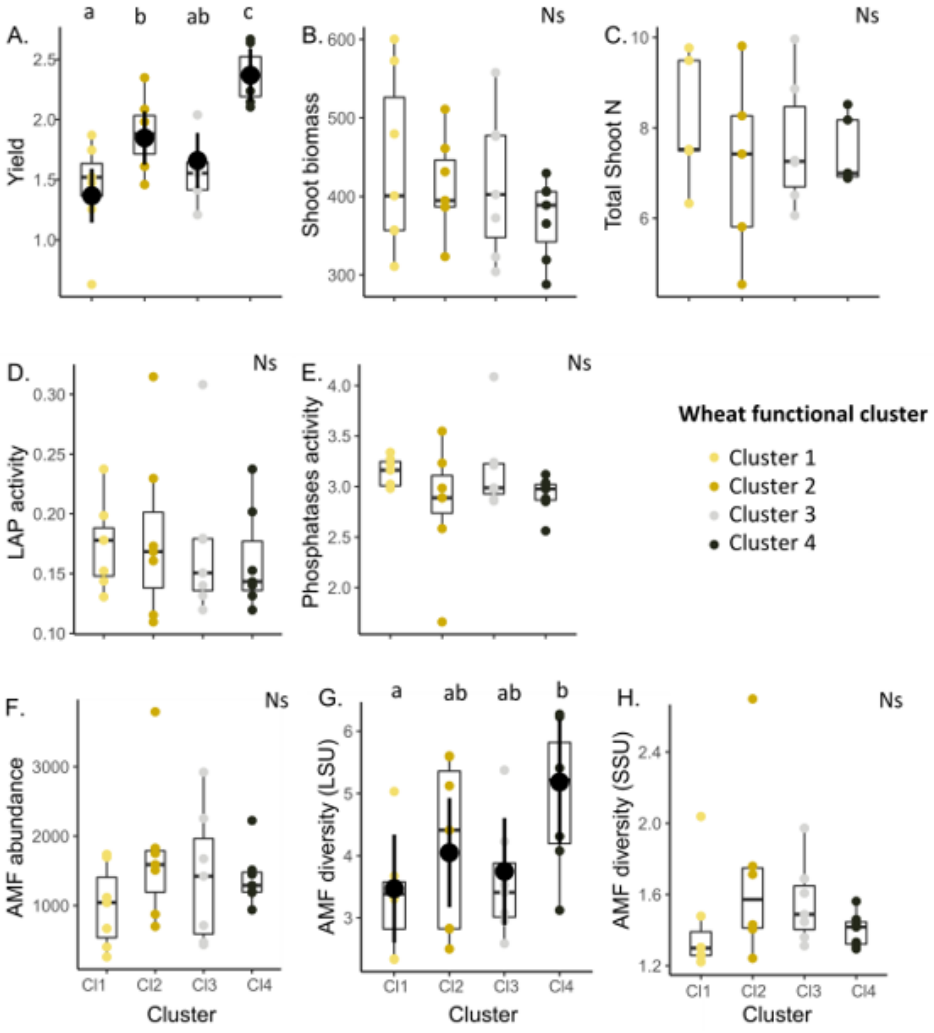

Figure 2

Boxplots presenting the values of: A. Grain yield (t ha^{-1}), B. Shoot dry biomass (g for 50 plants), C. Total N in dry shoot biomass (mg. on 50 plants), D. Leucine amino-peptidase activity (LAP; nmol. of substrate $g^{-1}.min^{-1}$), E. Phosphatases activity (µmol. of substrate $g^{-1}.min^{-1}$), F. AMF abundance in wheat roots (gene copy number ng^{-1} root DNA), G. AMF diversity in roots (Hill 1 index) on the LSU marker, H. AMF diversity in roots (Hill 1 index) on the SSU marker, for each of the four functional clusters. For each variable, significant differences between clusters are indicated by different letters ($p < 0.05$), and bold points represent the marginal means (with confidence interval at 0.95) from the linear mixed model.

