INTERCROPPING WITH GRAIN LEGUMES TO EXPLOIT PHOSPHORUS FOR ECOLOGICAL INTENSIFICATION OF MEDITERRANEAN CEREAL CROPPING SYSTEMS
DSS: AGR/02

Ph.D candidate
Emilio Lo Presti

Supervisor
Prof. Michele Monti

Ph.D. Coordinator
Prof. Marco Poiana

ACADEMIC YEARS 2016/17, 2017/18, 2018/19
Intercropped Grain Legumes to exploit phosphorus for an ecological intensification of Mediterranean Cereal Cropping Systems.

Abstract

Sustainable intensification (SI) is a new strategy proposed to satisfy the increasing global food request, to be profitable for the farmer and sustainable for agroecosystem, at the same time conserving resources for the next generations. Nowadays several researches suggest agroecological approach to promote SI in cropping systems. In this context the introduction of legumes in the cropping system is proposed to improve soil phosphorus (P) availability. The belowground interaction is considered the main cause of that ability.

In this thesis, the ability of three legume crops to facilitate phosphorus uptake of intercropped durum wheat was investigated. To achieve this goal three specific objectives were followed:

i) verify in agricultural soil the ability of three grain legumes to mobilize phosphorus through their specific root exudation (phosphatase activities and carboxylates composition) and confirm whether these facilitations are more expressed in phosphorus-limiting soil conditions;

ii) assess whether an increase in legume mass roots may influence phosphorus mobilization in the intercropping system;

iii) describe changes in soil bacterial community exerted in intercropping by the legume root activity mentioned above.

To realize these specific objectives lupin (*Lupinus albus* L.), pea (*Pisum sativum* L.) and faba (*Vicia faba* L.), were grown in pots on controlled climatic conditions as sole crop (SC) and intercropped (IC) with durum wheat (*Triticum turgidum* subsp. *durum* (Desf.) Husn.) in three separate experiments combined with the following treatments corresponding to each specific objective:

i) two levels of P supply (no P and adding 50 mg P/kg soil);

ii) two legume density (1:1 and 2:1 legume:wheat plants ratio);

iii) four different P availability levels corresponding to different P forms added to soil.

In the first and second experiments, the benefit for the main crop durum wheat was evaluated from its P uptake, as well as the variation of soil P pools (organic P, Olsen P) was compared to phosphomonoesterase (PME) activity and quantity, and quality of carboxylates exuded in soil. In the third experiment, the bacterial community structure of the rhizosphere was investigated.

The results from the first experiment showed that PME activity was greater in P1 than in P0 and in IC than in SC and PME activity and carboxylates exudation was greater in legumes than in wheat. Available P was more conserved in IC than in SC. The effect of intercropping on wheat P uptake was greater with pea and lupin at P0, and with pea at P1. The mixture wheat/pea was the most efficient in P uptaking. In IC, wheat growth was higher compared to SC, while biomass P concentration decreased.

In the second experiment, wheat P uptake increased in intercrop with two plants of faba and lupin and was associated to higher PME activity, while it decreased with pea. From the analysis of the third experiment, bacterial communities were affected primarily by the crop treatment followed by P availability. When P availability was low there was an enrichment of genera included in phosphate solubilizing bacteria (PSB) and plant growth-promoting rhizobacteria (PGPR) such as *Variovorax, Bradyrhizobium* and *Pseudomonas* in legume rhizosphere and intermingled rhizosphere of intercrop. Although intercropping was favorable for all the wheat-legume combination tested, a marked effect on wheat P uptake was confirmed only in pea intercrop and this advantage was more expressed in phosphorus-limiting soil conditions. The data from root exudates supported partially this result but
other aspects were involved, such as competition and complementary, varying legume density. The ability of legumes to enrich the bacterial community of the rhizosphere with the most favourable taxa in P limited condition was conserved in intermingled rhizosphere of both the intercrop partners contributing to the P facilitation.

Riassunto

L'intensificazione sostenibile (IS) è una nuova strategia proposta per soddisfare la crescente domanda di cibo nel mondo ma che sia allo stesso tempo redditizia per l'agricoltore e sostenibile per l'agroecosistema, conservando risorse per le generazioni future. In questo contesto, l’introduzione delle leguminose da granella all’interno dei sistemi colturali può rappresentare un valido strumento per aumentare la disponibilità di fosforo nel suolo (P). Si ritiene che questa abilità sia riconducibile ad interazioni che si generano a livello radicale. 

In questa tesi, è stata studiata la capacità di tre leguminose da granella di facilitare l’assorbimento del fosforo in frumento duro ad esse consociato. Con questa finalità sono stati perseguiti tre obiettivi specifici:

i) verificare in suolo la capacità di tre leguminose da granella di mobilitare il fosforo attraverso l’essudazione radicale (acidi organici e fosfatasi) e confermare se tali facilitazioni siano più espresse a bassa disponibilità dell’elemento;

ii) valutare se il raddoppio del numero di leguminose consociate abbia un effetto sulla disponibilità di fosforo;

iii) descrivere i cambiamenti nella comunità batterica del suolo dovuti alla sopra menzionata attività radicale delle leguminose consociate.

Per ottenere questi obiettivi specifici, lupino (Lupinus albus L.), pisello (Pisum sativum L.) e fava (Vicia faba L.), sono stati coltivati in vaso in condizioni climatiche controllate, sia in coltura pura (SC) che consociata (IC) a grano duro (Triticum turgidum subsp. durum (Desf.) Husn.) in tre esperimenti separati, ognuno corrispondente ai seguenti trattamenti così da soddisfare ciascuno degli obiettivi specifici prima enunciati:

i) due livelli di disponibilità di P (senza P e con l’aggiunta di 50 mg P / kg$^{-1}$ di terreno);

ii) grano duro consociato a una o due piante di leguminosa;

iii) quattro diversi livelli di fosforo disponibile corrispondenti a diverse forme di P aggiunte al suolo.

Nel primo e secondo esperimento, l’effetto favorevole per il grano duro è stato valutato attraverso il P assorbito, mentre la variazione dei pool di P del suolo (P organico, Olsen P) è stata confrontata con l'attività della fosfomonoesterasi (PME) e gli acidi organici (AO) nel suolo. Nel terzo esperimento, è stata studiata la struttura della comunità batterica della rizosfera.

Dai risultati del primo esperimento si evince una maggiore attività della PME in P1 rispetto a P0 e in IC rispetto a SC e valori di attività della PME e presenza di AO maggiori con le leguminose che col grano. Il P disponibile è risultato maggiore in IC che in SC. L’aumento di assorbimento di P in grano dovuto alla consociazione è stato maggiore con pisello e lupino a P0 e con pisello a P1. La combinazione grano-pisello è stata la più efficiente nell'assorbimento di P. In IC, il grano si è accresciuto più che in SC, mentre la concentrazione di P nella biomassa si è ridotta. Nel secondo esperimento, utilizzando due piante piuttosto che una, mentre con pisello l’assorbimento di P nel grano è diminuito, con fava e con lupino è aumentato e a ciò si è associata a una maggiore attività della PME. Nel terzo esperimento, le comunità batteriche sono state influenzate in primo luogo dalla specie e poi dalla disponibilità di P. A bassa disponibilità di P, la rizosfera delle leguminose e del grano, quando consociato ad esse, si è arricchita di generi noti come batteri fosfato solubilizzatori (PSB) e rizobatteri promotori della crescita delle piante (PGPR) come Variovorax, Bradyrhizobium e Pseudomonas. Sebbene la consociazione sia stata favorevole per tutte le combinazioni grano-
leguminosa studiate, l’effetto sull'assorbimento di P del grano è stato confermato solo con pisello e questo vantaggio è stato maggiore in condizioni di limitata disponibilità di fosforo. I dati provenienti dagli essudati radicali hanno giustificato solo parzialmente questo risultato, si suppone che altri aspetti come competizione e complementarietà mediati dal rapporto di semina tra le due specie siano coinvolti. La capacità dei legumi di arricchire la propria rizosfera di taxa batterici più favorevoli al proprio sviluppo in condizioni di P limitanti è stata mantenuta nella rizosfera intimamente connessa dei due partner della consociazione con effetti facilitativi sull’assorbimento del P.
## Contents

Introduction......................................................................................................................... 9

1 Intercropping.....................................................................................................................
   1.1 Biodiversity in cropping systems ........................................................................... 16
   1.2 Legume intercropping and agrobiodiversity ......................................................... 16
   1.3 Phosphorus exploitation by legumes ...................................................................... 17
   1.4 Facilitations in intercropping mediated by microorganisms ................................. 18

2 Aim Of Research And Thesis Outline........................................................................... 20

3 Grain legumes root exudates can help intercropped wheat to exploit phosphorus in P-limiting conditions........................................................................................................ 22
   3.1 Material and Methods ............................................................................................ 22
   3.2 Results .................................................................................................................... 25
   3.3 Discussion .............................................................................................................. 33
   3.4 Conclusions ........................................................................................................... 37
   3.5 Appendix ................................................................................................................ 38

4 Can the increase of the root mass of grain legume improve the facilitating effect on wheat uptake in intercropping system ............................................................... 39
   4.1 Material and Methods ............................................................................................ 39
   4.2 Results .................................................................................................................... 40
   4.3 Discussion .............................................................................................................. 44
   4.4 Conclusions ........................................................................................................... 46
   4.5 Appendix ................................................................................................................ 47

5 Intercropping affects structure of soil bacterial communities .................................... 50
   5.1 Material and Methods ............................................................................................ 50
   5.2 Results .................................................................................................................... 52
   5.3 Discussion .............................................................................................................. 55
   5.4 Conclusions ........................................................................................................... 56

6 General Conclusion ....................................................................................................... 58

7 References ...................................................................................................................... 59
List of Table

Table 3.1. Growth conditions in climate chamber .......................................................... 22

Table 3.2. Physical and chemical characteristics of soil used in the experiments .................. 23

Table 3.3. Soil organic phosphorus (mg kg$^{-1}$ 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 in subscript are sampling time corresponding to the different flowering time of legumes. Values are mean ± SE (n=4; n=8). Significance of P for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR) by Anova are reported in italic .......................................................... 26

Table 3.4. Total soil available phosphorus (PTAV) 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. Values are mean ± SE (n=4; n=8). Significance of P for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR) by Anova are reported in italic .......................................................... 28

Table S 3.1. Phosphorus concentration (mg g$^{-1}$ dry matter) in shoot and root of wheat and legumes 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 corresponding to the different flowering of legumes. Values are mean ± SE (n=4; n=8). Significance of P for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR) by Anova are reported in italic .......................................................... 33

Table S4.1. Dry matter (g plant$^{-1}$) in shoot and root of wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P=pea) and respective intercropping (WF; WL; WP) at two legume plant density (LD1 and LD2). -F, -L, and -P indicate the sampling time of wheat sole crop at the different flowering of legumes. Values are means ± SE (n=4; n=8). Significance of P for sowing ratios (LD), cropping treatments (CTR) and its interactions (LD x CTR) by Anova reported in italic .......................................................... 47

Table S 4.2. Phosphorus concentration (mg P kg$^{-1}$ dm) in shoot and root of legume and wheat grown in sole crop (W= wheat, F= faba; L=lupin; P=pea) and respective intercropping (WF; WL; WP) at two legume plant density (LD1 and LD2). -F, -L, and -P indicate the sampling time of wheat sole crop at the different flowering of legumes. Values are means ± SE (n=4; n=8). Significance of P by Anova reported in italic for sowing ratios (LD), cropping treatments (CTR) and its interactions (LD x CTR) .......................................................... 48

Table S 4.3. Phosphorus uptake (mg) in shoot and root of wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P=pea) and respective intercropping (WF; WL; WP) at two legume plant density (LD1 and LD2). -F, -L, and -P indicate the sampling time of wheat sole crop at the different flowering of legumes .......................................................... 50
List of figures

Figure 1.1. P dynamics in the soil/rhizosphere-plant continuum. C-P, Carbon-P; NO, nitric oxide; OA, organic acids

3.1 Phosphomonoesterase (PME) activity (μmol pNPP g⁻¹ soil h⁻¹) in sole crop (W= wheat, F= faba; L=lupin; P =pea), intercropping (WF; WL; WP) and in bare soils (BS). -F, -L, and -P indicate the sampling time of wheat corresponding to the different flowering of legumes. Values are means ±SE (n=8). PME activity at two-phosphorus level (P0 and P1) is also drawn. Values are means ±SE (n=48). For each part of the graph, the bars with a different letter above represent significantly different values (P≤ 0.05; Tukey’s HSD test)

3.2. Most representative carboxylates measured in rhizospheric soil of legume grown in sole crop (F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP)

3.3. Dry matter accumulation and partitioning in legume 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 means ± SE (n=4)

3.4. Relative dry matter accumulation 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 ±SE (n = 4)

3.5 Phosphorus uptake (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. Mean (n=4) ± SE

3.6. Phosphorus relative 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) ±SE. The vertical and horizontal lines represent all the points where dry matter accumulation in IC is equal to SC

3.7. Phosphomonoesterase (PME) activity plotted versus organic phosphorus (PORG) in bare soil (BS), in legumes and wheat grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP) without (closed) and with (open symbols) phosphorus supply. At P fertilized condition, PORG showed a linear and significant decrease as the PME activity increased (R²=0.890; P=0.0001)

3.8. Total carboxylates production, at limited phosphorus condition, in wheat rhizospheric soil plotted versus phosphorus concentration in intercropping (WIC) an sole crop (WSC) respectively indicated by (open) and with (closed symbols). Without phosphorus supply P
concentration in wheat dry matter showed a linear and significant decrease as the carboxylates increased. Vales are means ± SE (n=12)

4.1 Phosphomonoesterase (PME) activity (μmol pNPP g⁻¹ soil h⁻¹) in sole crop (W= wheat, F= faba; L=lupin; P =pea), intercropping (WF; WL; WP) and in bare soil (BS). -F, -L, and -P indicate the sampling time of wheat corresponding to the different flowering of legumes. Means (n=8; n=48) ± SE. PME activity at two phosphorus level (P0 and P1) is also drawn. Within the lower and upper cases, the different letters above each bar indicate significantly different values at P≤ 0.05; Tukey test

4.2. Organic and available (Olsen-P) phosphorus (mg P kg⁻¹ dry soil) in wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP). -F, -L, and -P indicate the sampling time of wheat at the different flowering of legumes. Values are means ± SE (n=8; n=24). The different letters above each bar indicate significantly different values at P≤ 0.05; Tukey test

4.3 Phosphorus concentration (mg P kg⁻¹ dm) in shoot of wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP) at two legume plant density (LD1 and LD2). -F, -L, and -P indicate the sampling time of wheat at the different flowering of legumes. Values are means ± SE (n=4; n=24)

4.5. Shoot and root relative dry matter (IC/SC) of wheat (W) and legumes (F= faba; L=lupin; P =pea) grown in mixture (WF; WL; WP), calculated as intercrop/sole crop ratio (IC/SC), both at one-plant (open symbols) and at two-plant (closed symbols) legume densities. Values are means ±SE (n=4). The vertical and horizontal lines represent all the points where dry matter accumulation in IC is equal to SC

4.6. Figure Phosphorus plant uptake (mg) by wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP) both at LD1 and LD2. -F, -L, and -P indicate the sampling time of wheat at the different flowering of legumes. Values are means ± SE (n=4).

4.7. Phosphorus relative uptake of wheat and legumes grown in mixtures, calculated as intercrop/sole crop ratio (IC/SC), both at one-plant (open symbols) and at two-plant (closed symbols) legume densities. Values are means ±SE (n = 4). The vertical and horizontal lines represent all the points where dry matter accumulation in IC is equal to SC

4.8. Phosphomonoesterase (PME) activity (μmol pNPP g⁻¹ soil h⁻¹) plotted versus phosphorus uptake (g plant⁻¹) of wheat intercropped with faba (WF), lupin (WL) and pea (WP) at 1:1 (open symbol) and 1:2 (closed symbol) sowing ratios. Values are means ±SE (n=4)

5.1. 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. Inverted triangles represent bulk soil samples; solid squares represent lupin rhizosphere; crosses represent lupin-wheat rhizosphere; solid triangles represent pea rhizosphere; stars represent pea-wheat
rhizosphere and solid circles represent wheat rhizosphere. Dark green color represents samples obtained from soil where P was added in available form; light green color represents samples obtained from soil added with NPK; red color represents samples obtained from soil where no P was added; and orange color represents samples from soil where P is unavailable. A - Samples were coloured by type (bulk soil, lupin rhizosphere, lupin-wheat rhizosphere, wheat rhizosphere, pea rhizosphere and pea-wheat rhizosphere) and treatment (available P, no P, NPK and unavailable P). B, C, D, E and F – Bacterial communities from the rhizosphere of different crops (lupin, lupin-wheat, wheat, pea and pea-wheat, respectively)

5.2. 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
Introduction

Within the current economic paradigm and given that the world’s population is increasing, dietary demands per capita are increasing, while at the same time the natural resource base (arable land of good quality, fresh water, nutrients, energy) (Valenzuela, 2016) as well as the human resources (experienced, resourceful, and innovative farmers and agronomists) (Struik et al., 2014) are eroding, it is likely that planetary boundaries will even be further exceeded. It is a moral imperative to ensure that enough food of adequate quality is produced for humankind, that all humans have access to the food of their preference in a fair manner, that production is taking place without eroding the natural resource base.

