Phosphorus availability drives the effect of legume-wheat intercropping on prokaryotic community interactions

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ABSTRACT

Phosphorus (P) is a finite and pivotal resource in determining plant yield. Intercropping with legumes is frequently proposed to improve P nutrition in many crops such as wheat, and the greater yield and P uptake observed are mostly attributed to legumes' root exudation of organic acids and phosphatases, which modify rhizosphere chemistry. The same rhizosphere modification drives the selection of specific bacterial communities by providing carbon sources such as organic acids and other metabolites. This study aimed to further understand the influence of P bioavailability on bacterial community selection and whether this can be extended to other crops through intercropping. Pea, lupin and wheat were grown as intercrops and as sole crops at four levels of P availability. This was achieved by using a low-P soil from the long-term experiment at Rothamsted Research, amended with available and low-available forms of P. After 62 days of growth, 16S rRNA gene amplicon sequencing was performed from rhizosphere samples, and acid and alkaline phosphomonoesterase (PME) activity was measured. The plant species was the main factor determining the structure of the bacterial community followed by P availability. When P was unavailable or depleted, legume monoculture as well as intercropping, was associated with reduced bacterial species richness and diversity, which was partly explained by an increased relative abundance of *Variovorax*, *Pseudomonas* and *Bradyrhizobium* spp. The complexity and interconnections of the bacterial community were increased in intercropping when P was unavailable as was alkaline PME activity, while the acid PME activity was more affected by the plant. In conclusion, wheat intercropping can generate a more complex and interconnected root-associated bacterial community, which can potentially contribute to the facilitation of P uptake.

1. Introduction

Phosphorus (P) is present in several key biological molecules and is the second most important mineral element for plant growth and development (Alori et al., 2017; Elser, 2012). However, due to its high retention in soils, P availability for uptake by plants is frequently low (Kochian, 2012; Shen et al., 2011). Consequently, modern agriculture is dependent on P derived from rock phosphate, a non-renewable resource which will likely be exhausted in 50–100 years (Cordell et al., 2009).

Before agricultural activities redistributed mined P in arable lands, plants evolved several strategies to improve P availability and colonize soils with low availability of P. Most of these strategies were based on the modification of the rhizosphere by acidification and the exudation of

carboxylates and phosphatases. This modification is part of the "rhizo-sphere effect" which is the combination of biological, chemical, and physical changes in soils driven by root exudates and rhizodeposition (Kuzyakov, 2002). Root exudates and rhizodeposition are pivotal in some complex and dynamic interactions between plants and networks of organisms, particularly microorganisms, which have been shaped by over 450 million years of co-evolution (Zhalnina et al., 2018). With these premises, it is logical that plants have developed the ability to select the most favourable microbial communities within the rhizo-sphere as a key strategy to improve nutrition in nutrient-poor soils.

To take advantage of these strategies, research has targeted the use of phosphate solubilizing and mineralizing microorganisms to amend soil P availability (Alori et al., 2017; Richardson and Simpson, 2011). In

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addition, P-mobilizing plant species, such as chickpea (*Cicer arietinum* L.), white lupin (*Lupinus albus* L.) and faba bean (*Vicia faba* L.), have been shown to improve the availability of macronutrients and micronutrients in cereals in the rhizosphere of cereal-legume intercropping systems (Faucon et al., 2017; Hinsinger et al., 2011; Wang et al., 2014; Xue et al., 2016) contributing, with other agroecological services, to the multifunctionality of the multi-cropping system based on legumes (Duchene et al., 2017). In the case of lupins (*Lupinus* spp.), cluster roots release large amounts of exudates, in particular carboxylates, which facilitate plant growth in soils where P is limited (Lambers and Shane, 2007). Furthermore, it has been demonstrated that intercropping with lupin increased wheat P uptake, without negatively affecting lupin P status (Cu et al., 2005). Pea (*Pisum sativum*) is reported to exude carboxylates and phosphatases from roots in lower quantities than lupin (Nuruzzaman et al., 2006), however, a consistent improvement of P uptake and growth in the target crop was reported through intercropping with this legume species (Lo Presti et al., 2021; Wu et al., 2021c). Furthermore, plant-exuded organic anions can provide a carbon source for soil microorganisms in the rhizosphere, including beneficial fungi and plant growth-promoting bacteria (Baudoin et al., 2003; Oburger et al., 2011; Wang and Lambers, 2020; Yuan et al., 2015). These, in turn, can release organic anions and phosphatases to mobilize soil P (e.g. phosphorus-solubilizing bacteria (PSB)), as well as plant hormones to promote root development further enhancing P uptake (Lugtenberg and Kamilova, 2009; Miransari, 2014; Vessey, 2003; Wang and Lambers, 2020).

It is known that plants select specific bacterial communities in their rhizosphere from bulk soil (Berg and Smalla, 2009). In this work we hypothesized that i) rhizosphere bacterial community composition is influenced by the bioavailability of P; ii) contrasting plant species differentially select their root microbiome in a P-dependent manner; iii) intercropping under contrasting P conditions influences microbiome selection compared to the cultivation of individual crop species; iv) root microbiome selection, in turn, influences soil phosphatase activity.

2. Material and methods

2.1. Soil preparation, addition of P treatments and plant growth

White lupin (*Lupinus albus* L. cv Multitalia), field pea (*Pisum sativum* L. cv Hardy) and durum wheat (*Triticum turgidum* subsp. *durum* (Desf.) Husn. cv Svevo) were grown in pots either as sole crop or as a wheat/legume intercrop. Bulk soil (BS) samples consisting of pots without plants served as controls (Table 1). The soil used for the experiment was from the exhausted land experiment at Rothamsted Research (Harpenden, Hertfordshire, UK) from the plot 054 (P-depleted soil) and plot 071 (P-enriched soil) with 3.8 mg and 26.4 mg Olsen P kg^{-1} of soil, respectively. The soil used is a Typic Paleudalf (Baillie, 2001) and differed significantly in the two plots only for the Olsen P due to the long-term treatment. The properties of the soil, referring to the 0–23 cm top layer collected for the experiment, were, respectively in the plot 054 and 071, as follows: 28 % of sand, 52 % of silt and 20 % of clay, pH 7.02 and 6.57, total carbon 8.49 g kg^{-1} and 10.84 g kg^{-1}, inorganic carbon 0.24 g kg^{-1} and 0.13 g kg^{-1}, organic carbon 8.26 g kg^{-1} and 10.71 g

kg^{-1}, total nitrogen 0.97 g kg^{-1} and 1.24 g kg^{-1}, exchangeable Ca 2.27 g kg^{-1} and 2.35 g kg^{-1}, exchangeable K 0.31 g kg^{-1} and 0.26 g kg^{-1}, exchangeable Mg 0.055 g kg^{-1} and 0.057 g kg^{-1}. The soil was collected, air-dried and sieved using a 4-mm sieve.

The P-depleted soil (from plot 054) was amended with KH_2PO_4 or $Ca_3(PO_4)_2$ to a total of 100 mg kg^{-1} of P in soil or not amended to obtain the following P treatments: available P (AP) (P-depleted soil amended with KH_2PO_4), unavailable P (UP) (P-depleted soil amended with Ca_3PO_4) and no P (NoP) (P-depleted soil, not amended). An additional P treatment was obtained with P-enriched soil (from plot 071), without any phosphate amendment, named NPK, which represents the soil after several years of balanced fertilization (Table 1).

