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# Does legume root exudation facilitate itself P uptake in intercropped wheat?

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## Keywords

Intercropping, Grain legume, Wheat, Root exudates, Acid phosphomonoesterase, Phosphorus availability.

## Abstract

*Purpose* To assess the ability of three different grain legume species to facilitate phosphorus (P) uptake by intercropped wheat and to evaluate the differences in root exudation and phosphatase activity that each grain legume species can induce in the soil.

*Methods* In a pot experiment under controlled climatic conditions, lupin, pea and faba bean intercropped with durum wheat and respective sole crops were grown at high and low phosphorus availability. At the flowering time of each grain legume, intercrop and sole crops were sampled.

*Results* Intercropping favored wheat P uptake more at low P availability than at high P availability. Compared to the sole crop, wheat P uptake was greater in intercropping with pea both without and with the addition of phosphorus and with lupin when phosphorus was not added. Phosphatase activity, which was generally high, was highest in soil with lupin and faba bean sole crops. In the rhizosphere of faba bean, the highest total carboxylate concentration was also measured, followed by pea and lupin. Root exudation was favored by P supply, showing greater values in intercropped wheat than in the respective sole crops.

*Conclusions* P uptake by wheat was not directly linked to greater root exudation in the tested intercropping systems. Even if root exudation and available phosphorus were generally greater with legumes both in sole crop and in intercropping, other interactions such as complementarity and competition were involved, hiding potential facilitation exerted by faba bean and bringing out the benefits from the wheat/pea combination.

## Abbreviations

**IC:** Intercrop

**SC:** Sole crop

**P:** Phosphorus

**W-P:** Wheat sole crop sampled at pea flowering time

**W-F:** Wheat sole crop sampled at faba bean flowering time

**W-L:** Wheat sole crop sampled at lupin flowering time

**BS-P:** Bare soil sampled at pea flowering time

**BS-F:** Bare soil sampled at faba bean flowering time

**BS-L:** Bare soil sampled at lupin flowering time

**WP:** Wheat/pea intercrop

**WF:** Wheat/faba bean intercrop

**WL:** Wheat/lupin intercrop

**P0:** No P supply

**P1:** With P supply

## **Introduction**

Phosphorus (P) is a major nutrient for all living organisms. It is essential for cellular activity since it plays a vital role in energy metabolism and the biosynthesis of nucleic acids and membranes, making it nonsubstitutable for life and a key production factor in agriculture. Its scarcity in soils is a limiting factor for crop production in many agroecosystems (Cordell et al. 2009). In most soils, due to its low mobility, the P concentration in the soil solution ranges between 0.1  $\mu\text{M}$  and 10  $\mu\text{M}$ , which is much lower than the adequate P concentration for optimal growth of many crop plants (Hinsinger 2001; Raghothama 1999). As a result, in the recent past, disproportionate quantities of P fertilizers and manure were needed to compensate for the low P use efficiency of crops in industrialized countries. The increasing use of P fertilizers in agriculture is controversial because phosphate rocks that are used to manufacture them are a finite resource with an estimated time frame of availability of approximately 100 years to 125 years (Heffer et al. 2006; USGS 2010; Smit et al. 2009). On the other hand, large amounts of surplus P were retained as residual P in soils because of fertilizer and manure supply (Syers et al. 2008). In soil, the P surplus is converted into increasingly less soluble forms over time. In particular, P is adsorbed by clay minerals and by iron (Fe) and aluminum (Al) oxides in acidic soil (Hinsinger 2001; Kizewski et al. 2011) or on the surface of calcium (Ca) carbonate (Larsen 1967) and clay minerals (Devau et al. 2010) in neutral to calcareous soil. P may subsequently precipitate in phosphate minerals (Lindsay et al. 1989) that slowly release phosphate ions into the soil solution by dissolution. Therefore, within this context, alternative management strategies are needed to exploit these residual soil P reserves by desorption and dissolution, improving P availability in the agroecosystem to reduce external input.

P-efficient plants develop many chemical, biological, and biochemical mechanisms to increase P acquisition in low-P soils (Shen et al. 2011). The exudation of organic anions (Bello 2021; George et al. 2008; Gerke et al. 1994; Li et al. 1997; Li et al. 2012; Neumann et al. 1999; Tawaraya et al. 2014) and phosphatase enzymes (Gianfreda 2015) is one of these strategies. Carboxylates released in the rhizosphere compete with phosphate groups for binding sites in the soil (Nuruzzaman et al. 2005b), forming strong complexes with aluminum and iron oxides, and P is released into the soil solution (Jones and Darrah 1994; Ryan et al. 2001). Substantial exudation of carboxylates is well documented among a number of grain legume crops, e.g., white lupin (*Lupinus albus* L., Cu et al. 2005; Funayama-Noguchi et al. 2021; Gardner and Boundy 1983; Hocking and Randall 2001; Tiziani et al. 2020), pigeon pea (*Cajanus cajan* (L.) Millsp., Ae et al. 1990; Vora et al. 2021), faba bean (*Vicia faba* L., Li et al. 2007; Li et al. 2016; Lv et al. 2020; Xiao et al. 2020) and chickpea (*Cicer arietinum* L., Neumann and Römheld 1999; Pang et al. 2018; Sharma et al. 2021; Veneklaas et al. 2003), with differences in the quality and quantity of these exudates among the species (Nuruzzaman et al. 2005a) and soil conditions (Ae et al. 1990; He et al. 2017; Mimmo et al. 2011; Veneklaas et al. 2003). Phosphatase enzymes released by plants and microorganisms contribute to the hydrolysis of organic P, which is not directly available to plants, to supply available P to the soil solution (Spohn and

Kuzyakov 2013). Many authors (Houlton et al. 2008; Yadav and Tarafdar 2001; Venterink 2011) have reported higher phosphatase activity in soil under legumes than under other plants. The exudation of organic anions and phosphatase enzymes seems to be the most important strategy adopted by legumes to face the low availability of P in soils.

It has been reported that some legume crops can facilitate P uptake and biomass production of cooccurring nonlegume crops, apparently by exuding organic acids, protons or acid phosphatase (Li et al. 2004, 2007; Messaoudi et al. 2020; Tang et al. 2021). Thus, the most promising agronomic approach to use root exudates to mobilize sparingly available P from the soil appears to integrate into the cropping system P-mobilizing plant species as intercrops.

To date, some of the studies have been carried out on hydroponic systems, which provide detailed information on root exudation. In this case, we cannot exclude that nutrient solution affects root exudation compared to field conditions. On the other hand, in the studies carried out in the field, it is difficult if not impossible to remove the roots without excessive damage, precluding any detailed analysis. Therefore, we carried out the experiment on pots filled with soil from the field to minimize the alteration of growth conditions and, at the same time, to allow a straightforward collection of root exudates. In this experiment, the beneficial belowground interactions (facilitation) of two legumes, pea and faba bean, essential sources of proteins and carbohydrates both for human and animal consumption in the Mediterranean area, and one species, lupin, thoroughly studied because of its root exudation, were compared in legume/wheat intercropping. Moreover, the ability to facilitate P uptake of wheat mediated by root exudates of the legumes was evaluated by comparing the three intercrops at high and low levels of P supply.

We hypothesized that (i) P uptake of wheat was facilitated by grain legume intercropping and that the facilitation varied due to legume species; ii) wheat P uptake was less facilitated by legume intercropping when P was supplied, with differences among the legume species. Belowground interactions involved in P mobilization are complex and mainly mediated by root exudation of phosphatase and carboxylates. Therefore, we also hypothesized that (iii) greater wheat P uptake generally corresponded to greater carboxylate concentrations and phosphatase activity in the soil.

The authors supposed that although the intermingling between the two plants is appreciable, the changes generated by root exudation are useful for the intercropped plant when they persist outside the rhizosphere, in the bulk soil between the two root systems. Consequently, the study focused on two soil regions: the rhizosphere, where the exudates responsible for facilitation are released, and the bulk soil between the two root systems, where the facilitative effect of the exudation can be helpful for the intercropped partner. The composition of carboxylates was measured in the rhizosphere and considered to result from root exudation. In contrast, phosphatase activity and P pools (Olsen P and organic P) were measured in bulk soil and considered the consequence of the activity of root exudates in soil that facilitates the partner plant.

## **Material and methods**

### *Plant growth and experimental design*

Durum wheat (*Triticum turgidum* subsp. *durum* (Desf.) Husn. cv. Svevo), lupin (*Lupinus albus* L. cv. Multitalia), faba bean (*Vicia faba* var. *minor* Beck cv. Sikelia) and pea (*Pisum sativum* L. cv. Hardy) were grown in pots as sole crops (SC) and as intercrops (IC). To sample at the flowering stage of each legume, three wheat sole crops were also established (W-F, W-L and W-P for faba, lupin and pea, respectively). Nine cropping treatments resulted from combining the three legumes and wheat in intercropping and sole cropping. In addition, three bare soil (BS) treatments were added and sampled

at the flowering time of faba (BS-F), lupin (BS-L) and pea (BS-P) as a control for soil variable analysis.