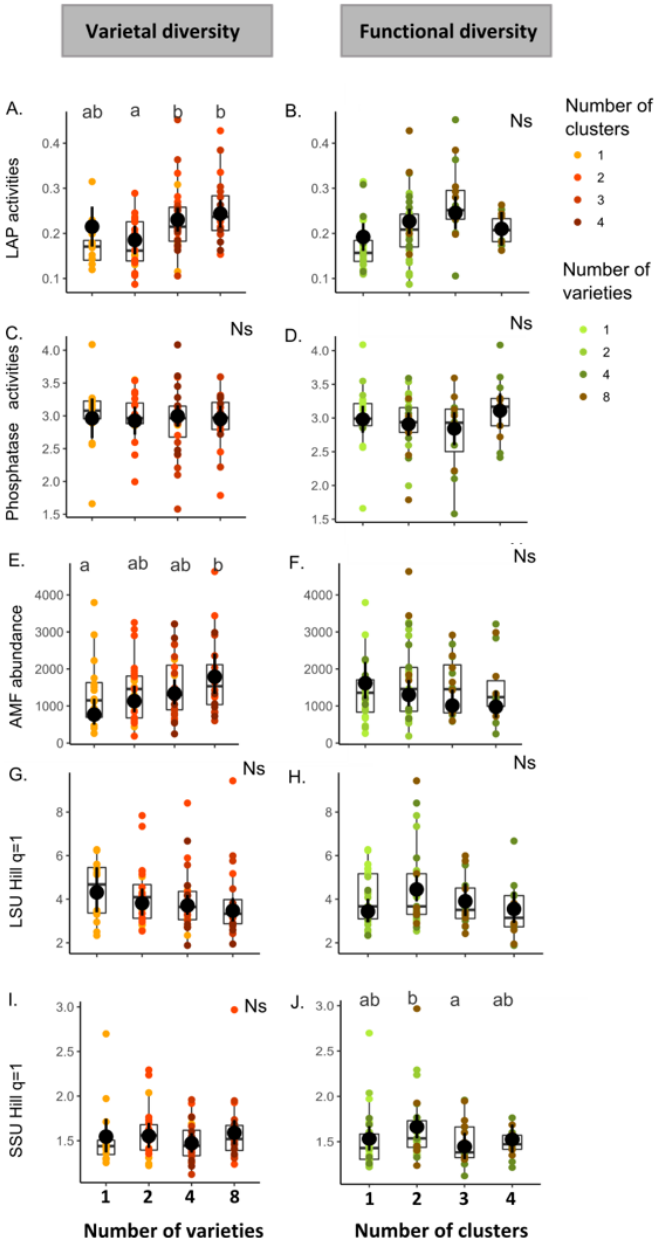

Figure 3

Boxplot showing variation according to the number of wheat varieties (varietal diversity, left panels) and the number of wheat functional clusters (functional diversity, right panels) of **A.B.** soil leucine amino-peptidase (LAP) activities (nmol. of substrate mineralized per gram of soil per minute) in the root zone, **C.D.** Phosphatase activities (nmol. of substrate $g^{-1}.min^{-1}$) in the root zone, **E.F.** AMF abundance in wheat roots (AMF gene copies ng^{-1}. root DNA), and alpha diversity of AMF index Hill 1 calculated from **G.H.** the LSU sequencing data and **I.J.** the SSU sequencing data. For each panel, significant differences between variety numbers or cluster numbers are indicated by different letters ($p < 0.05$; or Ns for non-significant according to Tukey tests), and bold points represent the marginal means from the used model.

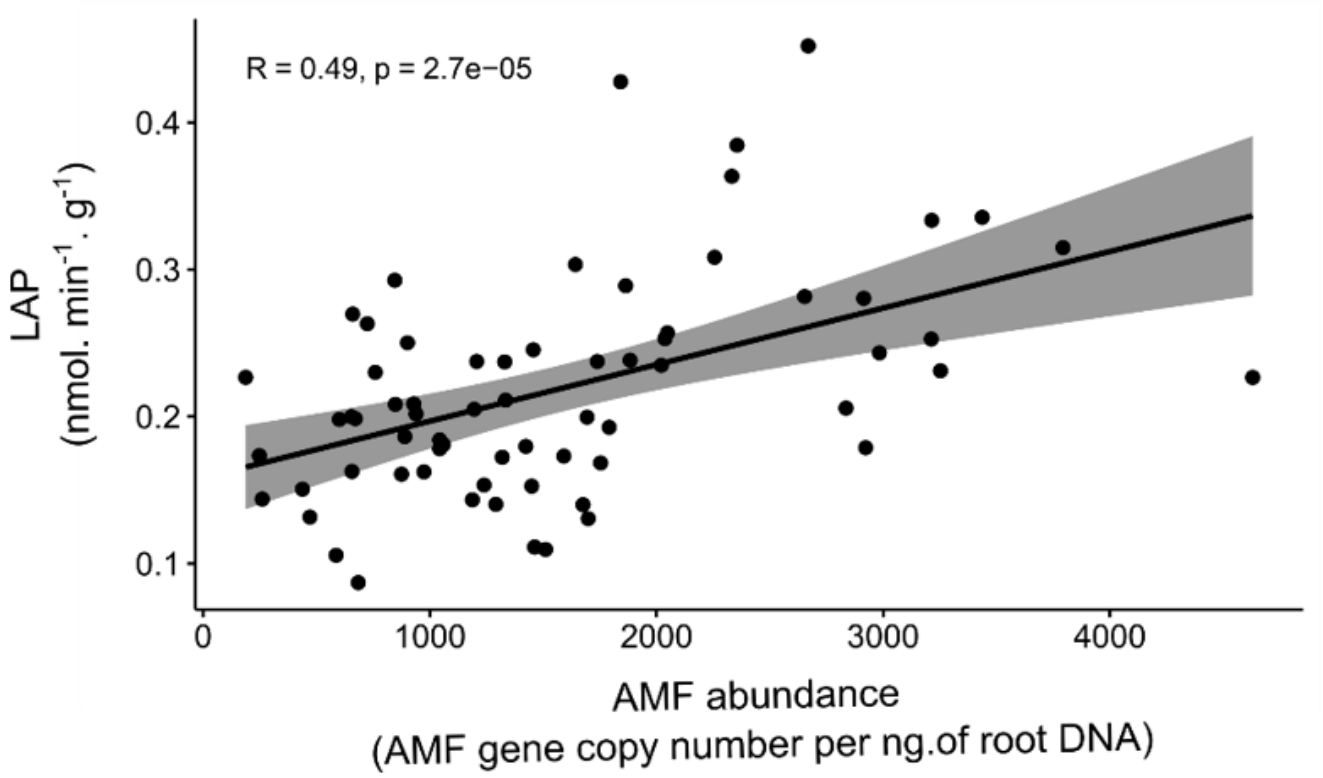

Figure 4

Correlation between the activity of leucine amino-peptidases (LAP) and arbuscular mycorrhizal fungi (AMF) abundance in roots, across all the 88 wheat plots. The Pearson coefficient (R) and p-value are indicated.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarytable.docx