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INTERCROPPING WITH GRAIN LEGUMES TO EXPLOIT PHOSPHORUS FOR ECOLOGICAL INTENSIFICATION OF MEDITERRANEAN CEREAL CROPPING SYSTEMS

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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^{-1} 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 consociate (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.

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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 CO_2 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 CO_2 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.

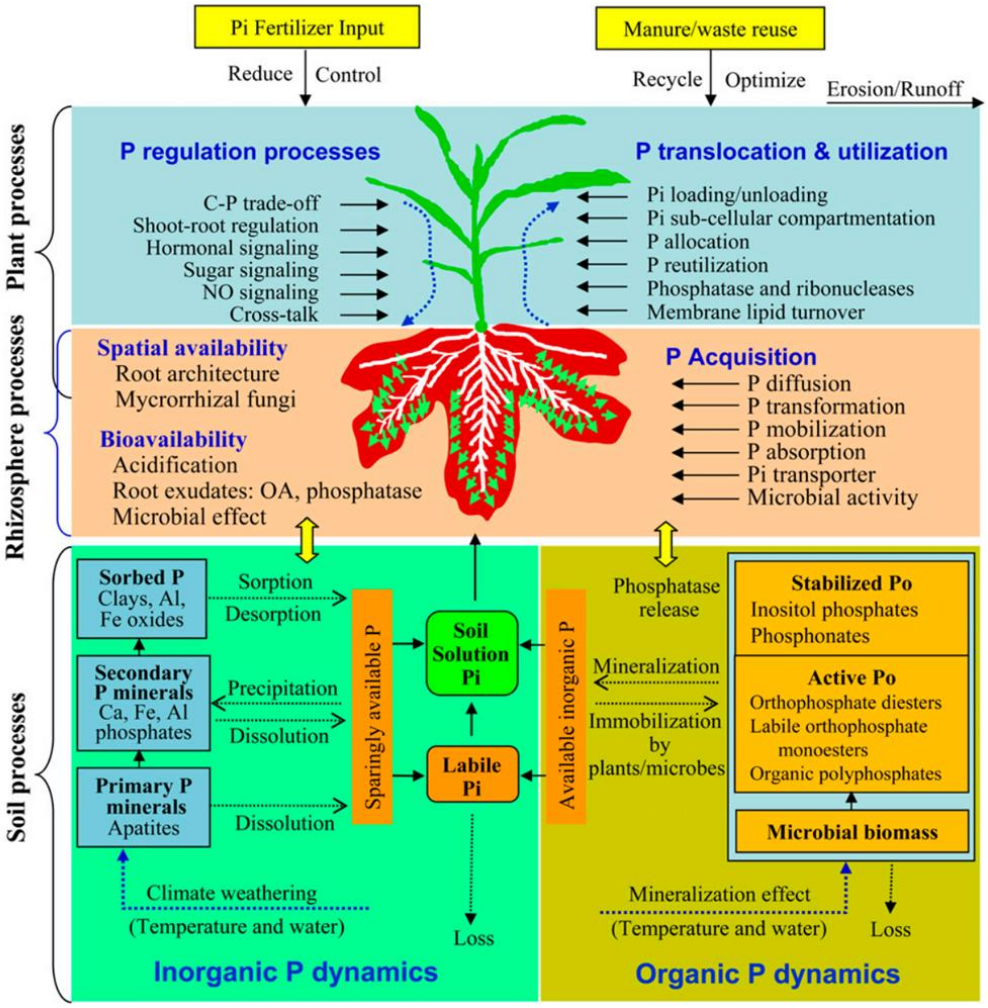

Figure 1.1. P dynamics in the soil/rhizosphere-plant continuum. C-P, Carbon-P; NO, nitric oxide; OA, organic acids (from Shen et al., 2011).

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; Nuruzzman 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 (Nuruzzmann 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 PO_4^{2-} 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

Period length (d)	*15*	*20*	*20*	*Until legume flowering*
Day (h)	8	8	10	14
Night (h)	16	16	14	10
Light intensity (µmoles/m^2 /s)	200	255	340	338
Temperature (°C)	7.5	15	20	25

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 KH_2PO_4,) 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

Bulk density (g cm^{-3})	1.23
Sand %	36
Silt %	32
Clay %	32
pH $_{CaCl2}$	6.6±*0.1*
Total organic carbon (g kg^{-1})	12.55±.*4.5*
Total nitrogen (g N kg^{-1})	1.38±*0.1*
C/N	9.07±*0.1*
$EC_{1:2}$ (dS m^{-1})	0.271±*0.021*
CEC ($cmol_{(+)}$ kg^{-1})	20.9±*1.3*
$CaCO_3$ (g kg^{-1})	8.4±*0.5*
P-Olsen (mg kg^{-1})	11.33±*0.2*
NH_4^+ - N (mg kg^{-1})	12.49±*0.2*
NO_3^- - N (mg kg^{-1})	26.16±*0.2*

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 $CaCl_2$ 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 (wv^{-1}) 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 H_2SO_4 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,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.

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

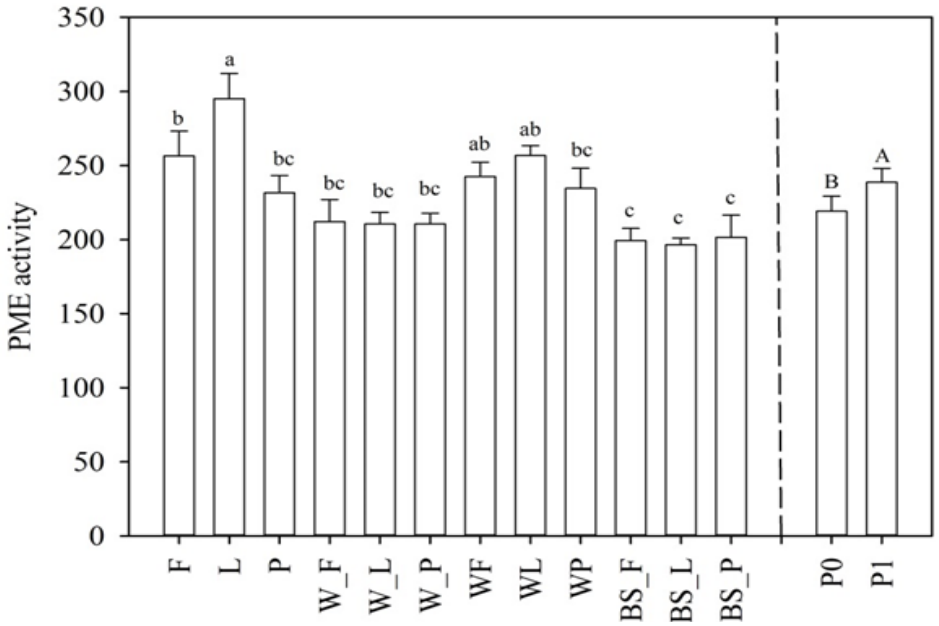

Figure 3.1. Phosphomonoesterase (PME) activity (µmol pNPP g^{-1} soil h^{-1}) in sole crop (W= wheat, F= faba; L=lupin; P =pea), intercropping (WF; WL; WP) and in bare soils (BS). -F, -L, and -P indicate the sampling time of wheat corresponding to the different flowering of legumes. Values are means ±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).

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} 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.

	P0		P1		Mean	
BS-F	86.070	±*9.62*	88.537	±*4.48*	87.304	±*4.94*
BS-L	87.833	±*4.78*	86.865	±*9.46*	87.349	±*4.91*
BS-P	80.560	±*6.11*	88.225	±*13.15*	84.393	±*6.87*
F	78.956	±*3.49*	75.546	±*13.64*	77.251	±*6.55*
L	77.726	±*6.20*	45.475	±*5.95*	61.601	±*7.28*
P	106.526	±*8.99*	56.808	±*10.65*	81.667	±*11.40*
W-F	92.242	±*4.43*	91.287	±*10.26*	91.765	±*5.18*
W-L	117.407	±*14.95*	92.504	±*11.56*	104.955	±*9.94*
W-P	77.514	±*9.52*	96.348	±*5.89*	86.931	±*6.29*
WF	75.852	±*8.78*	76.790	±*11.77*	76.321	±*6.80*
WL	84.042	±*2.98*	60.725	±*4.08*	72.384	±*4.99*
WP	86.966	±*12.39*	61.186	±*11.28*	74.076	±*9.16*
Mean	87.641	±*2.77*	76.691	±*3.42*	82.166	±*2.07*
P			*P=0.0047*			
CTR			*P=0.0036*			
P x CTR			*P=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.).

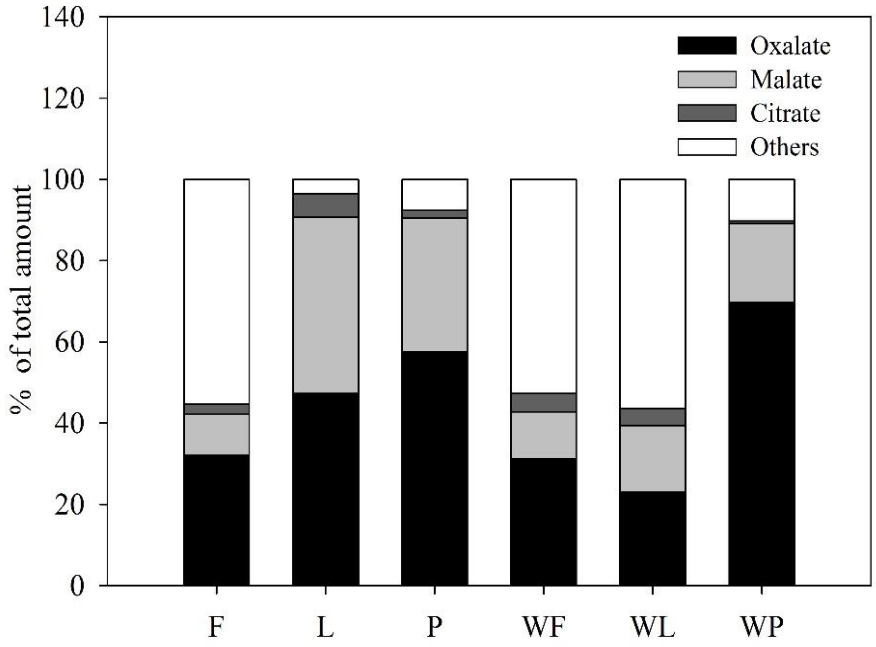

Figure 3.2. Most representative carboxylates measured in rizospheric soil of legume grown in sole crop (F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP).

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" (P_{TAV}) 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 P_{TAV}, P uptake by cropping treatment was added to Olsen phosphorus, measured in the soil after plant sampling:

$$PTAV = OlsenP + Pptake$$

As expected, P supply strongly affected the P_{TAV} ($P < 0.001$) 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.

	P0		P1		Mean
	(mg kg^{-1} dry soil)	%	(mg kg^{-1} dry soil)	%	(mg kg^{-1} dry soil)
F	17.11 ±*2.31*	78	85.82 ±*9.21*	92	51.46 ±*13.64*
L	14.73 ±*1.61*	88	77.92 ±*9.73*	87	46.32 ±*12.73*
P	21.10 ±*1.19*	78	81.03 ±*9.82*	90	51.06 ±*12.23*
W-F	18.41 ±*1.84*	72	65.54 ±*1.53*	90	41.98 ± *8.90*
W-L	14.85 ±*0.90*	71	59.31 ±*2.29*	89	37.08 ± *8.55*
W-P	20.08 ±*5.96*	85	75.17 ±*1.93*	96	47.63 ±*10.84*
WF	23.60 ±*1.74*	62	88.58 ±*3.36*	86	56.09 ±*12.24*
WL	22.74 ±*2.64*	66	86.40 ±*5.89*	84	54.57 ±*12.30*
WP	25.54 ±*2.47*	58	102.47 ±*6.46*	84	64.00 ±*14.87*
Mean	19.80 ±*0.76*	73	80.25 ±*2.87*	89	50.02 ± *3.25*
P			*P=<0.0001*		
CTR			*P=0.0003*		
PxCTR			*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).

