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RESEARCH ARTICLE

Wheat varietal diversity modulates nitrogen-related enzymatic activities but has limited impact on arbuscular mycorrhizal fungi

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Abstract

Background and aims High-input agriculture involves low within-field crop genetic diversity, while plant diversity in natural ecosystems was 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 soil biota. We investigated the effect of within-field diversity of bread wheat varieties on arbuscular mycorrhizal fungi (AMF) and two enzymatic activities involved in organic nitrogen and phosphorus mineralization.

Methods We set up a field experiment to test whether varietal or functional diversity modulate the abundance and diversity of AMF and the activity of leucine aminopeptidases and phosphatases in the root zone, considering the influence of root morphology. We used sixteen wheat varieties clustered into four groups according to previously measured traits. The abundance of AMF in roots was measured by qPCR, community composition was analyzed by Illumina metabarcoding on two AMF markers (SSU, LSU), and enzymatic activities were quantified by biochemical assays.

Results Soil properties were the primary drivers of all response variables. Varietal diversity affected nitrogen-related soil enzymatic activities but not those related to phosphorus, with a significant increase of leucine-aminopeptidase activities with increasing varietal diversity. Wheat varietal and

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functional diversity marginally impacted the abundance of AMF, and functional diversity negatively affected AMF diversity on the SSU marker. Mean root traits modulated enzymatic activities, but not AMF communities.

Conclusions Increasing intra-specific crop diversity affects essential soil microbial processes, providing valuable insights for studying the relationship between plant diversity and soil microbiota in agroecosystems.

Keywords *Triticum aestivum* L. · Intra-specific · 18S and 28S rDNA · Phosphatase activities · Leucine-aminopeptidase activities

Introduction

The diversification of cropping systems is a key constituent of a more sustainable, ecological intensification of agroecosystems (Gaba et al. 2015) or agroecology (Wezel et al. 2020). Despite a long-lasting debate (Wardle 1999), studies in natural and manipulated ecosystems have shown that increasing plant species richness generally enhances several ecosystem functions (Van der Plas 2019), in particular ecosystem productivity and stability (Hong et al. 2022; Tilman et al. 1997; Weisser et al. 2017). This type of positive relationship relies on two non-exclusive mechanisms, namely the selection (or sampling) effect and the complementarity effect (Loreau 1998; Hodapp et al. 2016). Functional diversity among plant species was shown to be a good predictor of ecosystem functioning (Garnier et al. 2016). 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 mixtures can lead to substantial overyielding (Litrico and Violle 2015) and new baskets of agroecosystem services (Dubs et al. 2023). In their meta-analyses including 91 studies on cereals and legumes, Reiss and Drinkwater (2018) found an overall yield increase of 2.2% for varietal mixtures relative to their single-variety components. Besides aboveground traits that can potentially improve light interception, disease dispersion, and other important processes (Borg et al. 2018), 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; Dubs et al. 2023; Hinsinger et al. 2011; Montazeaud et al. 2020).

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 (ie, specific root length, branching) to optimize soil exploration (foraging strategy; Lambers et al. 2006) and (ii) root physiology (release of protons, carboxylates and extracellular enzymes in particular) to mobilize inorganic nutrients or mineralize organic resources (mining strategy; Hinsinger et al. 2011). Tight interactions with rhizosphere microbiota (especially bacteria and fungi) are recognized for their contribution to both foraging and mining mechanisms (Campos et al. 2018). Amongst microorganisms, mycorrhizal fungi (AMF) considerably expand the rhizosphere volume as their external hyphae extend 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 (Nuccio et al. 2013) as related to specific enzymatic activities (Ezawa and Saito 2018).

Crop benefits from mycorrhiza can however vary considerably either stochastically or in relation to environmental conditions (e.g., nutrient availability; Ingraffia et al. 2020; Ryan and Graham 2018). They can also be determined by the identity of plant and fungal partners. It has been shown that wheat genotypes varied in their colonization rates, carbon investment into AMF and growth or nutrient uptake response (Elliott et al. 2021; Garcia de Leon et al. 2020; Graham and Abbott 2000). Despite limited host selectivity, AMF display host preferences, and the composition of AMF communities varies between plant species within the same genus (Pivato et al. 2007) as well as between genotypes of the same species as observed for wheat (Ercoli et al. 2017; Ellouze et al. 2018). If different genotypes harbor different AMF communities, increasing plant genotypic diversity within a field should enrich total AMF diversity. This 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.

Besides niche complementarity resulting from functional differences, average community characteristics can be important drivers of processes in plant mixtures (Dubs et al. 2023; Garnier et al. 2016). At the below ground level, root morphology can strongly influence enzymatic activities (Ma et al. 2018) and microbial community composition (Herms et al. 2022). Plant dependency on AMF association has also been proposed to be linked to root morphology, suggesting lower dependence for plant species characterized by finer roots and high specific root length (SRL; Bergmann et al. 2020; Hetrick 1991). At the intra-specific level, the influence of root morphology on enzymatic activities and AMF colonization have not yet been evaluated, but could prove valuable when formulating crop mixtures aimed at optimizing soil foraging and nutrient mining strategies.