Crop treatments and BS controls were combined with two P levels in the soil, with no P supply (P0) and adding 50 mg P kg<sup>-1</sup> soil as KH<sub>2</sub>PO<sub>4</sub> (P1) in a two-factorial completely randomized design with 4 replications. In each pot, one plant of each species and one legume plant with one wheat plant were grown for sole crop and intercropping, respectively. The pots were PVC tubes Ø 14 cm and 30 cm tall filled with soil mixed with perlite (80/20, v/v).

The soil was collected from the experimental farm (38°06'37" N, 15°39'40" E) of the Department AGRARIA at Mediterranea University of Reggio Calabria, Italy. The soil was selected for the low content of available P (11.33 mg of bicarbonate-extractable P kg<sup>-1</sup> soil). Main soil properties are listed in Table 1. Bare soil treatments were obtained by filling the pots with the same soil and perlite mix and leaving them without the crop.

The pots were placed from January to March 2018 under controlled conditions in a climate chamber, and plants were grown until legume flowering. Radiative and thermic regimes are reported in Table 2.

#### *Soil and plant sampling*

At flowering time of each legume species, plants and soil from the respective sole crop and intercropping pots were sampled for the analysis. The choice of sampling at flowering time was driven by the high release of root exudates, which occurs at flowering in grain legumes (García et al. 2001). Flowering occurred 58, 65 and 86 days after sowing in pea, faba bean and lupin, respectively, and corresponded to the sapling of each legume and the relative intercropping, wheat sole crops and bulk soils.

During sampling, the whole plants were gently removed from the soil, and the remaining bulk soil was carefully mixed and sampled for analyses in the laboratory. Shoots and roots were separated immediately, the roots were shaken slightly to remove excess soil, and the remaining adhering soil was defined as rhizosphere soil (Veneklaas et al. 2003). According to modified Pearse et al. (2007), the rhizosphere was sampled to determine carboxylate composition as follows: approximately 10 g of root with adhering soil was collected and transferred to a 100-mL vial containing 50 mL of 0.2 mM CaCl<sub>2</sub>. The root samples were gently dunked in the solution for 30 s to remove as much rhizosphere soil as possible. Care was taken to minimize root damage; however, it cannot be excluded that some carboxylates did not originate due to cellular damage. For bare soil, 10 g of bulk soil was used instead of root and adhering rhizosphere soil. A subsample of the extract was filtered through a 0.2-µm syringe filter into a 1-mL HPLC vial and transferred to a -20 °C freezer until HPLC analysis. After rhizosphere extraction, the root subsample was combined with the remaining root and accurately washed to remove any trace soil. Roots and shoots were placed in the oven and dried at 60 °C for 48 h, and weights were recorded.

#### *Plant analyses*

The P concentration of roots and shoots was obtained by digesting 100 mg of milled plant material with a mixture of nitric and perchloric acids (6:1). The P concentration in the digest was determined using a Lambda Fias UV/VIS Spectrophotometer (Perkin Elmer, Waltham, Massachusetts, U.S.) according to the molybdenum blue method (List et al. 1986; Ruzicka and Hansen 1981).

#### *Carboxylate analyses*

The analysis of rhizosphere extracts was performed according to the method suggested by Cawthray (2003) using HPLC with the PDA detector Altus A-10 (PerkinElmer, Waltham, Massachusetts, U.S.) and Kinetex 2.6  $\mu\text{m}$  F5 100  $\text{\AA}$  column (Phenomenex, CA, USA). The working standards included oxalic, tartaric, malic, malonic, acetic, maleic, citric, cis-aconitic, succinic, fumaric and trans-aconitic acids.

#### *Soil analyses*

The bulk soil samples were prepared and stored in three different ways according to the specific analysis: an aliquot was frozen immediately after sampling; another was air dried and sieved at 2 mm; and the last one was crushed to pass through a 0.2 mm sieve.

Frozen soil was used to measure phosphatase activity. Acid phosphomonoesterase (PME) activity was determined by the method proposed by Tabatabai and Bremner (1969), according to which PME activity in soil is expressed as the production of para-nitrophenol ( $\mu\text{g h}^{-1}$ ) (PNP). Briefly, a subsample of 1 g of soil was placed into a 15-mL vial, followed by the addition of 0.2 mL of toluene, 4 mL of modified universal buffer (pH 6.5) and 1 mL of 0.05 M p-nitrophenylphosphate (PNPP) made up in the modified universal buffer. Vials were gently shaken and incubated at 37 °C for 1 h. After incubation, 1 mL of 0.5 M  $\text{CaCl}_2$  and 4 mL of 0.5 M NaOH were added to stop the reaction. A subsample of the soil suspension was collected and filtered. The amount of PNP released by PME activity was measured via a spectrophotometer as the absorbance at 400 nm and expressed as  $\mu\text{g PNP g}^{-1} \text{ soil h}^{-1}$ . Controls were performed with each sample analyzed to allow for color not derived from PNP released from PME activity. Controls were made following the methodology described but adding PNPP solution after the additions of  $\text{CaCl}_2$  and NaOH solutions immediately before filtration. For each sample, PME activity as a mean of two 1 g subsamples was calculated.

Soil available P was extracted by 0.5 M  $\text{NaHCO}_3$  from 2 g of air-dried 2-mm sieved soil, according to the Olsen method (Olsen et al. 1954). Organic P was obtained as the difference of ignited at 550 °C and non-ignited soil samples extracted by 0.5 M  $\text{H}_2\text{SO}_4$  from 2 g of 0.2-mm sieved soil, according to Bowman (1989) and Kuo (1996). Both available P (Olsen P) and organic P were 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 analysis.*

Shapiro–Wilk and Levene’s tests were used to test the normality of the data and the homoscedasticity of its variance, respectively. According to the experimental design, a two-factorial completely randomized design with 4 replications, in order to assess the significance of the fixed effects of cropping treatments (CTR), phosphorus level (P) and their interactions (CTR  $\times$  P) experimental data were analyzed using the generalized linear model (GLM) by the GLM procedure embedded in SAS software (SAS Institute, Cary, USA, 2009). Tukey’s HSD (honestly significant difference) test at  $P \leq 0.05$  was used for pairwise multiple comparisons of treatment means. Reported data on soil chemical properties are mean values ( $n = 4$ ) expressed on a dry weight (DW) basis (105 °C).

## **Results**

#### *Dry matter accumulation in shoot and root.*

Soil phosphorus supply significantly affected plant dry matter accumulation and partitioning in intercropping, increasing in roots and decreasing in shoots.

Among cropping treatments, the shoot dry matter of wheat was higher in the intercrops with lupin (WL) and pea (WP) and significantly differed from that in the intercrop with faba (WF) and sole

crops. In contrast, root dry matter in intercropped legumes was not significantly different from that in the respective sole crops. In particular, the shoot dry matter of intercropped wheat greatly outweighed the respective sole crops in WP (+175%) and WL (+103%) but only slightly in WF (+7%) (Table S1). The highest wheat root dry matter increase, compared to the respective sole crop, was shown in intercropping with pea (+121%), whereas root dry matter slightly decreased in intercropping with faba bean (-9%) (Table S1).

In the WP, the shoot dry matter of both wheat and legumes was particularly limited by the P supply (P1); in this mixture, wheat and pea showed the most significant decreases. In the sole crop with P fertilization, shoot dry matter generally increased in wheat and lupin. The positive effect of P supply (P1) on root dry matter in intercropping was higher in WP than in other treatments, and this was observed both in wheat and pea. In the WF, P was added, and the root dry matter increase was more relevant in faba bean than in wheat. Detailed data are shown in Table S1.

To compare the intercropping versus the respective sole cropping, the relative dry matter of each intercropped partner was calculated as a ratio between dry matter absolute values (Fig. 1). Wheat relative dry matter in all mixtures was greater than one, which means wheat in intercropping accumulated more biomass than in sole cropping. In general, in wheat intercropped with pea and lupin, the dry matter was 121% and 74% higher, respectively, than that in sole crops. However, the advantage of intercropped legumes versus the respective sole crops was relatively low (+3%). In the intercrop WP with no P fertilization (P0), wheat showed the highest relative dry matter accompanied by a value of pea close to one. Under the same conditions, in WL, both wheat and lupin were favored by intercropping.

#### *Phosphorus concentration in shoot and root dry matter*

On average, the shoot P concentration was much higher in wheat than in legumes. In contrast, except for W-L, legumes exceeded wheat in terms of the P content of the root (Table S2).

Phosphorus supply (P1) caused a significant increase in shoot and root P content in intercropping and in sole cropping, higher in legume than in wheat. Wheat P concentration in the shoot was lower in intercropping than in sole cropping, but these differences narrowed when wheat was intercropped with pea. Compared to the sole crop, wheat intercropped with faba bean showed a considerably lower shoot P content in P0, but this did not occur in P1. Compared to the respective sole crop, intercropped pea showed higher shoot P content both in P0 and in P1. Intercropping greatly favored lupin under no P fertilization and faba bean under P fertilization (Table S2).

The P concentration in root dry matter was higher in wheat intercropped with faba bean than in the respective sole crop, both with and without P supply, and in intercropping with pea when P was added. In Supplementary Table S2, the data are presented in detail.