Fraser et al. (2016) identified four perspectives in the debate on global food security, and from these perspectives, they proposed four types of key strategies: (i) technological strategies to increase production, (ii) socio-economic strategies to achieve equitable food distribution, (iii) strategies to promote local food movements, and (iv) economic, political, and regulatory changes to correct current market and food system imperfections and failures.

Food demand and climate change The most recent projections reveal that world population can be expected to increase from the current 7.2 billion people to 9.6 billion in 2050 and 10.9 billion in 2100 and, the world population is unlikely to stop growing in this century (Gerland et al., 2014). Considering the expected per capita demand for food, measured as caloric or protein needs, it has been possible to predict an increase by 100–110% in the global food demand from 2005 to 2050 (Tilman et al., 2011). The environmental impacts of doubling global crop production will depend on how the increased production is achieved (Tilman et al., 2002; Foley et al. 2011). With this regard, strategies of global agricultural development that are directed to greater technology improvement and transfer would meet 2050 crop demand with much lower environmental impacts than the strategies applied in the past (Tilman et al., 2001).

Many studies have estimated the impacts of climate changes on crop yields. Based on these studies, there is medium confidence that climate trends have negatively affected wheat and maize production for many regions (-2% and -1% per decade, respectively for wheat and maize) (Porter et al., 2014). Warming has promoted crop production only in some high-latitude regions, such as northeast China or the UK (Jaggard et al., 2007; Chen et al., 2010; Supit et al., 2010; Gregory and Marshall, 2012) while drought stress persists as the main driver of losses for the crops. As a result, in low-yielding years due to environmental stress, the elevated CO₂ does not give any yield benefit. Therefore, use of autumn-sown crops, as wheat, escaping to the drought season enhances resilience of cropping systems to climate change in Europe and their negative effect are partially compensated by CO₂ fertilization (Webber et al., 2018). In the perspective of adaptation to climate change, cropping system diversification represents an innovation pathway to improve production, especially of wheat in marginal areas, adopting organic and low external input systems.
Agroecosystem disservices from agriculture. The green revolution permitted to double the world population and to triple the cereal production with only a 30% increase of the cultivated land area (Wik et al., 2008). Between 1960 and 2000, yields for all countries rose by 208% for wheat, 109% for rice, 157% for maize, 78% for potatoes, and 36% for cassava (FAO, 2004). It resulted from the adoption of higher-yielding crop varieties, increased use of pesticides and fertilizers and improved access to irrigation and mechanization. The consequences in water use, soil degradation, and chemical runoff have had serious environmental impacts (Burney et al., 2010). Chemical fertilizers have played a significant role in the green revolution but excessive use of them has led to a reduction in soil fertility and to environmental degradation. Moreover, the use of chemical fertilizers is reaching the theoretical maximum use beyond which there will be no further increase in yields (Ahmed, 1995). The slowdown in yield growth that has been observed since the mid-1980s can be partly attributed to the degradation of the agricultural resources (Pingali, 2012).

Agriculture accounts for 70 percent of water worldwide consumption and plays a major role in water pollution. Farms discharge large quantities of agrochemicals, organic matter, sediments and saline drainage into water bodies. The resultant water pollution poses risks to aquatic ecosystems, human health and productive activities (UNEP, 2016). Water pollution from agriculture has direct negative impacts on aquatic ecosystems due to eutrophication caused by the accumulation of nutrients in lakes and coastal waters that impacts on biodiversity and fisheries. Regarding this issue, more attention has been paid to agricultural nitrogen (N) in comparison to phosphorus (P) due to differences in the agronomic efficiency, and complexities of transportation processes, as well as, to the inadequate analytical methods (Gao et al., 2017). P due to his key role in all living systems has been largely considered one of the main global cause of eutrophication of water bodies (Faridmarandi and Naja, 2014; Foy et al., 1995; Oenema et al., 2005). After N, P is the major plant growth-limiting nutrient despite being abundant in soils in both inorganic and organic forms. While N could be obtained from the air, phosphorus and potassium must be mined. The world has enough potassium to last several centuries (Vaccari, 2009). The low distribution of the P mines in the world and the announced depletion of them make the question of P a problem for the next fifty years. Moreover, many soils throughout the world are P-deficient because the free phosphorus concentration (the form available to plants) even in fertile soils is generally not higher than 10 μM even at pH 6.5 where it is most soluble. At the same time, theoretical estimates have suggested that the accumulated P in agricultural soils is sufficient to sustain maximum crop yields worldwide for about 100 years (Goldstein et al., 1993).

During recent decades, fertilizer application increased by 35–40% worldwide, leading to an estimated 25.7% of global P losses (Smith et al., 1999; Vitousek et al., 2009). In many studies, the majority of agricultural P losses are driven by storm events and overland transport flow as non-point source (NPS) of pollution as a result of soil particles erosion (Chen et al., 2017, 2014). Phosphorus is characterized by a low availability to plants. Therefore, chemical fertilization is largely applied and often part of the
added amount of P fertilizer provided may reach the water table thus, causing water pollution and eutrophication, as well as, a significant economic loss.

The green revolution resulted in global food security and played an important role in transforming developing countries, such as India, from being food-deficient to having a food surplus. An important consequence of the green revolution was the huge use of natural resources. The increase of yield was possible thanks to the support of resources such as fuel, agrochemical in general and in particular fertilizer. In the green revolution, the research focused on the high use of external input employed in the environments where returns would be high in order to maximize the yield. The marginal environments and the optimal use of the resources were not considered as a target environment or reference topic by the green revolution.

An alternative way for modern agriculture: the sustainable intensification. The challenge of modern agriculture is to satisfy the increasing global food request, be profitable for farmers and, at the same time, ensure sustainability to the agroecosystem by the conservation of the natural resources for the next generations. An innovative approach in response to this challenge is the “sustainable intensification” (SI), aimed at producing more from the same area while conserving resources, reduce negative impacts on the environment and enhance natural capital and the flow of ecosystem services (Rai et al., 2011). In the SI the improvement of ecosystem services may lead to the reduction or removal of energetic and chemical inputs that are the basis of conventional agriculture.

The Millennium Ecosystem Assessment defined ecosystem services as the benefits humans obtain from ecosystems, and grouped them into four categories (Millennium Ecosystem Assessment, 2005):

- Supporting services, such as nutrient cycling and soil formation.
- Regulating services, such as pest control, crop pollination, climate regulation, and water purification.
- Provisioning services, such as food, fibre, fuel, and water.
- Cultural services, such as education, recreation, and aesthetic value.

During the past decades, the loss of habitat and simplification of agriculture landscape (Hoekstra et al., 2004; Tscharntke et al., 2005) in combination with other environmental changes such as climate change, pollution and species invasions determined a reduction of biodiversity that resulted in a reduction of ecosystem services (Hooper et al., 2005).

The management of ecosystem services delivered by biodiversity into crop production systems represents an interesting tool available to sustainable intensification to match or augment yield levels while minimizing negative impacts on the environment (Cassman, 1999; Dorè et al., 2011). The supporting and regulating ecosystem services provided by the organisms can be incorporated into cropping systems, such that production is maximized while environmental impacts are minimized through the decrease, but not necessarily exclusion, of anthropogenic inputs, such as inorganic fertilizers, pesticides, energy, and irrigation (Cassman, 1999; Dorè et al., 2011). It must be underlined that even intensively cultivated, crop production systems depend heavily on supporting and regulating
services that determine the primary production that can be harvested (Bommarco et al., 2013). For the farmer there are two possible ways to close the gap between real productivity and potential productivity, increasing conventional intensification with known negative externalities and a possible long-term decline in productivity or, alternatively, integrating and extending several natural supporting and regulating services (Bommarco et al., 2013). In most of the developed countries, the productivity closed the gap thanks to the high external input. Then the aim in these areas is the re-establishment of ecosystem services and the replacement of external input to preserve the resources maintaining the productivity. However, in large parts of the world, productivity is lower, with a wide gap between farm (actual) yield and potential yield (Neumann et al., 2010; Lobell et al., 2009); here, the challenge will be to ecologically enhance productivity by optimizing ecosystem services in low-input (but not necessarily no-input) farming systems. Ecological replacement by ecosystem services and productivity enhancement in fact are not mutually exclusive and both processes can be combined to close the yield gap (Bommarco et al., 2013).

Ecosystem services are strictly connected to the level of intactness, complexity, and/or species richness of ecosystems (Díaz et al., 2006). In fact, agrobiodiversity has the potential to improve soil physical stability and resilience of microbial processes mediating nutrient cycling as part of supporting services (Gregory et al., 2009; Garcia-Pausas et al., 2011; Peres et al., 2013). Some studies show that species communities, formed by the multiple pressures and drivers acting in human-dominated landscapes, generally function better with increasing diversity levels (Cardinale et al., 2012) and demonstrate, for example, that crop yield increases with increasing pollinator diversity (Hoehn et al., 2008) or with diversified crop rotations (Bennett et al., 2012).

However, even if the presence of rare species improves biodiversity their contribution as service providers maybe low. The contribution of individual species to regulating or supporting ecosystem services in agriculture varies markedly and is a function of the abundance of each species and the efficiency with which it provides the service (Balvanera et al., 2006).

The benefits derived from ecosystem services can increase the interest in biodiversity conservation. However, it is important to distinguish between promoting biodiversity for the services it delivers (functional biodiversity) or for the inherent conservation value (Kleijn et al., 2011).
Phosphorus dynamics in soil

Phosphorus exists in soils in many different forms: inorganic P (Pi) precipitated as phosphate minerals, slowly exchangeable adsorbed Pi, Pi in soil solution, rapidly exchangeable adsorbed Pi, organic P (Po) and microbial P (Bellon and Penvern, 2014). In arable soils, a major proportion of soil P (up to 80 %) is made up of inorganic P (Pi) (Pellerin et al., 2003). A part of Pi includes primary P minerals such as apatites, strengite, and variscite, which are very stable with a low release of available P from these minerals by weathering, generally too slow to meet the crop demand. Another part of soil Pi is adsorbed via surface complexation processes on positively-charged minerals and may be rapidly exchanged with the soil solution (Devau et al. 2011). The rest is bounded due to the pH of the soil with iron and aluminium (Hinsinger 2001; Kizewski et al. 2011), or with calcium, (Freeman and Rowell 1981; Lindsay et al., 1989) forming phosphate minerals that slowly release phosphate ions into the soil solution (Frossard et al., 2000; Hinsinger 2001; Kizewski et al., 2011). More in detail, in acidic soils, P can be dominantly adsorbed by Al/Fe oxides and hydroxides, such as gibbsite, hematite, and goethite (Parfitt, 1989) and clay minerals. Clay minerals and Fe/Al oxides have large specific surface areas, which provide large number of adsorption sites. The adsorption of soil P can be enhanced with increasing ionic strength. With further reactions, P may be occluded in nanopores that frequently occur in Fe/Al oxides, and thereby become unavailable to plants (Arai and Sparks, 2007).

In neutral to calcareous soils, P retention is dominated by precipitation reactions (Lindsay et al., 1989), although P can also be adsorbed on the surface of Ca carbonate (Larsen, 1967) and clay minerals (Devau et al., 2010). Phosphate can precipitate with Ca, generating dicalcium phosphate (DCP) that is available to plants. Ultimately, DCP can be transformed into more stable forms such as octocalcium phosphate and hydroxyapatite (HAP), which are less available to plants at alkaline pH (Arai and Sparks, 2007). HAP dissolution increases with decrease of soil pH (Wang and Nancollas, 2008), suggesting that rhizosphere acidification may be an efficient strategy to mobilize soil P from calcareous soil. Therefore, soil pH plays a major role in the availability of inorganic P (Devau et al., 2011; Hinsinger, 2001). With increasing soil pH, solubility of Fe and Al phosphates increases but solubility of Ca phosphate decreases, except for pH values above 8 (Hinsinger, 2001).

Po generally accounts for 30% to 65% of the total P in soils (Harrison, 1987). Soil Po mainly exists in stabilized forms as inositol phosphates and phosphonates, and active forms as orthophosphate diesters, labile orthophosphate monoesters, and organic polyphosphates (Turner et al., 2002; Condron et al., 2005). Organic P is not directly available to plants since it requires hydrolysis by phosphatase-like enzymes excreted by plants or microorganisms (Spohn and Kuzyakov, 2013). Another pool of soil P is the microbial biomass P, which amounts to only 0.4–2.5% of total P in arable soils (Bünemann et al., 2011).

Even in the more fertile soils, the Pi concentration of the soil solution is seldom higher than 10 μM (Bieleski, 1973). This low concentration of available Pi in soil is too far from the concentration in plant tissue (from 5 to 20 mM Pi, Raghothama, 1999). As a result, chemical P fertilizers are needed to
improve crop growth and yield. The major forms of phosphate used as fertilizers include monocalcium phosphate (MCP) and monopotassium phosphate. Contrary to monopotassium phosphate, which lightly influences soil physical and chemical properties (Lindsay et al., 1962), MCP can significantly alter soil physicochemical properties. In fact, MCP generates in soil large amounts of protons, phosphate, and dicalcium phosphate (DCP), and eventually forms a P-saturated patch (Benbi and Gilkes, 1987). This Pi-saturated patch forms three different reaction zones including direct reaction, precipitation reaction, and adsorption reaction zones (Shen et al., 2011).

The strong acidity of the direct reaction zone (pH = 1.0–1.6), results in an elevated mobilization of soil metal ions. These metal ions can also react with high concentrations of Pi in the zone thus causing further precipitation of Pi. The amorphous Fe-P and Al-P that thereby form can be partly available to plants. In calcareous soil, new complexes of MCP and DCP can be formed and with time DCP is gradually transformed into more stable forms of Ca phosphates (octocalcium phosphate or apatite) (Shen et al., 2011).

An important source for P fertilization is also manure. In fact, nearly 70% of total P in manure is labile. In manure, Pi accounts for 50% to 90% (Dou et al., 2000). Manure also contains large amounts of Po, such as phospholipids and nucleic acids (Turner and Leytem, 2004) which can be mineralized increasing the available fraction. Manure can also have effect on Ca phosphate in soil due to organic acids generated by mineralization of humic substances. Organic acids such as citrate can efficiently weaken the nanoparticle stability of hydroxyapatite, by controlling the free Ca availability and thereby the nucleation rate (Martins et al., 2008). P adsorption to soil particles can be greatly reduced through applying organic substances. The large numbers of negative charges of humic acids, carboxyl and hydroxyl groups, can strongly compete for the adsorption sites with Pi. Changes of soil P availability can also be generated by pH alteration caused by manure.

Although phosphate is strongly held by soil surfaces, it is not immobile (Heckrath et al., 1995), particularly if the material to which the phosphate is bound becomes detached from the soil matrix. A loss of P is possible by surface runoff and by rapid water movements through preferential pathways generated by large pores in soil (subsurface drainage). In arable land, elevated concentrations caused by P fertilizer increase the potential for P loss. The loss of P in dissolved and particulate forms is a function of, but not exclusively of, topography, soil type, soil test phosphorus (STP) concentration, and soil hydrology (McDowell et al., 2001). This aliquot of P, reaching the water table, is one of the main causes of water pollution and eutrophication, as well as, a significant economic loss.
Figure 1.1. P dynamics in the soil/rhizosphere-plant continuum. C-P, Carbon-P; NO, nitric oxide; OA, organic acids (from Shen et al., 2011).
1 INTERCROPPING

1.1 Biodiversity in cropping systems: the agrobiodiversity

Cropping system, more specifically conventional cropping system, was characterised by an interest in the reduction of diversity, and by an increase in use efficiency of external additional resources provided to agroecosystem. In these agroecological contexts, communities are not mainly formed by processes of natural competition and dispersal because agricultural management heavily interferes with the nature and intensity of these processes. The first interest in biodiversity in managed agroecosystems was in the selection of the more productive species, varieties and races, and in the reduction of the unproductive species. Therefore the approach to biodiversity conservation in agroecosystems should be different from the natural ecosystems (Moonen and Barberi, 2008). In an agroecosystem context are considered ‘functional groups’ the species traits (part of functional agrobiodiversity) which are the basis for the ‘ecosystem services’ provided by the communities. Farmers can try to influence the agroecosystem services provided through the manipulation of these ‘agroecosystem functional groups’.