In this study, we used a field trial set up using 16 bread wheat varieties grouped into four functional clusters according to previously acquired plant trait values. We aimed to understand how varietal and functional diversity of bread wheat affect AMF communities (abundance and diversity) and two enzymatic activities involved in organic N and P mineralization in the root zone (leucine-aminopeptidase (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. We also expect that, beside wheat diversity, mean root traits influence these processes.

Material and methods

Field experiment

Our study was conducted under field conditions in 2016 at the INRAE Experimental Station (UE VS) in Versailles, France (48°48′26″N, 02°05′13″E, elevation 114 m; Fig. 1a). We monitored the effect of intraspecific diversity on different ecosystem services in parallel (Dubs et al. 2018a, 2023; Vidal et al. 2020). Previous to this experiment, the field has been managed for decades under "conventional" farming, i.e. under short crop rotations, with annual and deep ploughing, use of synthetic chemical fertilizers, pesticides, and growth regulators. 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 (SD 11) mg kg^{-1} of available (Olsen) P. In a previous step, 58 bread wheat (*Triticum aestivum* L.) varieties 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, hereafter referred to as clusters (Dubs et al. 2018b). 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

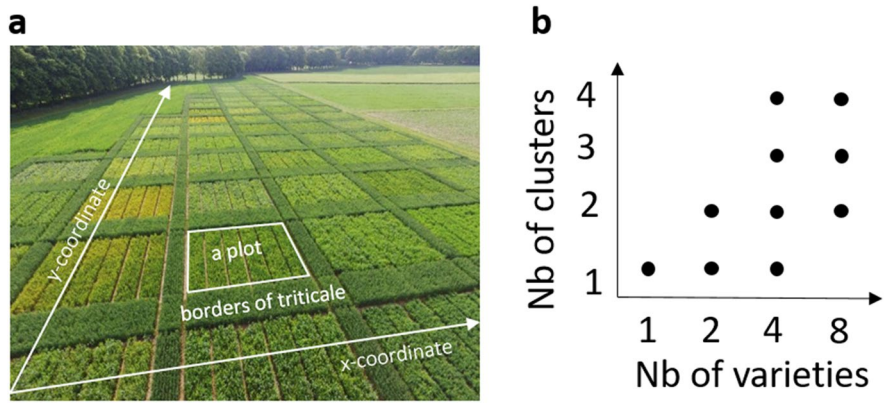

Fig. 1 Aerial picture and design of the field experiment. **A** Spatial distribution of the wheat plots in the field experiment (with x and y-coordinates), each plot being buffered by rows of triticale. **B** Scheme displaying how wheat diversity varied in terms of variety number and number of functional clusters of these varieties

the cluster 1 (cl1) represent a functional group characterized by the highest susceptibility to fungal diseases, low specific root length (SRL) and weak flag leaf N content. The cl2 cluster was composed of wheat varieties (Renan, Skerzzo, Midas, Alauda) that were less susceptible to fungal diseases (only one susceptible variety) but also with low SRL. The cl3 cluster 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 cluster contained elite varieties (Grapeli, Soissons, Arezzo, Boregar) with high SRL and the 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 level of NH_4^+ uptake capacity for 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. 1b). 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 × 8 m, sown at a density of 180 seeds m^{-2} with 17. 5 cm between rows (Fig. S1). 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 repetition (i.e. different variety compositions) for each variety number x functional group number combination (Fig. S1 b). This design is similar to prominent ecological experiments that explored biodiversity-ecosystem functioning relationships (e.g. Jena experiment; Weisser et al. 2017). Each plot was isolated from adjacent plot or field edge by 1.75 m wide strips of triticale (×Triticosecale Wittm. Ex A. Camus) at a density of 250 seeds m^{-2}. Wheat was sown in October (2015) after a preceding maize crop. The crop received relatively low input levels (170 kg N ha^{-1} in three doses of ammonium nitrate), matching with a wheat grain yield objective of 6 t ha^{-1}(instead of 9 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 for 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. Phenological stage was variable according to varieties, but all were close to heading stage. On each plot, 50 plants were sampled along four 50 cm rows (Fig. S1 c), to ensure that plot heterogeneity was accounted for and that sampling encompassed all the wheat varieties present in the plot considered. Plants were uprooted at 20 cm depth with a spade fork. Roots were washed, separated from above-ground parts by cutting at the base of the stem, dried on a tissue, and weighted fresh. To be representative, three segments of two centimeters were sampled from each of the 50 plants and pooled at the plot level. A composite soil sample of the plot was obtained by pooling ten soil cores (8-cm diameter, 0–8 cm deep; using a corer) sampled in the topsoil root zone at the same locations as for plant sampling, meaning, in the inter-row of the four 50 cm rows sampled for plants (Fig. S1 c). Vials containing either soil or root samples were directly covered by liquid N_2 and stored at -80 °C, for a total of 88 root samples and 88 soil samples.