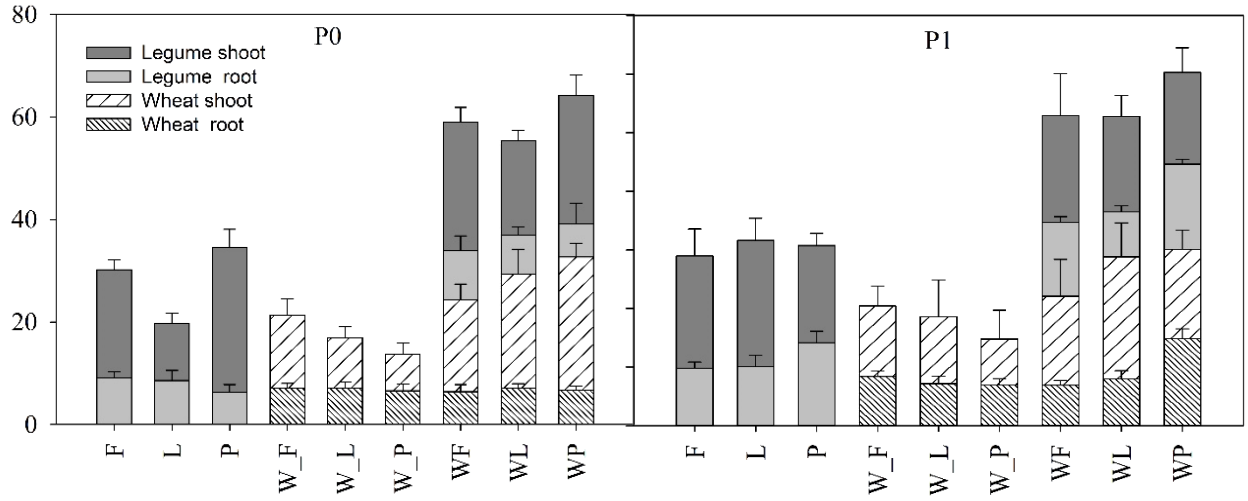

Figure 3.3. Dry matter accumulation and partitioning in legume and wheat grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP) at two phosphorus supply (P0 and P1). _F, _L, and _P indicate the sampling time of wheat corresponding to the different flowering of legumes. Values are means ± SE (n=4).

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).

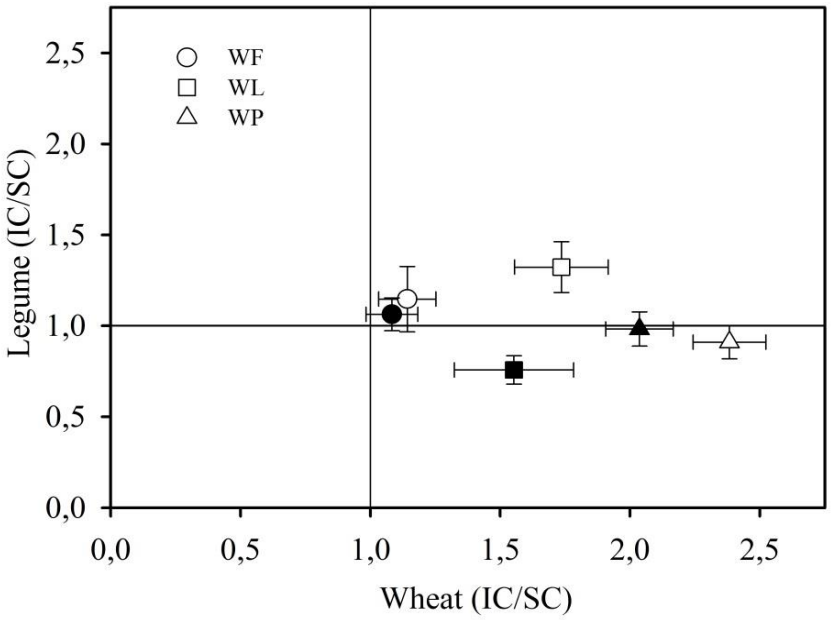

Figure 3.4. Relative dry matter accumulation of wheat and legumes grown in mixture, calculated as intercrop/sole crop ratio (IC/SC), without (open symbols) and with (closed symbols) phosphorus supply. Values are the mean ±SE (n = 4).

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

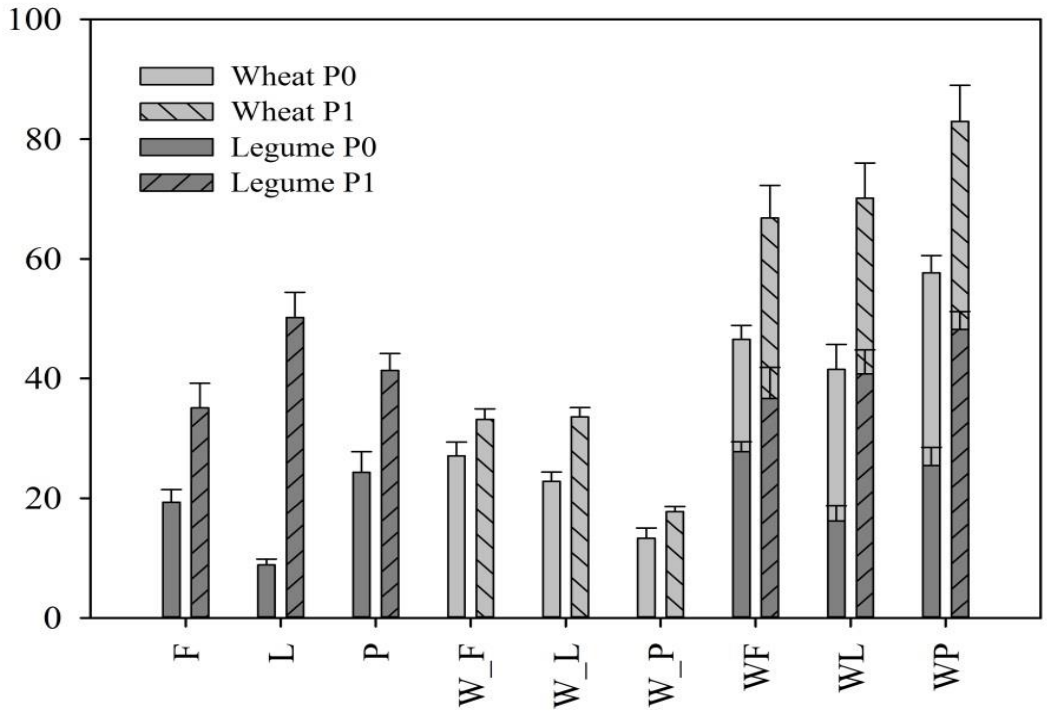

Figure 3.5 Phosphorus uptake (mg plant^{-1}) by legumes and wheat grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP) at two phosphorus supply (P0 and P1). _F, _L, and _P indicate the sampling time of wheat corresponding to the different flowering of legumes. Mean (n=4) ± SE

(+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).

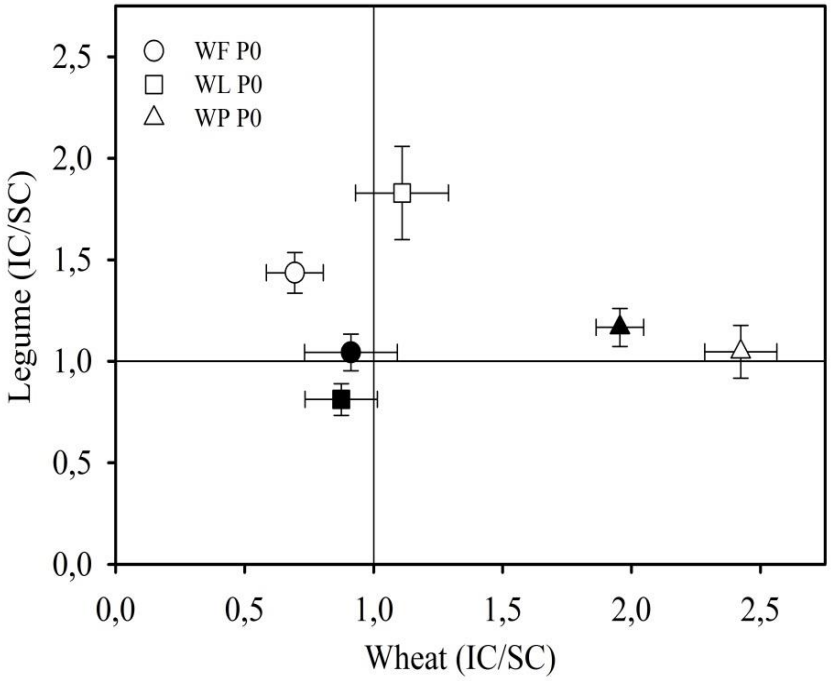

Figure 3.6. Phosphorus relative uptake of wheat and legumes grown in mixture, calculated as intercrop/sole crop ratio (IC/SC), without (open symbols) and with (closed symbols) phosphorus supply. Values are the mean (n = 4) ±SE. The vertical and horizontal lines represent all the points where dry matter 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.

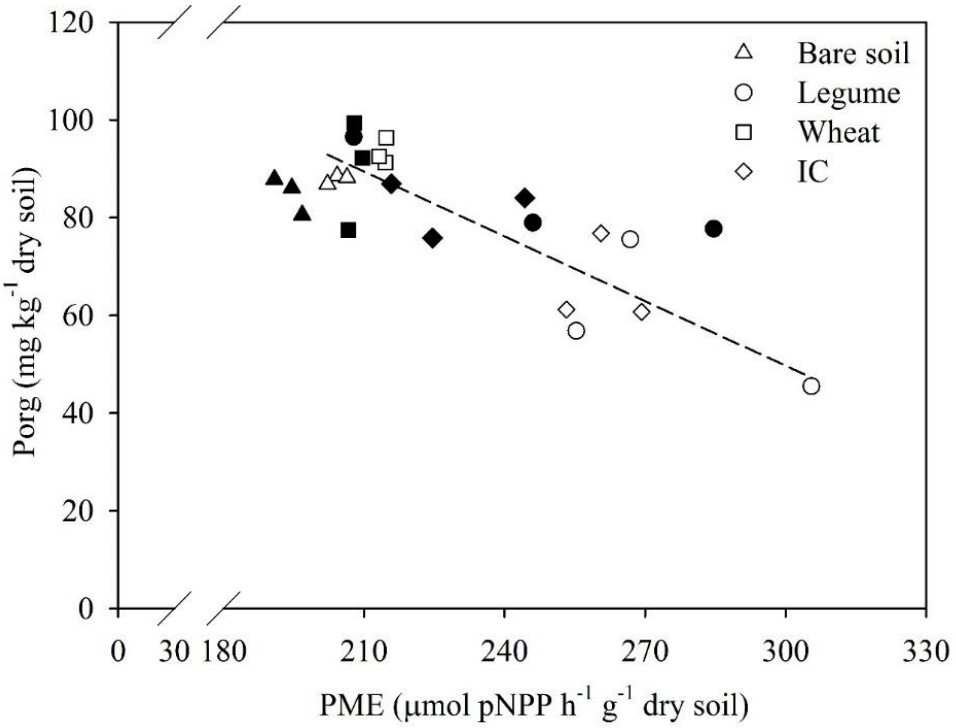

Figure 3.7. Phosphomonoesterase (PME) activity plotted versus organic phosphorus (P_{ORG}) in bare soil (BS), in legumes and wheat grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP) without (closed) and with (open symbols) phosphorus supply. At P fertilized condition, P_{ORG} showed a linear and significant decrease as the PME activity increased (R^2=0.890; P=0.0001). Phosphomonoesterase (PME) activity is expressed as paranitrophenol (pNP) h^{-1} g^{-1} of dry soil.

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).

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_2PO_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_2PO_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

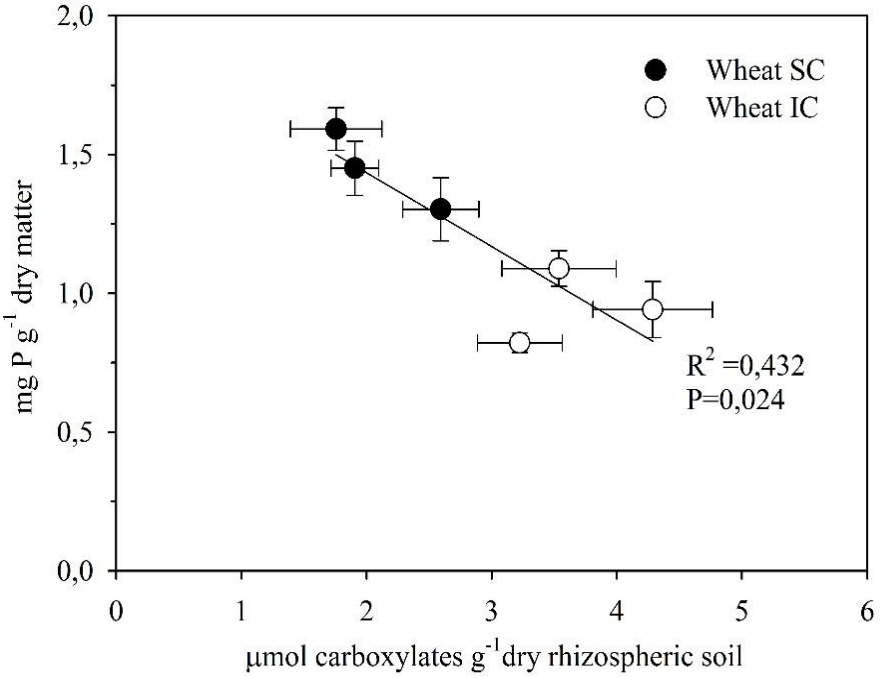

Figure 3.8. Total carboxylates production, at limited phosphorus condition, in wheat rhizospheric soil plotted versus phosphorus concentration in intercropping (WIC) an sole crop (WSC) respectively indicated by (open) and with (closed symbols). Without phosphorus supply P concentration in wheat dry matter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

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

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 (P_{TAV}), 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.