Seeds were surface-sterilized with 70 % ethanol for 30 s, followed by immersion in 1.25 % active chlorine for 20 min and washed five times under gentle shaking with sterile water. Before sowing, the seeds were imbibed overnight and germinated in aseptic conditions. One (for sole crops) or two (for intercrops) same-size pre-germinated seeds were sown in each pot filled with soil mixed with perlite (66/33, v/v). After transplanting, the seedlings were transferred to the glasshouse and grown at the following controlled environmental conditions: at 20/15 °C day/night temperature with 8/16 h darkness/light photoperiod, supplemented by artificial light when light intensity fell below 100 μmol photons m^{-2} s^{-1}, at 60 % of relative humidity.

N fertilization was performed three times: four, five and six weeks after transplanting, applying 18 mg N kg^{-1} soil of $Ca(NO_3)_2$ to all the pots. N fertilization aimed to limit nodulation of the legumes to avoid differences in N availability between intercropping and sole cropping and ensure that the plant growth was affected only by P. When, after 62 days of growth, wheat was at the heading stage, pea was at the early flowering stage and lupin was at the end of the vegetative stage, the experiment was terminated, and the roots with adhering soil were separated from non-rhizosphere soil. The legume roots were checked for the absence of nodules which were strongly limited by N fertilization. The two fractions of soil were kept separately, for rhizosphere processing and P analysis.

2.2. Rhizosphere processing

For each pot, the entire root system with adhering soil was collected from both sole cropping and intercropping, with roots from the two different crops considered as one sample. Loose soil was carefully shaken off so that only tightly-bound soil was used for DNA extraction (rhizosphere). The roots were transferred to 50 ml screw-cap Falcon tubes, filled with 30 ml of sterile water. The Falcon tubes were shaken on a flatbed shaker at 4 °C for 10 mins at full speed to release rhizosphere soil from the root system. After removing the roots, the tubes were centrifuged for 5 min at 4000 rpm and the roots were discarded. After that, most of the supernatant was removed, leaving 5 ml of water in the tube. The rhizosphere soil was re-suspended in the tube using a Vortex and 1.5 ml of the suspension was transferred into a new 2-ml microcentrifuge tube. The microcentrifuge tube was centrifuged at full speed for 2 min and the supernatant was discarded. The pellet was stored at −80 °C for further DNA extraction and assay of phosphatase activity.

2.3. Olsen P soil analysis

When the experiment was terminated, the effectiveness of P addition was assessed by analysing P availability in non-rhizosphere soil from each pot. Available P was extracted with 0.5 M $NaHCO_3$ from 2 g of air-dried 2-mm sieved soil, according to the Olsen method (Olsen, 1954). P from the extract was quantified via spectrophotometry using a Lambda 400 Fias UV/VIS Perkin Elmer (Waltham, Massachusetts, U.S.) according to the molybdenum blue method (List et al., 1986; Ruzicka and Hansen, 1981). Statistical comparison of Olsen P content was performed with the software RStudio (version 4.1.2), using two-way analysis of variance (ANOVA) (P treatment × cropping treatment) followed by

Table 1
Summary and description of the treatments and corresponding labels.

P treatments	Cropping treatments
AP: available P (in the form of KH_2PO_4)	P: pea sole crop
UP: unavailable P (in the form of $Ca_3(PO_4)_2$)	W: Wheat sole crop
NoP: no P (soil no amended with P)	L: Lupin sole crop
NPK: soil after several years of balanced fertilization	PW: Pea-wheat intercropping
	LW: Lupin-wheat intercropping
	BS: Bulk soil

Tukey's HSD test (*P*-value <0.05).

2.4. Soil DNA extraction and quantification

For each sample, DNA was extracted from 0.25 g of soil using the MoBio PowerSoil™ DNA Isolation Kit (Carlsbad, CA, USA). Extractions were performed according to the manufacturer's instructions, and the bead-beating step was performed with a MP Biomedicals FastPrep-24 machine twice for 30 s at 5.5 m s^{-1}. DNA purity and concentration were determined by NanoDrop spectrophotometry (Thermo Scientific, Wilmington, DE, USA) as well as a Qubit 2.0 Fluorimeter using the ds DNA HS assay kit (Thermo Fisher).

2.5. 16S rRNA gene amplicon sequencing and bioinformatic processing

Bacterial and archaeal 16S rRNA genes were amplified from bulk soil and rhizosphere DNA samples, using barcoded universal prokaryotic primers 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 907R (5′-CCG TCA ATT CCT TTG AGT TT-3′) for paired-end microbial community amplification, targeting the V4-V5 region, resulting in amplicons of approximately ~392 bp. The amplicons were subjected to Illumina® sequencing using the MiSeq platform to generate 2 × 300 bp paired-end reads at Novogene (China). 16S rRNA gene amplicon sequences were analysed using the pipeline proposed by Quantitative Insights Into Microbial Ecology (QIIME2) (version 2018.11.0) (Bolyen et al., 2019). DADA2 (Callahan et al., 2016) was performed on reads which had their barcodes and primers previously removed. Feature table, taxonomy table, metadata file and tree were uploaded into RStudio (version3.5.0) and the package phyloseq (McMurdie and Holmes, 2014) was used for downstream analysis. Chloroplasts and mitochondrial sequences and four outliers were removed from the dataset. Data was normalised using proportions (Total Sum Scaling (TSS)) method, which outperforms other normalization methods such as CSS, DESeq-VS, edgeR-TMM (McKnight et al., 2019). PCoA plots were obtained using the same package using Bray-Curtis similarity distance matrix. Permutational Multivariate Analysis of Variance (PERMANOVA) tests were performed using 'adonis' function in the *vegan* R package with 9999 permutations. The number of observed ASVs and diversity based on Simpson index were calculated in QIIME2. Statistical analyses of alpha diversity indexes were performed in RStudio (version3.5.0). Normality and homogeneity of variances were checked using Shapiro–Wilk test and Levene's test, respectively, and One-way ANOVA and post-hoc test (Tukey HSD) were used to assess differences.

2.6. Analysis of differentially abundant ASVs

MicrobiomeAnalyst (Dhariwal et al., 2017), an online tool for comprehensive statistical, visual and meta-analysis of microbiome data, was used for detecting features which were differentially abundant between different plant species, using Random Forest (RF) models which are versatile, have a high prediction accuracy and provide additional information such as variable importance (Touw et al., 2013). RF is a supervised machine-learning algorithm that has been applied to microbiome data to identify microbial taxa that differentiate between phenotypes (Chong et al., 2020). The mean decrease in classification is based on permutation; for each tree, the classification accuracy is determined both with and without random permutation of the values of the variable. Low abundance and low variance features, for features with <2 counts in <20 % of the samples and 10 % of the values below the determined inter-quantile range (IQR), were removed.

2.7. Network analysis

To assess the interactions among bacterial taxa in the microbiome, the feature table with absolute abundance values was imported into RStudio. Before network analysis, features with a read number of less than or equal to 5 and not occurring in 50 % of samples were removed. Network analyses were performed using the Molecular Ecological Network Analyses (MENA) pipeline (Deng et al., 2012) available at http://ieg4.rccc.ou.edu/mena/. Network construction was performed using default values except for two settings: the majority was set to 1 and logarithm transformation was not taken into account (or not used). Random Matrix Theory (RMT) was used to automatically identify the appropriate threshold value before network construction. Network topological properties were calculated and evaluated (Deng et al., 2012). Phylogenetic molecular ecological networks (pMENs) were visualised using Cytoscape (v. 3.4.0) (Shannon et al., 2003). Taxa with the highest betweenness centrality scores were considered keystone species (Vick-Majors et al., 2014). Hub taxa were defined as the nodes possessing a score of betweenness centrality >0.09 (Floc'h et al., 2020).