Quantification of soil chemical properties and enzymatic activities

Soil chemical properties and enzymatic activities were measured for each plot on the composite soil sample. Total C and total N 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 aminopeptidase (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 h 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.

Community weighted mean traits

Community-weighted mean traits of the specific root length (SRL) and root diameter (RD) were calculated from previously measured traits on the 16 wheat varieties (in pure stand; Cantarel et al. 2021) and considering the abundance (at sowing) of each variety within the wheat varietal mixtures. This approach allowed us to compute a representative trait value for the entire community, providing insights into the overall traits of the mixed wheat varieties.

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 with a MATAB/chloroform protocol, followed by an RNAse step and precipitation in isopropanol (Table S2). The 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; (Gollotte et al. 2004); Table S3) also used for Illumina sequencing of the 28S nuclear ribosomal Large Sub-Unit rDNA (LSU) region. 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 s at 98 °C, 30 s at 60 °C, 30 s 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. For absolute qPCR, standard curves were obtained by serial dilutions (10^{-8}–10^{-3}) of linearized plasmids containing the cloned fragment of the LSU region targeted by FLR3-FLR4 primers (certified as Glomeromycotina by Sanger sequencing). For conciseness, the number of AMF gene copies per ng of root DNA in wheat roots will hereafter be referred to as the abundance of AMF.

For metabarcoding, as we expected that intra-specific diversity of host plants might induce subtle variation in AMF community composition (compared to communities of different plant species), we used two different AMF markers. The Small Sub-Unit (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 (Delavaux et al. 2021; Hart et al. 2015; Krüger et al. 2012). 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 mentioned previously; approximatively 380 pb; (Gollotte et al. 2004) and NS31/AML2 (approximatively 480 pb; Simon et al. 1992; Lee et al. 2008), targeting respectively the LSU region and the SSU region of the rDNA. For the first round of PCR (PCR1), reactions were carried on two replicated dilutions of 15 ng mm^{-3} of DNA per sample. The PCR conditions are presented in Table S3. 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 given access to on a data repository (see link in the Data Availability section), and results through steps of the pipeline are shown in Fig. S2. In short, sequences were quality filtered and adapters removed using the filterAndTrim function from the dada2 R package (version 1.24.0; Callahan et al. 2016a), 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 (ASV). The ASV with sequences shorter than 200 bp were discarded and remaining ASV were taxonomically assigned using assignTaxonomy function from dada2 (Callahan et al. 2016a), which implements the RDP classifier of Wang et al. (2007). We used the LSU database from RDP classifier (Czaplicki 2017) for LSU and Maarjam (Öpik et al. 2010) databases for taxonomic assignation of SSU. The ASV were post-clustered using vsearch at 97% following recommendation of Tedersoo et al. (2022). To filter non-AMF sequences, the ASV were blasted (function filter blast pq() from the MiscMetabar package v.0.4; Taudière 2023) on the database previously used for taxonomic assignation, discarding taxa which did not match with Glomeromycota at 80% identity (with a minimum cover of 50% and a minimum bit score of 50). As verification, we also blasted all SSU to a curated database based on SILVA (McLaren and Callahan 2021) but limited to Glomeromycota. On the SSU dataset, two outlining samples with less than 50,000 sequences were discarded from the dataset. The ASV represented by less than two sequences were discarded from datasets.

AMF diversity analyses

All statistical analyses were carried out using R Studio (RStudio Team, 2016, version 4.0.4). Alpha-diversity was evaluated by Hill numbers using the function 'renyi' ('vegan' package; Oksanen et al. 2019) without previous normalization following (McMurdie and Holmes 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) was compared between the four wheat clusters cultivated as mono-cluster mixtures and between wheat diversity levels (functional and varietal) were analyzed by PERMANOVA on Bray–Curtis distances using the 'adonis' function from the 'vegan' R package (Oksanen et al 2019). PERMANOVA were done on both the LSU and SSU markers using rarefied datasets.

Statistical analyses

To quantify which field variables, including wheat diversity, abiotic soil properties and spatial position, mainly drove the observed response variables, i.e. enzymatic activities and the abundance and diversity of AMF, we fitted a full model including all the field variables as explanatory variables (lm() function; Table S4), as following: Response_variable ~ number of varieties + number of cluster + y coordinate + x coordinate + CWM-SRL (Community weighted Specific Root Length) + CWM-RD (Community weighted Root diameter) + shoot biomass + soil total N + soil total C + soil pH + soil Olsen P. Both the explanatory and response variables were z-score standardized ($\mu = 0$, $\sigma = 1$). Backward model selection, using the 'glmulti' function (glmulti package; Calcagno and de Mazancourt 2010), was used to rank the models according to their Akaike Information Criterion (AIC) values, corrected for small sample size (option of the function: crit = aicc). The N best models were selected with the criteria of AIC differences from the best model lower than two. Standardized parameter estimates with 95% unconditional confidence intervals, relative trait importance and adjusted mean R-squared were obtained by model-averaging of the N best models using the coef() function ('glmulti' package; Calcagno and de Mazancourt 2010). In the context of model averaging, the mean adjusted R-squared ($\overline{R^2_{adj}}$) is the average of the adjusted R-squared values across the models being considered, as done in Montazeaud et al. (2020).