#### *Phosphorus uptake in intercropping*

Considering the results from the whole plants (shoot and root), P uptake increased in all treatments with P supply, and the increase was more remarkable in legumes than in wheat (Fig. 2).

The highest total P amount was taken up by the WP system, which was significantly different from all other treatments. The WF and WL system uptakes were also significantly higher than their respective sole crops, which did not differ significantly from each other. With P supply, the wheat mixed with faba bean took up 60.7% more P than at P0, whereas with lupin and pea, the increase was much lower. The P uptake increased in intercropped legumes under P fertilization, mostly in lupin than in pea and faba bean (Fig. 2).

In addition to dry matter, the relative P uptake of each intercropped partner was calculated as a ratio between absolute values in intercropping and the respective sole crops. Wheat relative P uptake in WP averaged 2.19, which means it absorbed 119% more than its respective sole crop. In contrast, in both WF and WL, the average values of wheat relative P uptake were lower or close to unity, resulting in lower absorption than in WP. Without P supply, wheat intercropped with pea showed the highest relative P uptake value, and wheat intercropped with faba bean was considerably lower than one (Fig. 3).

#### *PME activity and organic phosphorus in soil*

The average value of PME activity was higher in legume-based systems than in wheat sole crops and bare soils. The PME activity in the sole crops of lupin and faba bean was significantly greater than that in the bare soils. PME activity was certainly promoted by cluster roots that were observed in lupin during sampling. In soil with P supply, the average PME activity was significantly ( $P = 0.0017$ ) higher (+8%) than in soil with natural content (Fig. 4). No cropping treatments  $\times$  P interaction was observed.

With P supply, the organic fraction of P in the soil ( $P_{ORG}$ ) significantly decreased compared to  $P_0$ , and this contraction was more marked in lupin and pea grown in both intercrop and sole crops. Only in the lupin sole crop and intercrop was  $P_{ORG}$  significantly lower than in the respective wheat sole crop (W-L) (Table 3).

#### *Carboxylate exudation in the rhizosphere*

The total carboxylate concentration in rhizospheric soil was influenced by the P supply and cropping treatments, and a cropping treatment  $\times$  P interaction was observed.

Total carboxylates were tenfold greater in the legume rhizosphere ( $43.1 \mu\text{mol g}^{-1}$  dry rhizospheric soil on average) than in the wheat rhizosphere ( $4.3 \mu\text{mol g}^{-1}$  dry rhizospheric soil on average). The maximum total accumulation of carboxylates was found in the rhizosphere of the faba bean. This result significantly differed from the values found in the rhizospheres of the other legumes and wheat (Fig. 5). Total carboxylates did not differ significantly between the rhizosphere of lupin and faba bean.

Phosphorus supply favored carboxylate accumulation in the rhizosphere of faba bean but did not affect lupin and pea. Similarly, carboxylates in wheat intercropped with faba bean and in the respective sole crop (W-F) were greater at  $P_1$  than at  $P_0$  (Fig. 5). In all other cropping treatments, no significant differences were shown between the levels of P availability. When P was supplied ( $P_1$ ) in the WF, intercropping acted to improve total carboxylate accumulation in wheat and reduce it in faba bean rhizospheres but did not affect the rhizosphere at  $P_0$ .

More than 99% of the total carboxylates released in the rhizosphere in wheat and legumes consisted of oxalate, acetate, succinate, malate, malonate, tartrate, and, in some cases, citrate, with high differences among cropping treatments and P levels. In faba bean-based systems (WF and F), the main carboxylates were oxalate, acetate and malate, accounting for up to 70% of the total concentration, with values generally higher than  $10 \mu\text{mol g}^{-1}$  dry soil, with the highest being oxalate ( $50.88 \mu\text{mol g}^{-1}$  dry soil). Citrate, cis-aconitate, malonate, tartrate and succinate were also detected in the faba bean rhizosphere, generally ranging between  $1 \mu\text{mol g}^{-1}$  and  $10 \mu\text{mol g}^{-1}$  dry soil. Maleate, trans-aconitate and fumarate were detected in traces ( $<1 \mu\text{mol g}^{-1}$  dry soil). In the lupin rhizosphere, 90% of the total carboxylates consisted of oxalate, acetate, malate and citrate, ranging from traces to  $8.76 \mu\text{mol g}^{-1}$  dry soil; traces of cis-aconitate, malonate, succinate and maleate were also found. In the pea rhizosphere, oxalate and malate ranged between  $1.86 \mu\text{mol g}^{-1}$  and  $10.96 \mu\text{mol g}^{-1}$  dry soil,

representing 90% of the total carboxylates with traces of malonate, acetate, citrate, cis-aconitate, succinate and maleate (Fig. S1).

The most identified carboxylate in the wheat rhizosphere (average concentration 2.46  $\mu\text{mol g}^{-1}$  dry soil) was oxalate (55%), followed by malate (14% of the total) and traces of acetate and tartrate. Traces of cis-aconitate, malonate, succinate, maleate, trans-aconitate and fumarate were found in both intercropped and sole cropped wheat at two P levels.

With P supply, both oxalate and malate considerably increased in the rhizosphere of intercropped legumes, and malate increased in lupin and pea sole crops. When P was supplied to faba bean, malonate and cis-aconitate were close to the concentrations capable of mobilizing P in the rhizosphere (Gerke et al. 2000) both in the intercrop (4.12  $\mu\text{mol g}^{-1}$  and 3.39  $\mu\text{mol g}^{-1}$  dry soil, respectively, of cis-aconitate and malonate) and in the sole crop (6.56 of cis-aconitate). In contrast, the concentration of acetate in sole cropped faba bean and citrate in sole cropped lupin and pea decreased when P was supplied.

The carboxylate composition in the wheat rhizosphere was affected by the P supply. At P1, the concentration of the single carboxylates was generally higher than that at P0. In sole cropped wheat, both oxalate (+132%) and malate (+300%) increased with P supply. In the WF, both oxalate and malate concentrations were higher in the wheat rhizosphere, while in the WP and WL, only malate increased with P supply.

In intercropping, the concentration of all the most abundant carboxylates in the legume rhizosphere was lower than that in cropping alone, excluding acetate, which increased four times. The concentration of all the carboxylates was greater in intercropped wheat than in the sole crop at both P0 and P1.

#### *Available phosphorus in soil*

Soil Olsen P was significantly different between P0 and P1 ( $P < 0.0001$ ) and among cropping treatments ( $P \leq 0.05$ ), but no interaction was found between P levels and cropping treatments.

At P0, the average Olsen P value was 15.19  $\text{mg kg}^{-1}$  dry soil, and in P1, it reached 69.07  $\text{mg kg}^{-1}$  dry soil. Among the treatments, the highest and lowest levels of Olsen P were 50.74  $\text{mg kg}^{-1}$  and 31.76  $\text{mg kg}^{-1}$  dry soil found in WP and W-L, respectively. Between these two extreme values, available P was higher in legume-based systems in W-P and BS-P. All cropping treatments depleted Olsen P compared to the respective bare soils at P0. The depletion was lower in intercropped wheat than in wheat cropped alone. In contrast, at P1, there was an enhancement (20% on average) of Olsen P in the legume-based systems over the bare soil (data not shown).

The authors proposed a concise way to summarize the amount of P absorbed by the plants and the fraction of P mobilized in the soil, named "Total available phosphorus" ( $P_{\text{TAV}}$ ).  $P_{\text{TAV}}$  reflects the system's ability to mobilize P, taking into account the fraction of P taken up by the plants. It relates to the total P fraction mobilized in the soil as available P that includes the measured Olsen P and the amount absorbed by plants until sampling. Phosphorus uptake by each cropping treatment was added to Olsen P measured in the soil at plant sampling:

$$P_{\text{TAV}} = \text{Olsen P} + P \text{ uptake}$$

$P_{\text{TAV}}$  should be read as a tool to understand the magnitude of P mobilization globally exerted by the system rather than a measure of available P released from the soil during the lifetime of the plants. As expected, the P supply strongly affected the  $P_{\text{TAV}}$ , and among the cropping treatments, intercropping showed the highest values. The P supply more markedly enhanced the  $P_{\text{TAV}}$  of WP, followed by the other intercropping systems and sole cropped faba bean, which showed similar

results. The  $P_{TAV}$  of wheat cropped alone was generally lower than that of legume-based systems. When P was not added (P0), the intercropping systems showed a lower percentage of residual P than the other cropping systems, which in WP was the lowest, while it was the highest in lupin and W-P sole crops, exceeding the 80%. Even with P fertilization, the residual P was on average lower in intercropping (<90%) than in the sole crop (Table 4).