1.2 Legume intercropping and agrobiodiversity

An agronomic strategy to enhance agrobiodiversity in cropping systems is growing two or more crops together on the same land and, at the same time in intercropping (Willey, 1990), or in sequent season in crop rotation (Bennett et al., 2012). When more than one species is grown in the same land, the interspecific interactions occurred could be negative, as competition, or positive, as complementarity and facilitation. Complementarity and facilitations represent the cause of the yield increase observed in intercropping (Duchene et al., 2017). The concept of “interspecific complementarity”, suggests that crops differ in the way they find and use resources, thereby limiting interspecific competition and, thus, optimizing the use of resources (Bedoussac et al., 2015). It is possible to distinguish complementarity in temporal, spatial or chemical partitioning (Justes et al., 2014). The time lag between the needs of two or more intercropped species generates a temporal complementary. Such as, when clover is sown in the spring under a winter wheat cash crop (Amossé et al., 2013), the greatest need of resources (water, nutrients, etc.) occurs in different moments for the two partners. Spatial complementarity means that processes, such as nutrient uptake, occurs in different locations. Root architecture and root depth are the most important traits in determining spatial complementary, related to water or to nutrient extraction depth (Hauggaard-Nielsen and Jensen, 2005). Chemical complementarity refers to the ability of species to mobilize different chemical forms of nutrients. This classification of complementarity is formal while most of the results observed in the field are the consequence of combined effect of temporal, spatial and chemical complementarity.

The improvement of plant growth or production quality observed in many field experiments (Jensen et al., 2006) cannot be explained only by complementarity. Cereal/legume systems can promote beneficial interactions (facilitation) in which plants benefit from additional services that partially
overcome competition (Duchene et al., 2017). Complementarity is mainly responsible for limiting competitive interactions by improving resources partitioning, while facilitation provides additional services by improving environmental growth conditions and resources availability (Duchene et al., 2017). Legumes, when used both in intercrop and in crop rotation, are able to increase biodiversity inside the cropping system and moreover, as these plants are able to establish symbiosis with certain types of bacteria (Rhizobium spp. and Bradyrhizobium spp.), are able to biologically fix nitrogen into the soil, which increases soil fertility (Nulik et al., 2013). However, these plants cannot improve on-farm diversity by their self but can be considered as crucial component of multiple cropping systems. Indeed, in multiple cropping systems, services as nutrient recycling and soil fertility are improved through the ability of legumes to fix nitrogen, release free phosphorous and their capacity to increase soil biodiversity and, at the same time, legume also help to curb and control pests and diseases. Additionally, since legumes often promote higher rates of accumulation of soil carbon than cereals or grasses, they can contribute to improve the soil carbon sequestration of agro-ecosystems (Jensen et al., 2012).

1.3 Phosphorus exploitation by legumes
Phosphorus (P) is a major nutrient for all living organisms and it is a key production factor in agriculture. Its scarcity in soils is a limiting factor for crop production in many soils (Cordell et al., 2009). There is evidence that grain legumes may improve P availability not only for themselves but also for crops grown in mixture and in rotation with them (Cu et al., 2005; Hinsinger et al., 2011). Many studies suggested that enhanced P availability is partly responsible for the positive effect of legumes observed in intercropping systems (Betencourt et al., 2011; Li et al., 2007) and in succeeding crops in rotation (Kamh et al., 1999; Nuruzzaman et al., 2005a).

Many plants use exudation of low molecular weight organic molecules into the rhizosphere to enhance the mobilisation of soil P (Gerke et al., 1994; Li et al., 1997). Carboxylates released in the rhizosphere compete with phosphate groups for binding sites in the soil (Nuruzzammam et al., 2005b), forming strong complexes with aluminium and iron oxides, and P is liberated into soil solution (Jones and Darrah, 1994; Ryan et al., 2001; Uren and Reisenauer, 1988). Substantial exudation of carboxylates is well documented amongst a number of grain legume crops, e.g., white lupin (Lupinus albus L., Gardner and Boundy, 1983; Hocking and Randall, 2001; Cu et al., 2005), pigeon pea (Cajanus cajan (L.) Millsp., Ae et al., 1990; Ae et al., 1991), faba bean (Vicia faba L., Li et al., 2007) and chickpea (Cicer arietinum L., Neumann and Römheld, 1999; Veneklaas et al., 2003) with differences in quality and quantity of this exudates among the species. Nuruzzmann et al. (2005a) found in field pea and in white lupin rhizospheres more carboxylates than in faba bean. It has also been reported that the rates and compositions of carboxylates vary considerably with soil conditions (Ae et al., 1990; Dinkelaker et al., 1989; Veneklaas et al., 2003). There is also evidence of higher phosphatase activity in soil under legumes than under other plants (Houlton et al., 2008; Yadav and Tarafdar, 2001; Veterink, 2011). All this partly explains P availability increasing showed under intercropping (Hinsinger et al., 2011; Latati
et al., 2014) and crop rotation (Kamh et al., 1999; Hocking and Randall, 2001), even if the phenomenon is more complex and involves other factors, such as niche complementarity (Hinsinger et al., 2011) and microbial activity of belowground communities attracted by root activity of intercropped species (Berg and Smalla, 2009; Marschner et al., 1986, 2001, 2004). It has been shown that some legumes crops, such as *Vicia faba* L. and *Cicer arietinum* L., facilitate P uptake and biomass production of co-occurring non-legumes crops (*Zea mays* and *Triticum aestivum*), apparently by exuding organic acids, protons or acid phosphatase (Li et al., 2004, 2007). Similar results were found in pot experiments with wheat intercropped with chickpea (Li et al., 2003), lupin (Kamh et al., 1999; Cu et al., 2005) and faba bean (Song et al., 2007). For Morel and Hinsinger (1999), the turnover of the organic root exudates and organic P fractions, and the equilibrium between readily and sparingly plant-available inorganic P fractions, are time-dependent. In crop rotation, during the vegetation-free period between cropping seasons, plant-available/mobilised P may be immobilised thus limiting the beneficial effect of P mobilisation by one crop to the next crop in the rotation. Therefore, a transfer of mobilised P from a P-efficient crop to an inefficient crop is more likely to occur in a mixed cropping system (Gardner and Boundy, 1983; Horst and Waschkies, 1987; Kamh et al., 1999). Then, it appears more likely that a positive rotational effect of P-mobilising crops is mainly due to transfer of readily available P via the crop residues (Kamh et al., 1999). Thus, to make mobilised P available to the main crop, the most promising agronomic approach appears to be the integration into the cropping system of P-mobilising plant species as intercrops or in rotation (Horst et al., 2001). In fact, Nuruzzmann et al. (2005b) observed that, after removal of the legume roots of white lupin, field pea and faba bean, the concentration of carboxylates in the soil declined gradually until no detectable amounts of carboxylates were found after four weeks.

1.4 Facilitations in intercropping mediated by microorganisms

Rhizosphere represents a volume of soil rich of a large amount of nutrients provided to microbial communities due to rhizodeposition and exudation by crop roots (Hinsinger et al., 2009; Wichern et al., 2007). In fact, plants exudates make possible the instauration of a rich microorganism community near the roots (Bais et al., 2006; Bertin et al., 2003; Morgan et al., 2005). The quality and quantity of root exudates significantly affect soil microbial community structure (Berg and Smalla, 2009; Hamilton and Frank, 2001; Qiang et al., 2004; Wieland et al., 2001). The alteration of microbial community structure can correspond to the selection of specific functional traits of soil microbial communities (Bartelt-Ryser et al., 2005; Fridley, 2001; Zak et al., 2003; Zhou et al., 2015). In many cases, the selection of specific microorganisms is not generated by a simple passive diffusion mechanism and the establishment of a microbe-favourable environment but involves a complex series of signals that mediate the interaction through complex molecular exchanges between plant and microorganisms (Zhou et al., 2015; Faure et al., 2009; Hirsch et al., 2003; Johansson et al., 2004). Interactions with microorganisms generate many positive effects for the plants, such as the providing of nutrients (Gianinazzi et al., 2010; Jeffries et al., 2003; van Kessel et al., 1985) and phytohormones.
(Bashan and de-Bashan, 2010), pest control and stimulation of plant resistance to pathogens (Audenaert et al., 2002; Lemanceau, 1992) and the attenuation of biotic and of abiotic plant stress factors (Vacheron et al., 2015).

It is possible that the community structure of the legume/cereal intercropping, which is shaped by one of the partners or by the combined activity of both the two species, generates advantages for the entire intercropping. In that case, the positive influences generated by plant-microbe interaction can be considered as part of the facilitations generated by the mixture of species. The main advantages of intercropping between legume and non-legume species appear to be due to the stimulation of rhizosphere activities based on legume N-fixing action, the associated exudates and the resulting changes in pH (Duchene et al., 2017).

Generally, the use of legumes in crop rotation (Alvey et al., 2003) or intercropping (Latati et al., 2014; Li et al., 2009; Qiang et al., 2004; Song et al., 2007; Tang et al., 2014; Wang et al., 2007) resulted in an improvement of microbial diversity. The intricate processes that regulate soil communities need to be more explored and involve the production and exudation of specific molecules by legumes capable to influence Plant Growth-Promoting Rhizobacteria (PGPR) mobility, improving root colonization and the phytobeneficial activity of these PGPR (Schelud’ko et al., 2009; Jain and Gupta, 2003). Confirmation that the composition of rhizosphere communities is species-specific (Marschner et al., 2001) also strongly supports the hypothesis that intercropping creates favourable conditions for belowground interactions. Indeed, legumes modify the chemical properties of the entire rhizosphere and, with their own group of specific bacteria, stimulate the rhizosphere for the potential benefit of both the legume and the cereal since their respective root systems are not separate but intermingled (Duchene et al., 2017). Many articles (Bernard et al., 2007, 2009; Blagodatskaya and Kuzyakov, 2008; Fontaine et al., 2003) reported also the increase of decomposition rates of organic matter (SOM) in intercrop due to the addition of fresh organic matter from legumes which stimulates the activities of soil bacteria communities involved in the mineralization of stable forms of SOM.

In the rhizosphere is concentrated the highest proportion of phosphate solubilizing microorganisms (PSM) that are more metabolically active than those isolated from sources other than the rhizosphere (Vazquez et al., 2000). Conversely, the salt-, pH- and temperature-tolerant phosphate-solubilizing bacteria have been reported to be maximum in the rhizoplane followed by the rhizosphere and root-free soil in alkaline soils (Johri et al., 1999). These organisms can convert the insoluble phosphate compounds into soluble forms in the soil, by their phosphate-solubilizing ability (Kang et al., 2002; Pradhan and Sukla, 2005), making more phosphorus available to the crops. The main solubilizing mechanism is the release of organic acids can either directly dissolve the mineral phosphate, as a result of anion exchange of PO₄³⁻ by acid anion, or can chelate both iron and aluminium ions associated with phosphate (Omar, 1998). Important genera of mineral phosphate solubilizing microorganisms include Bacillus and Pseudomonas (Illmer and Schinner, 1992), while Aspergillus and Penicillium are the most important fungal genera (Motsara et al., 1995).
2 AIM OF RESEARCH AND THESIS OUTLINE

The purpose of this PhD thesis is to assess in agricultural soil the ability of the three grain legumes (faba, lupin and pea) to facilitate phosphorus uptake by durum wheat in intercropping. To achieve this goal these three main objectives were pursued:

- verify in agricultural soil, the ability of the three grain legumes, different in root exudation, habitus and growth, to mobilize phosphorus through their specific root exudation (phosphatase activities and carboxylates composition) and confirm whether these facilitations is more expressed under phosphorus-limiting soil conditions;
- assess whether an increase in legume mass roots may influence phosphorus mobilization in the intercropping system;
and
- describe any changes in soil bacterial community exerted in intercropping by the legume root activity mentioned above.

To achieve these specific objectives, during a three-years period, three separate experiments were carried out in pots under controlled environment (two in climatic chamber and one in heated greenhouse). Wheat and the legumes were intercropped and the crops were sampled when each legume reached flowering. In this phase the highest release of root exudates in grain legume occurs (García et al., 2001). Therefore in order to compare intercropped wheat with respective sole crop at the same date (flowering of intercropped legume), three wheat sole crop were included in the experimental design, each corresponding to the three different legume flowering times.

The performance of legumes and wheat in intercropping were compared with the respective sole crops and to this end, in addition to the measured absolute values, were used the “relative values” (eg. relative dry matter accumulation) calculated as intercropping / sole crop ratio.

The experiments dedicated to the first two topics were carried out at the Department AGRARIA at the Mediterranean University of Reggio Calabria during 2017 and 2018, the third topic was investigated, during the October 2018- July 2019 period, at the Rothamested Resarch Center (Harpenden, UK) where a specific experiment was carried out.

In this thesis the three experiments are described, and the results are presented and discussed in three separate chapters that have been drafted in the form of a scientific article manuscript.

The first experiment was described in the third chapter, where are reported the results on the effects of root exudates (carboxylates and phosphatases) in legume/cereal intercropping at high and low soil P availability. The exudation was compared to the plant dry matter yield and P uptake of both the intercropping partners and was related to the efficiency of intercropping system compared to sole crop, and to the modification of the P pools in soil.
The fourth chapter contains a second experiment, where was studied the effect of legume density increase in wheat:legume sowing ratio (1:1 vs 1:2) on root exudation, on plant dry matter yield, on P uptake and on variation of soil P pools. This experiment was particularly aimed at deepening the study of the combined effects of interspecific competition and facilitation in intercropping on yield and P uptake of wheat.

A third experiment is placed in the fifth chapter and explores the role of soil bacteria in the facilitative interaction generated by legumes in intercrop. The variation of rhizospheric bacterial community structure was investigated varying P forms soil source in pea and lupin intercropped with durum wheat.
3 Grain legumes root exudates can help intercropped wheat to exploit phosphorus in P-limiting conditions.

The aim of this study is the evaluation of the beneficial interactions mediated by root exudates that occurred in a grain legume/wheat intercrop varying phosphorus availability in soil. The benefit for the main crop (wheat) was evaluated by measuring dry matter yield and by calculating P uptake. The relation among root exudates (phosphatases and carboxylates), phosphorus plant uptake and its pool in soil was also investigated. The hypothesis is to verify, in agricultural soil under P-limited conditions, the facilitative support exerted by different grain legumes on the wheat uptake in intercropping through root exudation and related modifications in soil P pools.

3.1 Material and methods

Plant growth and experimental design

Wheat (*Triticum turgidum* subsp. *durum* (Desf.) Husn. cv. Svevo) (W), lupin (*Lupinus albus* L. cv. Multitalia) (L), faba bean (*Vicia faba* var. minor Beck cv. Sikelia) (F) and pea (*Pisum sativum* L. cv. Hardy) (P) were grown in pots as sole crop (SC) and in intercropping (IC). In order to sample at the flowering of each legume three wheat sole crops were also considered (W-F, W-L and W-P for faba, lupin and pea respectively).

Table 3.1. Growth conditions in climate chamber

<table>
<thead>
<tr>
<th>Period length (d)</th>
<th>15</th>
<th>20</th>
<th>20</th>
<th>Until legume flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day (h)</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Night (h)</td>
<td>16</td>
<td>16</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>Light intensity (µmoles/m² /s)</td>
<td>200</td>
<td>255</td>
<td>340</td>
<td>338</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>7.5</td>
<td>15</td>
<td>20</td>
<td>25</td>
</tr>
</tbody>
</table>

Nine cropping treatments (CTR) resulted from the combination of the three legumes and wheat respectively grown in intercropping and sole crop. In addition, three bare soil treatments were added at the flowering time of faba (BS-F), lupin (BS-L) and pea (BS-P) as a control in soil variables analysis. Crop treatments and BS controls were combined with two levels of phosphorus (P) in the soil, P0 (with no P supply) and P1 (with 50 mg P kg⁻¹ soil as KH₂PO₄) in a factorial randomized block design with 4 replications. Cropping treatments were obtained growing in a pot one plant of each crop species (SC); one plants of legume plus one plant of wheat in the same pot (IC). The pot were PVC tube Ø 14 cm and 30 cm tall filled with the soil mixed with perlite (80/20, v/v). The pots were placed in a climate chamber and grown until legumes flowering. Radiation, and temperature regimes are reported in table 3.1.
Soil and plant sampling and analysis

Soil was collected from the experimental farm (37°E 51N°) 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⁻¹ soil). Soil properties are listed in Table 3.2.

Table 3.2. Physical and chemical characteristics of soil used in the experiments

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk density (g cm⁻³)</td>
<td>1.23</td>
</tr>
<tr>
<td>Sand %</td>
<td>36</td>
</tr>
<tr>
<td>Silt %</td>
<td>32</td>
</tr>
<tr>
<td>Clay %</td>
<td>32</td>
</tr>
<tr>
<td>pH CaCl₂</td>
<td>6.6±0.1</td>
</tr>
<tr>
<td>Total organic carbon (g kg⁻¹)</td>
<td>12.55±4.5</td>
</tr>
<tr>
<td>Total nitrogen (g N kg⁻¹)</td>
<td>1.38±0.1</td>
</tr>
<tr>
<td>C/N</td>
<td>9.07±0.1</td>
</tr>
<tr>
<td>EC₁₂ (dS m⁻¹)</td>
<td>0.271±0.021</td>
</tr>
<tr>
<td>CEC (cmol(+) kg⁻¹)</td>
<td>20.9±1.3</td>
</tr>
<tr>
<td>CaCO₃ (g kg⁻¹)</td>
<td>8.4±0.5</td>
</tr>
<tr>
<td>P-Olsen (mg kg⁻¹)</td>
<td>11.33±0.2</td>
</tr>
<tr>
<td>NH₄⁺ - N (mg kg⁻¹)</td>
<td>12.49±0.2</td>
</tr>
<tr>
<td>NO₃⁻ - N (mg kg⁻¹)</td>
<td>26.16±0.2</td>
</tr>
</tbody>
</table>

At flowering time of each legume, the pots were destroyed and the plants and the soil were collected for the analysis. After plants separation into shoot and root, about 10 g of root were immediately collected for carboxylates analysis. After the carboxylate extraction, the root subsample was combined with the rest of root and accurately washed to remove any trace of remaining soil. Roots and shoots were placed in oven and dried at 70°C and dry weights were recorded. P concentration of root and shoot was obtained digesting 100 mg of milled plant material with the mixture nitric and perchloric acids (6:1) (Johnson e Ulrich 1959) and the digested was measured via molybdate method (Westerman, 1990) modified for Lambda Fias UV/VIS Spectrophotometer Perkin Elmer.