Results

Abundance and community composition of AMF

The abundance of arbuscular mycorrhizal fungi (AMF) in wheat roots was quantified using absolute qPCR. Median values varied, ranging from 1152.75 AMF gene copies per ng of root DNA in single varietal plots to 1534.25 AMF gene ng^{-1}. root DNA in plots grown with eight varieties (Fig. 2e).

The AMF diversity was explored by Illumina metabarcoding on two AMF specific markers. On the SSU dataset, median sequencing depth was 206,596.5

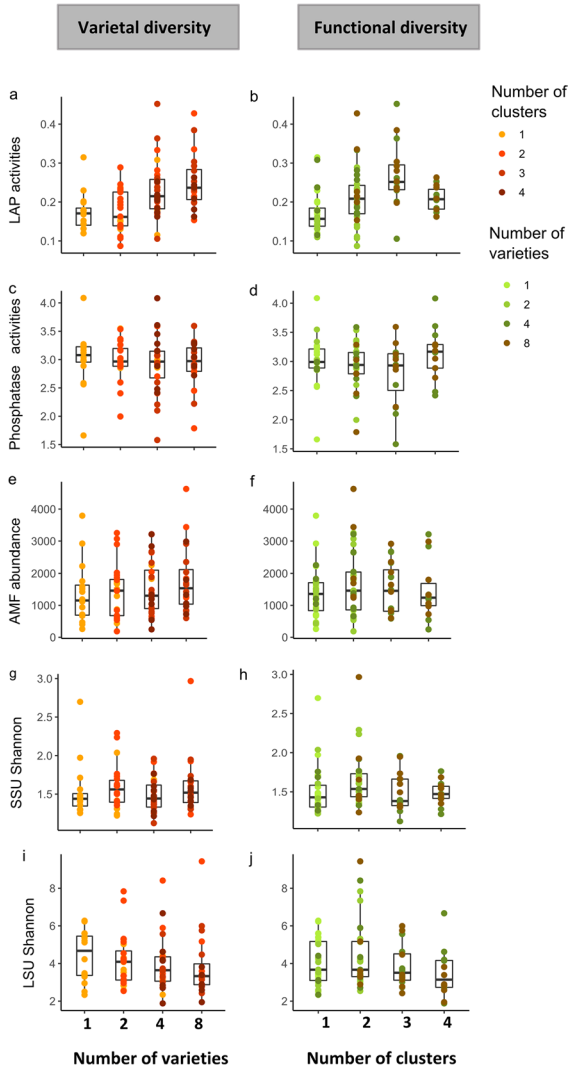

Fig. 2 Boxplot showing variation according to the number of wheat varieties (varietal diversity, left panels) and the number of wheat functional clusters (functional diversity, right panels) of (**a.b.**) soil leucine amino-peptidase (LAP) activities (nmol. of substrate mineralized per gram of soil per minute) in the root zone, **c.d.** Phosphatase activities (nmol. of substrate g^{-1}. min^{-1}) in the root zone, **e.f.** the molecular abundance of AMF in wheat roots (AMF gene copies ng^{-1}. root DNA), and Shannon diversity of AMF (Hill q = 1) calculated from (**g.h.**) the LSU and (**i.j.**) the SSU sequencing datasets

(max: 409,335; min: 109,875; upper quartile: 250,359; lower quartile: 171,051) and 243 ASV were identified. On the LSU dataset, median sequencing depth was 151,662.5 (max: 255,370.0; min: 35,809.0; upper quartile: 188,337.5; lower quartile: 119,067.2) and 238 ASV were identified (Fig. S2). The ASV