## Discussion

### *Dry matter and phosphorus accumulation by plant*

The biomass dry matter and P concentration were used to assess the facilitations exerted by intercropping with legumes on wheat. The resulting P uptake (dry matter  $\times$  P concentration) was considered the consequence of increased P mobility in the soil as direct facilitation dispensed by legumes (Duchene et al. 2017). Regardless of the level of P fertilization, total dry matter and shoot dry matter were greater in intercropped wheat than in sole cropped wheat, without any detrimental effect on the intercropped legume. However, the intercrop WF differed significantly from other mixtures without significantly limiting or improving effects on intercropped wheat. Our results confirmed the increase in biomass observed when cereals were intercropped with lupin (Cu et al. 2005; Dissanayaka et al. 2015) and with pea (Bedoussac and Justes 2010) and agree with Li et al. (2007), who reported no effect of intercropping with faba bean at low P levels. Contrary to our results, in field experiments (Messaoudi et al. 2020; Song et al. 2007), shoot dry matter and yield increased in wheat intercropped with faba bean, showing high variability among the years. When P availability was lower (P0), the difference in total dry matter between intercropped and sole cropped wheat was more pronounced than at high soil P (P1), according to the results from maize-lupin intercropping reported by Dissanayaka et al. (2015) at low and moderate levels of P supply. The increase in total biomass when wheat was intercropped was one of the most relevant outcomes of this study (Fig. 1). In our experiment, passing from sole cropping to intercropping wheat dry matter accumulation increased while the plant P concentration decreased. This decrease was less severe in wheat intercropped with pea than in other mixtures, contributing to the greater P uptake observed (Table S2). Some authors linked biomass accumulation and, consequently, the yield increase to improved soil P availability (Betencourt et al. 2012; Li et al. 2007). However, the direct relationship between P availability and P plant accumulation is arduous to demonstrate due to the combination of several factors (e.g., water, nutrients, pests, diseases) involved in plant growth that can be affected by intercropping. Apparently, our results suggest that dry matter contributed more than the P concentration to the observed P uptake. On the other hand, the increase in wheat dry matter in intercropping could be the consequence of the greater P availability during growth, which is also confirmed by the greater P uptake observed in wheat intercropped with lupin and pea.

We can state that only in WP did P become more available for wheat both with (P1) and without (P0) P supply. Instead, in WL, it occurred only at low P availability (P0), according to the stress-gradient hypothesis (Bertness and Callaway 1994). When pea was grown as the sole crop, its root dry matter was similar to that of faba bean and lupin, but in intercropping, pea and faba bean were greater than lupin (Table S1). Because of the main role in belowground interactions, root dry matter is fundamental for the interpretation of the results in this experiment. Our results disagree with Nuruzzaman et al. (2005b), who found that faba bean root dry matter was higher than that of the other legumes. Although the root length was not calculated in this experiment, the thinness of pea roots suggests a greater exploration of soil by pea than faba bean, improving the possibility of root intermingling between the partners of the intercrop. We hypothesize that a greater intermingling in

WP amplified the effect of the root exudates by shortening the distances between pea and wheat roots. In contrast, in faba bean and lupin intercrops, despite the greater root exudation, more time for growing or more legume plants is required to realize the same conditions.

The results confirm differences among the mixtures tested: unlike other legumes, pea facilitated P uptake of the mixed wheat, improving its dry matter accumulation without lowering the biomass P concentration. However, excluding WF, intercropping facilitated wheat growth, mainly when P was less available for the plants.

#### *PME activity and organic phosphorus in soil*

Phosphomonoesterase (PME) activity is considered, with carboxylate release, the reaction of the plants to low P availability. A reduction in PME activity is expected in the soil when P is available (Venterink 2011). In this study, PME activity was measured in bulk soil and considered the consequence of root activity in the soil, which facilitates the mixture. Consequently, the inhibitory effect expected with P supply was not shown because PME activity was more influenced by the increase in root biomass resulting from enhanced P availability than by the reduction in root exudation. The increase in PME activity resulting from the P supply in legume-based systems disagrees with other studies (Olander and Vitousek 2000; Venterink 2011) carried out analyzing soil from the rhizosphere, where P fertilization significantly inhibited phosphatase activity. To estimate the root efficiency in PME activity, including the aliquot from the indirect contribution of microorganisms, the ratio of PME activity/root biomass was calculated (data not shown). This calculation permitted us to compare different species and different P supplies independent of root growth. Our results showed that comparing legume root dry weight with the corresponding PME activity in the soil, the improvement of root growth, as an effect of P supply, compensated for the reduction of PME expressed as activity per root mass unit, as reported by other studies (Olander and Vitousek 2000; Venterink 2011, Sun et al. 2020). The decrease of PME efficiency with P fertilization was particularly marked in pea, both in sole crop and intercrop, suggesting that pea, in our experiment, was better able to modulate PME exudation than other legumes, depending on the availability of P in soil. However, contrasting results were shown by several other studies. For example, P fertilization did not affect PME activity in the lupin and faba bean rhizospheres (Lyu et al. 2016; Spohn and Kuzyakov 2013) and, in some cases, increased phosphatase activity in the rhizospheres of soybean (Lyu et al. 2016), canola and wheat (Solaiman et al. 2007). Moreover, our results are supported by Olander and Vitousek (2000). They observed significant inhibitory effects on the enzyme activity mediated by P supply, not in short- but in long-term fertilization. The authors explained the results through the ability of enzymes, particularly phosphatases, to persist in soils for a long time by binding to soil humics and clays (Olander and Vitousek 2000). This “binding to soil” hypothesis was also confirmed by our data highlighting high PME activity in bare soil. The persistence of phosphatases in soil over time and the compensation of the low rate of exudation by root proliferation suggest that the use of legumes for the mobilization of organic P is only slightly limited by P fertilization.

The convenience of intercropping legumes also stems from their considerable rhizosphere PME activity, which is considered higher than that of cereal crops (Nuruzzaman et al. 2006; Venterink 2011). In our study, PME efficiency, which is a direct consequence of rhizosphere PME activity, was higher in legumes than in wheat (+23%), and the difference was wide when P was added. Our study agrees with some authors (Venterink 2011; Nuruzzaman et al. 2006) who, comparing several legume and nonlegume species, found greater PME activity in legumes, with variable responses to P availability among the species. However, our results are in contrast with a recent study where PME

efficiency was greater in maize rhizosphere than in alfalfa when grown separately, but it increased in both partners' rhizospheres when intercropped (Sun et al. 2020). The higher PME activity in the soil of the studied legumes, particularly of lupin and faba bean, confirms the potential of the legumes to mobilize organic P for the main crop.

Phosphatases are exuded by plant roots to allow mobilization and utilization of soil organic P (Li et al. 2004; George et al. 2008). As a result, organic P is expected to be lowered by PME activity, finding a relationship between enzyme activity and organic P in the soil. However, in our study,  $P_{\text{ORG}}$  varied among the treatments, showing a significant negative relation with PME activity only when P was supplied ( $R^2 = 0.890$ ;  $P = 0.0001$ ) (Fig. S2). Without P fertilization, PME activity was less intense than with P supply (P1), and the increase in PME activity was not associated with a proportional  $P_{\text{ORG}}$  reduction. We found that  $P_{\text{ORG}}$  was generally lower in legume-based systems (intercropping and in sole crop) than in sole cropped wheat and bare soil, according to the higher PME activity associated with legumes. In fact, in bare soil and wheat cropped alone, PME activity was low, and consequently, the highest amount of  $P_{\text{ORG}}$  was found in those treatments.

#### *Carboxylates in rhizosphere*

The exudation of carboxylates from the roots has been considered the plant's reaction to P-limiting conditions for a long time (Marschner et al. 1986), representing one of the various adaptive strategies that plants evolved to enhance P-acquisition and -use efficiency. It is part of a P-mining strategy that also includes protonation and promotes desorption or solubilization of P from sparingly available sources in soil (Lambers et al. 2006; Richardson et al. 2011). Therefore, the influence of soil P availability on the regulation of carboxylate release is controversial. Many authors (Pearse et al. 2006; Nuruzzaman et al. 2006; Sun et al. 2020) reported that carboxylate exudation was suppressed when P was supplied. On the other hand, both qualitative and quantitative compositions of the rhizosphere carboxylates are affected by abiotic factors such as pH, type and nutrient availability of soil, as well as biotic factors such as microorganisms in the soil, plant species and plant age (He et al. 2017; Mimmo et al. 2011; Pang et al. 2015; Suriyagoda et al. 2012). When P was supplied in our experiment, total carboxylates did not show any significant difference in the rhizosphere of lupin and pea compared to P0. Only faba bean responded to the P supply, increasing its exudation (Fig. 5). Even if the reduction in carboxylates is expected in the rhizosphere of plants supplied with P, in many studies, no decrease was reported (He et al. 2017; Lyu et al. 2016; Pearse et al. 2007), and in some cases, carboxylate exudation increased due to the soil type (Lyu et al. 2016; Nuruzzaman et al. 2005a). Phosphorus sources and soil types are considered to have a central role in suppressing P-regulated exudation (He et al. 2017; Pearse et al. 2007; Nuruzzaman et al. 2005a). The weak response of lupin to the applied P fertilizer exhibited in our study was also observed by other authors adding P as  $\text{KH}_2\text{PO}_4$  (Lyu et al. 2016; Nuruzzaman et al. 2005a; Shu et al. 2007). Our results disagree with Pearse et al. (2007), who, adding the same P form, observed a significant suppression of carboxylate exudation in *Lupinus* species grown on washed sand. Shane et al. (2003) suggested regulation of citrate exudation by the shoot P status, but carboxylate exudation was only slightly downregulated at a very high shoot P status (Wouterlood et al. 2005). In our study, the supply of 50 mg P as  $\text{KH}_2\text{PO}_4$   $\text{kg}^{-1}$  of soil improved the P shoot concentration, but it was probably not enough to cause such downregulation. Indeed, the critical level of P concentration in lupin shoots at or below which cluster root formation and citrate exudation would be significantly upregulated is 2–3 mg  $\text{g}^{-1}$  DW (Li et al. 2008), higher than the values found in this experiment. However, this threshold varied due to experimental conditions. The reaction of carboxylate exudation to external or internal P varies in