Carboxylates analysis. Each 10 g root sample was transferred into a 100-ml vial and 50 ml of 0.2 mM CaCl₂ were applied. Roots were then gently dunked for 30 s to remove as much rhizosphere soil as possible (Pearse et al., 2003). 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. The analysis was performed according to method suggested by Cawthray, (2003) using HPLC with PDA detector Altus A-10 (PerkinElmer) and column Kinetex 2.6 µm F5 100 Å.

Chemical analysis. After roots were gently removed from the pots, the soil was carefully mixed and sampled for the analysis in the laboratory. The 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 while the last aliquot was crushed to pass through a 500 µm sieve. The frozen soil was used for the measure of the phosphatase activity and for the determination of ammonium and nitrate. The <2 mm fraction was used to determine soil pH and electrical conductivity (EC). While the <500 µm fraction was used for total organic C (TOC) and N (TN). Ammonium and nitrate were extracted by KCl 2 M solution from 5 g of fresh soil (Beemner and Keeney, 1966) and measured by Lambda Fias UV/VIS Spectrophotometer Perkin Elmer. Soil pH was measured in a 1:2.5 (w/v) soil: 0.01 M calcium chloride solution and soil EC was measured in a 1:2 (w/v) soil: water mixture, according to Sparks et al. (1996).
Acid phosphatase activity was determined by the method proposed by Tabatabai and Bremner (1969) and modified by Hedley et al. (1982) according to which phosphatase in soil is expressed as production of para-nitrophenol (μmol h⁻¹). One g of soil was incubated with para-nitrophenylphosphate (pNPP) in 4 ml of 0.04 M sodium maleate buffer (pH 6.5) at 37 °C for 1 h. After the reaction was stopped with 1 M NaOH and the amount of para-nitrophenol (pNP) released by phosphatase activity was measured via spectrophotometer as absorbance at 400 nm and expressed as phosphomonoesterase activity (μmol pNP g⁻¹ soil h⁻¹) (PME).

As an index of available form, was used phosphorus extracted by Olsen method (Olsen et al. 1954), and measured via spectrophotometer using Lambda Fias UV/VIS Spectrophotometer Perkin Elmer. Organic P was obtained as difference of ignited at 550 °C and no ignited soil sample H₂SO₄ extracts according to Bowman (1989) and Kuo (1996).

Data were processed by ANalysis Of VAriance using a PROC GLM in SAS v. 9.2 (SAS,Inc., Cary, NC, US, 2009) for the RCB design model to test for significance of treatments. For means comparison Tukey’s HSD test was performed.
3.2 Results

Phosphatase activity and organic phosphorus in soil

The statistical analysis showed significantly differences of phosphomonoesterase activity (PME) between the two levels of P (P= 0.0017) and among cropping treatments (CTR), BS-F, BS-L and BS-P included, (P<0.0001), but no interaction CTR x P was observed. Among treatment, PME varied from 192 (BS-L) to 295 μmol pNPP g⁻¹ soil h⁻¹ (LSC). Legume based systems was 252 μmol pNPP g⁻¹ h⁻¹, more than wheat sole crop (SC) (211) and bare soils. In soil with phosphorus supply, the average PME activity was 238.42 μmol pNPP g⁻¹ h⁻¹, i.e. 8% significantly higher than in soil with natural content (220.82 μmol pNPP g⁻¹ h⁻¹) (Fig. 3.1).

Organic fraction of phosphorus in the soil (P_{ORG}) between the P levels and cropping treatment significantly differed and ANOVA also showed a significant interaction. Average P_{ORG} at P0 was 87.64 mg/kg in soil, 14% more than in P1 (Tab 3.3). Among the cropping treatments, the highest P_{ORG} average value was observed in W-L sole crop that was significantly higher than WL and L. At P0 higher P_{ORG} in soil were observed under intercropping and sole crops, excluding faba in both cropping systems and wheat sole crops. In bare soil the P_{ORG} increased when P was supplied.
Table 3.3. Soil organic phosphorus (mg kg$^{-1}$ 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 in subscript are sampling time corresponding to the different flowering time of legumes. Values are mean ± SE (n=4; n=8). Significance of P for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR), by Anova are reported in italic.

<table>
<thead>
<tr>
<th></th>
<th>P0</th>
<th>P1</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS-F</td>
<td>86.070 ±9.62</td>
<td>88.537 ±4.48</td>
<td>87.304 ±4.94</td>
</tr>
<tr>
<td>BS-L</td>
<td>87.833 ±4.78</td>
<td>86.865 ±9.46</td>
<td>87.349 ±4.91</td>
</tr>
<tr>
<td>BS-P</td>
<td>80.560 ±6.11</td>
<td>88.225 ±13.15</td>
<td>84.393 ±6.87</td>
</tr>
<tr>
<td>F</td>
<td>78.956 ±3.49</td>
<td>75.546 ±13.64</td>
<td>77.251 ±6.55</td>
</tr>
<tr>
<td>L</td>
<td>77.726 ±6.20</td>
<td>45.475 ±5.95</td>
<td>61.601 ±7.28</td>
</tr>
<tr>
<td>P</td>
<td>106.526 ±8.99</td>
<td>56.808 ±10.65</td>
<td>81.667 ±11.40</td>
</tr>
<tr>
<td>W-F</td>
<td>92.242 ±4.43</td>
<td>91.287 ±10.26</td>
<td>91.765 ±5.18</td>
</tr>
<tr>
<td>W-L</td>
<td>117.407 ±14.95</td>
<td>92.504 ±11.56</td>
<td>104.955 ±9.94</td>
</tr>
<tr>
<td>W-P</td>
<td>77.514 ±9.52</td>
<td>96.348 ±5.89</td>
<td>86.931 ±6.29</td>
</tr>
<tr>
<td>WF</td>
<td>75.852 ±8.78</td>
<td>76.790 ±11.77</td>
<td>76.321 ±6.80</td>
</tr>
<tr>
<td>WL</td>
<td>84.042 ±2.98</td>
<td>60.725 ±4.08</td>
<td>72.384 ±4.99</td>
</tr>
<tr>
<td>WP</td>
<td>86.966 ±12.39</td>
<td>61.186 ±11.28</td>
<td>74.076 ±9.16</td>
</tr>
<tr>
<td>Mean</td>
<td>87.641 ±2.77</td>
<td>76.691 ±3.42</td>
<td>82.166 ±2.07</td>
</tr>
</tbody>
</table>

\[
P = 0.0047  \\
CTR = 0.0036  \\
P \times CTR = 0.0164
\]

Carboxylates exudation in rhizosphere

Total carboxylates production in rhizospheric soil was influenced by P supply and crop treatment. Total carboxylates were ten-fold greater in legume rhizosphere (43.1 μmol/g dry rhizospheric soil) than in wheat rhizosphere (4.3 μmol/g DW rhizospheric soil). The greatest carboxylates accumulation was observed in faba followed by pea, lupin and wheat. In legume rhizosphere carboxylates were always greater in P1 (+85%) compared to P0. In wheat they were greater at P1 only in the rhizosphere of wheat intercropped with faba and of the respective SC (respectively four- and five-fold higher) but no significant difference was shown between P0 and P1 in the other crop treatments. In wheat rhizosphere, the accumulation of carboxylates was greater in IC compared to SC both in P0 (+82%) and P1 (+75%). Carboxylates of legume rhizosphere were generally lower (-20%) in IC than in SC; on the contrary, between the two pea-systems no differences were shown at P0 but at P1 an 20% increase was found in intercropping.

Both in wheat and in legumes more than 99 % of the total carboxylates released in the rhizosphere consisted in oxalate, acetate, succinate, malate, malonate, tartrate and, in some cases, in citrate. High differences among the treatments and P levels were found.
In faba-based systems (WF and F) oxalate, acetate and malate were the most abundant carboxylates, representing the 70% of total. Citrate, cis-aconitate, malonate, tartrate and succinate were also detected in faba rhizosphere, ranging between 1 and 10 μmol g⁻¹ soil. Maleate, trans-aconitate and fumarate were detected in traces (<1μmol g⁻¹ DW soil). In lupin rhizosphere the carboxylates was represented by oxalate, acetate, malate and citrate accounting for 90% and cis-aconitate, malonate, succinate and maleate in traces were found. In pea rhizosphere oxalate and malate representing the 90% of total carboxylates and traces were detected of malonate, acetate, citrate, cis-aconitate, succinate and maleate (Fig. 3.2.).

The most identified carboxylate in wheat rhizosphere was oxalate (55% of the total) followed by malate (14%) and small amount of acetate and tartrate. Traces of cis-aconitate, malonate, succinate, maleate, trans-aconitate and fumarate were found both in IC and in SC at two P level.

Most abundant carboxylates detected in legumes were oxalate and malate. With P supply, both oxalate and malate increased in rhizosphere of intercropped legume (three and five times compared to P0 respectively), and malate also increased in lupin and pea sole crop. At concentration useful for P mobilization (Gerke, Römer and Beißner, 2000) were found malonate and cis-aconitate, they increased at P1 in WL and FSC. The concentration of acetate in FSC and citrate in LSC and PSC decreased when P was supplied.

Carboxylates in wheat rhizosphere were affected by P supply. In P1 the concentration of the single carboxylates was generally higher than in P0. In wheat SC, both oxalate (+132%) and malate (+300%) increased with P supply. In WF, both oxalate and malate were greater in wheat rhizosphere while in WP and WL only higher malate concentration were observed.

In IC the concentration of all the most abundant carboxylates in legumes rhizosphere was lower than in SC, excluding acetate that increased four times. The concentration of all the carboxylates was greater in IC wheat compared to SC, both at P0 and P1.

*Total available phosphorus in soil.*
The term "Total available phosphorus" (PTAV) was used to identify the total P fraction mobilized in the soil by legume root exudation activity and it included the P amount absorbed by plants until sampling. To calculate PTAV, P uptake by cropping treatment was added to Olsen phosphorus, measured in the soil after plant sampling:

$$PTAV = OlsenP + P_{ptake}$$

As expected, P supply strongly affected the PTAV (P< 0.001) showing an average increase of 60.45

Table 3.4. Total soil available phosphorus (PTAV) 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. Values are mean ± SE (n=4; n=8). Significance of P for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR) by Anova are reported in italic.

<table>
<thead>
<tr>
<th></th>
<th>P0 (mg kg(^{-1}) dry soil)</th>
<th>%</th>
<th>P1 (mg kg(^{-1}) dry soil)</th>
<th>%</th>
<th>Mean (mg kg(^{-1}) dry soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>17.11 ±2.31</td>
<td>78</td>
<td>85.82 ±9.21</td>
<td>92</td>
<td>51.46 ±13.64</td>
</tr>
<tr>
<td>L</td>
<td>14.73 ±1.61</td>
<td>88</td>
<td>77.92 ±9.73</td>
<td>87</td>
<td>46.32 ±12.73</td>
</tr>
<tr>
<td>P</td>
<td>21.10 ±1.19</td>
<td>78</td>
<td>81.03 ±9.82</td>
<td>90</td>
<td>51.06 ±12.23</td>
</tr>
<tr>
<td>W-F</td>
<td>18.41 ±1.84</td>
<td>72</td>
<td>65.54 ±1.53</td>
<td>90</td>
<td>41.98 ± 8.90</td>
</tr>
<tr>
<td>W-L</td>
<td>14.85 ±0.90</td>
<td>71</td>
<td>59.31 ±2.29</td>
<td>89</td>
<td>37.08 ± 8.55</td>
</tr>
<tr>
<td>W-P</td>
<td>20.08 ±5.96</td>
<td>85</td>
<td>75.17 ±1.93</td>
<td>96</td>
<td>47.63 ±10.84</td>
</tr>
<tr>
<td>WF</td>
<td>23.60 ±1.74</td>
<td>62</td>
<td>88.58 ±3.36</td>
<td>86</td>
<td>56.09 ±12.24</td>
</tr>
<tr>
<td>WL</td>
<td>22.74 ±2.64</td>
<td>66</td>
<td>86.40 ±5.89</td>
<td>84</td>
<td>54.57 ±12.30</td>
</tr>
<tr>
<td>WP</td>
<td>25.54 ±2.47</td>
<td>58</td>
<td>102.47 ±6.46</td>
<td>84</td>
<td>64.00 ±14.87</td>
</tr>
<tr>
<td>Mean</td>
<td>19.80 ±0.76</td>
<td>73</td>
<td>80.25 ±2.87</td>
<td>89</td>
<td>50.02 ± 3.25</td>
</tr>
</tbody>
</table>

|          | P=0.0001                       |    | P=0.0003                     |    | P=0.051                       |

mg kg\(^{-1}\) comparing unfertilized cropping treatments. Significant differences were also showed among the treatments (P=0.001) and interaction between P x CRT (P<0.03) was also found (Table 3.4).

The average value of PTAV in cropping treatments was 50.02 mg kg\(^{-1}\) dry soil; highest and lowest value were observed in intercropping and in wheat sole crop. The incremental effect of P supply were strongest in WP (+76.93) and F (+68.71 mg kg\(^{-1}\) dry soil) and varied between +64 and +60 mg kg\(^{-1}\) dry soil in intercropping WF and in L, grown both in IC and in SC. On average, CTRs left in the soil 73 and 89% of PTAV in P0 and P1 respectively. When phosphorus was not added, the intercropping showed a lower percentage (62%) of the residual available phosphorus, lowest in WP. Among sole crops, only in L and W-P exceeded 80%. Even with P fertilization the residual available phosphorus was on average lower in intercropping (<90%) than in the sole crop.

OLSEN-P in soil was significantly different between P0 and P1 (P<0.0001) and among cropping treatments (P<0.05) but no interaction was found between P level and cropping treatments. At P0, Olsen-P average value was 15.19 and in P1 reached 69.07. Among the treatments the highest level of Olsen-P was 50.74 in WP, the lowest 31.76 mg kg\(^{-1}\) dry soil in W-L was found. Between these two extreme values, the highest amount of available P was found in legume-based systems, in W-P and in BS-P (data not shown).
Phosphorus concentration in shoot and root dry matter

Phosphorus concentration of shoot and root dry matter were significantly affected by P and CTR both in legume and wheat and, excluding root in legume, by P x CTR interaction. On average, shoot P concentration was much higher in wheat than in legumes (+41.6%), but legume exceeded wheat in P content of root (+49.3%). Contrary to what was observed in the shoot, highest P contents in the roots of wheat sole crop (W-L) were found. In supplementary table 3.1 data in details are presented. Phosphorus supply caused a general increase of shoot and root P content both in IC and in SC that was clearly higher in legume than in wheat. Compared to SC, the wheat intercropped with pea demonstrated the lowest and similar P content reductions in shoot at both P0 and P1 (-16.2 and -15.0% respectively), whereas in wheat intercropped with faba the gap between IC and SC decreased from -48.4% (P0) to -1.9% (P1). Compared to respective sole crop, intercropped pea showed higher shoot P content both in P0 (+39.3%) and in P1 (+36.4%), intercropped lupin was greatly favored only under no P fertilization (+51.1%), as well as faba (+12.2%) under P fertilized condition.

The phosphorus concentration in root dry matter was greater in wheat intercropped with faba than in respective sole crop, both with and without P supply (+4.8 and +18.9% respectively), and in intercropping with pea (+56.8%) when P was added.

Dry matter accumulation in shoot and root.

Phosphorus supply, as well as cropping treatments, significantly affected dry matter accumulation in shoot (SDM) and root (RDM) both of wheat and of legumes. Significant interactions between P x CTR in SDM and RDM were also found.

Phosphorus supply differently affected plant dry matter partitioning in intercropping, increasing RDM and decreasing SDM (+27.8 and +44.0%; -10.6 and -16.8% in wheat and legume respectively) (Fig. 3.3).

Among cropping systems, SDM of wheat was highest both in WL and in WP and significantly differed from WF and sole crops. On the contrary, RDM in intercropped legumes was not significantly different from respective sole crops. SDM of intercropped wheat greatly overweighted the sole crop in WP (+175%) and in WL (+103%) but slightly in WF (+7%). Highest RDM increase, compared to respective sole crop, was shown in wheat intercropping with pea (+121%), on the contrary RDM slightly decreased in intercropping with faba (-9%).
P supply negatively affected SDM of wheat and of legume in intercropping, particularly in WP, in which wheat and legume showed higher decreases (-41.1 and -37.3 % respectively) than other treatments. With P fertilized sole crop, SDM of wheat increased (+13.9% sampling dates average) and significantly increased in lupin (+94.3%).