Table 1 Taxonomic assignment at the Order level with the number of ASVs and the proportion of sequences per Order on the SSU and LSU dataset

rDNA portion	Order	Number of ASVs	% of sequences
SSU	Glomerales	173	97
	Diversisporales	30	1.49
	Archaeosporales	23	1.49
	Paraglomerales	2	0.02
	NA	6	< 0.004
	Total	234	18,056,430 seq
LSU	Glomerales	200	99.26
	Diversisporales	10	0.37
	Archaeosporales	8	0.12
	Paraglomerales	0	0
	NA	20	0.25
	Total	238	13,171,912 seq

from the SSU and LSU regions were assigned to respectively four and three Orders (Table 1), including *Glomerales*, *Diversisporales*, *Archeosporales*, *Paraglomerales*, and non-identified ASV (belonging to Glomeromycota but not assigned to lower taxonomic ranks). Among all LSU sequences, the ten most abundant ASV accounted for 92.1%, with *Funneliformis* being the dominant genus, represented by 6 out of 10 ASV (Table 2). Similarly, for the SSU region, the ten most abundant ASV accounted for 96.6% of all sequences and included ASV from the genera formerly called *Glomus*, *Scutelospora*, *Archeospora*, *Diversispora*, and two uncultured Glomeromycota. As the taxonomy in the Maarjam database, which may be outdated, can be misleading on the proportion of the *Glomus* genus (Stefani et al. 2020a), we verified the assignment by blasting on NCBI. The most abundant ASV_1, and ASV_122 were further identified as *Funneliformis* (Table S6). In terms of alpha diversity, the median values of Shannon alpha-diversity (Hill q = 1 index) on the SSU marker ranged from 1.40 to 1.52 for wheat mixtures composed of four and two varieties, respectively (Fig. 2g). On the LSU marker, median Shannon values ranged from 3.08 to 2.63 for wheat mixtures of one and two varieties, respectively (Fig. 2i). In term of beta-diversity, there was no significant difference in AMF community composition between wheat varietal and functional diversity levels on neither the LSU nor the SSU data sets (PERMANOVA; Table S5).

Table 2 Blast results of the ten most abundant Amplicon Sequence Variants (ASV), representing 96.7% of the AMF sequences of the Small Sub-Unit (SSU) dataset using Maarjam database and 92.2% of the Large Sub-unit (LSU) dataset using RDP database

rDNA portion	ASV	% of sequences	Genus	Species	% id. match	Query cover	e-value
SSU	ASV_1	94	Glomus	Glomus_sp.	100	100	6.36e-131
	ASV_86	0.7	Scutellospora	Scutellospora_Shi14b_Scu-21	99.2	100	1.38e-127
	ASV_105	0.5	Archeospora	Archeospora_sp.	99.2	100	1.76e-126
	ASV_80	0.4	Archaeospora	Archaeospora_trappei	100	100	6.36e-131
	ASV_155	0.3	Archaeospora	Archaeospora_trappei	99.6	100	2.96e-129
	ASV_95	0.2	Diversispora	Diversispora_Zheng16_OTU52	100	100	6.36e-131
	ASV_78	0.2	Glomus	Glomus_sp.	99.2	100	1.76e-126
	ASV_138	0.2	Glomus	Glomus_sp.	100	100	8.15e-130
	ASV_206	0.1	Glomus	Glomus_sp.	98	100	1.39e-122
	ASV_122	0.1	Diversispora	Diversispora_spurca	99.6	100	2.96e-129
LSU	ASV_1	76.8	Funneliformis	uncult_Funneliformis	100	100	1.28e-131
	ASV_38	8.8	Funneliformis	Glomus_macrocarpum	99.6	100	5.93e-130
	ASV_73	2	Funneliformis	Funneliformis_caledonium	100	100	1.28e-131
	ASV_154	0.9	Funneliformis	Glomus_macrocarpum	97.6	100	1.29e-121
	ASV_105	0.8	Rhizophagus	uncultured_glomeromycete	96.8	100	3.61e-117
	ASV_114	0.7	Funneliformis	Glomus_macrocarpum	98	100	1.00e-122
	ASV_66	0.6	Funneliformis	Glomus_caledonium	98.1	63	6.41e-75
	ASV_97	0.6	Rhizophagus	Glomeromycota_sp._OTU8	100	100	1.28e-131
	ASV_91	0.5	Rhizophagus	uncultured_Rhizophagus	100	100	1.28e-131
	ASV_69	0.4	Rhizophagus	uncultured_glomeromycete	96.4	100	1.68e-115

Enzymatic activities in soil of the root zone

Leucine aminopeptidase (LAP) and phosphatase activities were assessed using a fluorimetric method. Median LAP activity displayed a range from 0.17 nmol of substrate mineralized per gram of soil per minute in single varietal plots to 0.24 nmol. of substrate $g^{-1}.min^{-1}$ in plots with eight varieties of wheat (Fig. 2a). In contrast, phosphatase activities in the soil remained relatively constant across plots with varying numbers of wheat varieties, with an overall median activity of 2.99 nmol of substrate $g^{-1}.min^{-1}$ (Fig. 2c).