SHOOT

	Wheat			Legume		
Cropping treatment	P0	P1	Mean	P0	P1	Mean
F				0,45±0.05	0,74±0.09	*0,59*±0.07
L				0,56±0.03	1,94±0.25	*1,25*±0.10
P				0,56±0.04	1,18±0.13	*0,87*±0.09
W-F	1,59±0.33	1,62±0.28	*1,61*±0.18			
W-L	1,45±0.18	1,75±0.35	*1,60*±0.20			
W-P	1,30±0.21	1,87±0.45	*1,59*±0.13			
WF	0,82±0.09	1,59±0.19	*1,21*±0.09	0,68±0.02	0,83±0.08	*0,75*±0.09
WL	0,94±0.08	1,13±0.08	*1,04*±0.07	0,54±0.07	1,92±0.22	*1,23*±
WP	1,09±0.25	1,59±0.16	*1,34*±0.12	0,78±0.03	1,61±0.14	*1,20*±0.04
Mean	*1,20*±0.17	*1,59*±0.22	*1,40*±0.15	*0,60*±0.02	*1,37*±0.10	*0.98*±0.07
P		*P<0.0001*			*P<0.0001*	
CS		*P<0.0001*			*P<0.0001*	
P x CS		*P<0.0304*			*P<0.0001*	

ROOT

	Wheat			Legume		
Cropping treatment	P0	P1	Mean	P0	P1	Mean
F				1,12±0.22	2,11±0.28	*1,62*±0.12
L				0,31±0.04	0,88±0.11	*0,59*±0.08
P				1,37±0.27	1,55±0.33	*1,46*±0.20
W-F	0,63±0.08	0,74±0.07	0,69±0.04			
W-L	1,21±0.28	1,90±1.22	1,55±0.18			
W-P	0,60±0.05	0,44±0.02	0,52±0.05			
WF	0,66±0.03	0,88±0.06	0,77±0.08	1,24±0.33	1,71±0.54	*1,47*±0.18
WL	0,66±0.04	0,73±0.05	0,69±0.09	0,82±0.09	1,23±0.18	*1,02*±0.09
WP	0,57±0.08	0,69±0.07	0,63±0.07	0,97±0.10	1,60±0.34	*1,29*±0.14
Mean	*0,72*±0.03	*0,90*±0.03	*0,81*±0.04	*0,97*±0.05	*1,51*±0.11	*1,21*±0.08
P		*P<0.0001*			*P<0.0001*	
CTR		*P<0.0001*			*P<0.0001*	
P x CTR		*P=0.0304*			*NS*	

4 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 that an higher interspecific competition for P could enhance the exudation of phosphatases from roots of the intercropped legumes.

4.1 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 (*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 drayed 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 (wv^{-1}) 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 H_2SO_4 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

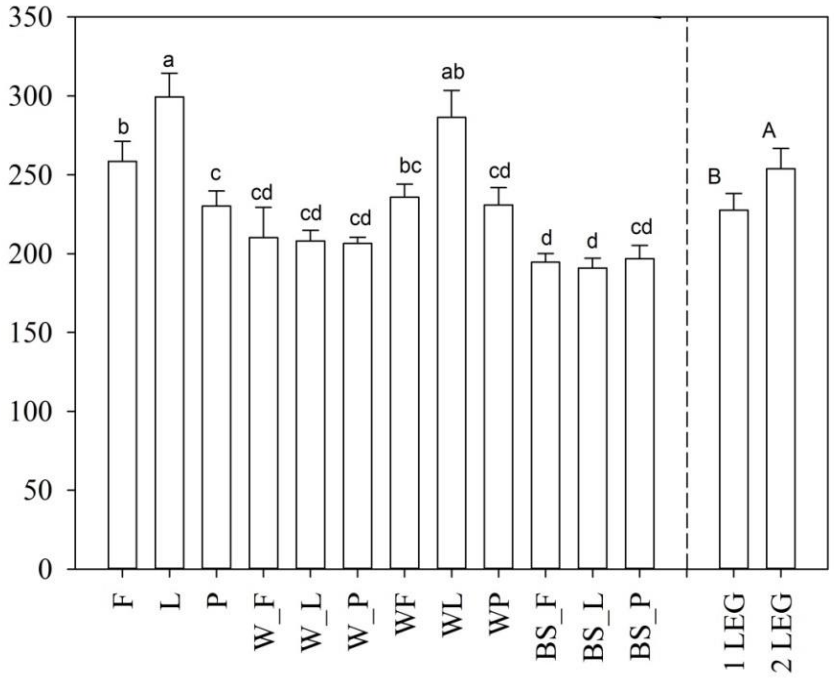

Figure 4.1 Phosphomonoesterase (PME) activity (µmol paranitrophenol g^{-1} soil h^{-1}) in sole crop (W= wheat, F= faba; L=lupin; P =pea), intercropping (WF; WL; WP) and in bare soil (BS). -F, -L, and -P indicate the sampling time of wheat corresponding to the different flowering of legumes. Means (n=8; 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 $P \leq 0.05$; Tukey test)

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} 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

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}

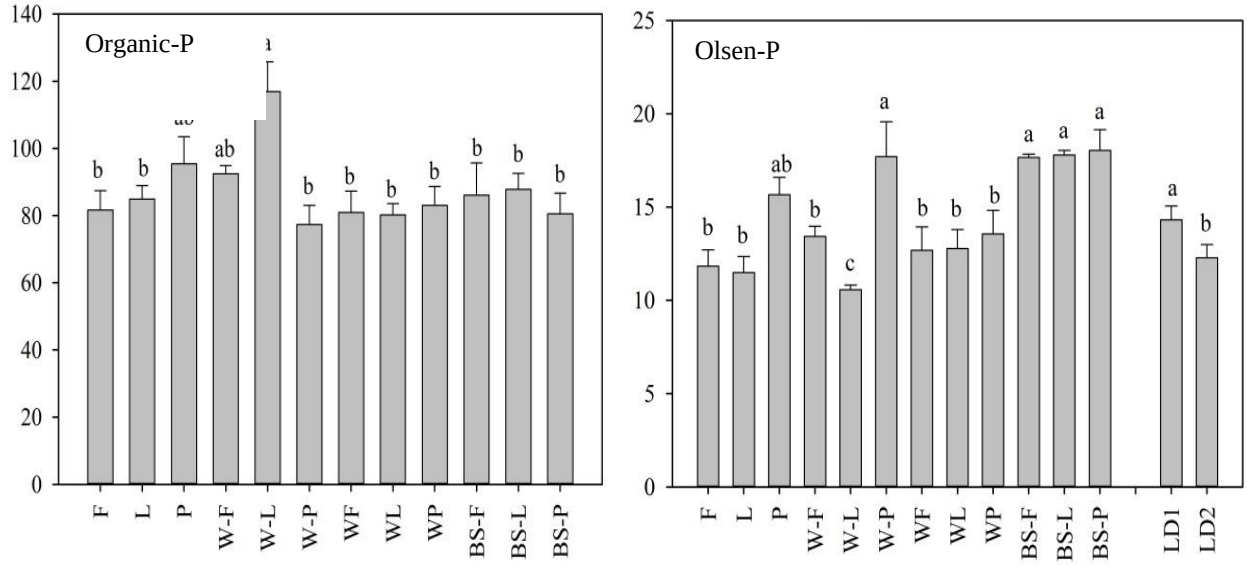

Figure 4.2. Organic and available (Olsen-P) phosphorus (mg P kg^{-1} dry soil) in wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP). -F, -L, and -P indicate the sampling time of wheat at the different flowering of legumes. Values are means ± SE (n=8;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

dry soil (W-F), in W-L organic phosphorus was significantly highest (116.9 mg kg^{-1} dry soil), (Fig. 4.2.).

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

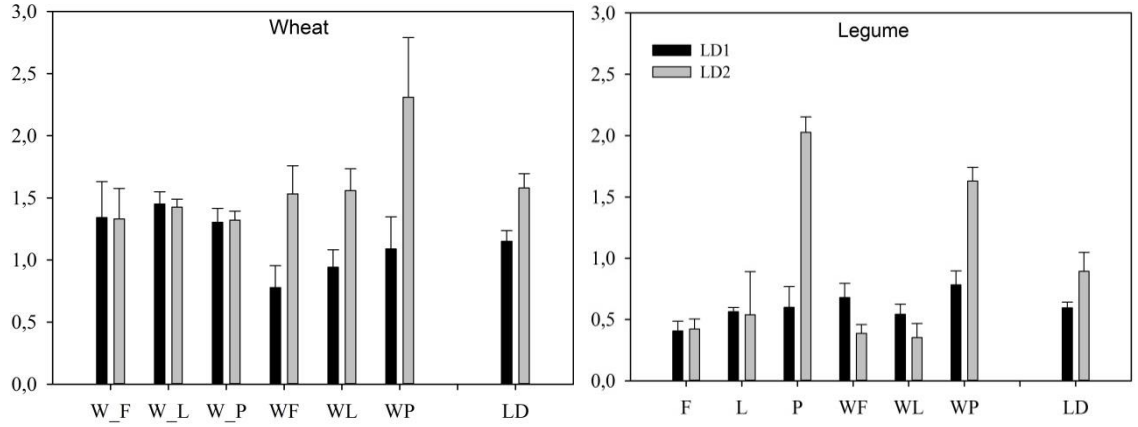

Figure 4.3 Phosphorus concentration (mg P kg^{-1} dm) in shoot of wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP) at two legume plant density (LD1 and LD2). -F, -L, and -P indicate the sampling time of wheat at the different flowering of legumes.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

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).

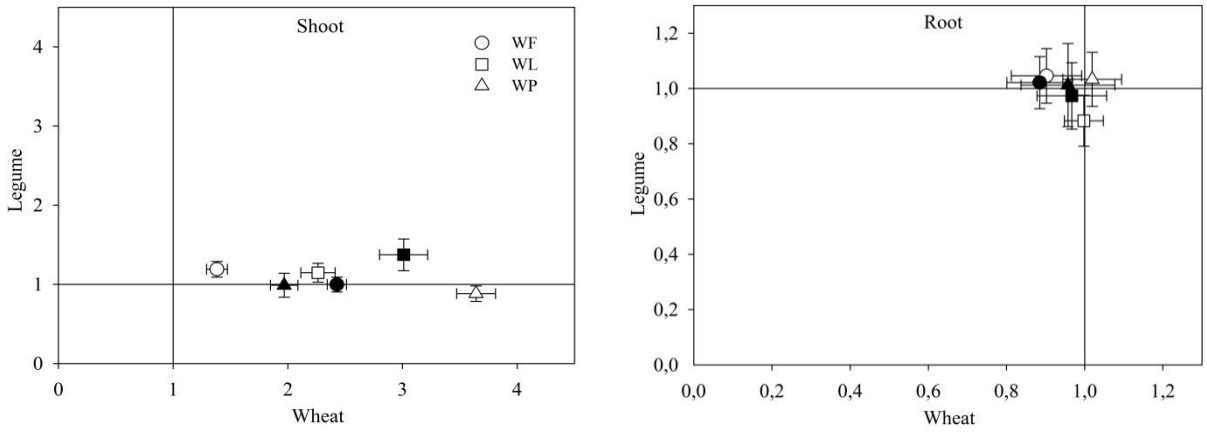

Figure 4.5. Shoot and root relative dry matter (IC/SC) of wheat (W) and legumes (F= faba; L=lupin; P =pea) grown in mixture (WF; WL; WP), calculated as intercrop/sole crop ratio (IC/SC), both at one-plant (open symbols) and at two-plant (closed symbols) legume densities. Values are means ±SE (n=4). The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

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

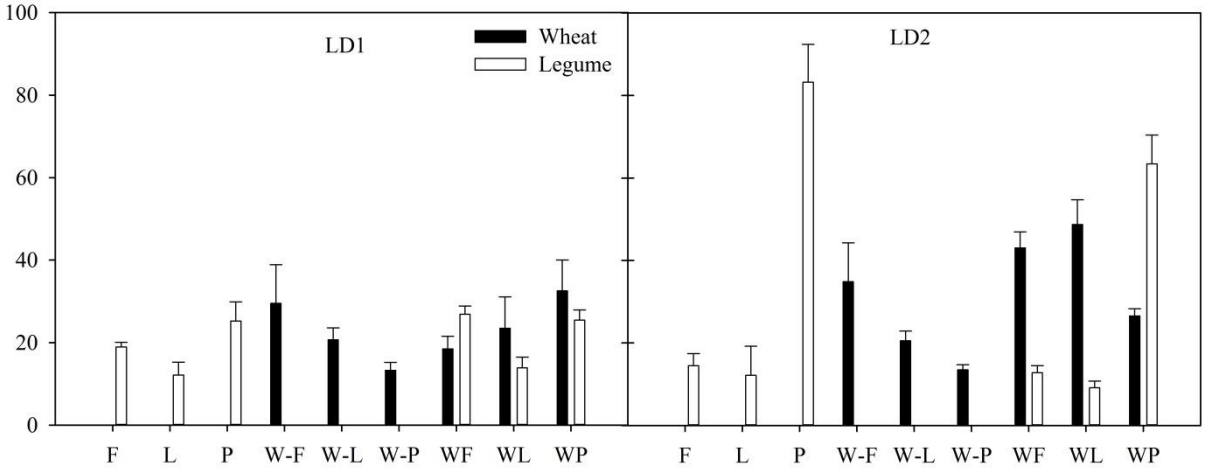

Figure 4.6. Phosphorus plant uptake (mg) by wheat and legume grown in sole crop (W= wheat, F= faba; L=lupin; P =pea) and intercropping (WF; WL; WP) both at LD1 and LD2. -F, -L, and -P indicate the sampling time of wheat at the different flowering of legumes. Values are means ± SE (n=4).