The positive effects of P supply on RDM in intercropping were higher in WP than in other treatments, observed both on wheat and pea (+120.8 and +121.3% respectively); in WF RDM was also greater with P supply than without but the increase was more relevant in faba (+31.9%) than in wheat (+7.8%).

In order to compare IC versus SC, relative dry matter accumulation (RDMA) of each intercropped partner was calculated as a ratio between dry matter absolute values (Fig. 3.4).

![Figure 3.4](image.png)

**Figure 3.4.** Relative dry matter accumulation 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 ±SE (n = 4).

Wheat RDMA in all mixture was higher than unit (1.66 on the two P levels average) which means wheat in intercropping was able to accumulate biomass 66% more than in sole crop. In general wheat accumulated dry matter 121 and 74% more than sole crop when intercropped with pea and lupin respectively. Quite lower was the advantage of intercropped legumes that exceeded sole crop by 3% on average. Without phosphorus fertilization wheat showed highest RDMA in WP (2.38) resulting in RDMA of pea close unit. At the same condition, in WL not only wheat (1.74), but also lupin (1.32) was favoured by intercropping.
Phosphorus uptake in intercropping

The P uptake increased significantly (P<0.0001) with the phosphorus supply resulting on average in 56.3% higher. In intercropping systems, the increase (+52.1%) was greater than that observed in wheat (+34.4%) but considerably lower than that in legumes (+87.4%) sole crops. Cropping treatments and cropping treatments x phosphorus interaction were also significant at P<0.0001. The highest total P amount by WP was acquired (70.33 mg plant\(^{-1}\) as average of P0 and P1), significantly different from all other treatments. Intercropping WF (56.71) and WL (55.83 mg plant\(^{-1}\)) 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 absorbed 60.7% more phosphorus than at P0, with lupin and pea the increase was much lower (+15.9 and +7.8% respectively) (Fig.3.5). The P uptake increased in intercropped legume under P fertilization and was extremely higher in lupine (+152%) than in pea (+89.5%) and faba (+32.0%).

As well as for biomass, relative phosphorus uptake (RPU) of each intercropped partner was calculated as a ratio between IC and SC absolute values. Wheat RPU in WP reached on average 2.19, which means it absorbed 119% more than wheat grown in sole crop. On the contrary, both with faba and lupin RPU average wheat values were lower than, or close, to unit, resulting in lower absorption by 20% (WF) and 1% (WL) than in sole crop. Without P supply wheat intercropped with pea showed the highest relative P uptake value (2.42) but in intercropping with faba it decreased to 0.69 (Fig. 3.6).
Figure 3.6. Phosphorus relative 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) ±SE. The vertical and horizontal lines represent all the points where dry matter accumulation in IC is equal to SC.
3.3 Discussion

Phosphatase and organic phosphorus in soil

PME activity increased with P supply in legume-based systems, wheat sole crop and bare soil. These results disagree with other studies (Olander and Vitousek 1999; Venterink 2011, Sun et al., 2019) where P fertilization significantly inhibited phosphatase activity. A possible explanation of different response is that cited studies were carried out in rhizospheric soil or in hydroponic systems. Otherwise, our experiment was conducted on agricultural soil and PME activity was detected in bulk soil. Indeed, comparing legume root dry weight with the corresponding PME activity in soil, our results showed that the greater root growth, as an effect of P supply, compensated the reduction of PME expressed as activity per root mass unit, as well as reported by other studies (Olander and Vitousek, 1999; Venterink, 2011, Sun et al., 2019). Anyway, contrasting results were shown by many researches. For example, Spohn and Kuzyakov (2013) reported that P fertilization strongly decreased alkaline phosphatase activity, but had no effect on acid phosphatase activity. In another study (Solaiman et al., 2007) P supply increased phosphatase activity at flowering time in the rhizosphere of two canola genotypes and one wheat genotype. Moreover, our results are supported by those obtained by Olander and Vitousek (2000), where significant inhibitory effects on the enzyme activity, mediated by P supply, were observed not in the short- but in a long-term fertilization. The authors explained the results by the ability of enzymes, particularly phosphatases, to persist in soils for long time by binding to soil humics and clays (Burns 1982; Sinsabaugh, 1994; Rojo et al., 1990 in Olander and Vitousek). This “binding to soil” hypothesis can also confirmed by our data reporting high PME activity in bare soil.

In order to estimate the root efficiency in PME activity, inclusive of the aliquot from the indirect contribution of microorganisms, the ratio of PME activity/root biomass was calculated. This calculation permitted to compare different species and different P supply independently to the root growth. Our results showed in legumes a higher PME efficiency than wheat (+23 %) and a larger difference was observed when P was added. Venterink (2011) comparing several legumes and non-legume species found a greater PME efficiency of the legumes with variable response to P availability among the species. Recent results (Sun et al., 2019) obtained on maize were in contrasting with ours as well as with those obtained by Nuruzzaman et al., 2006 and Venterink 2011, showing greater PME efficiency in maize rhizosphere than in alfalfa when grown separately and the increase in both the partners’ rhizospheres when grown in intercrop. In our experiment PME root efficiency of pea strongly, unlike other legumes, decreased with P fertilization both in sole crop and intercropping.

Organic phosphorus

It is known that organic P depletion must be considered as the consequence of bacterial and root PME activity. In our study, P_{ORG} resulted more influenced by PME activity under P supply, where the rates of enzyme activity were greater than in natural P soil (P0). We found that P_{ORG} was generally lower in legume-based systems (intercropping and in sole crop) than in wheat sole crop and in bare soil according to the higher PME activity observed. Contrary to what was expected, a significant negative relation between PME activity to the P_{ORG} (Fig.3.7), was found only when P was supplied (R^2=0.890; P=0.0001). Without P fertilization, the increase of PME activity was not associated to a proportional P_{ORG} reduction. In bare soil and in wheat sole crop PME activity was low and consequently the highest amount of P_{ORG} was found in that treatments.
Carboxylates in rhizosphere

In this study, the major fraction of all carboxylates in the rhizosphere was composed by oxalate and malate followed by acetate and citrate that were particularly abundant in faba. A low exudation rate observed in lupin can be considered an unusual results that disagree with other studies (Nuruzzaman et al., 2005a, b; Pearse et al., 2003, 2006) that reported a greater carboxylates production in lupin than in pea and in faba. In study of Nuruzzaman et al. (2005a) the carboxylates concentration in pea and in faba rhizosphere varied with soil type, in fact the author observed detectable amounts of carboxylate only in soil with low phosphorus-retention index. Our results confirmed that wheat had lower carboxylate concentrations in rhizosphere than grain legumes in all cropping treatments (Pearse et al., 2003, 2006; Hinsinger et al., 2003).

Another noticeable outcome is that total carboxylates amount was always greater in intercropped wheat than in the respective sole crop whereas in the study of Li et al., (2010) no significant differences in citrate and malate concentration between intercropped and sole crop cereal are reported. In our study the detected carboxylates in both IC and SC wheat rhizosphere was not influenced by P soil availability and at P-limited condition the wheat biomass P concentration significantly decreased (R2=0.432; P=0.024) when the carboxylates in wheat rhizosphere increased (Fig. 3.8).

Figure 3.7. Phosphomonooesterase (PME) activity plotted versus organic phosphorus (P\textsubscript{ORG}) in bare soil (BS), in legumes and wheat grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP) without (closed) and with (open symbols) phosphorus supply. At P fertilized condition, P\textsubscript{ORG} showed a linear and significant decrease as the PME activity increased (R\textsuperscript{2}=0.890; P=0.0001). Phosphomonoesterase (PME) activity is expressed as paranitrophenol (pNP) h\textsuperscript{-1} g\textsuperscript{-1} of dry soil.
Many authors (Pearse et al., 2006; Nuruzzaman et al., 2006; Sun et al., 2019) reported that carboxylate exudation was suppressed when P was supplied. The type of P source and soil could have a central role in suppressing phosphorus-regulated exudation (Pearse et al., 2003; Nuruzzaman et al., 2005a). Our results disagree with Pearse et al. (2003) who, adding KH$_2$PO$_4$, observed a significant suppression of carboxylates exudation in *Lupinus* species grown on washed sand but agree with Nuruzzaman et al. (2005a) who, adding the same P form, observed that variation of carboxylate exudation rate (increase or decrease) was related to the soil type. Another experiment (Shu et al., 2007) showed that using KH$_2$PO$_4$ as P form resulted in no significant differences between the carboxylate exudation of plants with and without P supply suggesting regulation of citrate exudation by the shoot P status (Shane et al., 2003). Therefore, the influence of P availability on carboxylates release is controversial. Wouterlood et al. (2005) found that carboxylate exudation was only slightly downregulated at a very high shoot P status. In our study, the supply of 50 mg P kg$^{-1}$ of soil improved P shoot concentration but it was not enough to cause such down-regulation. The reaction to low concentrations of external or internal P by increasing carboxylate exudation varies in the legume species. Legumes such as *C. arietinum* are not influenced by P availability and their exuding ability is constitutive (Wouterlood et al., 2004a, b, 2005).

Contrary to some studies (Pearse et al., 2003; Li et al., 2010; Nuruzzaman et al., 2006) that reported high amounts of citrate in the rhizosphere of pea and lupin, in our results only faba 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 phosphorus (Fox et al., 1990; Gerke, 1995). Below of 10 μmol g$^{-1}$ citrate or oxalate concentration in rhizospheric soil, the P mobilization is small or negligible (Gerke et al., 2000), in our experiment only oxalate reached that threshold. Among the intercrops the highest carboxylate

![Figure 3.8. Total carboxylates production, at limited phosphorus condition, in wheat rhizospheric soil plotted versus phosphorus concentration in intercropping (WIC) an sole crop (WSC) respectively indicated by (open) and with (closed symbols). Without phosphorus supply P concentration in wheat dry matter showed a linear and significant decrease as the carboxylates increased. Vales are means ± SE (n=12)](image)
concentration was found in faba but it was not associated with the improvement of wheat shoot P concentration or P uptake. In Pearse et al. (2006) and in Nuruzzaman et al. (2005a, b) rhizosphere of faba did not contain high concentrations of carboxylates and the authors stated that faba provided a beneficial effect to intercropped wheat attributing it to great root biomass accumulation. Similar positive effect exerted by faba was found in intercropping with maize (Li et al., 1999). Our results showed a higher root growth of faba than other legumes, not resulting in a roots intermingling suitable to facilitate intercropped wheat P uptake. In this conditions competition overcome the facilitation between fava and wheat, penalising the cereal growth.

Available phosphorus in soil
In all the treatments, available phosphorus (OLSEN-P) was greater with than without P supply due to the fertilization that exceeded P amount taken up by the plants. In our study OLSEN-P was generally higher in intercrop than in sole crop and this is proved greater wheat growth in IC than in SC. The highest value of soil available P was found in WP where wheat showed the best yield performance. Analysing the rhizospheric soil, some recent studies (Sun et al., 2019; Latati et al., 2014; Betancourt et al., 2012) agree with ours reporting an increase of P availability in other intercropping systems (cowpea and maize intercropping). As well as the results of Betancourt et al. (2012) obtained in a pot experiment, under controlled conditions by intercrop durum wheat with chickpea. In our experiment, results showed that studied legume species have an effect in mobilizing P from soil and not only in taking up it at very low concentrations in the soil solution (Hinsinger, 2001). In many cases, the increase of P availability promoted the growth of the plants generating 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 towards the root (Hinsinger, 2001).

Considering the total amount of P mobilized by the plant-soil system (Ptav), WP resulted the most efficient intercrop, taking up 32.23 and 34.75 mg of P from the soil and leaving 25.54 and 102.47 mg of P kg⁻¹ of soil respectively in P0 and in P1.

Dry matter and phosphorus accumulation by plant.
This study showed that intercropped wheat compared to sole crop resulted in a general increase of total and shoot dry matter both in P0 and in P1 without any detrimental effect on the intercropped legume. In WF differed significantly from other mixtures, without significantly limiting or improving wheat growth in intercropping. Our results confirmed the increase of biomass observed in other studies on cereals intercropped with lupin (Cu et al., 2005; Dissanayaka et al., 2015) and with pea (Bedoussac and Justes, 2010) and agree with the results obtained by Li et al., (2007) who reported no effect in maize intercropped with faba bean at low P level. Contrary to our results, in a field experiment (Song et al., 2007) the cereals yield increased in intercropping with faba bean showing an high variability among the years. The difference of total dry matter between IC and SC wheat was greater at low than at high P soil availability, according with the results from maize-lupin intercropping reported by Dissanayaka et al. (2015). Some authors linked the biomass accumulation, and consequently, the yield increase to the improved soil P availability (Betencourt et al., 2012; Li et al., 2007) but the direct relationship between P availability and P plant accumulation is arduous to demonstrate due to the combination of factors (e.g. water, nitrogen) that are involved in plant growth. On the other hand, our results showed that dry matter accumulation increased in wheat grown in intercropping compared to sole crop but that plant P concentration decreased. However, this decrease was less severe in wheat intercropped with pea than in other mixtures contributing to a greater P uptake.

Our results revealed that dry matter, more than P concentration, generally influenced P uptake. Wheat P uptake was greater in IC than in SC only in WP and in WL at P0, and in WP at P1. We can state that
only in WP an increase of P availability for wheat was obtained both with and without P supply, in the last condition only in WL this increase occurred, according to the stress-gradient hypothesis (Bertness, 1993). At P1, wheat P uptake was related to the increase of soil P availability (OLSEN-P) while at P0 wheat intercropped with pea showed the highest P uptake without difference of soil P availability compared to other IC. We assumed that soil sampled was not rhizospheric, consequently not involved by the P reduction of the rhizosphere caused by plant nutrition (Hinsinger, 2001). Therefore, the high wheat P uptake observed in WP and in WL at P0 and the greater biomass accumulation observed in wheat intercropped with pea and lupin cannot be explained only by the increase of P availability but other facilitations were involved.

3.4 Conclusions

In this study PME activity was generally high in all the treatments, due to the ability of phosphatases to persist in soils for long periods of time by binding to soil humics and clays. These results were obtained from bulk soil and for that reason this outcome would be in partial disagreement with those conducted on rhizosphere soil or in hydroponic system. PME activity and carboxylate exudation were mostly higher in legume-based systems than in wheat sole crop. Contrary to some previous studies, in our experiment both PME activity and carboxylate concentration increased with P supply but the decrease of PME root efficiency (PME activity per gram of root dry weight) was confirmed.

Wheat/pea intercropping resulted more efficient in mobilising phosphorus from the soil producing a noticeable benefit for intercropped wheat in terms of phosphorus uptake and growth. However, this combination of effects was not associated neither to the highest carboxylates release nor to the highest PME activity. On the contrary, the highest carboxylate accumulation was found in rhizosphere of intercropped faba but this condition did not facilitate the phosphorus uptake of wheat partner. However, soil available phosphorus was greater in intercropping than in sole crop and dry matter accumulation of intercropped wheat was mostly greater than sole crop.

Intercropped wheat compared to sole crop resulted in a general increase of total and shoot dry matter and our results revealed that dry matter, more than biomass P concentration, influenced P uptake. The increase of growth observed in intercropping was greater at limited phosphorus supply confirming the favourable effect of intercropping at limiting conditions (stress gradient hypothesis).

The beneficial effect on growth and on P uptake observed in intercropping cannot be completely explained by the increase of P availability in the soil, confirming the complexity of above ground interactions involved in the plant-plant facilitation and, consequently, the difficulty in explaining this type of interaction within the soil biota using only the cause-effect relationships between plant and soil.
3.5 Appendix

Table S 3.1. Phosphorus concentration (mg g⁻¹ dry matter) in shoot and root of wheat and legumes 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 corresponding to the different flowering of legumes. Values are mean ± SE (n=4; n=8). Significance of P for phosphorus (P), cropping treatments (CTR) and its interactions (P x CTR) by Anova are reported in italic.