Variables affecting enzymatic activities in the root zone, abundance and diversity of AMF in roots

The mean adjusted R-squared values for the iterated models ranged from 0.23 for LSU Shannon diversity to 0.50 and 0.51 for respectively LAP activities and the abundance of AMF in roots, which were better explained by the included explanatory variables (Fig. 3). Soil characteristics consistently emerged as highly influential variables, with total N exhibiting the highest relative importance and significant effect on all response variables. Spatial position (mainly on the y-axis) in the field also influenced responses, except for AMF diversity. Community weighted mean root traits (root diameter and specific root length) did not significantly impact any response variables (Fig. 3), although their relative importance was above 0.75 for both enzymatic activities. The relative importance of varietal and/or functional wheat diversity was above 0.75 for all response variables but for phosphatase activities and LSU alpha-diversity. The model selection approach enabled us to decipher the relative importance of both, non-independent diversity aspects, namely varietal and functional. The number of wheat varieties in the mixture had a significant positive effect on LAP activities, as indicated by positive non-zero standardized estimates (Figs. 2a and 3a). The number of wheat functional clusters had a significant negative effect on SSU Shannon (Figs. 2j and 3d). The abundance of AMF in the roots was only marginally affected by wheat diversity (Figs. 2e–f and

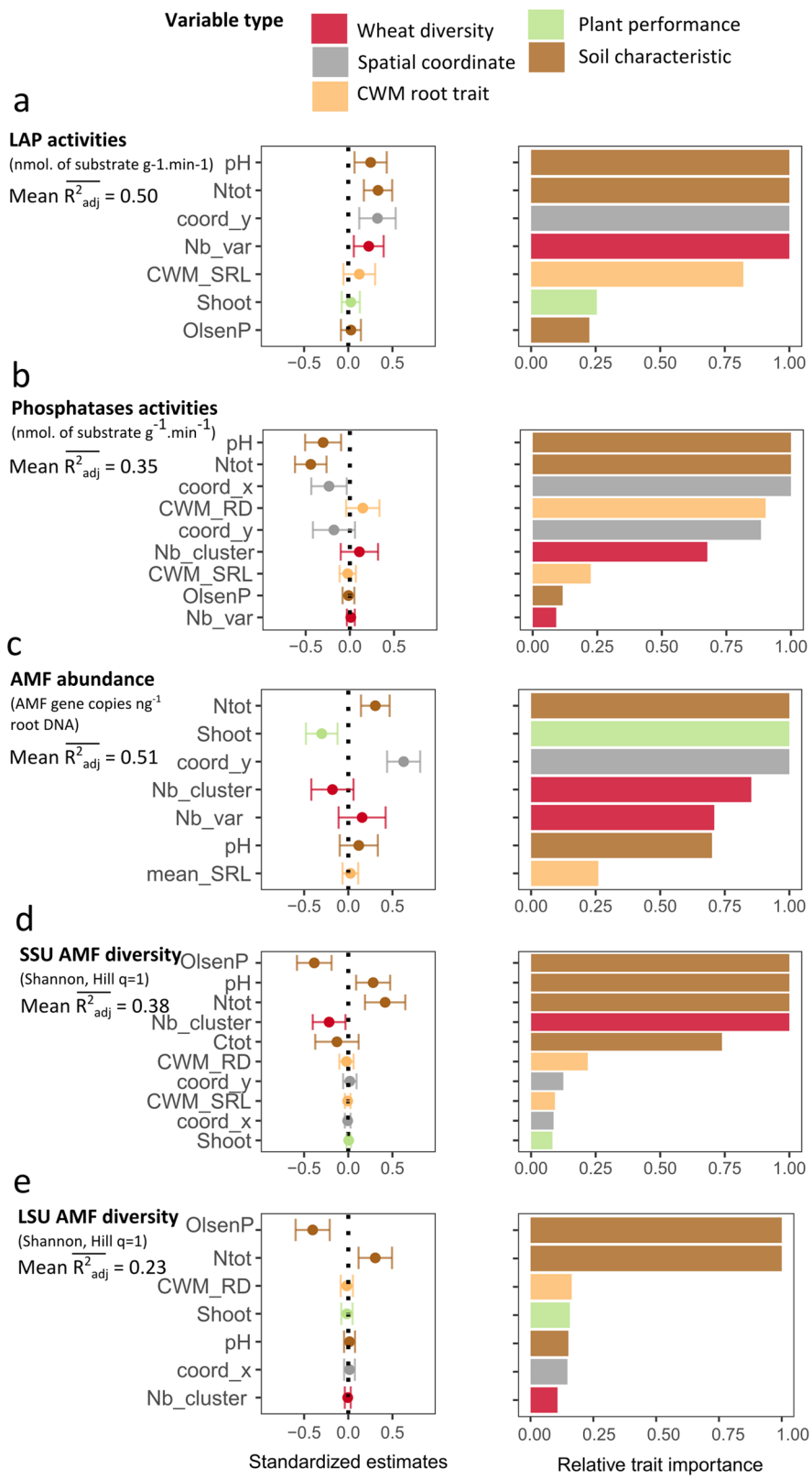

Fig. 3 Standardized effects on leucine-aminopeptidase activities (**a.**) phosphatases activities (**b.**) in soil of the root zone, AMF abundance in roots (**c.**), SSU diversity Hill q = 1 (**d.**) LSU diversity Hill q = 1 (**e.**) Backward model selection was performed on a full model on the above cited response variable and field variables (wheat diversities, soil proprieties, spatial position, community weighted mean root) as explanatory variables. Based on AICC, the N best models were retained to compute model-averaged parameter estimates (panels on the left side) with their 95% unconditional confidence intervals. The relative importance of the variables (panels of the right side) can be interpreted as the probability that the variable appears in the best model. Adjusted mean R-squared averaged across the N best models $\left(\overline{R^2_{adj}}\right)$ are also reported. (Abbreviations: Nb_var: number of wheat varieties; Nb_cluster: number of wheat functional cluster; coord_x and coord_y: spatial coordinates x,y; Shoot: shoot biomass; Ntot: soil total N; Ctot: soil total C; CWM_SRL: community weighted Specific Root Length; CWM_RD: Community weighted Root diameter)

3c), with an antagonistic tendency between varietal and functional diversity. As the impact of the spatial position was very pronounced, we further analyzed the best model by an additional lme model (with y coordinate as random effect; Table S2b) to understand better the biological effect of wheat diversity. When removing the spatial effect, varietal diversity had a significant and positive impact on the abundance of AMF in the roots, while the number of functional clusters had a negative impact (both *p.-values* < 0.01; Table S2b).

Discussion

Our study explores the effect of wheat varietal and functional diversity on microbial processes, focusing on arbuscular mycorrhizal fungi (AMF) and enzymes involved in the mineralization of soil organic N or organic P. While both varietal and functional diversity had notable impacts, these effects were not consistent across variables, primarily driven by soil characteristics. In our discussion, we contextualize these findings in light of other studies, exploring the underlying mechanisms at play.

Soil characteristics were main drivers of belowground processes, while the influence of root morphology was limited