2.8. Phosphomonoesterase activity

Acid and alkaline phosphomonoesterase (PME) activity analysis was performed following the method of Alvey et al. (2001) with modifications to be suitable for the rhizosphere pellet. The pellet was resuspended in 1 ml of deionized water. 0.25 ml of the soil suspension was used for the determination of alkaline and acid PME activity as described by Tabatabai and Bremner (1969). After analysis, the dry weight of the soil in the suspension used for the assay was determined at 105 °C to report PME activities per mass unit. Values were expressed as micromoles of p-nitrophenol released per gram of soil (dry weight) per hour. Data were subjected to a two-way analysis ANOVA (P treatment × cropping treatment) followed by Tukey's HSD test (*P*-value <0.05) performed with the software RStudio (version 4.1.2), to assess the crop and P availability effects. Values (which had a Gaussian distribution) were then correlated (Pearson rho coefficient) to the relative abundance of the ASVs (described above) in the rhizosphere using the command "cor" in RStudio (version 4.1.2), and the package Corrplot (Wei and Simko, 2021) for graphical displaying.

3. Results

3.1. Available P in the soil

The effectiveness of P addition to the soil was checked by measuring P availability in non-rhizosphere soil. The results confirmed significant (*P* < 0.001) differences among P treatments. Olsen P was significantly higher in AP, followed by NPK and the lowest in No P and UP treatments, which did not significantly differ from one another. There was an interaction between P treatment × cropping treatment (*P* < 0.01). Indeed, when P was available (AP), sole cropped lupin significantly lowered Olsen P compared to BS (Table S1).

3.2. Main factors driving differences in bacterial community structure

Overall, bacterial communities were affected primarily by the cropping treatment (bulk soil, lupin rhizosphere, lupin-wheat rhizosphere, wheat rhizosphere, pea rhizosphere and pea-wheat rhizosphere) (ADONIS, R^2 = 0.23311, *P* = 0.001), followed by the P treatment (AP, NoP, NPK and UP) (R^2 = 0.13787, *P* = 0.001) and niche (bulk soil, rhizosphere) (R^2 = 0.13245, *P* = 0.001) (Fig. 1A). Significant interactions between P treatment and cropping treatment (R^2 = 0.11100, *P* = 0.001) and P treatment and niche were observed (R^2 = 0.03419, *P* = 0.001). As cropping treatment was the main factor, PCoA plots were constructed for each rhizosphere type to assess the effect of different P treatments on bacterial community structure. Rhizosphere bacterial communities from all samples were significantly affected by different P treatments. For lupin (Fig. 1B), 46.17 % of the total variability in the bacterial composition is explained by the different P treatments (ADONIS, *P* = 0.001) and 40.94 %, 42.72 %, 45.47 % and 44.69 % of the total variability in bacterial communities of lupin-wheat, wheat, pea and
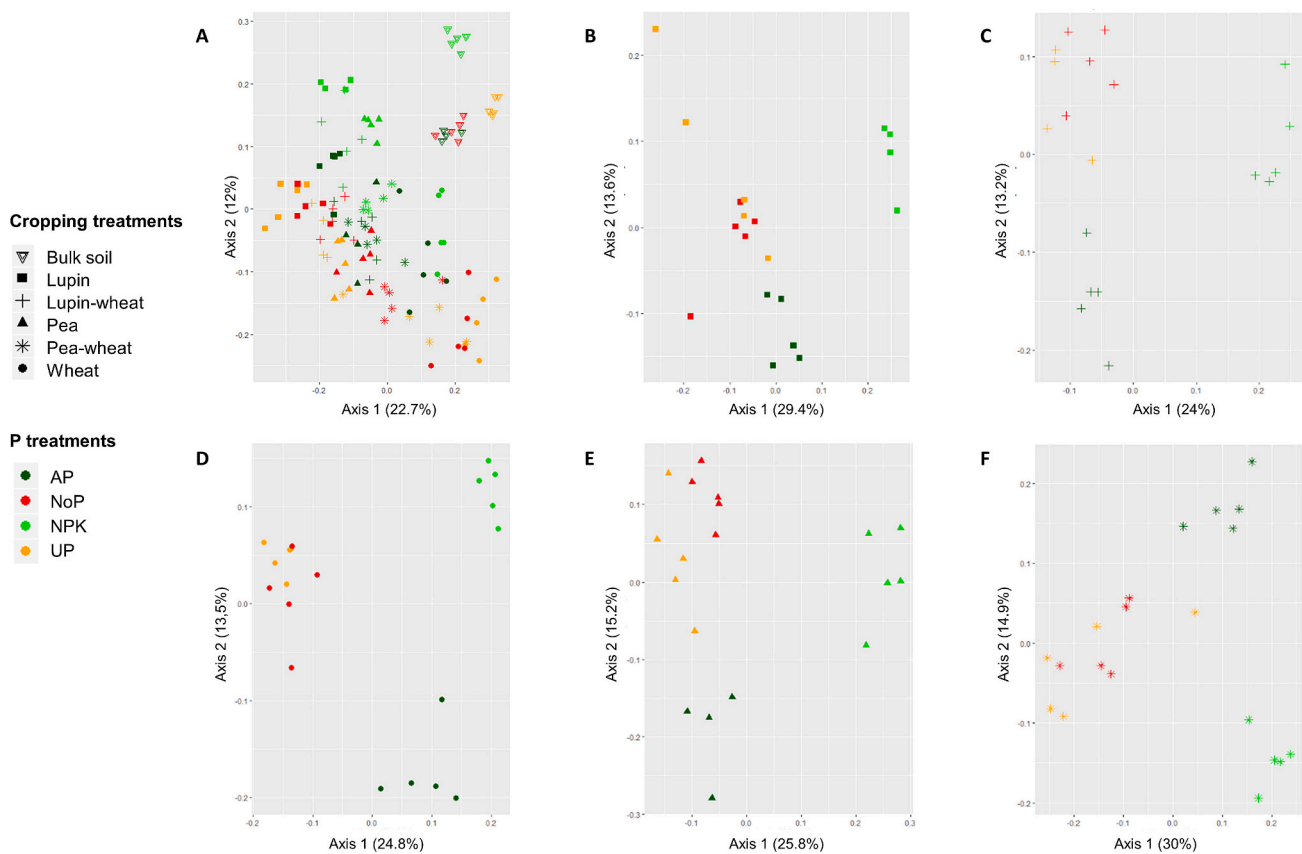
Fig. 1. PCoA plots based on Bray-Curtis distance matrix of bacterial communities from bulk soil and rhizosphere of different crops grown in soil with different P treatments. The percentage shown on each axis corresponds to the proportion of variation explained. Different types are represented by shapes. Inverted triangles = bulk soil samples; solid squares = lupin rhizosphere; crosses = lupin-wheat rhizosphere; solid triangles = pea rhizosphere; stars = pea-wheat rhizosphere and solid circles = wheat rhizosphere. Colours correspond to different P treatments. Dark green = AP; light green = NPK; red = NoP; and orange = UP. (A) Bacterial communities from all samples, showing the main separation per type. (B) Bacterial communities from lupin rhizosphere. (C) Bacterial communities from lupin-wheat rhizosphere. (D) Bacterial communities from wheat rhizosphere. (E) Bacterial communities from pea rhizosphere. (F) Bacterial communities from pea-wheat rhizosphere. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

pea-wheat can be explained by different P treatments, respectively (Fig. 1C–F) (ADONIS, $P = 0.001$). For all cropping treatments, bacterial communities from the NPK treatment were the most different compared to the other treatments. When P was supplied in unavailable form (UP) as tricalcium phosphate, bacterial communities were more similar to the treatment where P was not supplied (NoP). However, for lupin rhizosphere, bacterial communities from UP and NoP are more similar to bacterial communities amended with KH_2PO_4 (AP).