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]

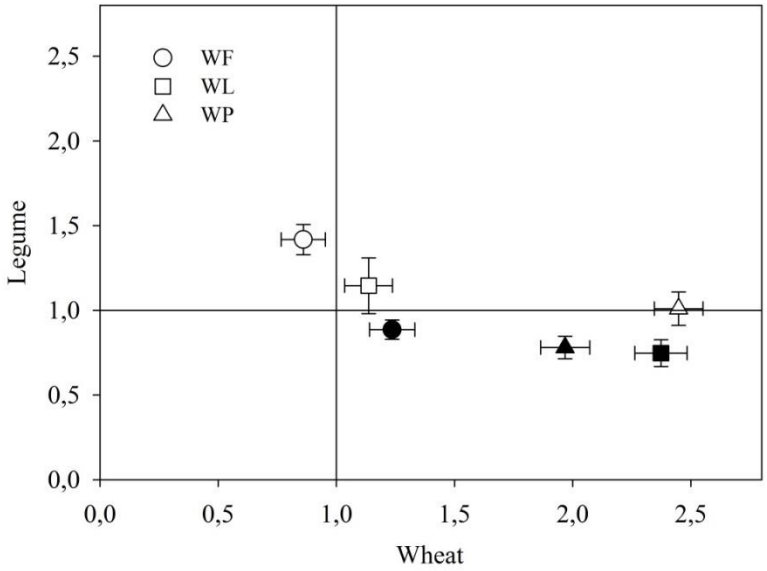

Figure 4.7. Phosphorus relative uptake of wheat and legumes grown in mixtures, calculated as intercrop/sole crop ratio (IC/SC), both at one-plant (open symbols) and at two-plant (closed symbols) legume densities. Values are means ±SE (n = 4). The vertical and horizontal lines represent all the points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

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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

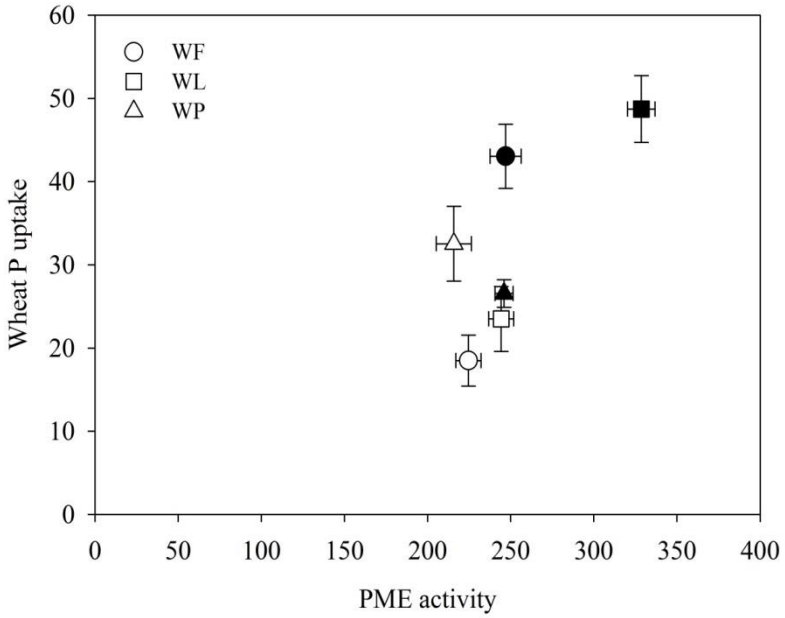

Figure 4.8. Phosphomonoesterase (PME) activity (µmol paranitrophenol g^{-1} soil h^{-1}) plotted versus phosphorus uptake (g $plant^{-1}$) of wheat intercropped with faba (WF), lupin (WL) and pea (WP) at 1:1 (open symbol) and 1:2 (closed symbol) sowing ratios. Values are means ±SE (n=4).

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

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.

4.5 Appendix

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.

SHOOT						
Cropping treatments	Wheat			Legume		
	LD1	LD2	Mean	LD1	LD2	Mean
F				21.13±2.08	15.55±1.09	18.34±1.33
L				16.10±1.54	17.46±1.82	16.78±1.09
P				28.27±1.97	31.53±2.24	29.90±1.53
W-F	13.01±2.13		12.23±2.02			
W-L	9.86±0.72		9.85±0.90			
W-P	7.13±1.08		7.17±0.94			
WF	17.97±3.04	27.80±5.16	22.89±1.89	25.16±1.33	15.52±1.41	20.34±1.30
WL	22.31±5.04	29.61±6.41	25.95±2.09	18.46±1.08	23.97±2.04	21.22±1.07
WP	25.96±3.25	14.20±3.12	20.08±1.97	24.96±2.05	31.19±2.01	28.08±1.20
Mean	17.28±3.14	18.21±3.08	17.74±1.16	22.35±1.16	22.54±1.27	22.44±1.12
LD		*P<0.0001*			*NS*	
CTR		*NS*			*P=0.005*	
LD x CT		*P<0.002*			*NS*	

ROOT						
	Wheat			Legume		
Cropping treatments	LD1	LD2	Mean	LD1	LD2	Mean
F				9.12±0.18	4.73±0.09	6.93±0.10
L				8.59±0.16	4.41±0.13	6.50±0.13
P				6.36±0.14	6.23±0.07	6.29±0.11
W-F	7.10±0.84	7.12±0.83	7.11±0.88			
W-L	7.03±0.71	7.01±0.43	7.02±1.02			
W-P	6.58±0.55	6.55±0.19	6.56±0.75			
WF	6.41±1.08	6.30±0.71	6.36±0.66	9.54±0.08	4.83±0.06	7.18±0.16
WL	7.02±1.00	6.78±0.25	6.90±0.54	7.59±0.13	4.30±0.12	5.94±0.15
WP	6.71±1.17	6.27±0.31	6.49±0.48	6.57±0.15	6.31±0.12	6.44±0.11
Mean	6.81±0.95	6.67±0.47	6.74±0.33	7.96±0.08	5.13±0.07	6.55±0.09
LD		*P=0.004*			*P<0.0001*	
CTR		*NS*			*P<0.0001*	
LD x CTR		*NS*			*NS*	

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).

Legume						
	Shoot			Root		
	LD1	LD2	Mean	LD1	LD2	Mean
F	0.41 ±*0.08*	0.42 ±*0.08*	0.41 ±*0.05*	1.17 ±*0.23*	0.86 ±*0.25*	1.02 ±*0.17*
L	0.56 ±*0.03*	0.54 ±*0.35*	0.55 ±*0.16*	0.36 ±*0.09*	0.23 ±*0.06*	0.29 ±*0.06*
P	0.60 ±*0.17*	2.03 ±*0.13*	1.31 ±*0.29*	1.36 ±*0.19*	1.54 ±*0.29*	1.45 ±*0.17*
WF	0.68 ±*0.12*	0.39 ±*0.07*	0.53 ±*0.08*	1.24 ±*0.25*	0.72 ±*0.15*	0.98 ±*0.16*
WL	0.54 ±*0.08*	0.35 ±*0.11*	0.45 ±*0.07*	0.62 ±*0.35*	0.18 ±*0.04*	0.40 ±*0.18*
WP	0.78 ±*0.11*	1.63 ±*0.11*	1.21 ±*0.18*	0.97 ±*0.14*	0.95 ±*0.10*	0.96 ±*0.08*
Mean	0.60 ±*0.05*	0.89 ±*0.15*	0.74 ±*0.08*	0.95 ±*0.11*	0.75 ±*0.12*	0.85 ±*0.08*
LD		*P<0.0001*			*P<0.0001*	
CTR		*P=0.009*			*NS*	
LDxCTR		*P<0.0001*			*NS*	
Wheat						
	Shoot			Root		
	LD1	LD2	Mean	LD1	LD2	Mean
W-F	1.34 ±*0.29*	1.33 ±*0.25*	1.34 ±*0.18*	0.63 ±*0.07*	0.64 ±*0.04*	0.63 ±*0.04*
W-L	1.45 ±*0.10*	1.43 ±*0.06*	1.44 ±*0.05*	0.92 ±*0.29*	0.91 ±*0.24*	0.92 ±*0.17*
W-P	1.30 ±*0.11*	1.32 ±*0.07*	1.31 ±*0.06*	0.60 ±*0.17*	0.61 ±*0.16*	0.61 ±*0.11*
WF	0.78 ±*0.18*	1.53 ±*0.23*	1.16 ±*0.19*	0.78 ±*0.16*	0.26 ±*0.08*	0.52 ±*0.13*
WL	0.94 ±*0.14*	1.56 ±*0.17*	1.25 ±*0.16*	0.66 ±*0.16*	0.37 ±*0.10*	0.52 ±*0.10*
WP	1.09 ±*0.26*	1.76 ±*0.10*	1.70 ±*0.34*	0.57 ±*0.08*	0.24 ±*0.02*	0.41 ±*0.07*
Mean	1.15 ±*0.09*	1.58 ±*0 .12*	1.37 ±*0.08*	0.69 ±*0.07*	0.51 ±*0.07*	0.60 ±*0.05*
LD		*P=0.0021*			*P<0.0001*	
CTR		*NS*			*NS*	
LDxCTR		*P=0.0387*			*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

Wheat												
	Shoot						Root					
Cropping treatments	LD1		LD2		Mean		LD1		LD2		Mean	
W-F	25.059	*9.04*	30.255	*9.19*	29.510	25.059	4.451	*±0.48*	4.580	*±0.42*	4.515	*±0.42*
W-L	14.362	*1.35*	14.058	*0.93*	20.678	14.362	6.316	*±1.90*	6.472	*±1.75*	6.394	*±1.69*
W-P	9.339	*1.04*	9.499	*0.30*	13.290	9.339	3.951	*±1.12*	3.991	*±1.03*	3.971	*±1.00*
WF	13.529	*2.69*	41.430	*4.05*	18.486	13.529	4.957	*±0.93*	1.619	*±0.48*	3.288	*±1.12*
WL	18.972	*6.68*	46.240	*5.44*	23.498	18.972	4.526	*±0.93*	2.491	*±0.64*	3.508	*±0.92*
WP	28.672	*7.53*	25.048	*1.67*	32.539	28.672	3.867	*±0.65*	1.502	*±0.13*	2.684	*±0.77*
Mean	18.322	*2.48*	27.755	*3.26*	23.000	18.322	4.678	*±0.43*	3.443	*±0.49*	4.060	*±0.48*
LD			*P<0.001*						*P<0.001*			
CT			*P=NS*						*P=NS*			
LD x CT			*P=0.0024*						*P=NS*			
Legume												
	Shoot						Root					
Cropping treatments	LD1		LD2		Mean		LD1		LD2		Mean	
F	8.602	*1.90*	6.501	*1.14*	7.55	*1.10*	10.331	*±1.35*	7.991	*±2.26*	9.161	*±1.30*
L	9.092	*2.95*	10.144	*7.05*	9.62	*3.54*	3.029	*±0.70*	1.994	*±0.48*	2.512	*±0.44*
P	16.539	*3.97*	64.015	*5.77*	40.28	*9.54*	8.671	*±1.22*	19.213	*±3.61*	13.942	*±2.66*
WF	15.420	*0.41*	5.827	*0.91*	10.62	*1.87*	11.431	*±1.64*	6.944	*±1.35*	9.187	*±1.30*
WL	9.155	*2.55*	7.533	*1.67*	8.34	*1.45*	4.726	*±2.68*	1.588	*±0.35*	3.157	*±1.39*
WP	19.115	*1.90*	51.442	*6.54*	35.28	*6.87*	6.338	*±0.88*	11.981	*±1.16*	9.159	*±1.26*
Mean	12.987	*1.26*	24.244	*5.28*	18.62	*2.81*	7.421	*±0.84*	8.285	*±1.44*	7.853	*±0.82*
LD			*P<0.0001*						*P<0.0001*			
CT			*P<0.0001*						*P<0.0394*			
LD x CT			*P<0.0001*						*P<0.0005*			

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 structure 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 KH_2PO_4 and $Ca_3(PO_4)_2$ obtaining the following four treatments: unav P (-P soil amended with Ca_3PO_4), ava P (-P soil amended with KH_2PO_4), 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, $p = 0.001$).