legume species. Legumes such as chickpea are not influenced by P availability, and their exuding ability is constitutive (Wouterlood et al. 2004a, b, 2005). Many authors (Nuruzzaman et al. 2005a; Pearse et al. 2007) reported in some soil types no significant variation in carboxylate exudation due to the P supply in pea, which is consistent with our results. Similarly, faba bean roots showed changes in neither morphological nor physiological responses to P supply (Lyu et al. 2016), suggesting that in faba bean, unlike in lupin, the exudation of organic anions is not induced by P deficiency (Wang and Lambers 2020). In our research, the increased exudation of carboxylates in P-supplied faba bean can be explained by the intensification of root metabolic activity due to fertilization since carboxylate exudation was not downregulated by P availability. The ability of faba bean to exude carboxylates without being conditioned by P availability is potentially helpful to access sparingly available sources of P even when P is available in the soil. Particularly in intercropping, root exudates can facilitate the access of sparingly available sources of P to low P-mobilizing crops such as wheat not only without but also with P supply.

Our results confirmed that wheat had a lower rhizosphere carboxylate concentration than grain legumes in all cropping treatments (Hinsinger et al. 2003; Pearse et al. 2006, 2007; Rose et al. 2010). Nevertheless, when no P was applied to wheat (P<sub>0</sub>) in our study, the rhizosphere carboxylates were negatively and significantly related to the shoot P concentration ( $R^2 = 0.432$ ;  $P = 0.024$ ) (Fig. S 3), suggesting the response of the root exudation rate to the shoot P status in wheat. Another noticeable outcome is that at P<sub>1</sub>, the total carboxylate concentration of wheat was greater in intercropping with faba bean than in the respective sole crop. An increase in malate in the rhizosphere of wheat intercropped with faba bean (Li et al. 2016) and in total carboxylates in maize with alfalfa (Sun et al. 2020) was reported in other studies. Li et al. (2014), who found the same results in maize intercropped with faba bean, suggested that diffusion of protons and carboxylates may extend the interaction zone between different plant species and ultimately enhance the growth of the neighboring plant. As a result, the increase in P uptake observed in our study, consequent to P supply, was more remarkable in wheat intercropped with faba bean than in the other mixtures. Thus, WF was the mixture where the P supply was most effective in increasing the P uptake of wheat compared to P<sub>0</sub> (Fig. 2).

Among the studied legumes, the highest carboxylate concentration, both in sole crops and intercropping systems, was found in faba bean, but this result was not associated with the improvement of shoot P concentration and P uptake in intercropped wheat. As reported by previous studies, the concentration of carboxylates in the rhizosphere of the faba bean was generally not high, and its beneficial effect provided to intercropped maize (Li et al. 1999) or wheat was attributed to considerable root biomass accumulation (Pearse et al. 2006; Nuruzzaman et al. 2005a, b). In our experiment, the root of the faba bean was as large as that of other legumes. Although the carboxylate concentration in the wheat rhizosphere was favored by faba bean intercropping, we hypothesized that root intermingling with wheat was too weak to facilitate P uptake. Under these conditions, competition overcame the facilitation between faba and wheat, penalizing cereal growth.

The exudation rate observed in lupin can be considered an unusual result that disagrees with other studies (Lyu et al. 2016; Nuruzzaman et al. 2005a, b; Pearse et al. 2006, 2007) that reported greater carboxylate production in lupin than in pea and in faba bean. The values are comparable to those observed by Lyu et al. (2016), who reported that the leaves showed chlorosis. The authors' opinion is that the low exudation rate observed in lupin is due to its low adaptability to the soil, which also explains its weak response to P supply.

In this study, the major fraction of all carboxylates in the rhizosphere was composed of oxalate and malate, followed by acetate and citrate, which were particularly abundant in faba bean. Contrary to

some studies (Pearse et al. 2007; Li et al. 2010; Nuruzzaman et al. 2006) that reported high amounts of citrate in the rhizosphere of pea and lupin, in our study, only faba bean released considerable citrate amounts. Oxalate and malate were the most abundant carboxylates detected in the legumes studied in this experiment. Citrate and oxalate seem to be the most efficient anions in mobilizing P (Fox et al. 1990; Gerke 1995). P mobilization is small or negligible below 10  $\mu\text{mol g}^{-1}$  citrate or oxalate in rhizospheric soil (Gerke et al. 2000). In our experiment, only oxalate reached that threshold and exclusively in the rhizosphere of the faba bean. Faba bean showed high potential for carboxylate exudation that did not result in the appropriate P uptake of wheat. As previously mentioned, we hypothesized that there was not enough root intermingling between faba bean and wheat to exhibit facilitation. Not excluding that sampling was too early to shift from complementary to facilitation on P uptake, as previously observed by Li et al. (2016).

#### *Available phosphorus in soil*

In this study, available phosphorus was assessed using Olsen P, and its variation was considered the result of root exudate activity on the sparingly available P source of the soil. On the other hand, Olsen P is expected to be lowered by plant uptake. When high Olsen P was associated with high P uptake, it is supposed that complementarity in the use of resources occurred. Therefore, different species exhibited different abilities to access soil sparingly phosphorus compounds, which supports the hypothesis of Turner (2008) concerning resource partitioning for soil phosphorus. In fact, Olsen P was generally higher in intercrops than in sole crops in our study and was related to increasing wheat growth. The highest value of soil available P was found in WP, where wheat showed the best yield performance. Analyzing the rhizospheric soil, some recent studies (Sun et al. 2020; Latati et al. 2014) agree with ours, reporting increased P availability in legume intercropping systems (cowpea and maize intercropping). The same result was obtained by Betancourt et al. (2012) in a pot experiment under controlled conditions by intercropping durum wheat with chickpea. Our experiment showed that legume species mobilized low available P from the soil and not only took it up at low concentrations from the soil solution (Hinsinger 2001).

In many cases, the increase in P availability promoted the growth of the plants and, consequently, the reduction of P in the rhizosphere. The decrease in the concentration of P ions should be expected in the rhizosphere in most cases, generating a concentration gradient that is the driving force for the diffusion of P ions toward the root (Hinsinger 2001). In our study, at P1, the values of P availability in the soil of legume-based systems were generally higher than those in the respective bare soils, suggesting that legumes allowed P mobilization even if P was available in the soil solution. At the same time, at P0, the depletion of Olsen P observed in cropping treatments compared to the respective bare soils was smaller in the intercrops than in sole cropped wheat according to the better P resource use in mixed culture than in monocropping under limiting conditions.

#### **Conclusions**

The first hypothesis that phosphorus uptake of wheat was facilitated by grain legumes was confirmed only in intercropping with pea. The second hypothesis that intercropping facilitated phosphorus uptake of wheat more so at low phosphorus availability than with phosphorus supply was also confirmed, but only in pea and lupin intercropping. The results showed that faba bean did not facilitate wheat phosphorus uptake either with or without phosphorus supply. Phosphorus uptake of wheat was not directly linked to root exudation of the three intercropped grain legumes, which disagrees with the third hypothesis.

Although the highest concentration of carboxylates and the highest PME activity were found in faba bean and lupin, respectively, phosphorus uptake in wheat was enhanced in intercropping with pea, which showed higher root biomass but lower exudates than in faba bean and lupin. This result suggests that legume root development and intermingling in the mixture may modulate the facilitative effect exerted by exudates on wheat phosphorus uptake.

Our study suggested that root exudation alone is not exhaustive for evaluating legume species. We cannot exclude that other factors, such as microbial activity, were involved in the facilitation exerted on wheat by pea. Further studies to deepen the understanding of the role of root distribution and the ratio of legumes and wheat sown in intercropping are needed. Interesting findings, such as the greater carboxylate concentration in the rhizosphere of intercropped wheat and the correlation between biomass phosphorus concentration of wheat and exuded carboxylates in the wheat rhizosphere, suggest a response of wheat root exudation to intercropping and phosphorus availability that needs further investigation.

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**Table 1.** Physical and chemical characteristics of soil used in the experiment.