3.3. Differentially abundant taxa present in each P treatment

Different P treatments affected the differential abundance of taxa in the rhizosphere of the tested crops (Fig. 2). Some features assigned to certain genera were found to be enriched when no P was added, such as *Variovorax* for lupin rhizosphere and *Bradyrhizobium* and *Pseudomonas* for pea-wheat rhizosphere (Fig. 2A and E, respectively). In the case of P being unavailable for plant absorption, *Variovorax* was enriched in the rhizosphere of lupin-wheat and pea (Fig. 2B and D) and *Pseudomonas* was enriched in the rhizosphere of pea (Fig. 2D).

When P was added in the available form (AP), it resulted in increased abundance of several genera, such as *Xanthomonas* in the rhizosphere of lupin-wheat and pea (Fi. 2B and 2D), *Lentzea* in pea and in pea-wheat rhizosphere (Fig. 2D and E), *Saccharothrix* and *Pseudonocardia* in pea-wheat rhizosphere (Fig. 2E). In NPK soil, *Catenulispora*, *Leifsonia* and *Arthrobacter* were enriched in lupin and lupin-wheat rhizosphere (Fig. 2A and B), *Pedobacter* was enriched in wheat, pea and pea-wheat (Fig. 2C, D and E).

3.4. Alpha-diversity

To compare alpha diversity in the bulk soil and rhizosphere samples of intercropped and sole-cropped plants, the number of observed ASVs and the Simpson indices were used to estimate the richness and the evenness of bacterial communities, respectively. The values of both indices in bulk soils were always greater or at least equal to that of the rhizosphere regardless of P treatment. In the wheat rhizosphere, the alpha diversity of the bacterial community did not differ significantly from the bulk soil, while in the legume rhizosphere, it was generally lower than in bulk soil. In comparison to the bulk soil, the lupin rhizosphere Simpson index was lower for all P treatments except for AP while the Observed species were lower than bulk soil in the UP treatment (Fig. S1A). In pea, the evenness indicator (Simpson) of bacterial communities from rhizosphere samples was lower than bulk soil for all treatments except for the AP treatment. In addition, the species richness was lower than bulk soil in all treatments except for NoP (Fig. S1B). When compared to sole cropped wheat, microbial diversity was lower when intercropped with lupin, for all P treatments. In contrast, the observed species richness was only lower in the UP treatment (Fig. S1A). When wheat was intercropped with pea, bacterial species richness was lower than sole cropped wheat only in the NPK treatment (Fig. S1B).

3.5. Intercropping and limited P affect network structure

Both the cropping system and P treatments influenced co-occurrence patterns of bacterial communities (Figs. S2 to S6). When P was
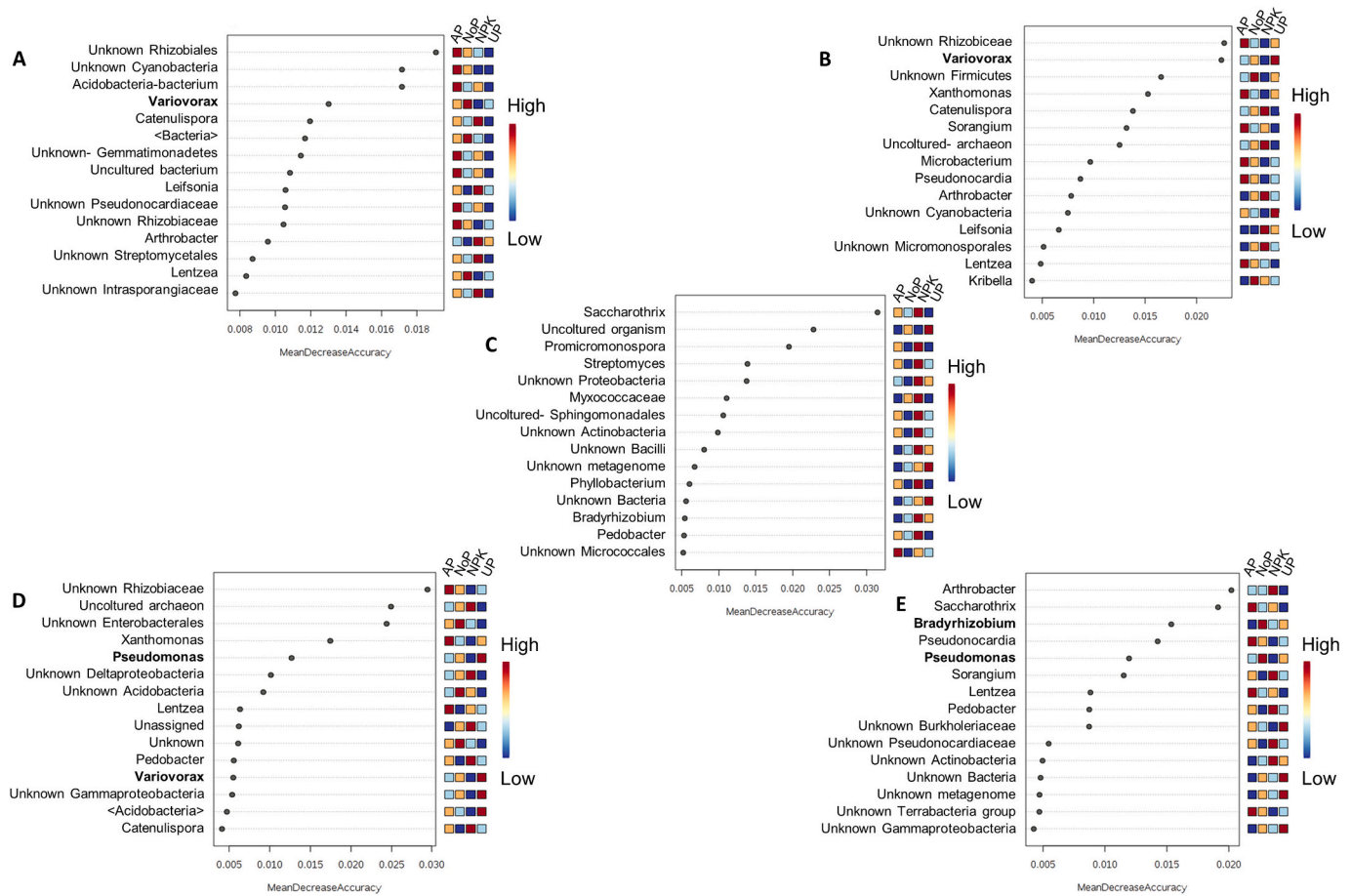
Fig. 2. Random forest analysis on each plant type to check for differentially abundant taxa at genus level, comparing different P treatments, only showing the top 15 taxa for lupin, lupin-wheat, wheat, pea and pea-wheat rhizosphere (A, B, C, D and E, respectively). X-axis shows the mean decrease accuracy (variable importance) and Y-axis shows the taxa which were found to be differentially abundant.

unavailable (UP), the number of edges, observed values of average degree and graph density were higher in intercropping than in the respective sole crops. This was particularly the case in the lupin-wheat intercropping, for example, the sum of edges for sole-cropped lupin and wheat was only 3028, whereas there were 9504 edges detected in lupin-wheat intercropped systems under this condition (Table 2). Under the same P treatment (UP), the clustering coefficient for wheat when intercropped with lupin was considerably higher than sole-cropped wheat. The clustering coefficient also increased in lupin-wheat intercropping when P was available (NPK and AP) compared to wheat and lupin sole crops.