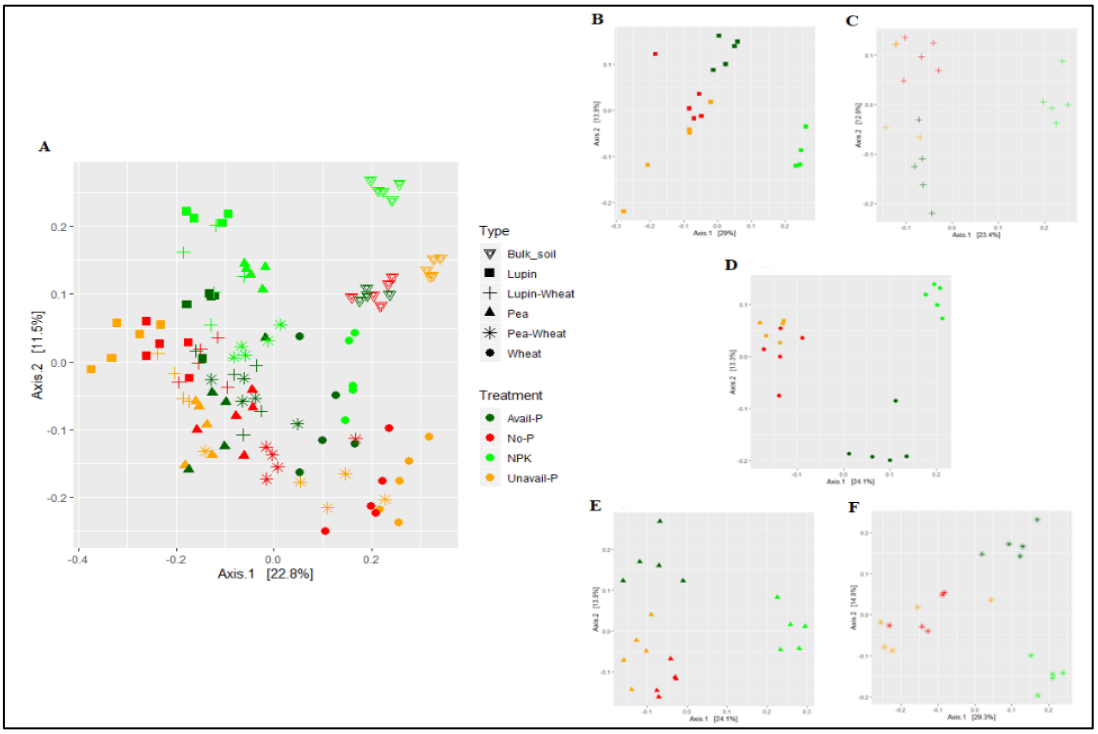

Figure 5.1. PCoA plots based on Bray-Curtis distance matrix of bacterial communities from bulk soil and rhizosphere of different crops grown in soil with different P treatments. The percentage shown on each axis corresponds to the proportion of variation explained. Inverted triangles represent bulk soil 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).

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 2 D) 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 and5.2E).

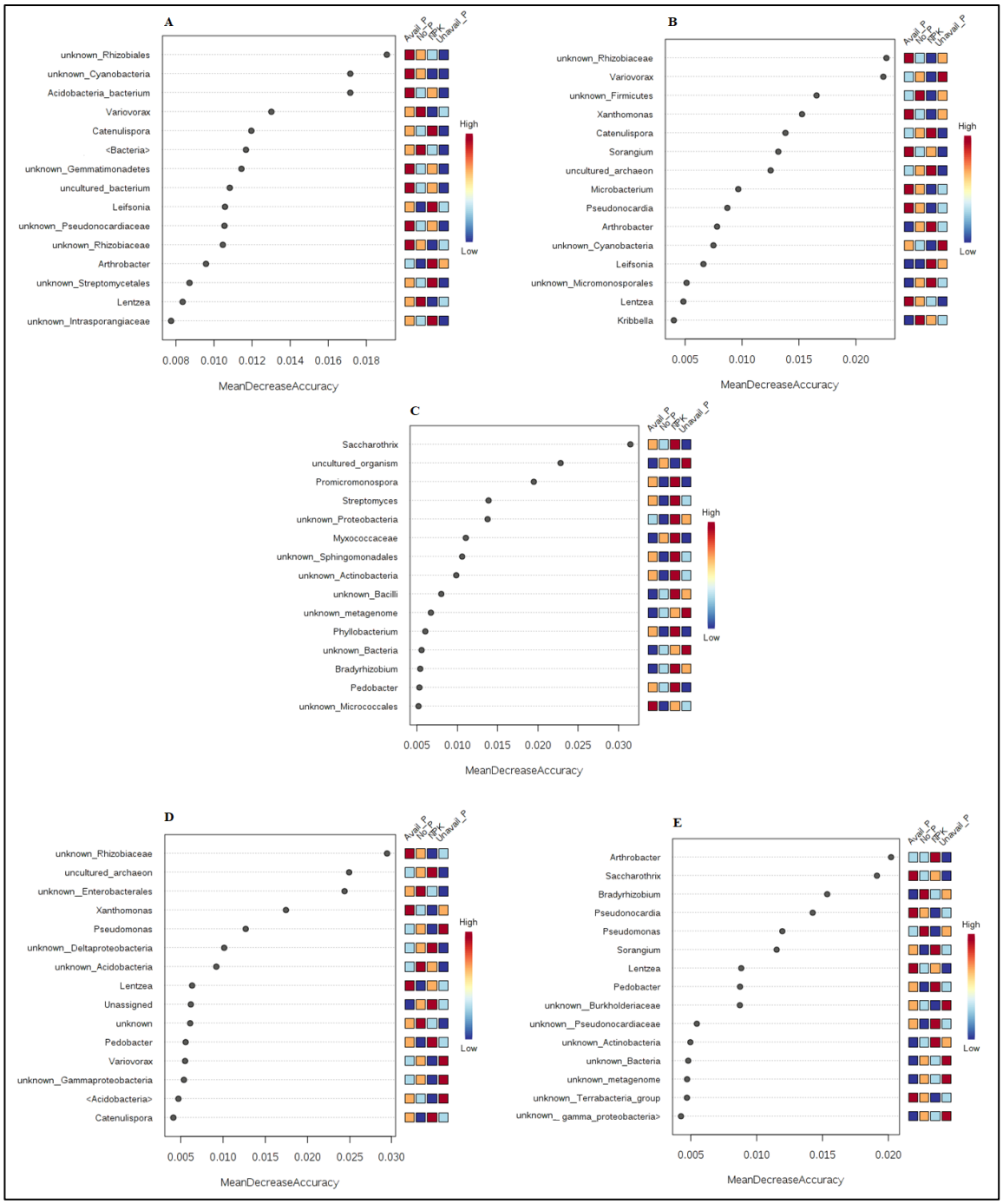

Figure 5.2. Random forest analysis on each plant type to check for differentially abundant taxa at genus level, comparing different P treatments, only showing the top 15 taxa for lupin, lupin-wheat, wheat, pea and pea-wheat rhizosphere (A, B, C, D and E, respectively). X-axis shows the mean decrease 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, differencing 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 pea when a calcium phosphate source was added (Pearse et al., 2007). Adding available P as KH_2PO_4, 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 Ca^{2+} 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-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick et al. 2007; Arshad et al. 2008; Zahir et al. 2008; Shakir 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 $[H_2PO_4]_2$). 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 e 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

Ae N, Arihara J and Okada K, Yoshihara T, Johansen C (1990) Phosphorus uptake by pigeon pea and its role in cropping systems of the Indian subcontinent. *Science* **248**: 477–480.

Ae N, Arihara J and Okada K (1991) Phosphorus uptake mechanisms of pigeon pea grown in alfisols and vertisols. In Phosphorus nutrition of grain legumes in the semi-arid tropics. Eds. C Johansen, K K Lee and K L Sahrawat. ICRISAT, Andra Pradesh pp. 91–98.

Afzal AFTAB., Ashraf M, Asad SA and Farooq M (2005) Effect of phosphate solubilizing microorganisms on phosphorus uptake, yield and yield traits of wheat (*Triticum aestivum* L.) in rainfed area. *Int. J. Agric. Biol*, *7*(2), pp.207-209.

Ahmed S (1995) Agriculture-Fertilizer Interface in Asia-Issues of Growth and Sustainability. Oxford and IBH Publ. Co. New Delhi.

Alvey S, Yang C-H, Buerkert A, Crowley DE (2003) Cereal/legume rotation effects on rhizosphere bacterial community structure in West African soils. *Biol. Fertil. Soils* **37**: 73–82. doi:http://dx.doi.org/10.1007/s00374-002-0573-2.

Alori ET, Glick BR, Babalola OO (2017) Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Front. Microbiol. 8:971. doi: 0.3389/fmicb.2017.00971.

Amossé C, Jeuffroy M-H, Celette F, David C (2013) Relay-intercropped forage legumes help to control weeds in organic grain production. *Eur. J. Agron*. **49**: 158–167. doi:http://dx.doi.org/10.1016/j.eja.2013.04.002.

Antoun H, Beauchamp C, Goussard N, Chabot R, Lalande R (1998) Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effect on radishes (*Raphanus sativus* L.), *Plant Soil* **204**: 57–67.

Assessment, M. E. (2005). *Ecosystems and human well-being* (Vol. 5). Washington, DC:Island press.

Arai Y, Sparks DL (2007) Phosphate reaction dynamics in soils and soil minerals: a multiscale approach. *Adv Agron* **94**: 135–179

Argaw A (2012) Evaluation of co-inoculation of *Bradyrhizobium japonicum* and Phosphate solubilizing *Pseudomonas* spp. effect on soybean (*Glycine max* L. Merr.) in Assossa Area. *Journal of Agricultural Science and Technology*, *14*(1), pp.213-224.

Audenaert K, Pattery T, Cornelis P, Höfte M (2002) Induction of systemic resistance to botrytis cinerea in tomato by Pseudomonas aeruginosa 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. *Mol. Plant. Microbe Interact*. **15**: 1147–1156. doi:http://dx.doi.org/10.1094/MPMI.2002.15.11.1147.

Badri D V and Vivanco J M (2009) Regulation and function of root exudates. *Plant, Cell and Environment* **32**:666–681.

Badri DV, Chaparro JM, Zhang R, Shen Q, Vivanco JM (2013) Application of natural blends of phytochemicals derived from the root exudates of Arabidopsis to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *J Biol Chem*, **288**:4502-4512.

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol*. **57**: 233–266. doi:http://dx.doi.org/10.1146/annurev. arplant.57.032905.105159

Baliah, NT and Begum PJ (2015) Isolation, identification and characterization of phosphate solubilizing bacteria (PSB) isolated from economically important crop plants. *Int. J. Curr. Microbiol. App. Sci*, *4*(3), pp.915-924.

Balvanera P, Pfisterer AB, Buchmann N, He JS, Nakashizuka T, Raffaelli D, Schmid B (2006) Quantifying the evidence for biodiversity effects on ecosystem functioning and services. *Ecology letters*, *9*(10), 1146-1156.

Bartelt-Ryser J, Joshi J, Schmid B, Brandl H, Balser T (2005) Soil feedbacks of plant diversity on soil microbial communities and subsequent plant growth. *Perspect. Plant Ecol. Evol. Syst.* 7, 27–49. doi:http://dx.doi.org/10.1016/j. ppees.2004.11.002.

Bashan Y, de-Bashan LE (2010) How the plant growth-promoting bacterium *Azospirillum* promotes plant growth—a critical assessment. Advances in Agronomy. Elsevier, 77-136.

Bedoussac L, Justes E (2010) 'The efficiency of a durum wheat-winter pea intercrop to improve yield and wheat grain protein concentration depends on N availability during early growth', pp. 19–35. doi: 10.1007/s11104-009-0082-2.