In the context of this field trial, spatial location and the variations in soil properties, in particular total N, emerged as significant factors influencing all response variables. In both enzymatic activities, pH emerged as a key explanatory factor, consistent with prior research at broader scales (Štursová and Baldrian 2011). Root morphological traits, treated as community-weighted mean traits, were prominent variables for both enzymatic activities within the root zone, although their positive influence was not statistically significant. While not conclusive, these results suggest a tendency aligning with the significance of root morphology in influencing enzymatic activities, as previously observed by Ma et al. (2018). Our results at the intraspecific plant diversity level thus extend observations made at the interspecific level.

Wheat varietal diversity influenced leucine aminopeptidase activities

Neither the number of wheat varieties nor the number of wheat functional clusters impacted phosphatase activities in the root zone (Figs. 2c, d and 3b). In contrast, increasing varietal diversity had a positive effect on LAP activities in the root zone (Fig. 3a), with a 1.5-fold increase in plots grown with eight varieties, relative to those with one variety. Leucine aminopeptidases (LAP) 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 N in soils (Schulten and Schnitzer 1997). Microbial enzymatic activities in the soil are strongly driven by the availability of labile C supply, such as root exudates (Averill and Finzi 2011). It has been shown that intra-specific diversity of *Anthoxanthum odoratum* can modify the quantity and quality of rhizodeposits, supporting higher microbial activities in the soil (Semchenko et al. 2021). The underlying mechanisms remain unknown, but changes in rhizodeposition could be due to an overall complementarity effect between wheat varieties possibly leading to greater photosynthesis and/or belowground C allocation, although these were not measured in our study.

Wheat diversity marginally affected the abundance of AMF in roots

Wheat diversity, both varietal and functional, did not significantly impact the abundance of AMF in roots, in a context of strong spatial structure of this response variable (Fig. 3c). In further investigations through an additional model where the spatial position was accounted for as a random effect factor, wheat varietal and functional diversity displayed a significant effect on the abundance of AMF (Table S2b). This discrepancy between models showed that wheat diversity have a biological effect on the abundance of AMF, but spatial variation at the metric scale of the field is predominant. Mycorrhizal colonization is known to be highly influenced by environmental conditions (ie. nutrient availability, inoculum legacy in the soil). However, soil P availability, known as a major driver, did not significantly affect the abundance of AMF here. Interestingly, we found that the shoot biomass

of wheat had a significant negative relationship with the abundance of AMF in roots. This suggests a trade-off in plant strategies, possibly suggesting a trade-off in resource allocation: root resource allocation beneficial to AMF is made at the cost of aboveground development.

The molecular method we used to estimate the abundance of AMF in root, which relies on AMF gene copy numbers, does not directly correspond to visually determined root colonization rates. Several factors can influence this quantification method, including intra-radical spores, variations in RNA operon numbers among AMF species, species-specific amplification preferences, and limitations in taxonomic coverage, as reported in previous studies (Gamper et al. 2008; Jansa et al. 2008). It is worth noting that the two primers pairs we employed in this study do not amplify fungi from the *Mucoromycotina* subphylum, which has been found to be abundant in wheat according to Orchard et al. (2017). However, the LSU primers we used have shown a reasonable correlation between quantitative PCR (qPCR) results and visual observations (Arruda et al. 2022). This correlation may be further enhanced through the use of relative qPCR methods, as demonstrated by Bodenhausen et al. (2021), however, less suitable for comparing different plant genotypes due to potential variations in the sequences of the plant-quantifying region.