3.6. Intercropping and P treatments affect keystone features

Putative keystone taxa were considered as the top 10 % of the taxa with the highest betweenness centrality, corresponding to the 31 common features. Most of the 31 putative keystone taxa identified per rhizosphere communities were different between intercrop and the respective sole crops. The number of putative keystone taxa shared between intercrops and sole crops was lower in the treatments with no or low P availability (NoP and UP) than when P was available (AP), except in the case of wheat-pea intercrop at NoP, which shared 7 keystone taxa with pea as a sole crop (Fig. S7). Under low P availability (UP), the intercropping shared two features, belonging to *Novosphingobium* and Methylophilaceae, with sole cropped lupin and the other two, Rhodanobacteraceae and *Streptosporangium*, with wheat, in the case of intercropping with lupin, while one feature, belonging Gemmatimonadaceae, was shared with both pea and wheat cropped

alone and other two, Pedosphaeraceae and *Solirubrobacter*, with wheat, in the case of intercropping with pea. In the treatment without P supply (NoP), the lupin-wheat intercropping shared three features, belonging to *Afipia*, Rhodanobacteraceae and Gammaproteobacteria, with sole cropped wheat and only *Kribbella* with lupin, while the pea-wheat intercropping shared a total of eight features with the sole crops: *Bradyrhizobium* with both pea and wheat, Thermomicrobiales with pea and *Marmoricola*, Roseiflexaceae, Nitrososphaeraceae, *Lysobacter* and three features belonged to Gaiellales with wheat. The complete lists of the putative keystone features identified in each network are reported in the Supplementary Tables 2 to 9.

3.7. Phosphatase activity in the rhizosphere

Acid PME activity was affected both by cropping treatments ($P <$ 0.001) and P treatments ($P <$ 0.05). The values were always greater in rhizosphere than in bulk soil (Fig. 3A). Among the cropping treatments, acid PME activity was significantly higher in the rhizosphere of lupin as sole crop and intercrop, followed by pea as sole crop. No significant differences were observed for pea-wheat intercropping and wheat sole cropping, which were only slightly higher than the values observed in the bulk soil (Fig. 3A). Among the P treatments, NPK showed the highest acid PME activity, but it was not statistically different to the values at UP and NoP and significantly differed only compared to AP, in which the lowest activity was recorded (Fig. 3B). Alkaline PME activity was affected by cropping treatments ($P <$ 0.001) and by P treatments ($P <$ 0.001). Unlike acid PME, alkaline PME activity in the rhizosphere was not different from that of bulk soil except in intercropping, where the
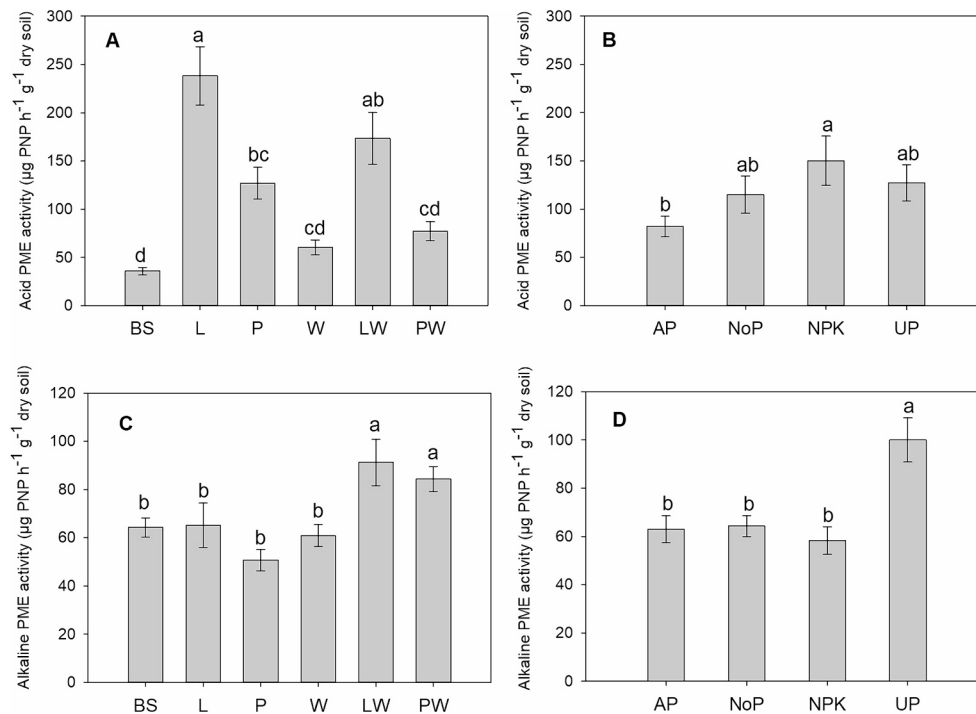
Fig. 3. Effect of cropping treatments and P treatments on acid and alkaline phosphomonoesterase (PME) activity expressed as µg of para-nitrophenol (PNP) g^{-1} soil h^{-1}: (A) acid PME activity at different cropping treatments (in rhizosphere of sole crop (W, wheat; L, lupin; P, pea), of intercropping (LW, lupin-wheat; PW, pea-wheat) and in bulk soil (BS)). Values are means ($n = 20$) $\pm$ SE; (B) acid PME activity at different P treatments (AP, available P; NoP, no P supply; NPK, balanced fertilization; UP, unavailable P). Values are means ($n = 30$) $\pm$ SE; (C) alkaline PME activity at different cropping treatments (in rhizosphere of sole crop (W, wheat; L, lupin; P, pea), of intercropping (LW, lupin-wheat; PW, pea-wheat) and in bulk soil (BS)). Values are means ($n = 20$) $\pm$ SE; (D) alkaline PME activity at different P treatments (AP, available P; NoP, no P supply; NPK, balanced fertilization; UP, unavailable P). Values are means ($n = 30$) $\pm$ SE. Different letters above the bars indicate significantly different values ($P \leq 0.05$; Tukey's HSD test).

values were significantly higher (Fig. 3C). Among the P treatments, alkaline PME activity was significantly higher in UP than in all other P treatments which did not differ significantly from each other (Fig. 3D). Acid and alkaline PME activity results are shown more in detail in Fig. S9.

Acid PME activity was significantly ($P < 0.01$) and positively correlated with the relative abundance of the classes Actinobacteria and Gammaproteobacteria (data not shown) and the orders Acidobacteriales, Betaproteobacteriales, Catenulisporales, Chitinophagales, Enterobacteriales, Glycomycetales, Isosphaerales, Micrococcales, Micromonosporales, Rickettsiales, Streptomycetales, Streptosporangiales (Fig. S8). Alkaline PME activity was significantly ($P < 0.01$) and positively correlated with the relative abundance of the class Gammaproteobacteria (data not shown) and the orders Flavobacteriales, Isosphaerales and Pseudomonadales (Fig. S8).

4. Discussion

4.1. Wheat and legumes harbour different bacterial communities

The effect of plants in structuring bacterial communities is well known, and this study further supports this, as bulk soil samples clustered apart from all rhizosphere samples (Fig. 1A). Our results are in accordance with the concept that different plants select for specific bacterial communities in their rhizosphere (Berg and Smalla, 2009; Costa et al., 2006; Garbeva et al., 2008; Lundberg et al., 2012) and that plants are one of the most important selective factors for microbial community composition in soil (Badri and Vivanco, 2009; Costa et al., 2006; Garbeva et al., 2004; Marschner et al., 2004). Plants can influence the rhizosphere through rhizodeposition of exudates, mucilage and sloughed cells. There are a variety of compounds exuded by roots and these include organic acids, sugars, amino acids, fatty acids, vitamins,

growth factors, hormones and antimicrobial compounds (Bertin et al., 2003). Root exudates are one of the main factors that influence the rhizosphere microbiome structure, altering the rhizosphere environmental conditions and offering nutrient sources for microbial growth (Badri et al., 2013; Shi et al., 2011).