Bedoussac L, Journet E-P, Hauggaard-Nielsen H, Naudin C, Corre-Hellou G, Jensen ES, Prieur L, Justes E (2015) Ecological principles underlying the increase of productivity achieved by cereal-grain legume intercrops in organic farming. A review. *Agron. Sustain. Dev*. 35, 911–935. doi:http://dx.doi.org/ 10.1007/s13593-014-0277-7.

Bellon S, Penvern S (2014) Organic Farming, Prototype for Sustainable Agricultures. Springer pp. 25.

Benbi DK, Gilkes RJ (1987) The movement into soil of P from superphosphate grains and its available to plants. *Fert Res* **12**: 21–36

Bennett AJ, Bending GD, Chandler D, Hilton S, Mills P (2012) Meeting the demand for crop production: the challenge of yield decline in crops grown in short rotations. *Biological Reviews*, **87**: 52-71.

Berg G, Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol. Ecol. 68, 1–13. doi:http://dx.doi.org/10.1111/j.1574-6941.2009.00654. x.

Bertin C, Yang X, Weston LA (2003) The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* **256**: 67–83. doi:http://dx.doi.org/10.1023/ A:1026290508166

Bertness MD (1993) 'Positive interactions in communities', (1990), pp. 27–29.

Betencourt E, Duputel M, Colomb B, Desclaux D, Hinsinger P (2011) Intercropping promotes the ability of durum wheat and chickpea to increase rhizosphere phosphorus availability in a low P soil. *Soil Biol. Biochem*. **46**:181–190.

Betencourt E, Duputel M, Colomb B, Desclaux D, Hinsinger P (2012) Intercropping promotes the ability of durum wheat and chickpea to increase rhizosphere phosphorus availability in a low P soil. *Soil biology and Biochemistry*, *46*, 181-190. doi: 10.1016/j.soilbio.2011.11.015.

Bieleski RL (1973) Phosphate pools, phosphate transport, and phosphate availability. *Annu Rev Plant Physiol* **24**: 225–252

Boiero L, Perrig D, Masciarelli O, Penna C, Cassàn F, Luna V (2006) Phytohormone production by three strains of *Bradyrhizobium japonicum*, and possible physiological and technological implications, *Appl. Microbiol. Biotechnol*. **74**:874–880.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolek T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Pruesse E, Rasmussen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, and Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology* 37: 852–857. https://doi.org/10.1038/s41587-019-0209-9

Bommarco R, Kleijn D, Potts SG (2013) Ecological intensification : harnessing ecosystem services for food security, *Trends in Ecology & Evolution*. Elsevier Ltd, 28(4), pp. 230–238. doi: 10.1016/j.tree.2012.10.012.

Bowman RA (1989) A sequential extraction procedure with concentrated sulfuric acid and dilute base for soil organic phosphorus. *Soil. Sci. Soc. Am. J.* **53**:362-366.

Bremner, J M, Keeney DR (1966) Determination and isotope-ratio analysis of different forms of nitrogen in soils: 3. exchangeable ammonium, nitrate, and nitrite by extraction-distillation methods 1. *Soil Science Society of America Journal*, *30*(5), 577-582.

Bünemann EK, Prusisz B, Ehlers K (2011) Characterization of Phosphorus forms in soil microorganisms. In: Bünemann EK, Oberson A, Frossard E (eds.) Phosphorus in Action. Springer-Verlag, Berlin.

Burney JA, Davis SJ, Lobell DB (2010) Greenhouse gas mitigation by agricultural intensification. *Proc Natl Acad Sci* USA **107**:12052–12057.

Callaway RM (2007) Direct mechanisms for facilitation. In RM Callaway, ed, Positive Interactions and Interdependence in Plant Communities. Springer, Dordrecht, The Netherlands, pp 15–59

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods, **13**:581-583.

Cardinale BJ, Duffy E, Gonzalez A, Hooper DU, Perrings C, Venail P, Narwani A, Mace GM, Tilman D, Wardle DA, Kinzig AP, Daily GC, Loreau M, Grace JB, Larigauderie A, Srivastava DS, Naeem S (2012) Biodiversity loss and its impact on humanity. *Nature*, **486:** 59-67.

Cassman KG (1999) Ecological intensification of cereal production systems: yield potential, soil quality, and precision agriculture. *Proc. Natl. Acad. Sci*. U.S.A. **96**: 5952–5959

Cassan F, Perrig D, Sgroy V, Masciarelli O, Penna C, Luna V (2009) *Azospirillum brasilense* Az39 and *Bradyrhizobium japonicum* E109, inoculated singly or in combination, promote seed germination and early seedling growth in corn (*Zea mays* L.) and soybean (*Glycine max* L.). *European journal of soil biology*, *45*(1), 28-35.

Cawthray GR (2003) Short communication A n improved reversed-phase liquid chromatographic method for the analysis of low-molecular mass organic acids in plant root exudates, 1011, pp. 233–240.

Chandra D, Srivastava R Gupta VVSR (2019) Field performance of bacterial inoculants to alleviate water stress effects in wheat (*Triticum aestivum* L .), pp. 261–281.

Chen C, Wang E, Yu Q, Zhang Y (2010) Quantifying the effects of climate trends in the past 43 years (1961-2003) on crop growth and water demand in the North China Plain. *Climatic Change*, **100**: 559-578.

Chen L, Zhong Y, Wei G, Cai Y, Shen Z (2014) Development of an integrated modelling approach for identifying multilevel non-point-source priority management areas at the watershed scale. *Water Resour. Res*. **50:** 4095–4109 4015.

Chen C, Zhang J, Lu M, Qin C, Chen Y, Yang L, Huang Q, Wang J, Shen Z, Shen Q (2016) Microbial communities of an arable soil treated for 8 years with organic and inorganic fertilizers. *Biol. Fertil. Soils* 52, 455–467. doi: 10.1007/s00374-016- 1089-5

Chen L, Sun C, Wang G, Xie H, Shen Z (2017). Event-based nonpoint source pollution prediction in a scarce data catchment. *J. Hydrol*. **552**: 13–27.

Chhabra S, Brazil D, Morrissey J, Burke J, O'Gara F, Dowling DN (2013) Fertilization management affects the alkaline phosphatase bacterial community in barley rhizosphere soil. *Biol Fertil Soil* **49**:31–39.

Collavino MM, Sansberro PA (2010) Comparison of in vitro solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth, pp. 727–738. doi: 10.1007/s00374-010-0480-x.

Condron LM, Turner BL, Cade-Menun BJ (2005) Chemistry and dynamics of soil organic phosphorus. In JT Sims, AN Sharpley, eds, Phosphorus: Agriculture and the Environment. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Inc., Madison, WI, pp 87–121

Cordell D, Drangert JO, White S (2008) The story of phosphorus: global security and food for thought. *Global Environmental Change*, **19**:292-305. doi:10.1016/j.gloenvcha.2008.10.009.

Cordell D, Drangert JO, White S (2009) The story of phosphorus: global food security and food for thought. *Global Environmental Change* **19**: 292–305.

Costa R, Götz M, Mrotzek N, Lottmann J, Berg G, Smalla K (2005) Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. FEMS Microbiology Ecology **56**:236–249.

Cu STT, Hutson J, Schuller KA (2005) Mixed culture of wheat (*Triticum aestivum* L.) with white lupin (*Lupinus albus* L.) improves the growth and phosphorus nutrition of the wheat. *Plant and Soil*, 272 (1/2), 143-151.

Devau N, Le Cadre E, Hinsinger P, Ge´rard F (2010) A mechanistic model for understanding root-induced chemical changes controlling phosphorus availability. *Ann Bot* (Lond) **105**: 1183–1197

Devau N, Le Cadre E, Hinsinger P, Colomb B, Gérard F (2011) Fertilization and pH effects on processes and mechanisms controlling dissolved inorganic phosphorus in soils. *Geochim Cosmochim Acta* **75**:2980–2996.

Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J (2017) MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. Nucleic acids research, 45(W1), pp.W180-W188.

Díaz S, Fargione J, Chapin III F S, Tilman D (2006). Biodiversity loss threatens human well being. PLoS biology, 4(8), e277.

Ding J, Jiang X, Ma M, Zhou B, Guan D, Zhao B, et al. Zhou J, Cao F, Li L, Li J (2016). Effect of 35 years inorganic fertilizer and manure amendment on structure of bacterial and archaeal communities in black soil of northeast China. Appl. Soil Ecol. 105, 187–195. doi: 10.1016/j.apsoil.2016.04.010.

Dinkelaker B, Römheld V, Marschner H (1989) Citric acid excretion and precipitation of calcium citrate in rhizosphere of white lupin (*Lupinus albus* L.). *Plant Cell Environ*. **12**: 285–292.

Dissanayaka, D. M. S. B. *et al.* (2015) 'Interspecific facilitation of P acquisition in intercropping of maize with white lupin in two contrasting soils as influenced by different rates and forms of P supply', *Plant and Soil*, 390(1–2), pp. 223–236. doi: 10.1007/s11104-015-2392-x.

Dissanayaka DMSB, Maruyama H, Masuda G, Wasaki J (2015) Interspecific facilitation of P acquisition in intercropping of maize with white lupin in two contrasting soils as influenced by different rates and forms of P supply. *Plant and Soil*, 390(1-2), 223-236.

Dou Z, Toth JD, Galligan DT, Ramberg CF, Ferguson JD (2000) Laboratory procedures for characterizing manure phosphorus. *J Environ Qu*al **29**: 508–514

Doré T, Makowski D, Malézieux E, Munier-Jolain N, Tchamitchian M, Tittonell P (2011) Facing up to the paradigm of ecological intensification in agronomy: revisiting methods, concepts and knowledge. *European journal of agronomy*, **34**: 197-210.

Duchene O, Vian JF Celette F (2017) Intercropping with legume for agroecological cropping systems: Complementarity and facilitation processes and the importance of soil microorganisms. A review, *Agriculture, Ecosystems & Environment*. Elsevier B.V., 240, pp. 148–161. doi: 10.1016/j.agee.2017.02.019.

Elser JJ (2012) Phosphorus: a limiting nutrient for humanity? Current Opinion in Biotechnology, 23:833-838.

FAO (2004) The State of Food and Agriculture 2003–2004 (Food and Agriculture Organization of the United Nations, Rome).

Faridmarandi, S Naja GM (2014) Phosphorus and water budgets in an agricultural basin. *Environ. Sci. Technol*. **48**: 8481–8490.

Faure D, Vereecke D, Leveau JHJ (2009) Molecular communication in the rhizosphere. *Plant Soil* **321**: 279–303. doi:http://dx.doi.org/10.1007/s11104- 008-9839-2.

Faucon MP, Houben D, Lambers H (2017) Plant functional traits: soil and ecosystem services. *Trends Plant Sci* **22**:385-394.

Foley JA, Ramankutty N, Brauman KA, Cassidy ES, Gerber JS, Johnston M, Mueller ND, O'Connell C, Ray DK, West PC, Balzer C, Bennett EM, Carpenter SR, Hill J, Monfreda C, Polasky S,

Rockström J, Sheehan J, Siebert S, Tilman D, Zaks DPM (2011) Solutions for a cultivated planet. *Nature* **478**:337–342.

Fox T, Comerford N, McFee W (1990) Phosphorus and aluminum release from a spodic horizon mediated by organic acids. *Soil Sci. Soc. Am. J*. 54, 1763±1767.

Foy RH, Smith RV, Jordan C, Lennox SD (1995) Upward trend in soluble phosphorus loadings to louch-neach despite phosphorus reduction at sewage-treatment works. *Water Res*. **29:** 1051–1063.

Fraser E, Legwegoh A, Krishna KC, CoDyre, M, Dias G, Hazen S, Johnson R, Martin R, Ohberg L, Sethuratnam S, Sneyd L, Smithers J, Van Acker R, Vansteenkiste J, Wittman H Yada R (2016) Biotechnology or organic? Extensive or intensive? Global or local? A critical review of potential pathways to resolve the global food crisis. *Trends in food science & technology*, *48*, 78-87.

Francioli D, Schulz E, Lentendu G, Wubet T, Buscot F, Reitz T (2016) Mineral vs. organic amendments: microbial community structure, activity and abundance of agriculturally relevant microbes are driven by long-term fertilization strategies. *Front. Microbiol.* 7:1446. doi: 10.3389/fmicb.2016.01446

Fridley JD (2001) The influence of species diversity on ecosystem productivity: how, where, and why? Oikos **93**: 514–526.

Freeman JS, Rowell DL (1981) The adsorption and precipitation of phosphate onto calcite. *J Soil Sci* **32**:75–84.

Frossard E, Condron LM, Oberson A, Sinaj S, Fardeau JC (2000) Processes governing phosphorus availability in temperate soils. *J Environ Qual* **29**:12–53.