<table>
<thead>
<tr>
<th>Cropping treatment</th>
<th>SHOOT</th>
<th></th>
<th>Legume</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wheat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P0</td>
<td>P1</td>
<td>Mean</td>
<td>P0</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td>0.45±0.05</td>
</tr>
<tr>
<td>L</td>
<td></td>
<td></td>
<td></td>
<td>0.56±0.03</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.56±0.04</td>
</tr>
<tr>
<td>W-F</td>
<td>1.59±0.33</td>
<td>1.62±0.28</td>
<td>1.61±0.18</td>
<td></td>
</tr>
<tr>
<td>W-L</td>
<td>1.45±0.18</td>
<td>1.75±0.35</td>
<td>1.60±0.20</td>
<td></td>
</tr>
<tr>
<td>W-P</td>
<td>1.30±0.21</td>
<td>1.87±0.45</td>
<td>1.59±0.13</td>
<td></td>
</tr>
<tr>
<td>WF</td>
<td>0.82±0.09</td>
<td>1.59±0.19</td>
<td>1.21±0.09</td>
<td>0.68±0.02</td>
</tr>
<tr>
<td>WL</td>
<td>0.94±0.08</td>
<td>1.13±0.08</td>
<td>1.04±0.07</td>
<td>0.54±0.07</td>
</tr>
<tr>
<td>WP</td>
<td>1.09±0.25</td>
<td>1.59±0.16</td>
<td>1.34±0.12</td>
<td>0.78±0.03</td>
</tr>
<tr>
<td>Mean</td>
<td>1.20±0.17</td>
<td>1.59±0.22</td>
<td>1.40±0.15</td>
<td>0.60±0.02</td>
</tr>
</tbody>
</table>

P  P<0.0001  P<0.0001
CS  P<0.0001  P<0.0001
P x CS  P<0.0304  P<0.0001

<table>
<thead>
<tr>
<th>Cropping treatment</th>
<th>ROOT</th>
<th></th>
<th>Legume</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wheat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P0</td>
<td>P1</td>
<td>Mean</td>
<td>P0</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td>1.12±0.22</td>
</tr>
<tr>
<td>L</td>
<td></td>
<td></td>
<td></td>
<td>0.31±0.04</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>1.37±0.27</td>
</tr>
<tr>
<td>W-F</td>
<td>0.63±0.08</td>
<td>0.74±0.07</td>
<td>0.69±0.04</td>
<td></td>
</tr>
<tr>
<td>W-L</td>
<td>1.21±0.28</td>
<td>1.90±1.22</td>
<td>1.55±0.18</td>
<td></td>
</tr>
<tr>
<td>W-P</td>
<td>0.60±0.05</td>
<td>0.44±0.02</td>
<td>0.52±0.05</td>
<td></td>
</tr>
<tr>
<td>WF</td>
<td>0.66±0.03</td>
<td>0.88±0.06</td>
<td>0.77±0.08</td>
<td>1.24±0.33</td>
</tr>
<tr>
<td>WL</td>
<td>0.66±0.04</td>
<td>0.73±0.05</td>
<td>0.69±0.09</td>
<td>0.82±0.09</td>
</tr>
<tr>
<td>WP</td>
<td>0.57±0.08</td>
<td>0.69±0.07</td>
<td>0.63±0.07</td>
<td>0.97±0.10</td>
</tr>
<tr>
<td>Mean</td>
<td>0.72±0.03</td>
<td>0.90±0.03</td>
<td>0.81±0.04</td>
<td>0.97±0.05</td>
</tr>
</tbody>
</table>

P  P<0.0001  P<0.0001
CTR  P<0.0001  P<0.0001
P x CTR  P=0.0304  NS
Can the increase of the root mass of grain legume improve the facilitating effect on wheat uptake in intercropping system

Interspecific competition for phosphorus may enhance the exudation of phosphatases from roots of the intercropped species. However, overlap of rhizospheres (depletion zone around roots) of the species is required for facilitation of immobile phosphorus to occur, which is not always the case. The aim of the study was to investigate in intercropping wheat/grain legume how an increase in legume density in mixture can stimulate phosphatase activity and whether it may also vary in relation to legume species. The hypothesis is that increasing root legume biomass in intercropping at P-limiting soil supply a greater overlap of the rhizospheric zones may occur (P-depletion zones) so an higher interspecific competition for P could enhance the exudation of phosphatases from roots of the intercropped legumes.

Material and methods

Plant growth and experimental design

Wheat (*Triticum turgidum* subsp. *durum* (Desf.) Husn. cv. Svevo) (W), lupin (*Lupinus albus* L. cv. Multitalia) (L), faba (*Vicia faba* var. minor Beck cv. Sikelia) (F) and pea (*Pisum sativum* L. cv. Hardy) (P) were grown in pots as sole crop (SC) and in intercropping (IC). The cropping treatments (CTR) resulted from the combination of the three legumes and wheat respectively grown in intercropping and sole crop. In order to sample at the flowering date of each legume, three wheat sole crops were also considered (W-F, W-L and W-P for faba, lupin and pea respectively). In this experiment, the wheat:legume sowing ratio of 1:1 (LD1), as used in intercropping in the previous experiment, was compared to 1:2 ratio (LD2), in which two legume plants were intercropped with wheat. Also in legume sole crops the sowing density was doubled. Crop treatments and sowing ratio were arranged in a factorial randomized block design with 4 replications. In addition, three bare soil treatments were added as a control for soil variables at the flowering time of faba (BS-F), lupin (BS-L) and pea (BS-P). The pots adopted were PVC tube Ø 14 cm and 30 cm tall filled with the soil mixed with perlite (80/20, v/v). The pots were placed in a climate chamber and grown until legumes flowering using the same growth parameters described in chapter 3 and shown in table 3.1.

Soil and plant sampling and analysis

The same soil of the experiment reported in chapter 3 was used and the its properties have been previous reported in table 3.2. At flowering time of each legume, the pots were destroyed and the plants and the soil were collected for the analysis. After, roots and shoots were placed in oven and dried at 70°C until dry weights were recorded. P concentration of root and shoot, was obtained digesting 100 mg of milled plant material with the mixture nitric and perchloric acid (6:1) (Johnson and Ulrich, 1959) and the digested was measured via molybdate method (Westerman, 1990) modified for Lambda Fias UV/VIS Spectrophotometer Perkin Elmer. After roots were gently removed from the pots, the soil was carefully mixed and sampled for the analysis in the laboratory. The samples were prepared and stored in three different way according to the specific analysis. An aliquot of the soil was frozen immediately after sampling, another aliquot was air dried and sieved at 2 mm while the last one was crushed to pass through a 500 µm sieve. The frozen soil was used for the measure of the phosphatase activity and the determination of ammonium and nitrate. The <2 mm fraction was used to determine soil pH and electrical conductivity (EC). While the <500 µm fraction was used for total organic C (TOC) and N (TN). Ammonium and nitrate were extracted by KCl 2 M solution from 5 g of fresh soil (Beemner and Keeney, 1966) and measured by Lambda Fias UV/VIS Spectrophotometer Perkin Elmer. Soil pH was measured in a 1:2.5 (w/v) soil: 0.01 M calcium chloride solution and soil EC was measured in a 1:2 (w/v) soil: water mixture, according to Sparks et al. (1996). Acid phosphatase activity was determined by the method proposed by Tabatabai and Bremner (1969) and modified by Hedley et al. (1982) according to which phosphatase in soil is expressed as production of para-nitrophenol (μmol h⁻¹). One g of soil was incubated, at 37 °C for 1 h, with para-nitrophenylphosphate in 4 ml of 0.04 M sodium maleate buffer (pH 6.5). After the reaction was
stopped with 1 M NaOH and the amount of para-nitrophenol released by phosphatase activity was measured via spectrophotometer as absorbance at 400 nm. As an index of available form, was used phosphorus extracted by Olsen method (Olsen et al. 1954), and measured via spectrophotometer using Lambda Fias UV/VIS Spectrophotometer Perkin Elmer. Organic P was obtained, according to Bowman (1989) and Kuo (1996) methods, as difference of ignited at 550 °C and no ignited soil sample H2SO4 extracts. Data were processed by ANalysis Of VAriance using a PROC GLM in SAS v. 9.2 (SAS, Institute Inc., Cary, NC, US, 2009) for the RCB design model to test for significance of treatments. For means comparison Tukey’s HSD test was performed.

4.2 Results

**Phosphatase activity, organic and available phosphorus in soil**

Phosphomonoesterase (PME) activity, measured at flowering time of each legume, significantly differed between cropping treatments (P < 0.001) and as average was 11.6 % significantly higher (P= 0.0009) in doubled legume density (LD2). ANOVA also did not highlight CTR x LD interaction effects. PME activity was in average 35.5, 29.5 and 7.2% larger than bare soil respectively in legume sole crop, intercropping and wheat sole crop. Bare soil at different sampling time showed similar values indicating that soil PME activity in this experimental conditions did not significantly varied over time. PME activity at LD1 was higher in legume sole crop (246.19) than legume intercropping (228.26 µmol pNPP g⁻¹ soil h⁻¹), on the contrary in LD2 it was greater in intercropping, particularly in WL (+34.5%) and WP (+14%). A higher PME activity in LD2 than LD1 was also shown in soil where pea

Figure 4.1 Phosphomonoesterase (PME) activity (µmol paranitrophenol g⁻¹ soil h⁻¹) in sole crop (W= wheat, F= faba; L=lupin; P =pea), intercropping (WF; WL; WP) and in bare soil (BS). -F, -L, and -P indicate the sampling time of wheat corresponding to the different flowering of legumes. Means (n=8; n=48) ± SE. PME activity at two phosphorus level (P0 and P1) is also drawn. Within the lower and upper cases, the different letters above each bar indicate significantly different values at P≤ 0.05; Tukey test)
was grown in sole crop (+21.6%). The lowest values in wheat sole crop at different sampling time were observed (208.26 µmol pNPP g⁻¹ h⁻¹ as average) (Fig. 4.1.). Only cropping treatments significantly (P=0.022) affected soil organic phosphorus. In all cropping treatments and bare soils values were similar ranging between 95.39 (pea sole crop) and 77.39 mg kg⁻¹ dry soil (W-F), in W-L organic phosphorus was significantly highest (116.9 mg kg⁻¹ dry soil) (Fig. 4.2.).

Figure 4.2. Organic and available (Olsen-P) phosphorus (mg P kg⁻¹ dry soil) in wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP). -F, -L, and -P indicate the sampling time of wheat at the different flowering of legumes. Values are means ± SE (n=8; n=24). The different letters above each bar indicate significantly different values at P≤ 0.05; Tukey test)

Available phosphorus (OLSEN-P) showed significant differences among cropping systems (P=0.0469) as well as between the two sowing ratios (P=0.0462). In bare soil OLSEN-P showed high and similar values on the three sampling dates. It was negatively influenced (on IC and SC average -24.2%) by the increased sowing legume density. Between crop treatments highest significant values were observed in wheat sampled at pea flowering time and in pea sole crops. The average OLSEN-P soil content in the cropping treatments both at LD1 and at LD2 which did not significantly differ from the bare soil. (Fig.4.2).
Phosphorus concentration in shoot and root dry matter

Compared to 1:1 sowing ratio, at 1:2 the shoot P concentration significantly increased both in intercropped legume (P=0.009) and wheat (P=0.0021) by +48.3 % and +37.4 respectively. On the contrary in root, P concentration significantly decreased in both species, resulting significantly lower in wheat (-26.1% on average) at LD2. Interaction between LD x CTR only in shoot was observed, both in wheat (P=0.0387) and legume (P<0.0001). Shoot P content in intercropped wheat showed a larger increase at LD2 compared to LD1 sowing ratio, reaching +97.0% in WF. In supplementary table S4.1 data are reported in detail.

The effect of the LD2 was contrasting in legumes by showing a decrease of -42.9 and -35.0 % respectively in intercropped faba and lupin; conversely, a very strong increase in pea, both in intercropping (+181%) and in sole crop (+239%) was observed. (Fig.4.3).

Dry matter accumulation and partitioning

Among crop treatments significantly highest values of root dry matter (RDM) in faba both in intercrop and sole crop were observed, intercropped lupin showed significant lowest value. ANOVA results also showed a high significant (P<0.0001) negative effect of LD2 on RDM in the legume resulting in an average decrease of -35.5% and this effect was similar on faba and lupin both in intercropping and in sole crop. However, the low RDM decrease (-4.0%) presented by the pea in both growth systems should be marked in LD2 where pea showed the highest values.

Among crop treatments, pea showed the highest values of shoot dry matter (SDM) that were not significantly different between intercropping and sole crop. Faba and lupin showed the lowest values in intercropping and sole crop respectively. In general, adopting LD2 did not result in a significant average effect on SDM in the legumes. However a considerable increase in LD2 was obtained from intercropped lupin (+29.8) and intercropped pea (+25.0%). SDM in pea sole crop also increased (+11.5%). Significant effects of interaction LD x CTR on SDM were found in wheat (P=0.002). In wheat intercropped with faba and lupin SDM increased at LD2 compared to LD1 by +54.7 and +32.7% respectively. On the contrary in intercropping with pea a large decrease in wheat (-45.3%) was highlighted. In supplementary table S4.2 data are reported in detail.

Considering the relative dry matter of each partners in mixture, calculated as intercropping/sole crop ratio, average values > 1 in SDMA were observed, but higher in wheat (2.45) than in legumes (1.10). However, the two partners did not differ in root RDMA and their respective average values were lower than unit (0.96 and 0.99).
The relative dry matter of intercropped wheat showed on average slight variations between LD1 and LD2, increasing in shoot and decreasing in root. In intercropped legume, an average increase of relative dry matter only in shoot was observed, larger than in wheat. It should be noted that in WP intercropping at LD2 the wheat SDMA suffered a significant reduction compared to LD1. On the contrary in WF wheat showed a significant increase (Fig. 4.5).

**Phosphorus uptake**

In general, by increasing the legume sowing density in crop treatments, average increase of phosphorus plant uptake was observed and it was significant (P<0.0001) both in legume (20.41 and 28.39 mg plant\(^{-1}\) in LD1 and LD2 respectively) and wheat (23.00 and 31.20 mg plant\(^{-1}\) in LD1 and LD2 respectively). However, the significant (P<0.0001) increase at LD2 occurred only in the legume shoot (+86.7%), on the contrary a significant decrease (-44.2%) in root was found. Among the crop treatments, pea showed the significantly (P<0.0001) higher values of phosphorus plant uptake both in intercropping and in sole crop (49.42 and 41.44 mg plant\(^{-1}\) respectively) whereas in lupin the lowest values were observed (11.10 and 11.63 mg plant\(^{-1}\) respectively). In pea most of the amounts of phosphorus adsorbed by plant was found in shoot, both in mixture (85%) and in sole crop (82%). The
shoot P uptake fraction were lowest in faba (51% and 59% in sole and intercropped plant respectively). [Data on shoot and root P uptake partitioning in Table S 4.3 are reported]

Figure 4.7. Phosphorus relative uptake of wheat and legumes grown in mixtures, calculated as intercrop/sole crop ratio (IC/SC), both at one-plant (open symbols) and at two-plant (closed symbols) legume densities. Values are means ±SE (n = 4). The vertical and horizontal lines represent all the points where dry matter accumulation in IC is equal to SC.

The adoption of 1:2 sowing ratio in mixture resulted in a significant average increase in phosphorus uptake by wheat (39.44 mg plant\(^{-1}\) in LD2), that was 45\% higher than LD1. The P uptakes of wheat intercropped both with lupin and faba bean were doubled in LD2 compared to LD1, whereas in wheat intercropped with pea P uptake was 18\% lower. In the same WP mixture, pea increased significantly (+149\%) the P uptake at LD2, while intercropped faba bean and lupin showed a decrease of 53 and 34\% respectively in LD2 compared to LD1. The effects of the increased sowing density on legumes grown in sole crops were quite different among the three species, resulting in an even more marked increase of pea in LD2 than in intercropping (+21\%), while less evident were the differences between LD1 and LD2 in lupin and faba bean. (Fig. 4.6.).

The relative phosphorus uptake (RPU) of wheat and legumes in intercropping, plotted in figures 4.7, varied more in wheat than in legumes. The relative legumes uptake in all three mixtures was lower in LD2 than in LD1, showing values < 1. Intercropped faba (1.42) and lupin (1.15) exceeded their respective sole crop while pea showed a similar uptake. The RPU in WF and WL were higher in LD2 (2.15 and 2.37 respectively) than in LD1, whereas in WP at LD1 (2.45) the absolute highest value was observed.

The relative legumes uptake in all three mixtures was lower in LD2 than in LD1, showing values < 1. Intercropped faba (1.42) and lupin (1.15) exceeded their respective sole crop while pea showed a similar uptake. The RPU in WF and WL were higher in LD2 (2.15 and 2.37 respectively) than in LD1, whereas in WP at LD1 (2.45) the absolute highest value was observed.

4.3 Discussion

PME activity observed in our experiment was considerably higher compared to the studies conducted on inert substrate (Olde Venterink, 2011) but comparable to others carried out on natural soil from the field (Nuruzzaman, et al., 2006). The high PME activity rate generally found in our study is explained by the persisting of phosphatases in soil for long time by binding to soil humics and clays (Burns,
1982; Sinsabaugh, 1994; Rojo et al., 1990 in Olander and Vitousek 2000). Furthermore, PME activity in bare soil did not result to vary significantly over the flowering sampling dates of legume, supporting the persisting of phosphatases over time. However, despite the high PME activity rate observed, the differences among the treatments were significant. Our results on PME activity confirmed the higher importance of legumes compared to cereals (Olde Venterink, 2011) and the major role of lupin (Todano et al., 1993), followed by faba bean and pea (Nuruzzaman et al., 2006). An interesting result is that pea, despite the great root growth and the high phosphorus uptake, in SC depleted less available phosphorus compared to other legumes. At LD1, according with Latati and Blavet (2014), Olsen-P was less depleted in WF and WL than in the respective SC, due to a complementary use of the resources (Hinsinger et al., 2011).