4.2. Effect of P treatment on the structure of bacterial communities

When separating the data by crop type [either as sole crops (SC) or intercrops (IC)], NPK treatment had a clear impact on bacterial communities for all crops. This could have been a result of long-term NPK application as the soil was recovered from an experiment in which NPK had been amended regularly during the last 164 years. Bacterial communities may have been selected by the systematic availability of the nutrients, differentiating from the other treatments where P depletion was the selective factor during the years. The effect of long-term organic or inorganic amendment applications on the structure of bulk soil microbial communities has been previously reported (Chen et al., 2016; Ding et al., 2016; Francioli et al., 2016; Soman et al., 2017). For the rhizosphere in particular, high levels of inorganic nitrogen (N) fertilizers negatively affected bacterial richness, diversity and functions in wheat (Kavamura et al., 2018; Reid et al., 2021). The differences between bacterial communities from NoP and UP treatments were low for all crops, supporting the notion that Ca_3PO_4 can be a recalcitrant P form for the plant host as well as the majority of the soil microbiome. However, in the lupin rhizosphere, NoP and UP bacterial communities converged with AP treatment to a greater extent than the other cropping treatments (Fig. 1B). This might be due to the greater ability of lupins to release carboxylates and to lower the pH compared to wheat and pea, and when Ca_3PO_4 is added P is consequently more available in the system (Pearse et al., 2007). Interestingly, the lupin-wheat intercrop community plot for these treatments was not as disparate as the corresponding wheat

Table 2

Topological features of co-occurrence networks for different groups of microbial taxa in lupin (L), wheat (W) and pea (P) as sole crops and as intercrops (LW, PW) under different P treatments: AP, UP, NoP and NPK.

	Samples	Nodes[a]	Edges[b]	Average degree[c]	Clustering coefficient[d]	Graph density[e]
AP	L	319	1131	7.09	0.38	0.02
	P	319	**6246**	39.16	0.68	0.12
	W	319	1590	9.97	0.51	0.03
	LW	319	1669	10.46	0.60	0.03
	PW	319	1691	10.60	0.69	0.03
UP	L	319	2068	12.97	0.70	0.04
	P	319	1681	10.54	0.62	0.03
	W	319	960	6.02	0.40	0.02
	LW	319	**9504**	59.59	0.71	0.19
	PW	319	2412	15.12	0.49	0.05
NoP	L	319	**5942**	37.25	0.72	0.12
	P	319	1132	7.10	0.57	0.02
	W	319	2380	14.92	0.74	0.05
	LW	319	1099	6.89	0.55	0.02
	PW	319	1312	8.23	0.64	0.03
NPK	L	319	**3492**	21.89	0.36	0.07
	P	319	1158	7.26	0.60	0.02
	W	319	973	6.10	0.32	0.02
	LW	319	1587	9.94	0.74	0.03
	PW	319	1094	6.86	0.53	0.02

Higher values are highlighted in bold.

[a] Number of nodes.

[b] Number of edges.

[c] Node connectivity - shows how many connections (on average) each node has to another unique node in the network.

[d] How nodes are embedded in their neighbourhood, and thus the degree to which they tend to cluster together.

[e] Average network distance between all pairs of nodes. It indicates the number of steps one needs to make on average in the graph in order to connect two randomly selected nodes.

monoculture plot. This could suggest that lupin intercropping with wheat improves P availability for wheat in this system. In general, when adding available P as KH_2PO_4, bacterial communities differed from the other treatments, which might support the hypothesis that the availability of P influenced root biomass and the release of root exudates, structuring microbial communities (Wasaki et al., 2018).

4.3. Effect of P treatment on putative plant growth-promoting taxa

When P was omitted (NoP) or added as unavailable Ca_3PO_4 (UP), the rhizosphere of legumes and wheat-legume intercrops was enriched for several species that have previously been considered plant growth-promoting rhizobacteria (PGPR) and/or phosphate solubilizing bacteria (PSB), which can alleviate the P-deficient conditions (Amy et al., 2022; Emami et al., 2018). However, it is important to acknowledge that amplicon sequencing alone is insufficient to prove PGPR activity. Further studies should be performed on microbial isolates from these systems to characterise their functional ability for P mineralisation and solubilization. Ultimately, these approaches should be overlaid with paired-omics approaches (e.g. metabolomics and genomics), mutagenesis of candidate beneficial genes as well as *in planta* studies to demonstrate plant growth promotion.

Variovorax was enriched in the lupin rhizosphere when no P was added as well as in the rhizosphere of lupin-wheat and pea (Fig. 2B and D) when P was unavailable for plant absorption (Fig. 2A). Even though our data provide detailed information only up to genus level and a reliable distinction of the species is not possible, it has been reported that several species within *Variovorax* genus are beneficial for plant growth and nutrient absorption. The P-solubilizing ability of species from the genus *Variovorax* is supported by many studies (Collavino et al., 2010; Zheng et al., 2018) and is mainly correlated to organic acid release (Magadlela et al., 2023) but also to phosphatase activity (Toukabri et al.,

2021). These bacteria are known specifically to colonize root tissues and to interact with plants through the exchange of signalling molecules and utilization of readily secreted compounds (Haichar et al., 2008). *Variovorax* may be considered a specialist, found in species such as bread wheat (Cordero et al., 2020; Rfaki et al., 2014), rape (*Brassica napus* L.) (Haichar et al., 2008) and *Avena barbata,* responding to plant growth with the increasing of relative abundance (Zhalnina et al., 2018).

In this study, the pea rhizosphere was enriched by *Pseudomonas* spp. in plants grown as sole crop when P was added in the unavailable form (UP) and as intercrop when no P was added. This agrees with the reduction in the relative abundance of *Pseudomonas* spp. in the rhizosphere observed in other studies in response to fertilization with available P (Chhabra et al., 2013; Tan et al., 2013). The ability of certain species of *Pseudomonas* isolates to solubilize P and improve P status has been previously demonstrated (Israr et al., 2016; Masters-Clark et al., 2020; Zabihi et al., 2011). As with other phosphate solubilizing bacteria (PSB), this function is thought to be due to the release of organic acids (Collavino et al., 2010; Rashid et al., 2004; Trivedi and Sa, 2008; Vyas and Gulati, 2009) and acid and alkaline phosphatase activity (Rodríguez and Fraga, 1999; Krey et al., 2011).

In our experiment, when no P was added, the abundance of *Bradyrhizobium* and *Pseudomonas* spp. increased in the pea-wheat rhizosphere. Some species from the Rhizobiaceae family are interesting, not only because they perform biological nitrogen fixation in association with legumes, but because certain free-living members of this family can also be considered as PGPR (Antoun et al., 1998; Boiero et al., 2007). *Bradyrhizobium japonicum* is considered to have plant growth-promoting capacity (Cassán et al., 2009) through siderophore production, P solubilization and IAA production (Antoun et al., 1998). It is reported that co-inoculation of *Pseudomonas* and *Bradyrhizobium* significantly increased P content and improved growth in *Bradyrhizobium japonicum* host plants (Argaw, 2012; Rotaru, 2018). It has also been reported that this genus increased its relative abundance in response to plant growth in non-symbiotic plants such as *Avena barbata* (Zhalnina et al., 2018).

It is important to acknowledge that although these bacteria may have similar identification by amplicon sequencing at the ASV level based on the 16S rRNA gene, it is not possible to confirm by this method whether they are the same species due to the lack of resolution associated with the amplicon sequencing of a single gene, which does not consider the accessory genome of community members.