Gamez R, Cardinale M, Montes M, Ramirez S, Schnell S, Rodriguez F (2019) Screening, plant growth promotion and root colonization pattern of two rhizobacteria (*Pseudomonas fluorescens* Ps006 and *Bacillus amyloliquefaciens* Bs006) on banana cv. Williams (*Musa acuminata* Colla). *Microbiological research*, *220*, pp.12-20.

Gao B, Huang Y, Huang W, Shi Y, Bai X, Cui S (2017) Driving forces and impacts of food system nitrogen flows in China, 1990 to 2012. *Sci. Total Environ*. **430**: 610–611.

Garbeva P, van Veen JA, van Elsas JD (2004) Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annual Review of Phytopathology **42**:243– 270.

Garbeva P, van Elsas JD, van Veen JA (2008) Rhizosphere microbial community and its response to plant species and soil history. *Plant and Soil* **302**:19–32.

García LJA, Barbas C, Probanza A, Barrientos ML Gutierrez Manero FJ (2001) Low molecular weight organic acids and fatty acids in root exudates of two Lupinus cultivars at flowering and fruiting stages. *Phytochemical Analysis: An International Journal of Plant Chemical and Biochemical Techniques*, *12*(5), pp.305-311.

Gardner WK, Boundy KA (1983) The acquisition of phosphorus by *Lupinus albus* L. IV. The effect of interplanting wheat and white lupin on the growth and mineral composition of the two species. *Plant Soil* **70**:391–402.

Garcia-Pausas J, Paterson E (2011) Microbial community abundance and structure are determinants of soil organic matter mineralisation in the presence of labile carbon. *Soil Biol Biochem* **43**: 1705–1713.

Gerland P, Raftery AE, Sevcikova H, Li N, Gu D, Spoorenberg T, Alkema L, Fosdick BK, Chunn J, Lalic N, Bay G, Buettner T, Heilig GK,Wilmoth J (2014)World population stabilization unlikely this century. *Science* **346**: 234–237.

Gerke J, Römer W, Beißner L (2000) The quantitative effect of chemical phosphate mobilization by carboxylate anions on P uptake by a single root. II. The importance of soil and plant parameters for uptake of mobilized P, *Journal of Plant Nutrition and Soil Science*, 163(2), pp. 213–219. doi: 10.1002/(SICI)1522-2624(200004)163:2<213::AID-JPLN213>3.0.CO;2-0.

Gianinazzi S, Gollotte A, Binet MN, van Tuinen D, Redecker D, Wipf D (2010) Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. *Mycorrhiza* **20**: 519–530. doi:http://dx.doi.org/10.1007/s00572-010-0333-3.

Gerke J, Römer W, Jungk A (1994) The excretion of citric and malic acid by proteoid roots of *Lupinus albus* L.: effect on soil solution concentrations of phosphate, iron and aluminium in the proteoid rhizosphere in samples of an oxisol and a luvisoil. *Z. Pflanzenernähr. Bodenk*. **157**: 289–294.

Gerke, J. (1995): Chemische Prozesse der Nährstoffmobilisierung in der Rhizosphäre und ihre Bedeutung für den Übergang vom Boden in die Pflanze. Cuvillier Verlag, Göttingen.

Goldstein A H, Rogers R D and Mead G (1993) Mining by microbe. *Bio/Technol*. **11**: 1250-1254.

Gregory AS, Watts CW, Griffiths BS, Hallett PD, Kuan HL, Whitmore AP (2009) The effect of long-term soil management on the physical and biological resilience of a range of arable and grassland soils in England. *Geoderma* **153**: 172–185.

Gregory PJ, BE Marshall (2012) Attribution of climate change: a methodology to estimate the potential contribution to increases in potato yield in Scotland since 1960. *Global Change Biology*, **18**: 1372-1388.

Hamilton EW, Frank DA (2001) Can plants stimulate soil microbes and their own nutrient supply? evidence from a grazing tolerant grass. *Ecology* **82**: 2397– 2402. doi:http://dx.doi.org/10.1890/0012-9658 (2001)082[2397:CPSSMA]2.0. CO;2.

Haichar FEZ, Marol C, Berge O, Rangel-Castro JI, Prosser JI, Balesdent J, Heulin T, Achouak W (2008) Plant host habitat and root exudates shape soil bacterial community structure, *ISME Journal*, 2(12), pp. 1221–1230. doi: 10.1038/ismej.2008.80.

Harrison AF (1987) Soil Organic Phosphorus—A Review of World Literature. CAB International, Wallingford, Oxon, UK, p 257

Hauggaard-Nielsen H, Ambus P and Jensen ES (2001) Temporal and spatial distribution of roots and competition for nitrogen in pea-barley intercrops - a field study employing P-32 technique. *Plant Soil* 236, 63–74

Hauggaard-Nielsen H, Jensen ES (2005) Facilitative root interactions in intercrops. *Plant Soil* **274**: 237–250. doi:http://dx.doi.org/10.1007/s11104-004-1305-1.

Heckrath G, Brookes PC, Poulton PR, Goulding KWT (1995) Phosphorus leaching from soils containing different phosphorus concentrations in the Broadbalk experiment. *Journal of environmental quality*, *24*(5), 904-910.

Hinsinger P (2001) Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant Soil* **237**:173–195.

Hinsinger P, Bengough AG, Vetterlein D, Young IM (2009) Rhizosphere: biophysics, biogeochemistry and ecological relevance. *Plant Soil* **321**: 117–152. doi:http://dx.doi.org/10.1007/s11104-008-9885-9

Hinsinger P, Betencourt E, Bernard L, Brauman A, Plassard C, Shen J, Tang X, Zhang F (2011) P for two, sharing a scarce resource: soil phosphorus acquisition in the rhizosphere of intercropped species. *Plant Physiol.* **156**: 1078–1086. doi:http://dx.doi.org/10.1104/pp.111.175331.

Hirsch AM, Bauer WD, Bird DM, Cullimore J, Tyler B, Yoder JI (2003) Molecular signals and receptors: controlling rhizosphere interactions between plants and other organisms. *Ecology* **84**: 858–868. doi:http://dx.doi.org/ 10.1890/0012-9658(2003)084[0858:MSARCR]2.0.CO;2.

Hocking PJ, Randall PJ (2001) Better growth and phosphorus nutrition of sorghum and wheat following organic acid secreting crops. In: Horst WJ, Schenk MK, Burkert A, Claassen N, Flessa H, Frommer WB, Goldbach H, Olfs HW, Romheld V, Sattlemacher B, Schmidhalter U, Schubert S, Wiren NV, Wittenmayer L (eds) Plant nutrition—food security and sustainability of agro-ecosystems through basic and applied research. Kluwer, Dordrecht, pp 548–549.

Hoehn P, Tscharntke T, Tylianakis JM, Steffan-Dewenter I (2008). Functional group diversity of bee pollinators increases crop yield. Proceedings of the Royal Society B: Biological Sciences, 275(1648), 2283-2291.

Hoehn P, Tscharntke T, Tylianakis JM, Steffan-Dewenter I (2008) Functional group diversity of bee pollinators increases crop yield. *Proceedings of the Royal Society B: Biological Sciences*, **275**: 2283-2291.

Hoekstra JM, Boucher TM, Ricketts TH, Roberts C (2005) Confronting a biome crisis: global disparities of habitat loss and protection. Ecology letters, 8(1), 23-29.

Hooper DU, Chapin FS, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S, Schmid B (2005) Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. *Ecological monographs*, **75:** 3-35.

Horst W J, Waschkies C (1987) Phosphorus nutrition of spring wheat in mixed culture with white lupin. *Z. Pflanzenernähr. Bodenk*. **150**: 1–8.

Horst WJ, Kamh M, Jibrin JM, Chude VO (2001) Agronomic measures for increasing P availability to crops. *Plant and Soil*. Kluwer Academic Publishers. Printed in the Netherlands. **237**: 211–223.

Houlton BZ, Wang YP, Vitousek PM, Field CB (2008) A unifying framework for dinitrogen fixation in the terrestrial biosphere. *Nature* **454**: 327–334.

Illmer P, Schinner F (1992) Solubilization of inorganic phosphates by microorganisms isolated from forest soil, *Soil Biol. Biochem*. **24**: 389–395.

Israr D, Mustafa G, Khan KS, Shahzad M, Ahmad N, Masood S (2016) Interactive effects of phosphorus and *Pseudomonas putida* on chickpea (*Cicer arietinum* L.) growth, nutrient uptake, antioxidant enzymes and organic acids exudation. *Plant physiology and biochemistry*, *108*, pp.304-312.

Jaggard K, Qi A, Semenov MA (2007) The impact of climate change on sugarbeet yield in the UK: 1976-2004. *The Journal of Agricultural Science*, **145**: 367-375.

Jain V, Gupta K (2003) The flavonoid naringenin enhances intercellular colonization of rice roots by *Azorhizobium caulinodans*. *Biol. Fertil. Soils* **38**: 119–123. doi:http://dx.doi.org/10.1007/s00374-003-0599-0.

Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea JM (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biol. Fertil. Soils* **37**: 1–16.

Jensen ES, Ambus P, Bellostas N, Brisson N, Corre-Hellou G, Crozat Y, Dahlmann C, Dibet A, von Fragstein P, Gooding M, Hauggaard-Nielsen H, Kasyanova E, Launay M, Monti M, Pristeri A, (2006) Intercropping of cereals and grain legumes for increased production, weed control, improved product quality and prevention of N –losses in European organic farming systems. *Eur. Org. Farming Syst*.

Jensen ES, Peoples MB, Boddey RM, Gresshoff PM, Hauggaard-Nielsen H, Alves BJR, Morrison MJ (2012) Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. *Agronomy for Sustainable Development* **32**: 329–364.

Jiang F, Chen L, Belimov AA, Shaposhnikov AI, Gong F, Meng X, Hartung W, Jeschke DW, Davies WJ, Dodd IC (2012) Multiple impacts of the plant growth-promoting rhizobacterium *Variovorax paradoxus* 5C-2 on nutrient and ABA relations of *Pisum sativum*. *J Exp Bot* 63:6421–6430. https://doi. org/10.1093/jxb/ers301

Johansson JF, Paul LR, Finlay RD (2004) Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. FEMS *Microbiol. Ecol*. **48**: 1–13. doi:http://dx.doi.org/10.1016/j.femsec.2003.11.012.

Johnson CM, Ulrich A (1959) Analytical methods for use in plant analysis. *Calif Agri Exp Stat Bull* **767**:25–78.

Johri JK, Surange S, Nautiyal CS (1999) Occurrence of salt, pH and temperature tolerant phosphate solubilizing bacteria in alkaline soils, *Curr. Microbiol*. **39**: 89–93.

Jones DL, Darrah PR (1994) Role of root derived organic acids in the mobilization of nutrients from rhizosphere. *Plant Soil* **166**: 247–257.

Justes E, Bedoussac L, Corre-Hellou G, Fustec J, Hinsinger P, Jeuffroy MH, Journet EP, Louarn G, Naudin C, Pelzer E (2014) Les processus de complémentarité de niche et de facilitation déterminent le fonctionnement des associations végétales et leur efficacité pour l'acquisition des ressources abiotiques. *Innov. Agron*. 1–24

Kamh M, Horst WJ, Amer F, Mostafa H, Maier P (1999) Mobilization of soil and fertilizer phosphate by cover crops. *Plant Soil* **211**: 19–27.

Kang SC, Ha CG, Lee TG, Maheshwari DK (2002) Solubilization of insoluble inorganic phosphates by a soil-inhabiting fungus *Fomitopsis* sp. PS 102, *Curr. Sci*. **82**: 439–442.

Kizewski F, Liu YT, Morris A, Hesterberg D (2011) Spectroscopic approaches for phosphorus speciation in soils and other environmental systems. *J Environ Qual* **40**: 751–776.

Kleijn D, Rundlöf M, Scheper J, Smith HG, Tscharntke T (2011) Does conservation on farmland contribute to halting the biodiversity decline?. *Trends in ecology & evolution*, *26*(9), 474-481.

Kochian LV (2012). Rooting for more phosphorus. *Nature*, 488:466-467.

Kowalchuk GA, Buma DS, de Boer W, Klinkhamer PG, van Veen JA (2002) Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie Van Leeuwenhoek 81 (1–4), 509.

Krey T, Caus M, Baum C, Ruppel S, Eichler-Löbermann B (2011) Interactive effects of plant growth–promoting rhizobacteria and organic fertilization on P nutrition of *Zea mays* L. and *Brassica napus* L. *Journal of Plant Nutrition and Soil Science*, *174*(4), 602-613.

Kuo S (1996) Phosphorus. In: D.L. Sparks (ed.) Methods of soil analysis. Agronomy 9. ASASSSA, Madison, WI.