Intercropping enhanced phosphorus concentration only at high legume density and at the associated high PME activity. The results showed that at 1:1 sowing ratio, the phosphorus uptake facilitation by legume was still too small compared to interspecific competition in mixture. Increasing the legumes in mixture, PME activity increased and intraspecific competition occurred, limiting legume but not wheat phosphorus uptake in mixture (Fig.4.8). In WL and WF legume facilitations occurring at 1:2 sowing ratio overcomes the existing effects of interspecific competition (observed at 1:1 sowing ratio). In pea, the limited differences of DM between LD 2 and LD 1, suggests a very low belowground intraspecific competition, which did not counteract the effects of the interspecific facilitation in intercropping at 1:1 sowing ratio. Interspecific competition occurring between wheat and pea in intercropping at 1:2 sowing ratio resulted in a high detrimental effect on wheat, also masking possible facilitating effects of the legume with respect to the cereal.

On the contrary, lupin and faba bean root dry weight drastically decreased at LD2 in comparison with LD1. In pea-based systems, the increase of total root biomass did not corresponded to a proportionally PME activity increase, on the contrary in faba bean- and lupin-based systems the slight increase of PME activity was in accordance with the slight total root dry weight increase observed doubling the number of plants in LD2. Wheat phosphorus uptake resulted linked to PME activity because the greater uptake observed at LD2 in intercropping compared to sole crop corresponded to a PME

Figure 4.8. Phosphomonoesterase (PME) activity (μmol paranitrophenol g⁻¹ soil h⁻¹) plotted versus phosphorus uptake (g plant⁻¹) of wheat intercropped with faba (WF), lupin (WL) and pea (WP) at 1:1 (open symbol) and 1:2 (closed symbol) sowing ratios. Values are means ±SE (n=4).
activity improvement. We can assume that phosphatase activity of intercropped legumes increased the mineralization of organic phosphorus and its absorption by the intercropped wheat. This occurred in WL and WF at LD2 where higher values of wheat phosphorus uptake and PME activity were observed, on the contrary in WP, growth and phosphorus uptake of wheat were limited by the competition with pea. This assumption was supported by differences in uptake between intercropped legumes at the two sowing ratios. In fact, at LD2, two pea plants resulted a huge sink, increasing by 5-folds P uptake and penalizing the intercropped wheat. Indeed, the high P uptake detected in pea at LD2 was linked to its root proliferation that, contrary to lupin and faba bean, did not decrease at high legume density, with a greater volume of soil explored. In fact, the results suggested that P uptake observed in pea was more a consequence of great soil exploration by root than PME activity. Our results are in discordance with Nuruzzaman et al. (2005, 2006) who found the highest P uptake in faba bean and explained this finding by the larger root dry matter observed in the legume. Also the high dry matter observed in pea is in discordance with Nuruzzaman et al. (2005, 2006) who reported the greater growth of faba bean and lupin in comparison with pea. Focusing on the effect of intercropping on wheat P uptake, the improvement observed in WP, at LD1 and in WL and in WF, at LD2 supported the hypothesis that in intercropping facilitations occurred as a result of the ability of the legumes to increase soil P availability is a benefit for the intercropped species (Callaway, 2007). The increase of P availability cannot explain entirely the benefit generated in intercropped wheat but other facilitations occurred and the increase of PME activity explained partially the increase of P uptake.

4.4 Conclusion

From the results previously described it can be stated that varying from 1:1 to 1:2 sowing ratio in the wheat/grain legume mixtures affects plant growth and phosphorus uptake in both partners and modify available phosphorus in the soil through the PME activity. The most interesting finding is that phosphorus uptake increased in wheat intercropped with faba bean and lupin at 1:2 sowing ratio without great detrimental effect for the intercropped legumes. On the contrary, in pea/wheat mixture the increase of sowing ratio strongly negative affected wheat P uptake (-50%) but by five times increased pea P uptake. Pea grown at sole crop took up more P in LD2 than in LD1, showing a lower intraspecific competition compared to other tested legumes and a slightly interspecific competition, supported by the negative effect on intercropped wheat growth, both of shoot and root. At LD2, despite the slight decrease of Olsen P measured in soil, in WP corresponded a great P uptake in the legume (127 mg by the two legumes) while the cereal showed a very low P uptake (17 mg/plant). The reduction of wheat P uptake observed in WP at LL was the result of the limited growth of the cereal, then the higher P concentration observed in wheat tissue can be interpreted as consequence of concentration effect.
Table S4.1. Dry matter (g plant\(^{-1}\)) in shoot and root of wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and respective intercropping (WF; WL; WP) at two legume plant density (LD1 and LD2). -F, -L, and -P indicate the sampling time of wheat sole crop at the different flowering of legumes. Values are means ± SE (n=4; n=8). Significance of P for sowing ratios (LD), cropping treatments (CTR) and its interactions (LD x CTR) by Anova reported in italic.

<table>
<thead>
<tr>
<th>Cropping treatments</th>
<th>SHOOT</th>
<th>Legume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wheat</td>
<td>LD1</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W-F</td>
<td>13.01±2.13</td>
<td>12.23±2.02</td>
</tr>
<tr>
<td>W-L</td>
<td>9.86±0.72</td>
<td>9.85±0.90</td>
</tr>
<tr>
<td>W-P</td>
<td>7.13±1.08</td>
<td>7.17±0.94</td>
</tr>
<tr>
<td>WF</td>
<td>17.97±3.04</td>
<td>27.80±5.16</td>
</tr>
<tr>
<td>WL</td>
<td>22.31±5.04</td>
<td>29.61±6.41</td>
</tr>
<tr>
<td>WP</td>
<td>25.96±3.25</td>
<td>14.20±3.12</td>
</tr>
<tr>
<td>Mean</td>
<td>17.28±3.14</td>
<td>18.21±3.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Legume Mean</td>
<td>21.13±2.08</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>16.10±1.54</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>28.27±1.97</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>LD</td>
<td></td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>CTR</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>LD x CTR</td>
<td></td>
<td>P&lt;0.002</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cropping treatments</th>
<th>ROOT</th>
<th>Legume</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wheat</td>
<td>LD1</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W-F</td>
<td>7.10±0.84</td>
<td>7.12±0.83</td>
</tr>
<tr>
<td>W-L</td>
<td>7.03±0.71</td>
<td>7.01±0.43</td>
</tr>
<tr>
<td>W-P</td>
<td>6.58±0.55</td>
<td>6.55±0.19</td>
</tr>
<tr>
<td>WF</td>
<td>6.41±1.08</td>
<td>6.30±0.71</td>
</tr>
<tr>
<td>WL</td>
<td>7.02±1.00</td>
<td>6.78±0.25</td>
</tr>
<tr>
<td>WP</td>
<td>6.71±1.17</td>
<td>6.27±0.31</td>
</tr>
<tr>
<td>Mean</td>
<td>6.81±0.95</td>
<td>6.67±0.47</td>
</tr>
<tr>
<td></td>
<td>Legume Mean</td>
<td>9.12±0.18</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>8.59±0.16</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>6.36±0.14</td>
</tr>
<tr>
<td>LD</td>
<td></td>
<td>P=0.004</td>
</tr>
<tr>
<td>CTR</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>LD x CTR</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>
Table S4.2. Phosphorus concentration (mg P kg⁻¹ dm) in shoot and root of legume and wheat grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and respective intercropping (WF; WL; WP) at two legume plant density (LD1 and LD2). -F, -L, and -P indicate the sampling time of wheat sole crop at the different flowering of legumes. Values are means ± SE (n=4; n=8). Significance of P by Anova reported in italic for sowing ratios (LD), cropping treatments (CTR) and its interactions (LD x CTR).

<table>
<thead>
<tr>
<th>Legume</th>
<th>Shoot</th>
<th>Root</th>
<th>Shoot</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD1</td>
<td>LD2</td>
<td>Mean</td>
<td>LD1</td>
</tr>
<tr>
<td>F</td>
<td>0.41 ±0.08</td>
<td>0.42 ±0.08</td>
<td>0.41 ±0.05</td>
<td>1.17 ±0.23</td>
</tr>
<tr>
<td>L</td>
<td>0.56 ±0.03</td>
<td>0.54 ±0.35</td>
<td>0.55 ±0.16</td>
<td>0.36 ±0.09</td>
</tr>
<tr>
<td>P</td>
<td>0.60 ±0.17</td>
<td>2.03 ±0.13</td>
<td>1.31 ±0.29</td>
<td>1.36 ±0.19</td>
</tr>
<tr>
<td>WF</td>
<td>0.68 ±0.12</td>
<td>0.39 ±0.07</td>
<td>0.53 ±0.08</td>
<td>1.24 ±0.25</td>
</tr>
<tr>
<td>WL</td>
<td>0.54 ±0.08</td>
<td>0.35 ±0.11</td>
<td>0.45 ±0.07</td>
<td>0.62 ±0.35</td>
</tr>
<tr>
<td>WP</td>
<td>0.78 ±0.11</td>
<td>1.63 ±0.11</td>
<td>1.21 ±0.18</td>
<td>0.97 ±0.14</td>
</tr>
<tr>
<td>Mean</td>
<td>0.60 ±0.05</td>
<td>0.89 ±0.15</td>
<td>0.74 ±0.08</td>
<td>0.95 ±0.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Wheat</th>
<th>Shoot</th>
<th>Root</th>
<th>Shoot</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD1</td>
<td>LD2</td>
<td>Mean</td>
<td>LD1</td>
</tr>
<tr>
<td>W-F</td>
<td>1.34 ±0.29</td>
<td>1.33 ±0.25</td>
<td>1.34 ±0.18</td>
<td>0.63 ±0.07</td>
</tr>
<tr>
<td>W-L</td>
<td>1.45 ±0.10</td>
<td>1.43 ±0.06</td>
<td>1.44 ±0.05</td>
<td>0.92 ±0.29</td>
</tr>
<tr>
<td>W-P</td>
<td>1.30 ±0.11</td>
<td>1.32 ±0.07</td>
<td>1.31 ±0.06</td>
<td>0.60 ±0.17</td>
</tr>
<tr>
<td>WF</td>
<td>0.78 ±0.18</td>
<td>1.53 ±0.23</td>
<td>1.16 ±0.19</td>
<td>0.78 ±0.16</td>
</tr>
<tr>
<td>WL</td>
<td>0.94 ±0.14</td>
<td>1.56 ±0.17</td>
<td>1.25 ±0.16</td>
<td>0.66 ±0.16</td>
</tr>
<tr>
<td>WP</td>
<td>1.09 ±0.26</td>
<td>1.76 ±0.10</td>
<td>1.70 ±0.34</td>
<td>0.57 ±0.08</td>
</tr>
<tr>
<td>Mean</td>
<td>1.15 ±0.09</td>
<td>1.58 ±0.12</td>
<td>1.37 ±0.08</td>
<td>0.69 ±0.07</td>
</tr>
</tbody>
</table>

| LD     | P<0.0001 |     |     |
| CTR    | P<0.0001 | NS  | NS  |
| LDxCTR | P<0.0001 | NS  | NS  |
Table S. 4.3. Phosphorus uptake (mg) in shoot and root of wheat and legume grown in sole crop (W= wheat, F= faba; L= lupin; P = pea) and respective intercropping (WF; WL; WP) at two legume plant density (LD1 and LD2). -F, -L, and -P indicate the sampling time of wheat sole crop at the different flowering of legumes.

<table>
<thead>
<tr>
<th>Cropping treatments</th>
<th>Shoot</th>
<th>Wheat</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD1</td>
<td>LD2</td>
<td>Mean</td>
</tr>
<tr>
<td>WF</td>
<td>13.529</td>
<td>2.69</td>
<td>41.430</td>
</tr>
<tr>
<td>WL</td>
<td>18.972</td>
<td>6.68</td>
<td>46.240</td>
</tr>
<tr>
<td>WP</td>
<td>28.672</td>
<td>7.53</td>
<td>25.048</td>
</tr>
<tr>
<td>Mean</td>
<td>18.322</td>
<td>2.48</td>
<td>27.755</td>
</tr>
</tbody>
</table>

| LD | P<0.001 | P<0.001 | P<0.001 |
| CT | P=NS    | P=NS    | P=NS    |
| LD x CT | P=0.0024 | P=0.0024 | P=0.0024 |

<table>
<thead>
<tr>
<th>Legume</th>
<th>Shoot</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD1</td>
<td>LD2</td>
</tr>
<tr>
<td>F</td>
<td>8.602</td>
<td>1.90</td>
</tr>
<tr>
<td>L</td>
<td>9.092</td>
<td>2.95</td>
</tr>
<tr>
<td>P</td>
<td>16.539</td>
<td>3.97</td>
</tr>
<tr>
<td>WF</td>
<td>15.420</td>
<td>0.41</td>
</tr>
<tr>
<td>WL</td>
<td>9.155</td>
<td>2.55</td>
</tr>
<tr>
<td>WP</td>
<td>19.115</td>
<td>1.90</td>
</tr>
<tr>
<td>Mean</td>
<td>12.987</td>
<td>1.26</td>
</tr>
</tbody>
</table>

| LD | P<0.0001 | P<0.0001 | P<0.0001 |
| CT | P<0.0001 | P<0.0394 | P<0.0005 |
| LD x CT | P<0.0001 | P<0.0001 | P<0.0001 |
5 Intercropping affects structure of soil bacterial communities

Phosphorus (P) is the second most crucial mineral element for plant growth and development (Alori et al. 2017), being present in several key biological molecules (Elser, 2012). However, due to its high retention to soils, it is little available for absorption by plants (Shen et al. 2011). Kochian (2012) pointed out that many agricultural soils belong to regions where phosphorus retention is high. Thus, modern agriculture is dependent on phosphorus derived from rock phosphate, which is a non-renewable resource that could be exhausted in 50-100 years (Cordell et al. 2009). As a way of circumventing this problem, research has been made towards the use of microorganisms to change phosphorus availability either via mineralization or solubilization (Richardson; Simpson, 2011) and screening for phosphate-solubilizing microorganisms (Alori et al., 2017); the use of phosphorus-mobilising plant species which improve P nutrition for themselves and for other plants (Faucon et al., 2017) and the use of cereal-legume intercropping to increase P uptake (Xue et al., 2016). It has been shown that legume intercropping can improve the mobilization of several macronutrients and micronutrients in the rhizosphere of different crops (Cu et al., 2005; Hinsinger et al., 2011). Lupin is able to form root clusters that allow plants to grow in soils where P is low or unavailable and they might even benefit other crops (Lambers and Shane, 2007). Intercropping wheat with lupin increased uptake of phosphorus by wheat, without significantly affecting the growth or uptake of P by lupin (Cu et al. 2005). It is reported in many studies (Kowalchuck et al. 2002, Marschner et al. 2004, Costa et al. 2005, Garbeva et al. 2008, Berg and Smalla 2009, Lundberg et al. 2012) that plants harbor specific bacterial community in their rhizosphere. The aim of this experiment is to describe any changes in rhizospheric bacterial community exerted in intercropping by the legume root exudation, evaluating the abundance of phosphate solubilizing bacteria (PSB) and plant growth-promoting rhizobacteria (PGPR) at P limited conditions with the purpose of better understanding the role of soil bacteria in the facilitative interaction generated by legumes in intercrop.

5.1 Material and Methods

Plant growth, soil preparation and addition of P treatments

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 a pot experiment as sole crop (SC) and as intercrop (IC) combining each legume species with the cereal in a pot. Pots containing bulk soil were also added as a control. The soil used for the experiment was from the exhausted land experiment at Rothamsted Research (Harpenden, Hertfordshire, UK): from the plot 054 (-P soil) and plot 071(+P soil) with respectively 3.8 mg and 26.4 mg Olsen P/kg of soil. After sampling, the soil was air dried and sieved using a 4 mm sieve. In order to achieve different P availability levels, -P and +P soils were amended with 100 P mg/kg soil as KH₂PO₄ and Ca₃(PO₄)₂ obtaining the following four treatments: unav P (-P soil amended with Ca₃PO₄), ava P (-P soil amended with KH₂PO₄), no P (-P soil with no phosphate amendment) and NPK (+P soil with no phosphate amendment ).

The seeds were surface sterilized with 70% ethanol for 30 seconds and with 1.25% active chlorine for 20 minutes and washed five times under gentle shaking with sterile water. Before sowing, the seeds were imbibed overnight and germinated in aseptic conditions. One or two (in the IC plant treatment) same size seedlings were transplanted in each 1 l pots filled with soil, mixed with perlite (66/33, v/v). One pot for each soil treatment was added to the experiment and analysed after watering as time zero (T0). After transplanting the plants were transferred in a greenhouse at controlled environmental conditions. The plants grew at 21.5 °C with 8/16 hours darkness/light photoperiod, supplemented by artificial light, at 60 % of relative humidity and watered by sprinkler irrigation.
Four, five and six weeks after transplanting the pots were fertilized by fertigation, with 18 mg N/kg of substrate, not watering the leaves. This, limited legumes nodulation and the differences of N availability between wheat grown in intercropping and in sole crop, ensured that the plant growth was limited only by P.

After 62 days of growth, shoots were harvested and dried for 72 h at 80 °C for dry weight, while the pot with the soil has been collected for DNA extraction and chemical analysis.