Soil properties	
Bulk density (g cm <sup>-3</sup> )	1.23
Sand %	36
Silt %	32
Clay %	32
pH <sub>CaCl2</sub>	6.6±0.1
Total organic carbon (g kg <sup>-1</sup> )	12.55±4.5
Total nitrogen (g N kg <sup>-1</sup> )	1.38±0.1
C/N	9.07±0.1
EC <sub>1:2</sub> (dS m <sup>-1</sup> )	0.271±0.021
CEC (cmol <sub>(+)</sub> kg <sup>-1</sup> )	20.9±1.3
CaCO <sub>3</sub> (g kg <sup>-1</sup> )	8.4±0.5
P-Olsen (mg kg <sup>-1</sup> )	11.33±0.2
NH <sub>4</sub> <sup>+</sup> - N (mg kg <sup>-1</sup> )	12.49±0.2
NO <sub>3</sub> <sup>-</sup> - N (mg kg <sup>-1</sup> )	26.16±0.2

**Table 2.** Growth conditions in climate chamber.

<i>Period length (d)</i>	<i>15</i>	<i>20</i>	<i>20</i>	<i>Until legume flowering</i>
Day (h)	8	8	10	14
Night (h)	16	16	14	10
Light intensity (μmoles/m <sup>2</sup> /s)	200	255	340	340
Temperature (°C)	8	15	20	25

**Table 3.** Soil organic phosphorus (mg kg<sup>-1</sup> dry soil) in sole crop (W= wheat, F= faba; L=lupin; P =pea), intercropping (WF; WL; WP) and bare soil (BS) at two phosphorus supply (P0 and P1). -F, -L, and -P indicate the sampling time corresponding to the different flowering time of legumes.

	P0			P1			Mean		
BS-F	86.1	±9.6	abcd	88.5	±4.5	abcd	87.3	±4.9	AB
BS-L	87.8	±4.8	abcd	86.9	±9.5	abcd	87.3	±4.9	AB
BS-P	80.6	±6.1	abcd	88.2	±13.1	abcd	84.4	±6.9	AB
F	79.0	±3.5	abcd	75.5	±13.6	abcd	77.2	±6.5	AB
L	77.7	±6.2	abcd	45.5	±5.9	d	61.6	±7.3	B
P	106.5	±9.0	ab	56.8	±10.6	cd	81.7	±11.4	AB
W-F	92.2	±4.4	abcd	91.3	±10.3	abcd	91.8	±5.2	AB
W-L	117.4	±14.9	a	92.5	±11.6	abcd	104.9	±9.9	A
W-P	77.5	±9.5	abcd	96.3	±5.9	abc	86.9	±6.3	AB
WF	75.8	±8.8	abcd	76.8	±11.8	abcd	76.3	±6.8	AB
WL	84.0	±3.0	abcd	60.7	±4.1	bcd	72.4	±5.0	B
WP	87.0	±12.4	abcd	61.2	±11.3	bcd	74.1	±9.2	AB
Mean	87.6	±2.8		76.7	±3.4		82.2	±2.1	
<i>P</i>									<i>P=0.0047</i>
<i>CTR</i>									<i>P=0.0036</i>
<i>P x CTR</i>									<i>P=0.0164</i>

The values shown are means ± standard errors. Significance of *p* for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR) by Anova are reported in italic. On both columns P0 and P1 values followed by different lower-case are significant different; Mean values followed by different capital letters indicate the significant differences at  $P \leq 0.05$  (Tukey's HSD test).

**Table 4.** Total soil available phosphorus (P<sub>TAV</sub>, mg kg<sup>-1</sup> dry soil) in sole crop (W= wheat, F= faba; L=lupin; P =pea), intercropping (WF, WL, WP) and residual amount (%) after crop uptake at two phosphorus supply (P0 and P1). -F, -L, and -P indicate the sampling time corresponding to the different flowering of legumes.

	P0			P1			Mean		Residual P at P0	Residual P at P1	
									(%)	(%)	
F	17.1	±2.3	d	85.8	±9.2	ab	51.5	±13.6	ABC	78	92
L	14.7	±1.6	d	77.9	±9.7	bc	46.3	±12.7	BC	88	87
P	21.1	±1.2	d	81.0	±9.8	abc	51.1	±12.2	ABC	78	90
W-F	18.4	±1.8	d	65.5	±1.5	bc	42.0	± 8.9	BC	72	90
W-L	14.8	±0.9	d	59.3	±2.3	c	37.1	± 8.5	C	71	89
W-P	20.1	±6.0	d	75.2	±1.9	bc	47.6	±10.8	BC	85	96
WF	23.6	±1.7	d	88.6	±3.4	ab	56.1	±12.2	AB	62	86
WL	22.7	±2.6	d	86.4	±5.9	ab	54.6	±12.3	AB	66	84
WP	25.5	±2.5	d	102.5	±6.5	a	64.0	±14.9	A	58	84
Mean	19.8	±0.8		80.2	±2.9		50.0	± 3.2		73	89
<i>P</i>									<i>P&lt;0.0001</i>		
<i>CTR</i>									<i>P&lt;0.0001</i>		
<i>PxCTR</i>									<i>P=0.0285</i>		

The values shown are means ± standard errors. Significance of *p* for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR) by Anova are reported in italic. On both columns P0 and P1 values followed by different lower-case are significant different. Mean values followed by different capital letters indicate the significant differences at  $P \leq 0.05$  (Tukey's HSD test).

**Table S1.** Dry matter (g) in shoot and root of wheat and legumes grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and in intercropping (WF; WL; WP) at two phosphorus supply (P0 and P1). -F, -L, and -P indicate the sampling time corresponding to the different flowering of legumes.

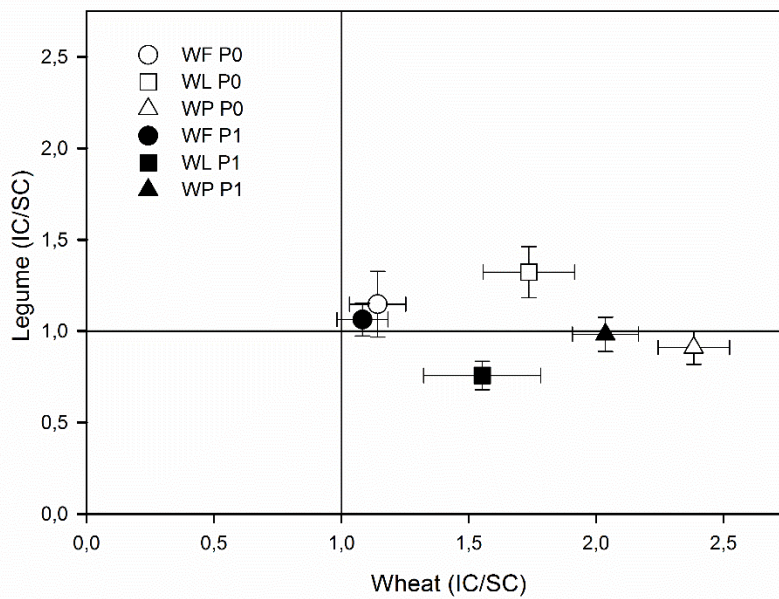
		P0		P1		Mean	
Wheat shoot	W-F	14.25	de	16.52	d	15.38	B
	W-L	9.86	fg	11.40	ef	10.63	C
	W-P	7.13	g	7.85	fg	7.49	D
	WF	17.97	cd	15.20	de	16.58	B
	WL	22.31	ab	20.85	bc	21.58	A
	WP	25.96	a	15.28	de	20.62	A
	<b>Mean</b>	<b>16.25</b>		<b>14.52</b>		<b>15.38</b>	
<i>P</i>		<i>p=0.001</i>					
<i>CTR</i>		<i>p&lt;0.0001</i>					
<i>P x CTR</i>		<i>p&lt;0.0001</i>					
Wheat root	W-F	7.10	bcd	8.41	b	7.76	B
	W-L	7.03	bcd	7.17	bcd	7.10	BC
	W-P	6.58	cd	6.93	bcd	6.76	C
	WF	6.41	d	6.91	bcd	6.66	C
	WL	7.02	bcd	7.98	bc	7.50	BC
	WP	6.71	cd	14.82	a	10.77	A
	<b>Mean</b>	<b>6.81</b>		<b>8.70</b>		<b>7.76</b>	
<i>P</i>		<i>p&lt;0.0001</i>					
<i>CTR</i>		<i>p&lt;0.0001</i>					
<i>P x CTR</i>		<i>p&lt;0.0001</i>					
Legumes shoot	F	21.13	bcd	19.13	bcd	20.13	AB
	L	11.11	e	21.58	bcd	16.34	B
	P	28.27	a	16.59	de	22.43	A
	WF	25.16	ab	18.20	d	21.68	A
	WL	18.46	cd	16.31	de	17.39	B
	WP	24.96	abc	15.64	de	20.30	AB
	<b>Mean</b>	<b>21.52</b>		<b>17.91</b>		<b>19.71</b>	
<i>P</i>		<i>p&lt;0.0001</i>					
<i>CTR</i>		<i>p=0.0003</i>					
<i>P x CTR</i>		<i>p&lt;0.0001</i>					
Legumes root	F	9.12	cd	9.84	c	9.48	AB
	L	8.59	cde	10.04	bc	9.31	B
	P	6.36	e	14.14	a	10.25	AB
	WF	9.54	c	12.59	ab	11.06	A
	WL	7.59	cde	7.64	cde	7.61	C
	WP	6.57	de	14.54	a	10.55	AB
	<b>Mean</b>	<b>7.96</b>		<b>11.46</b>		<b>9.71</b>	
<i>P</i>		<i>p&lt;0.0001</i>					
<i>CTR</i>		<i>p&lt;0.0001</i>					
<i>P x CTR</i>		<i>p&lt;0.0001</i>					

Significance of p for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR) by Anova are reported in italic. On both columns P0 and P1 values followed by different lower-case are significant different at  $P \leq 0.05$ ; Mean values followed by different capital letters are significant different at  $P \leq 0.05$  (Tukey's HSD test).