It is interesting to highlight that the genera, whose relative abundance increased at low P availability in our experiment (*Variovorax*, *Pseudomonas* and *Bradyrhizobium*), have been previously specifically associated with some plants through the assimilation of root exudates (Haichar et al., 2008). The enrichment of these genera occurred specifically in the rhizosphere of legumes (both sole crop and intercrop), which are considered to accumulate more carboxylates in the rhizosphere than cereals (Hinsinger et al., 2003; Pearse et al., 2006) and vary their carboxylate composition in response to P availability in soils (different P forms) (Pearse et al., 2007). It is possible that at low P availability, plants respond by modifying their exudate composition to select a modified bacterial community that contains a higher proportion of PSB and PGPR species (Ludueña et al., 2023). This has previously been supported by observations that root exudate production and composition are the key drivers for differences in microbial community structure and function (Marschner et al., 2004; Tiziani et al., 2022).

4.4. Intercropping reduces microbial richness and diversity

We found that in legume-based systems the diversity of the bacterial rhizosphere community was generally lower than in the other samples, except for the available P treatment where diversity was more similar to bulk soil and other planting combinations. Our results support the hypothesis that, when P is in the unavailable form, certain plants, in our case legumes, can select specific root-associated bacteria resulting in reduced community diversity. In other studies, this phenomenon has

been observed in response to high N fertilization status (Kavamura et al., 2018; Wu et al., 2021b) as well as heat, drought and salt stress in the leaf endosphere (Vescio et al., 2021; Vita et al., 2022).

Sole-cropped wheat showed generally higher richness and diversity of rhizosphere community than legumes, similarly to what was observed by Cordero et al. (2020). In the rhizosphere and passing through the rhizosphere to the endosphere, there are intensive selection pressures caused by the host immune system and plant exudates that increasingly reduce the number of bacteria able to colonize these compartments (Mardani-Korrani et al., 2021; Xiong et al., 2021). Our results suggest that wheat is less able than legumes to select a rhizosphere community since its alpha diversity did not significantly differ when compared to the bulk soil.

Our findings show that legume intercropping reduces rhizobacterial diversity in comparison to wheat sole cropping, but the lowest richness and diversity were found in the rhizosphere of sole-cropped legumes. Furthermore, because the richness (Observed) was lower in pea rhizosphere than in bulk soil at all P levels (except NoP), pea seems to be more effective than lupin in selecting a less complex bacterial community. On the other hand, lupin seems more effective than pea in extending to the intercropped wheat the reduction in evenness. In lupin, the reduction of the number of observed taxa is well documented and explained by the complex strategy developed by the plant to protect secreted organic anions from microbial degradation (Weisskopf et al., 2006), while Pivato et al. (2021) found the reduction of several alpha diversity indices (Observed, Shannon and Simpson) in the rhizosphere of pea compared to that of wheat. An interesting outcome is that when P was added in the unavailable form (UP), sole cropped pea and lupin showed values of richness and diversity (Observed and Simpson) markedly lower than wheat (Fig. S1A, B). The variation of rhizodeposition, as a response of the plant to the conditions of the environment (P in the unavailable form), could explain the observed reduction of rhizobacterial diversity (Bulgarelli et al., 2013).

Our research suggests that plants differ in their ability to select their bacterial community by reducing the richness and evenness of the rhizosphere microbial community. The intensity of the selection is affected by P availability in legumes while in wheat the response is weak. When wheat and legumes are intercropped, the selection of bacterial rhizosphere community by legumes can be transferred to wheat by the intermingling of partners roots.

4.5. Intercropping alters network structure under P unavailability

Both intercropping and P treatments influenced co-occurrence patterns of bacterial communities. This is in agreement with Li and Wu (2018) who observed that clover, wheat and mustard intercropping had a complex connection between bacterial taxa. Since higher values of average degree, clustering coefficient and graph density indicate that features are more interconnected (Jiao et al., 2019) and more edges correspond to a more complex network, the greater number of edges, observed values of average degree and graph density in intercropping than in sole crop when P was unavailable (UP) resulted in the rhizosphere bacterial community becoming more complex. Indeed, the pivotal role of P in stimulating soil microbial metabolic activity and the greatest impact of P deficiency on community assembly are well documented (Feng et al., 2017; Zheng et al., 2009). Thus, our results suggest that intercropping compared to sole cropping favoured co-occurrence patterns of bacterial communities mostly when soil was rich in P but in the unavailable form of tricalcium phosphate. When P has low availability, a more intricate and cooperative network could alleviate nutrient competition and create more trophic levels or resource cascades (Banerjee et al., 2016), because bacteria can use nutrient resources more efficiently through resource complementarity (Dai et al., 2018).

In all other P treatments, the influence of legume intercropping on network complexity was weak (at AP on lupin and wheat; NoP on pea; NPK on wheat) or acted negatively (at AP on pea; at NoP on lupin and

wheat; at NPK on lupin and pea) (Table 2). Our results are consistent with those from several interspecific intercrops (Li and Wu, 2018), legume-wheat intercrops (Pivato et al., 2021) and intraspecific intercrops (Wu et al., 2020), which suggest more complex networks within bacterial communities in intercropping than in sole cropping rhizosphere. In contrast to the mentioned studies, which did not investigate the influence of intercropping varying the availability of P source, we found a consistent effect of intercropping only when P was added in the unavailable form.

4.6. Intercropping and P treatment affect keystone features

Keystone species have a disproportionate deleterious effect on the community upon their removal (Berry and Widder, 2014). The low number of keystone features shared between the intercrops and sole crops (Fig. S7) implies that intercropping markedly affected network complexity and connections. The keystone features in common between intercrops and sole crops were lower when P was limited (NoP and UP) suggesting that the reorganization of the putative keystone occurs in a P-dependent manner (Fig. S7). Most keystone taxa are associated with plant-beneficial traits, including P mobilization, and were associated with the rhizosphere of the legumes, as well as wheat.

Furthermore, when intercropping wheat with lupin under P limitation (UP and NoP) some keystone members of the root microbiome, that are in common with sole cropped lupin, are assigned to bacterial groups previously shown to be capable of degrading xenobiotics (Macey et al., 2020; Waigi et al., 2015) and having plant growth-promotion properties (Fraser et al., 2015; Krishnan et al., 2016). Others in common with sole cropped wheat, in previous studies were specifically found in the rhizosphere of bread wheat (Liu et al., 2022): one within Gemmatimonadaceae, two within Rhodanobacteraceae, which are generalist hydrocarbon-degraders (Gutierrez, 2019) and another, non-specified, also belonging to Gammaproteobacteria. Under the same P availability levels, it was also found that in pea-wheat as well as sole wheat cropping that some keystone taxa implicated in P metabolism were common e.g. *Marmoricola* spp. and *Lysobacter* spp. (Yang et al., 2023; Zhang et al., 2021). In terms of N cycling it was found that Nitrososphaeraceae, capable of ammonia oxidation (Kerou and Schleper, 2016), three taxa assigned to Gaiellales, which are putative nitrate reducers (Tatariw et al., 2021), and N$_2$ fixing *Bradyrhizobium*, were common keystone taxa for both sole cropped wheat and pea (Antoun et al., 1998) (Tables S2-S9). It is plausible that common keystone taxa are selected for under non-optimal conditions such as N and P starvation.

4.7. Phosphatase activity in the rhizosphere

Acid PME activity was more affected by cropping treatment than by P treatment, which is consistent with the idea that the acid PME exudation in low-P soil is mostly ascribed to roots than microbial activity, even if the contribution of soil microflora in response to P-deficiency should not be excluded (Nannipieri et al., 2011). The highest acid PME activity was found in lupin rhizosphere, confirming the higher values found in lupin compared to that in other legumes and non-legume crops (Nuruzzaman et al., 2006; Olde Venterink, 2011) (Fig. 3A). Lupin was more able than pea in maintaining high acid PME activity in intercropping (Fig. 3A) and it also profoundly influenced the diversity of bacterial communities in intercropping (Fig. S1). Because both the diversity of bacterial communities (selection) and the acid PME activity are linked to root exudation, these results could be explained by the higher root exudation reported for lupin (Lyu et al., 2016).