Increasing functional diversity of wheat alters AMF diversity

To our knowledge, this is the first study addressing how plant intra-specific diversity modifies AMF diversity. While increasing wheat varietal diversity had no significant effect on AMF alpha-diversity (Fig. 3d), there was a significant effect of wheat functional diversity noted on the SSU marker (Shannon, Hill q = 1) (Fig. 3d), although remaining weak (Fig. 2h). The observed negative impact of wheat functional diversity on AMF diversity contradicts our initial hypothesis. Two potential explanations could account for this phenomenon. It may be a result of a sampling effect, where the inclusion of wheat clusters with less diverse AMF communities influenced the overall outcome. Alternatively, it could be driven by increased competition for plant carbon resources, favoring the proliferation of more generalist AMF fungi. Globally, both markers showed a convergent dominance of a single *Funneliformis* ASV. The sharing of a few AMF ASV raises questions about the competitive dynamics for nutrients in common mycorrhizal networks, which would be worth exploring further. *Funneliformis* was also the dominating genus in two field studies in Canada on large sets of durum wheat genotypes including landraces and commercial varieties, with low differences of alpha and beta diversity (Ellouze et al. 2018; Stefani et al. 2020b). Stefani et al. (2020b) found no difference, while Ellouze et al. (2018) did not find any difference when looking at AMF in roots, but observed that durum wheat genotype differently shaped the composition of AMF communities in their rhizosphere. The phenotypic plasticity exhibited by wheat varieties in response to varying environmental conditions should be considered though. 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 (as related to fertilization level).

Our observations were carried out within a specific environment, in a field under conventional management (ie., annual tillage, application of mineral fertilizers and chemical pesticides). In the realm on AMF, these practices might have already selected for a restricted community (hence, the dominance of *Funneliformis* genus) that exhibits a limited response to changes in wheat functional clusters or diversity. Indeed, mycorrhizal community is known to be different in fields under minimum-tillage (Brito et al. 2012; Jansa et al. 2006; Liu et al. 2022) or organic fertilization (Kozjek et al. 2021; Verbruggen et al. 2010).

Conclusion & perspectives

In the past decade, farmers have shown a growing interest for using varietal mixtures in wheat cultivation. In 2023, 19.5% of bread wheat cultivation in France comprised such mixtures (Arvalis 2023). However, there remains a significant gap in valuable knowledge necessary for informed decision-making in designing varietal mixtures (Borg et al. 2018). This emphasizes the need for a deeper understanding of the

ecological mechanisms that govern the performance of these mixtures (Litrico and Violle 2015). Our field study sheds light on the multifaceted relationships between wheat diversity, soil characteristics, and ecological processes involved in nutrient cycling and acquisition. Soil properties and spatial positioning in the field emerged as primary drivers of the response variables, with soil total N appearing as a key player. Wheat varietal diversity significantly influenced soil enzymatic activities related to the mineralization of soil organic N, particularly leucine-aminopeptidase activity, with a 1.5-fold increase in mixtures containing eight wheat varieties compared to pure stands. Conversely, wheat diversity did not impact soil enzymatic activities related to the mineralization of soil organic P (phosphatases). Mean root traits marginally influenced these enzymatic activities but showed no interaction with AMF. Regarding the symbiotic associations between wheat and AMF, wheat varietal diversity significantly increased the abundance of AMF in roots, although as a second order effect after soil spatial variability. Additionally, an increase in wheat functional diversity, ie. cluster number, negatively but weakly influenced AMF diversity, found only for the SSU marker. The AMF communities was strongly dominated by the *Funneliformis* genus. Overall, our results contribute to the understanding of the potential of intra-specific diversity in determining the strategies related to nutrient cycling and acquisition, and point to the need for further studies under various environmental conditions and agricultural practices or systems, in particular conservation agriculture and organic farming systems.

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Authors contribution Jérôme Enjalbert and Xavier Le Roux contributed to the field experiment 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 analyses were conducted by Elisa Taschen, who authored the first draft of the manuscript. Claude Plassard, Esther Guillot and Cyrille Violle provided valuable input through discussions and support for the statistical analyses, and all authors contributed to the manuscript writing and revisions. 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.

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Data availability The datasets generated and analysed in the study are available in DRYAD: doi:https://doi.org/10.5061/dryad.2jm63xswb.

Private link while awaiting DRYAD approval for publication: https://datadryad.org/stash/share/SfANlUsD31QRbG6Qc6lNS-hofUbkopvtRlWlDz7-Esw

Declarations

Competing interest The authors have no relevant financial or non-financial interests to disclose.

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