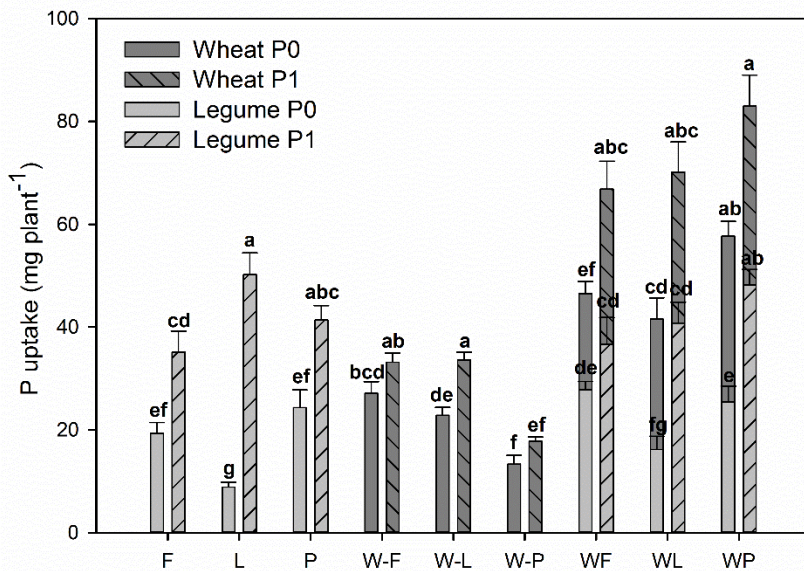
**Table S2.** Phosphorus concentration (mg g<sup>-1</sup> dry matter) in shoot and root of wheat and legumes grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and in intercropping (WF; WL; WP) at two phosphorus supply (P0 and P1). -F, -L, and -P indicate the sampling time corresponding to the different flowering of legumes.

	CTR	P0	P1	Mean
Wheat shoot	W-F	1.59 abc	1.62 abc	1.61 A
	W-L	1.45 abcd	1.75 ab	1.60 A
	W-P	1.30 bcde	1.87 a	1.59 A
	WF	0.82 e	1.59 abc	1.21 B
	WL	0.94 de	1.13 cde	1.04 B
	WP	1.09 cde	1.59 abc	1.34 AB
	<b>Mean</b>	<b>1.20</b>	<b>1.59</b>	<b>1.40</b>
	<i>P</i>		<i>p&lt;0.0001</i>	
	<i>CTR</i>		<i>p&lt;0.0001</i>	
	<i>P x CTR</i>		<i>p&lt;0.0296</i>	
Wheat root	W-F	0.63 cd	0.74 cd	0.69 BC
	W-L	1.21 b	1.90 a	1.55 A
	W-P	0.60 cd	0.44 d	0.52 C
	WF	0.66 cd	0.88 bc	0.77 B
	WL	0.66 cd	0.73 cd	0.69 BC
	WP	0.57 cd	0.69 cd	0.63 BC
	<b>Mean</b>	<b>0.72</b>	<b>0.90</b>	<b>0.81</b>
	<i>P</i>		<i>p=0.0001</i>	
	<i>CTR</i>		<i>p&lt;0.0001</i>	
	<i>P x CTR</i>		<i>p&lt;0.0001</i>	
Legumes shoot	F	0.45 d	0.74 cd	0.59 C
	L	0.56 d	1.94 a	1.25 A
	P	0.56 d	1.18 bc	0.87 BC
	WF	0.68 cd	0.83 cd	0.75 C
	WL	0.54 d	1.92 a	1.23 AB
	WP	0.78 cd	1.61 ab	1.20 AB
	<b>Mean</b>	<b>0.60</b>	<b>1.37</b>	<b>0.98</b>
	<i>P</i>		<i>p&lt;0.0001</i>	
	<i>CTR</i>		<i>p&lt;0.0001</i>	
	<i>P x CTR</i>		<i>p&lt;0.0001</i>	
Legumes root	F	1.12	2.11	1.62 A
	L	0.31	0.88	0.59 C
	P	1.37	1.55	1.46 AB
	WF	1.24	1.71	1.47 AB
	WL	0.82	1.23	1.02 BC
	WP	0.97	1.60	1.29 AB
	<b>Mean</b>	<b>0.97</b>	<b>1.51</b>	<b>1.21</b>
	<i>P</i>		<i>p&lt;0.0001</i>	
	<i>CTR</i>		<i>p&lt;0.0001</i>	
	<i>P x CTR</i>		<i>NS</i>	

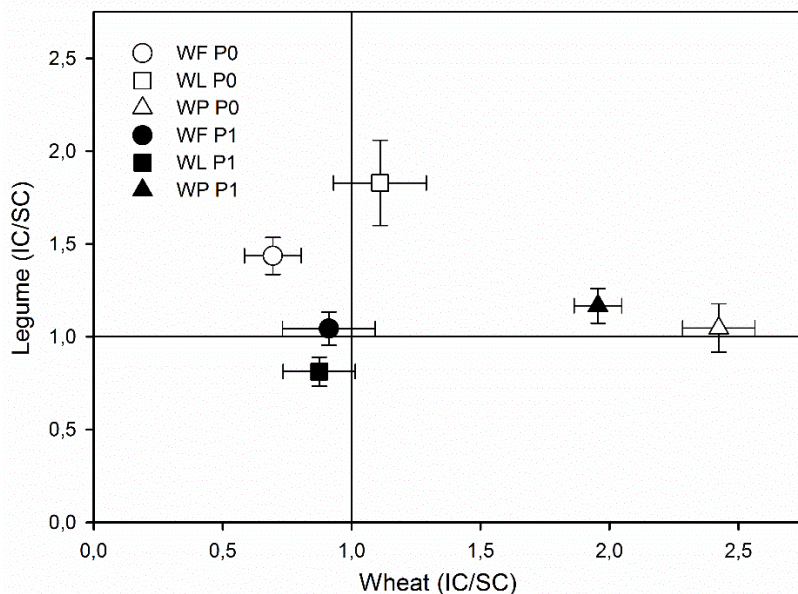
Significance of p for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR) by Anova are reported in italic. On both columns P0 and P1 values followed by different lower-case are significant different at  $P \leq 0.05$ ; Mean values followed by different capital letters are significant different at  $P \leq 0.05$  (Tukey's HSD test).



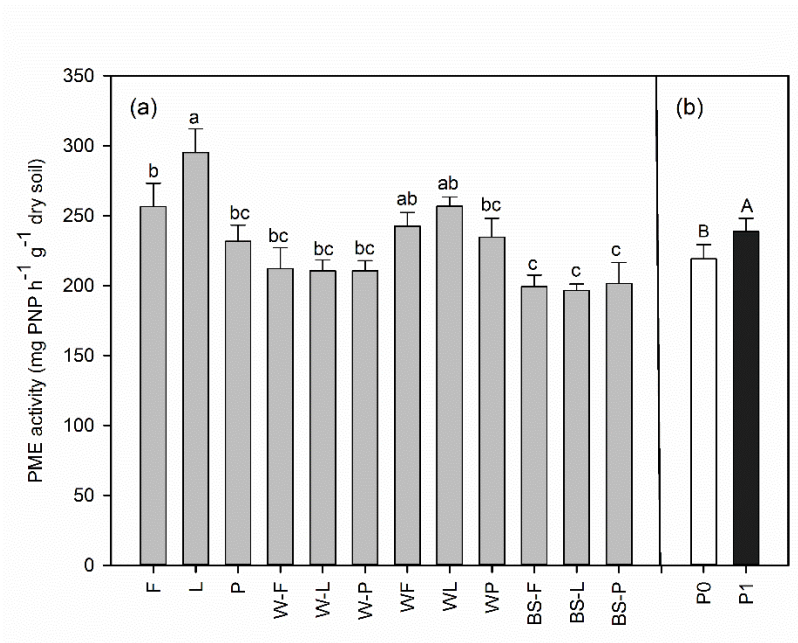
**Fig. 1** Relative dry matter accumulation of wheat and legumes grown in intercropping, calculated as intercrop/sole crop ratio (IC/SC), without (open symbols) and with (closed symbols) phosphorus supply. Values are the mean ( $n = 4$ )  $\pm$ SE. The vertical and horizontal lines represent all the points where dry matter accumulation in IC is equal to SC.