In our study, when P was available (AP) the rates of acid PME activity were lower (Fig. 3B), which is consistent with the suppression of phosphatase activity reported by a rich body of the literature when P is added to the soil (Olander and Vitousek, 2000; Olde Venterink, 2011; Wu et al., 2021a). It is difficult to interpret the measurement of PME activities of rhizosphere soil if the contribution of plant and microbial phosphatase

activities are not resolved (Nannipieri et al., 2011). However, high PME activity in our data is positively correlated with the abundance of several bacterial orders in the root microbiome ($P < 0.01$), such as Streptomycetales, Streptosporangiales, Betaproteobacteriales, Catenulisporales, Micrococcales and Micromonosporales (Fig. S8). This finding suggests that these bacteria might be involved in regulating the P cycling in the rhizosphere.

However, several orders were negatively correlated ($P < 0.01$) with acid PME activity (data not shown). This decrease could be explained by the strategies that certain plant species such as lupin evolved to limit microbial degradation of carboxylates which are secreted with phosphatase to improve phosphate acquisition (Weisskopf et al., 2006). These strategies involve the reduction of rhizosphere pH in the cluster-roots (Lambers et al., 2009) and the exudation of isoflavonoids and fungal cell wall degrading enzymes, both lead to a decrease in bacterial abundance and fungal sporulation (Weisskopf et al., 2006). Furthermore, root exudation profiles have been linked to P status and amended root microbiome structure (Weisskopf et al., 2011).

The effect of roots in increasing enzyme activity in the rhizosphere in comparison to surrounding bulk soil (Cheng and Kuzyakov, 2005; Nannipieri et al., 2012) was more evident for acid PME than for alkaline PME activity, which was greater than bulk soil only in intercropping ($P < 0.001$). This last finding can suggest more favourable conditions for bacteria involved in organic P cycling in intercropping than in sole cropping.

Alkaline PME activity was also affected by P treatments ($P < 0.001$) but its value was significantly higher only when P was in the unavailable form (Fig. 3D). An increase in alkaline phosphatase activity was found by Malik et al. (2012) in response to the addition of organic matter, which is explained by the increase of microbial growth. On the other hand, the increase in alkaline PME activity observed in this study with the supply of an inorganic low-available source of P could be explained by the modification of root exudates and the consequent changes in bacterial community structure (Dunfield and Germida, 2003; Tesfaye et al., 2003). This last statement is supported by the positive correlation ($P < 0.01$) of alkaline PME activity with the class Gammaproteobacteria

and the relative order Pseudomonadales (Fig. S8) which contains *phoD*-harboring bacteria (Wang et al., 2022; Zheng et al., 2021).

5. Conclusions

Our work suggests that the plant is one of the main selective factors driving rhizobacterial community composition. Furthermore, P availability further shapes the composition, selecting the taxa to mitigate P starvation, confirming our first hypothesis that rhizosphere bacterial community composition is influenced by the bioavailability of soil P. In general, legumes were more effective than wheat in reducing the bacterial richness and diversity (Fig. 4) and in selecting putative PSB and PGPR, while, in wheat, alpha diversity did not differ from that of bulk soil, confirming the second hypothesis that plant species differentially select their root microbiomes. When wheat is grown with legumes in intercropping there is a reduction of alpha diversity (Fig. 4) accompanied by an increase of taxa containing putative PSB and PGPR, confirming the third hypothesis that the selective ability of some species can be extended to others through intercropping. An interesting outcome is the increase of complexity and co-occurrence patterns of bacterial communities in intercropping compared to the respective sole crops when P was unavailable in the form of tricalcium phosphate (Fig. 4). This corresponded with an increase of alkaline PME activity, which positively correlated with the abundance of putative PSB and PGPR taxa. Acid PME activity was linked to plant species, potentially indicating the greater contribution of the plant than bacteria in exuding this enzyme, although its correlation with some taxa suggests their involvement in regulating the P turnover. The conclusions above indicate that some of the facilitations responsible for the ecosystem multifunctionality of legumes in intercropping can be partially ascribed to their ability to interact with soil microbiome favouring the growth and activity of some taxa. The extent of this contribution and how it varies due to environmental conditions are still unknown and remain a starting point for future studies.

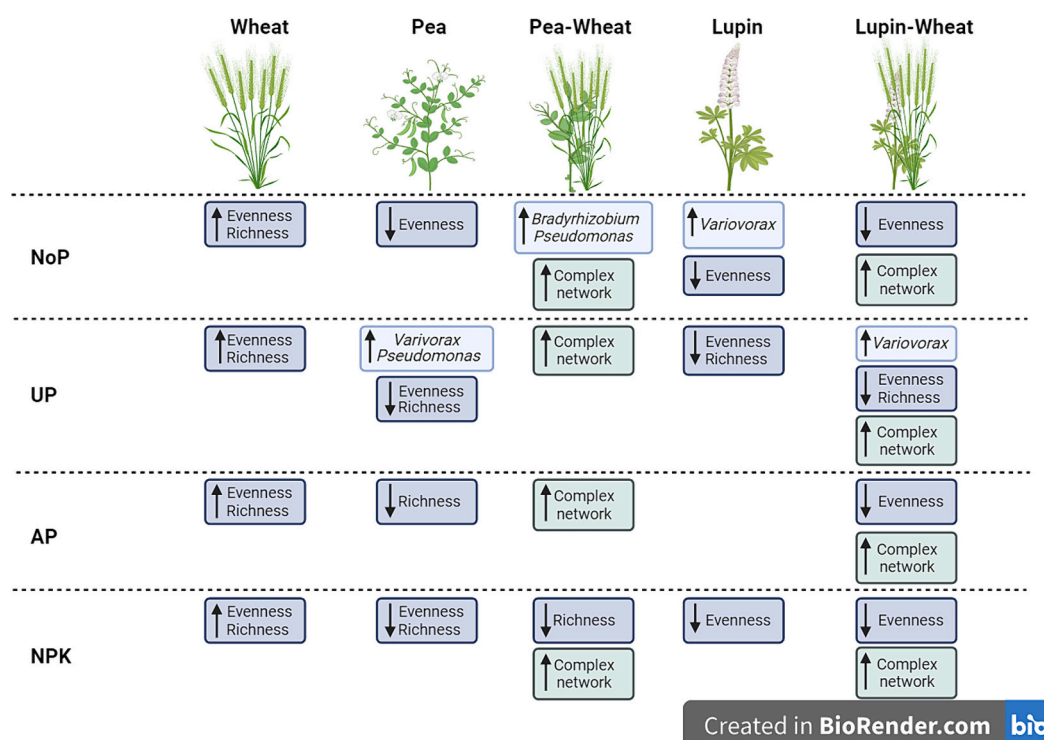Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2024.105414.

Fig. 4. Graphical representation of the main research findings synthetically summarized.

CRediT authorship contribution statement

Emilio Lo Presti: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Vanessa N. Kavamura:** Data curation, Formal analysis, Visualization, Writing – review & editing. **Maïder Abadie:** Data curation, Formal analysis. **Maurizio Romeo:** Formal analysis. **Tessa E. Reid:** Formal analysis. **Sigrid Heuer:** Conceptualization, Resources, Supervision. **Michele Monti:** Conceptualization, Resources, Supervision. **Tim H. Mauchline:** Writing – review & editing, Supervision, Conceptualization, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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