Soil sampling
During the sampling, for each pot the entire roots with adhering soil were collected from all the plants grown, taking both the connected partners in IC without split them into two samples. The rhizosphere soil was carefully shacked off from the samples so that only the tightly bound soils attached will be used for rhizoplane extraction. The roots were transferred to a 50 ml screw-cap tube (falcon tube), and thirty milliliters of sterile water were added. The falcon tube was shacked on the flatbed shaker at 4 °C for 10 mins at full speed. After removing the roots, the tubes were centrifuged for 5 min at 4,000 rpm and the roots discarded. After that, most of the supernatant was removed living five milliliters of water in the tube. The rhizoplane soil was re-suspended in the tube using Vortex and 1.5 milliliters of the suspension were transferred in a 2 ml microfuge tube. The microfuge tube was centrifuged at full speed in a microfuge for 2 mins and the supernatant was removed. The pellet (rhizoplane) was stored at -80°C until the DNA extraction.

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 but with the use of the MP Biomedicals FastPrep-24 machine twice for 30 s at 5.5 m.s⁻¹. DNA purity and concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) as well as a Qubit 2.0 Fluorimeter using ds DNA HS assay kit (Thermo Fisher).

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, and subjected to Illumina® sequencing using the MiSeq platform to generate 2 x 300 bp paired-end reads at Novogene (China). 16S rRNA gene sequences were analyzed using the pipeline proposed 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 package phyloseq (McMurdie; Holmes 2013) was used for downstream analysis. Eukaryotes and three outliers were removed from the dataset. Data were normalised using proportions (Total Sum Scaling (TSS)) method, which according to McKnight et al. (2019), outperformed other normalization methods such as CSS, DESeq- VS, edgeR-TMM.

Analysis of differentially abundant OTUs
The online tool for comprehensive statistical, visual and meta-analysis of microbiome data called Microbiome Analyst (Dhariwal et al., 2017) was used for detecting features that were differentially abundant between different plant species, using Random forest analysis. Random Forest (RF) is a supervised machine-learning algorithm that has been applied to microbiome data to identify microbial taxa that differentiate between phenotypes (72, 73). The filtered feature table was arranged as the required format and it was uploaded with the mapping and taxonomy files. Low abundance and low variance features were removed using default values, where features with less than 2 counts in less
than 20% of the samples and 10% of the values below the determined inter-quantile range (IQR) were removed.

5.2 Results

Main factors driving differences in bacterial community structure

Overall, bacterial communities were affected primarily by the type of sample (bulk soil, lupin rhizosphere, lupin-wheat rhizosphere, wheat rhizosphere, pea rhizosphere and pea-wheat rhizosphere) (Figure 1A; ADONIS, $R^2 = 0.22539, p = 0.001$), followed by treatment (available P, no P, NPK and unavailable P) (Figure 1A; ADONIS, $R^2 = 0.13476, p = 0.001$) and niche (bulk soil x rhizosphere) (Figure 1A; ADONIS, $R^2 = 0.13245, p = 0.001$). Significant interactions between treatment and type (ADONIS, $R^2 = 0.11209, p = 0.001$) and treatment and niche were observed (ADONIS, $R^2 = 0.03291, p = 0.001$). As “type” was the main factor, PCoA plots were constructed for each rhizosphere type to check 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 (Figure 1B), 46.08% of total variability in bacterial composition is explained by different P treatments (ADONIS, $p = 0.001$) and 40.28%, 41.96%, 42.29% and 44.02% of total variability in bacterial communities of lupin-wheat, wheat, pea and pea-wheat is explained by different P treatments, respectively (Figures 1C, 1D, 1E and 1F) (ADONIS, $p = 0.001$).
Differentially abundant taxa present in each P treatment

Different P treatments affected differential abundance of taxa in the rhizosphere of the tested crops (Figure 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 (Figure 2A and 2E, respectively). In the case of P being unavailable for plant absorption, *Variovorax* was enriched in the rhizosphere of lupin-wheat and pea (Figure 2B and 2D) and *Pseudomonas* was enriched in the rhizosphere of pea (Figure 2D). P, when added in the available form, increased the abundance of several genera, such as *Xanthomonas* in the rhizosphere of lupin-wheat and pea (Figure 2B and 2D), *Lentzea* in pea and in pea-wheat rhizosphere (Figure 2D and 2E), *Saccharothrix* and *Pseudonocardia* in pea-wheat rhizosphere (Figure 2E). In NPK soil, *Catenulispora*, *Leifsonia* and *Arthrobacter* were enriched in lupin and lupin-wheat rhizosphere (Figure 5.2A and 5.2B) *Pedobacter* was enriched in wheat, pea and pea-wheat (Fig. 5.2C, 5.2D and 5.2E).
Figure 5.2. 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.
5.3 Discussion

Main factors driving differences in bacterial community structure

Our results are in accordance with the concept that different plants harbor specific bacterial community in their rhizosphere (Kowalchuck et al. 2002, Marschner et al. 2004, Costa et al. 2005, Garbeva et al. 2008, Berg and Smalla 2009, Lundberg et al. 2012) and that plants are the primary selective factors for microbial community composition in soil (Garbeva et al. 2004, Marschner et al. 2004, Costa et al. 2005, Badri and Vivanco 2009). Root exudates are the main factor that influences the rhizosphere microbiome structure (Badri et al., 2013; Shi et al., 2011), altering the rhizosphere environmental conditions and offering nutrient sources for microbial growth. There is a variety of compounds exuded by roots, maximally organic acids and sugars, but also amino acids, fatty acids, vitamins, growth factors, hormones and antimicrobial compounds (Bertin et al., 2003). Separating the data by type of sample (crop treatments), bacterial community from NPK treatment was different from the microbial community of other treatments. The soil used in NPK treatment is from a long-term experiment that was amended with a regular amount of nitrogen, phosphorus and potassium every year. The microbial community may have been selected by the systematic availability of the nutrients, differenting from the other treatments where P depletion was the selective factor during the years. It is reported the effect of long-term organic or inorganic amendment applications on the structure of bulk soil microbial communities (Chen et al., 2016; Ding et al., 2016; Francioli et al., 2016; Soman et al., 2017). In particular, high levels of inorganic nitrogen fertilizers negatively affect bacterial richness and diversity (Kavamura et al., 2018). The differences between bacterial community from no P and unavailable P community were low in all the types of samples (crop treatments). The results showed that P, supplied in the unavailable form (tricalcium phosphate), slightly affected the microbial community that was similar to no P supply. Only in lupin rhizosphere, this separation was more evident, maybe due to its greater ability to lower pH and to release carboxylates compared to wheat and pease when a calcium phosphate source was added (Pearse et al., 2007). Adding available P as KH2PO4, the bacterial community of pea-based systems and wheat sole crop rhizosphere, differed to the other community. The results support the idea that the availability of P, influenced root biomass and the release of root exudates that shaped the microbial community (Wasaki et al., 2018).

Differentially abundant taxa present in each P treatment

When P was added as tricalcium phosphate (unavailable P) or was not added to soil there was an enrichment of some genera considered plant growth-promoting rhizobacteria (PGPR) in the rhizosphere of legumes and wheat-legume intercrop. Variovorax was enriched in lupin rhizosphere when no P was added and in the rhizosphere of lupin-wheat and pea (Figure 2B and 2 D) when P being unavailable for plant absorption (Figure 2A). It is reported that the inoculation of Variovorax paradoxus increased root and shoot biomass of pea (Jiang et al., 2012) and had positive effects on foliar N, Ca, S, and Fe concentrations, but not on foliar P and K concentrations (Safronova et al., 2006). The inoculation of Variovorax paradoxus isolate on wheat (Triticum aestivum L.) produced higher crop yields and significantly higher N, P, K Ca2+ and Na contents (straw and grain) (Chandra et al., 2019). Variovorax sp. is considered useful to alleviate abiotic stress such as water stress due to the production of 1-amincyclopropane-1-carboxylate (ACC) deaminase (Glick et al. 2007; Arshad et al. 2008; Zahir et al. 2008; Shakeri et al. 2012; Naveed et al. 2014) and other plant growth-promoting properties (e.g. siderophore production and phosphate solubilisation) (Chandra et al., 2019; Kurth et al., 2016). P solubilizing ability of species from the genus Variovorax is supported by many studies (Collavino and Sansberro, 2010; Zheng et al., 2018). These bacteria are known specifically to colonize root tissues and to interact with plants through exchange signaling molecules and utilize readily secreted compounds (Haichar et al., 2008). Variovorax may be considered as specialist, found in species such as rape (Haichar et al., 2008) and Avena barbata responding to plant growth with the
increase of relative abundance (Zhalnina et al., 2018). In this study pea rhizosphere, was enriched by *Pseudomonas* in plants grew as sole crop when P was added in the unavailable form and in intercrop when no P was added. That is in agreement with the reduction of the relative abundance of *Pseudomonas* in rhizosphere observed in some study (Chhabra et al., 2013; Tan et al., 2013) as a result of P fertilization (Ca \([\text{H}_2\text{PO}_4]_2\)). The ability of certain species of *Pseudomonas* to solubilise P has been proved for a long time in vitro (Illmer and Schinner, 1992; Collavino and Sansberro, 2010; Baliah and Begum, 2015) and in vivo as capacity to improve plant P uptake (Lifshitz et al., 1987; Afzal et al., 2005; Zabihi et al., 2011; Israr et al., 2016). As other phosphate solubilising bacteria (PSB), that ability is due to the release of organic acids (Vyas et al., 2009; Rashid et al., 2004; Trivedi and Sa, 2008) and of acid and alkaline phosphatases (Rodríguez and Fraga, 1999; Krey et al., 2011). Several *Pseudomonas* strains, as well as phosphorus solubilizing bacteria, are considered also interesting plant growth-promoting rhizobacteria (PGPR) improving growth, yield (Gamez et al., 2019; Pal et al., 2016; Zarei et al., 2019) and nutrients uptake, tolerance to biotic (Sahu et al., 2018) and abiotic stress (Singh et al., 2019) in plants.

In our experiment, when no P was added, the abundance of *Bradyrhizobium* in pea-wheat rhizosphere increased in combination with *Pseudomonas*. Some species from the *Rhizobiaceae* are interesting not only because they perform biological nitrogen fixation in association with legumes but certain free-living members of this family can also be considered as PGPR (Boiero et al., 2006; Antoun et al., 1998). *Bradyrhizobium japonicum* is considered to have plant growth-promoting capacity (Cassan et al., 2009) through siderophore production, phosphorus solubilization and IAA production (Antoun et al., 1998). Our results showed an increase in the abundance of *Bradyrhizobium* in pea-wheat rhizosphere in combination with *Pseudomonas*. It is reported that co-inoculation of *Pseudomonas* and *Bradyrhizobium* significantly increased phosphorus content and improved growth in *Bradyrhizobium japonicum*-host plants (Rotaru, 2018; Argaw et al., 2012). It has also been reported that this genus increased relative abundance in response to the plant growth in no symbiotic plants such as *Avena barbata* (Zhalnina et al., 2018).

It is interesting to underline that the PSB and PGPR (*Variovorax, Pseudomonas* and *Bradyrhizobium*) that increased their relative abundance at low P availability in our experiment, are specifically associated with some plants through the assimilation of root exudates (Haichar et al., 2008). The enrichment of these genera occurred in legumes rhizosphere grown both in sole crop and in intercrop. Legumes are considered to accumulate more carboxylates in the rhizosphere than cereal (Pearse et al., 2003, 2006; Hinsinger et al., 2003) and to vary their carboxylate composition due to P availability in soil (different P forms in soil) (Pearse et al., 2007). Our results suggested that at low P availability the plants reacted modifying exudate composition that shaped the bacterial community structure favoring the improvement of PSB and PGPR relative abundance, confirming that root exudate amount and composition are the key drivers for the differences in community structure (Marschner et al., 2004). That resulted in the changes in bacterial community composition observed in our and another study (Lagos et al., 2016) due to P addition.

### 5.4 Conclusions

The plant species was the main factor driving structure of rhizosphere microbial communities. For each plant species, rhizosphere community structure varied due to P availability in soil. When the availability of phosphorus in the soil was low (no P and unavailable P) the relative abundance of some taxa increased in legume rhizosphere, particularly of some notorious plant growth-promoting rhizobacteria (PGPR) and phosphate solubilizing bacteria (PSB). In wheat, bacterial community structure was affected by P availability but not any PGPR or PSB increase or decrease of abundance was detected when P availability changed. The “ability” of legumes to enrich their rhizosphere favouring PGPR and PSB was conserved in intermingled rhizosphere of intercrop. Our results support the idea that legumes can shape the bacterial community structure selecting the most favourable taxa.
in P limited condition. The key role of root exudates in this selection is supported by many authors. Although root exudates were not detected in this experiment, their quality and quantity varied in the first experiment due to P availability and the value were higher in legume rhizosphere. A direct correspondence of plant-root exudates profile-specific bacterial community structure is difficult to find because of the influence of other factors, such as soil type, plant genotype, root exudates not detected, microbial exudates etc. but the results support that roots exudates may be considered the main cause of the bacterial community structure variation in rhizosphere.
6 General Conclusions

Intercropping influenced soil available phosphorus that was generally greater than in sole crop and this effect was more evident in legume-based systems where this P-form was related to the higher PME activity and carboxylate exudation.

The greater increase of plant growth in intercropping treatments at low P availability, particularly observed in wheat, confirmed the favourable effect of intercropping at limiting conditions is in agreement with the stress gradient hypothesis for competition and facilitation in plant communities proposed by Maestre et al. (2009). Increasing grain legume density in intercropping positive affected phosphorus uptake of both partners and available phosphorus in the soil by increasing the PME activity.

Wheat/pea intercropping was more effective to mobilise phosphorus from the soil, producing a noticeable benefit for intercropped wheat in terms of phosphorus uptake and growth. However, this combination of effects was not directly attributable neither to the highest carboxylates release nor to the highest PME activity but rather to the different growth pattern root of pea and wheat in intercropping that enhanced wheat growth and P uptake at P-limited condition. This interference of pea was confirmed by results obtained when 1:2 sowing ratio was used in wheat/pea where cereal, despite the increase of the pea root mass in intercropping, was able to exceed the P uptake of sole crop by 2.5 times. As reported by Hauggaard-Nielsen et al. (2001) for barley/peas, this behaviour of wheat in intercropping with pea can probably be justified by the presence of nutrient greater depletion zone create by pea roots at more superficial layers that forced wheat roots downwards. The existence of a different root growth pattern was also justified by the slight detriment of pea growth observed in wheat/pea intercropping, particularly at limited P supply.

In addition, our results support the hypothesis that legumes can shape the bacterial community structure selecting the most favourable taxa in P limited condition.

Combining the results of the three experiments, we can also conclude that the legume in intercropping, through the shaping of bacterial community, is able to provide a greater amount of available phosphorus in the soil and consequently allow a greater uptake. This indirect effect could occur when a high uptake was not associated with a high production of exudates.

Finally the obtained results can be considered a useful contribution to deepen knowledge on the agroecological role of three classic grain legumes of the Mediterranean environment, (faba bean, lupin and pea) in intercropping with one of the most widespread cash crops of arable farming systems in that area, such as durum wheat. They can help to explain the different performance of grain legume and wheat when the complementarity use of resources and/or facilitation in intercropping occurred and how they vary when the sowing ratio in mixture is changed.
7 REFERENCES


doi:http://dx.doi.org/10.1094/MPMI.2002.15.11.1147.


doi:http://dx.doi.org/10.1146/annurev.arplant.57.032905.105159


61


Nuruzzaman M, Lambers H, Bolland MDA, Veneklaas EJ (2005b) Phosphorus uptake by grain legumes and subsequently grown wheat at different levels of residual phosphorus fertiliser. Aust J Agric Res 56:1041–1047


Sparks DL, Page AL, Helmke PA, Loeppert RH, Soltanpour PN, Tabatai MA, Johnson CT, Sumner ME (1996) Methods of soil analysis: part 3 - chemical methods. ASA-CSSA- SSSA, Madison, WI.


Acknowledgements

I would like to express my gratitude for the precious help to my research group, Dr. Giuseppe Badagliacca and Dr. Maurizio Romeo, and to Dr. Beatrix Petrovicova for the precious help.

I would like to express my sincere gratitude to Prof. Antonio Gelsomino, Prof. Francesco Sunseri, and Dr. Antonio Lupini, for the useful suggestion during the planning of the research project.

A special thanks goes to Dr. Tim Mauchline and Dr. Sigrid Heuer for their guidance and scientific support during my visit in Rothamsted Research and to Maïder Abadie, Dr. Ian Clark, Emily Masters-Clark, Dr. Vanessa Nessner Kavamura-Noguchi, Tessa Reid, Dr. Adriana Torres who helped me to carried out field and lab experiments.

I would also like to thank Prof. Antonio Delgado Garcia and Prof. Robert M. Rees for their accurate comments and remarks and also for their very valuable suggestions.

Last but not least, thanks to my friend Federico, who, not only as a bioinformatician, has been close to me in these three years of thesis.