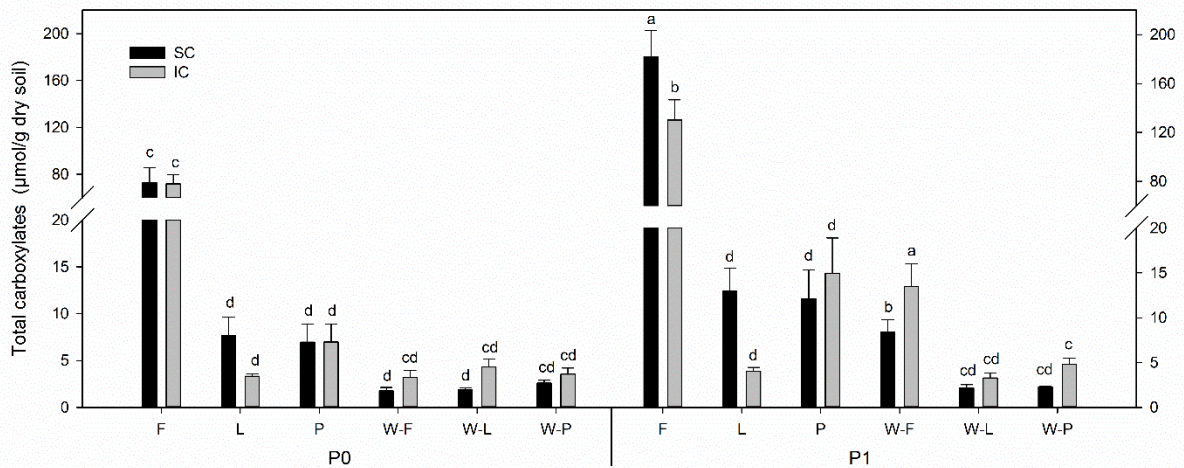
**Fig. 2** Phosphorus uptake ( $\text{mg plant}^{-1}$ ) by legumes and wheat grown in sole crop (W = wheat, F = faba; L = lupin; P = pea) and intercropping (WF; WL; WP) at two phosphorus supply (P0 and P1). -F, -L, and -P indicate the sampling time of wheat corresponding to the different flowering of legumes. Values are the mean ( $n = 4$ )  $\pm$ SE. Different letters above the bars indicate significantly different values ( $P \leq 0.05$ ; Tukey's HSD test) among legumes (light gray filled bars) or wheat (dark gray filled bars).



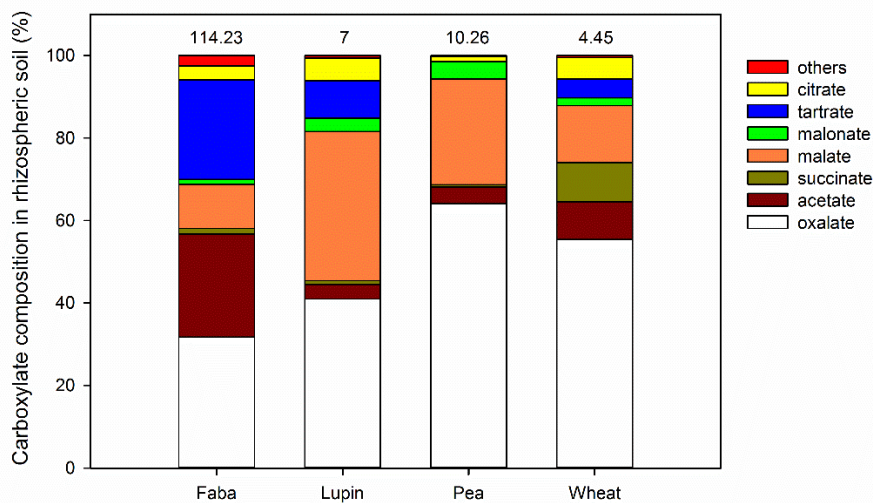
**Fig. 3** Relative phosphorus uptake of wheat and legumes grown in mixture, calculated as intercrop/sole crop ratio (IC/SC), without (open symbols) and with (closed symbols) phosphorus supply. Values are the mean ( $n = 4$ )  $\pm$ SE. The vertical and horizontal lines represent all the points where phosphorus uptake in IC is equal to SC.



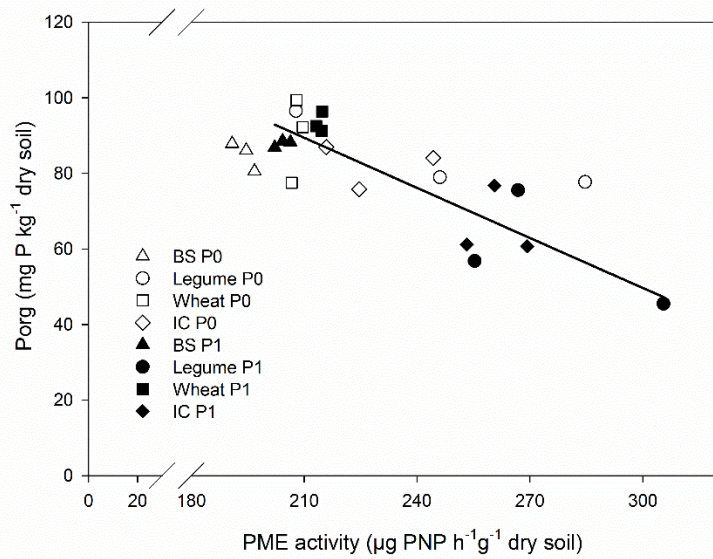
**Fig. 4 (a)** Phosphomonoesterase (PME) activity ( $\mu\text{g PNP g}^{-1} \text{ soil h}^{-1}$ ) in sole crop (W = wheat, F = faba; L = lupin; P = pea), in intercropping (WF; WL; WP) and in bare soils (BS). -F, -L, and -P indicate the sampling time of wheat and bare soil corresponding to the different flowering of legumes. Values are means ( $n = 8$ )  $\pm$  SE. **(b)** PME activity at two-phosphorus level (P0 and P1). Values are means ( $n = 48$ )  $\pm$  SE. Different letters above the bars indicate significantly different values ( $P \leq 0.05$ ; Tukey's HSD test).



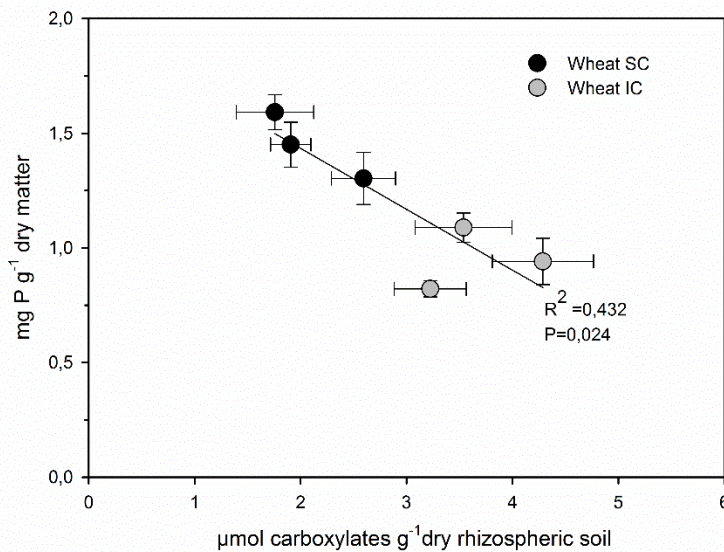
**Fig. 5** Total carboxylates in the rhizosphere ( $\mu\text{mol/g}$  dry soil) of faba bean (F), lupin (L), pea (P) and wheat (W) grown in sole (SC) and intercropping (IC) at two phosphorus supply (P0 and P1). -F, -L, -P indicate the sampling time of wheat corresponding to the different flowering of legumes. Values are means ( $n = 4$ )  $\pm$  SE. Different letters above the bars indicate significantly different values ( $P < 0.0001$ ; Tukey's HSD test) among legumes (F, L, P) or wheat (W-F, W-L, W-P).



**Fig. S1** The most representative carboxylates in the rhizospheric soil of wheat, faba bean, lupin and pea as a percentage of the average content (values on top of bars) of sole crops and intercropping systems 'Other' includes carboxylates (maleate, cis-aconitate, fumarate and trans-aconitate) at concentration below  $1\mu\text{mol g}^{-1}$  dry soil.



**Fig. S2** Phosphomonoesterase (PME) activity plotted versus organic phosphorus  $P_{ORG}$  in bare soil (BS), in legumes and wheat grown in sole crop (SC) and intercropping (IC) with (closed) and without (open symbols) phosphorus supply. Under P supply,  $P_{ORG}$  linearly and significantly decreased as the PME activity increased ( $R^2 = 0.890$ ;  $P = 0.0001$ ). PME activity is reported as paranitrophenol (PNP)  $\mu$ g h<sup>-1</sup> g<sup>-1</sup> of dry soil.



**Fig. S3** Total carboxylates in rhizospheric soil of wheat plotted versus phosphorus concentration in shoot of wheat grown in intercrop (IC) and sole crop (SC) at phosphorus limited condition. Without phosphorus supply, dry matter P content in wheat showed a linear and significant decrease as the carboxylates increased. Values are means ( $n = 12$ )  $\pm$  SE.