Kurth C, Schieferdecker S, Athanasopoulou K, Seccareccia I, Nett M (2016) Variochelins, lipopeptide siderophores from *Variovorax boronicumulans* discovered by genome mining. *Journal of natural products*, *79*(4), pp.865-872.

Lambers H, Shane MW (2007) Role of root clusters in phosphorus acquisition and increasing biological diversity in agriculture. In: J.H.J. Spiertz, P.C. Struik and H.H. van Laar (eds.), Scale and Complexity in Plant Systems Research: Gene-Plant-Crop Relations, 237-250.

Larsen S (1967) Soil phosphorus. *Adv Agron* **19**: 151–210

Latati M, Blavet D, Alkama N, Laoufi H, Drevon JJ, Gérard F, Pansu M, Ounane SM (2014) The intercropping cowpea-maize improves soil phosphorus availability and maize yields in an alkaline soil. *Plant Soil* **385**: 181–191. doi: http://dx.doi.org/10.1007/s11104-014-2214-6.

Lifshitz R, Kloepper JW, Kozlowski M, Simonson C, Carlson J, Tipping EM, Zaleska I (1987) Growth promotion of canola (rapeseed) seedlings by a strain of *Pseudomonas putida* under gnotobiotic conditions. *Canadian Journal of Microbiology*, *33*(5), pp.390-395.

Lindsay WL, Frazier AW, Stephenson HF (1962) Identification of reaction products from phosphate fertilizers in soils. *Soil Sci Soc* Proc 26: 446–452

Lindsay WL, Vlek PLG, Chien SH (1989) Phosphate minerals. In: J.B. Dixon and S.B. Weed (Editors), Minerals in soil environment, 2nd edn. Soil Science Society of America, Madison, pp. 1089–1130.

Li M, Shinano T, Tadano T (1997) Distribution of exudates of lupin roots in the rhizosphere under phosphorus deficient conditions. *Soil Sci. Plant Nutr*. **43**: 237–245.

Li L, Tang C, Rengel Z, Zhang F (2003) Chickpea facilitates phosphorus uptake by intercropped wheat from an organic phosphorus source. *Plant Soil* **248**: 297–303.

Li SM, Li L, Zhang FS, Tang C (2004) Acid phosphatase role in chickpea/maize intercropping. *Ann Bot* **94**:297–303.

Li L, Li S, Sun J, Zhou L, Bao X, Zhang H, Zhang F (2007) Diversity enhances agricultural productivity via rhizosphere phosphorus facilitation on phosphorus deficient soils. *Proc Natl Acad Sci* **104**: 11192–11196.

Li H, Shen J, Zhang F, Marschner P, Cawthray G, Rengel Z (2009) Phosphorus uptake and rhizosphere properties of intercropped and monocropped maize, faba bean, and white lupin in acidic soil. *Biol. Fertil. Soils* **46**: 79–91. doi:http:// dx.doi.org/10.1007/s00374-009-0411-x.

Li C, Kuyper TW, van der Werf W, Zhang J, Li H, Zhang F, Hoffland E (2019) Testing for complementarity in phosphorus resource use by mixtures of crop species. *Plant and Soil*, *439*(1-2), 163-177.

Lobell DB, Cassman KG, Field CB (2009) Crop yield gaps: their importance, magnitudes, and causes. *Annual review of environment and resources*, *34*, 179-204.

Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, Tremblay J, Engelbrektson A, Kunin V, Glavina del Rio T, Edgar RC, Eickhorst T, Ley RE, Hugenholtz P, Green Tringe S, Dangl JL (2012) Defining the core Arabidopsis thaliana root microbiome. *Nature*, *488*(7409), 86-90.

Maestre FT, Callaway RM, Valladares F, Lortie CJ (2009) Refining the stress-gradient hypothesis for competition and facilitation in plant communities. *Journal of Ecology*, *97*(2), pp.199-205.

Malhotra H, Sharma S, Pandey R (2018). Phosphorus Nutrition: Plant Growth in Response to Deficiency and Excess. Plant Nutrients and Abiotic Stress Tolerance, 171–190. doi:10.1007/978-981-10-9044-8_7.

Marschner H, Römheld V, Horst WJ, Martin P (1986) Root-induced changes in the rhizosphere: importance for the mineral nutrition of plants. *Z. Für Pflanzenernähr. Bodenkd*. **149**: 441–456. doi:http://dx.doi.org/10.1002/ jpln.19861490408.

Marschner P, Yang CH, Lieberei R, Crowley DE (2001) Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biol. Biochem*. **33**: 1437–1445. doi:http://dx.doi.org/10.1016/S0038-0717(01)00052- 9.

Marschner P, Crowley D, Yang CH (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. *Plant Soil* **261**: 199–208.

Martins MA, Santos C, Almeida MM, Costa MEV (2008) Hydroxyapatite micro- and nanoparticles: nucleation and growth mechanisms in the presence of citrate species. *J Colloid Interface Sci* **318**: 210–216.

Matson PA (1997) Agricultural intensification and ecosystem properties. Science **277**: 504–509

McDowell, R. W., & Sharpley, A. N. (2001). Approximating phosphorus release from soils to surface runoff and subsurface drainage. *Journal of environmental quality*, *30*(2), 508-520.

McKnight DT, Huerlimann R, Bower DS, Schwarzkopf L, Alford RA, Zenger KR (2019) Methods for normalizing microbiome data: an ecological perspective. Methods in Ecology and Evolution, 10(3):389-400. doi: 10.1111/2041-210X.13115.

McMurdie PJ, Holmes S (2014) Waste not, want not: why rarefying microbiome data is inadmissible. PLoS Comput Biol 10(4): e1003531. doi:10.1371/ journal.pcbi.1003531.

Morel C, Hinsinger P (1999) Root-induced modification of the exchange of phosphate ion between soil solution and soil solid phase. *Plant Soil* **211**: 103–110.

Motsara MR, Bhattacharyya PB, Srivastava B (1995) Biofertilizers their description and characteristics. *Biofertilizer Technology, Marketing and Usage, A sourcebook-cum-Glossary, Fertilizer development and consultation organisation*, 204-204.

Millennium Ecosystem Assessment (2005) Ecosystems and Human Well-Being: Synthesis, Island Press

Moonen A C, Barberi P (2008) Functional biodiversity: an agroecosystem approach. *Agriculture, ecosystems & environment*, *127*(1-2), 7-21. doi: 10.1016/j.agee.2008.02.013

Morgan JAW, Bending GD, White PJ (2005) Biological costs and benefits to plant-microbe interactions in the rhizosphere. *J. Exp. Bot.* 56, 1729–1739. doi: http://dx.doi.org/10.1093/jxb/eri205.

Moss B (2008) Water pollution by agriculture. Philos. Trans. R. Soc. Lond. B: Biol. Sci. **363**: 659–666

Nessner Kavamura V, Hayat R, Clark IM, Rossmann M, Mendes R, Hirsch PR, Mauchline TH (2018) Inorganic nitrogen application affects both taxonomical and predicted functional structure of wheat rhizosphere bacterial communities. *Frontiers in microbiology*, *9*, 1074.

Neumann G, Römheld V (1999) Root excretion of carboxylic acids and protons in phosphorus deficient plants. *Plant Soil* **211**: 121–130.

Neumann K, Verburg P H, Stehfest E, Müller C (2010) The yield gap of global grain production: A spatial analysis. *Agricultural systems*, *103*(5), 316-326.

Nulik J, Dalgliesh N, Cox K, Gabb S (2013) Integrating herbaceous legumes into crop and livestock systems in eastern Indonesia. Australian Centre for International Agricultural Research (ACIAR), Canberra.

Nuruzzaman M, Lambers H, Bolland MDA, Veneklaas EJ (2005a) Phosphorus benefits of different legume crops to subsequent wheat grown in different soils of Western Australia. *Plant and Soil*. Springer **271**: 175–187

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

Nuruzzaman M, Lambers H, Bolland MD, Veneklaas EJ (2006). Distribution of carboxylates and acid phosphatase and depletion of different phosphorus fractions in the rhizosphere of a cereal and three grain legumes. *Plant and Soil*, *281*(1-2), 109-120.

Oenema O, van Liere L, Schoumans O (2005) Effects of lowering nitrogen and phosphorus surpluses in agriculture on the quality of groundwater and surface water in the Netherlands. *J. Hydrol*. **304**: 289–301.

Olander LP, Vitousek PM (2000) Regulation of soil phosphatase and chitinase activity by N and P availability. *Biogeochemistry*, *49*(2), 175-190.

Olde Venterink H (2011) Legumes have a higher root phosphatase activity than other forbs, particularly under low inorganic P and N supply. *Plant and Soil*, 347(1), pp. 137–146. doi: 10.1007/s11104-011-0834-7.

Olsen SR, Cole CV, Watanabe FS, Dean LA (1954) Estimation of available phosphorus in soils by extraction with sodium bicarbonate. United States Department of Agriculture Circular No. 939.

Omar SA (1998) The role of rock phosphate solubilizing fungi and vesicular arbuscular mycorrhiza (VAM) in growth of wheat plants fer- tilized with rock phosphate, *World J. Microb. Biot*. **14**: 211–219.

Pal D, Kotesthane A, Dey U (2016) Screening for Plant Growth Promoting Activity (PGPA) of fluorescent *Pseudomonas* spp. *Int. J. Pure App. Biosci*, *4*(2), pp.156-162.

Pellerin S, Le Clech B, Morel C, Linères M (2003) Gestion de la fertilité phospho-potassique en agriculture biologique: questions posées et premiers résultats. *Compte Rendu de l'Académie d'Agriculture de France* **89**(1): 30–34

Parfitt RL (1989) Phosphate reactions with natural allophone, ferrihydrite and goethite. *J Soil Sci* **40**: 359–369

Pearse SJ, Veneklaas EJ, Cawthray GR, Bolland MD, Lambers H (2006) Carboxylate release of wheat, canola and 11 grain legume species as affected by phosphorus status. *Plant and Soil*, *288*(1-2), 127-139.

Pearse SJ, Veneklaas EJ, Cawthray G, Bolland MD, Lambers H (2007) Carboxylate composition of root exudates does not relate consistently to a crop species' ability to use phosphorus from aluminium, iron or calcium phosphate sources. *New Phytologist*, *173*(1), 181-190.

Peres G, Cluzeau D, Menasseri S, Soussana JF, Bessler H, Engels C, Habekost M, Gleixner G, Weigelt A, Weisser WW (2013) Mechanisms linking plant community properties to soil aggregate stability in an experimental grassland diversity gradient. *Plant Soil* **373**: 285–299.

Pingali PL (2012) Green revolution: impacts, limits, and the path ahead *Proc. Natl. Acad. Sci.* U.S.A., **109**: 12302-12308.

Porter JR, Xie L, Challinor AJ, Cochrane K, Howden SM, Iqbal MM, DB Lobell, Travasso MI (2014) Food security and food production systems. In: *Climate Change 2014: Impacts, Adaptation, and Vulnerability.Part A: Global and Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Field CB, Barros VR, Dokken DJ, Mach KJ, Mastrandrea MD, Bilir TE, Chatterjee M, Ebi KL, Estrada YO, Genova RC, Girma B, Kissel ES, Levy AN, MacCracken S, Mastrandrea PR, White LL (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, pp. 485-533.

Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE (2010) Global pollinator declines: trends, impacts and drivers. *Trends in ecology & evolution*, **25**: 345-353.

Pradhan N, Sukla LB (2005) Solubilization of inorganic phosphate by fungi isolated from agriculture soil, *African J. Biotechnol*. **5:** 850– 854.

Qiang C, Peng H, Gaobao H, (2004) Effect of intercropping on soil microbial and enzyme activity in the rhizosphere. *Acta Pratacult. Sin*. **14**: 105–110.

Raghothama KG (1999) Phosphate acquisition. *Annu Rev Plant Physiol Plant Mol Biol* **50**: 665–693

Rashid M, Khalil S, Ayub N, Alam S, Latif F (2004) Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms (PSM) under in vitro conditions. *Pak J Biol Sci*, *7*(2), pp.187-196.

Rai M, Reeves T, Collette L, Allara M (2011) Save and grow: a policymaker's guide to sustainable intensification of smallholder crop production. Rome FAO.

Richardson AE, Simpson RJ (2011) Soil Microorganisms Mediating Phosphorus Availability. *Plant Physiol*. 156, 989– 996

Richardson AE, Simpson RJ (2011) Soil Microorganisms Mediating Phosphorus Availability. *Plant Physiol*. 156, 989– 996

Rodríguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*, 17(4–5), pp. 319–339. doi: 10.1016/S0734-9750(99)00014-2.

Rotaru, V. (2018) Effects of Combined Application of *Bradyrhizobium japonicum* and *Pseudomonas putida* on Nutrients and Water Contents of Soybean in Relation to Soil Moisture Regime. In *"Agriculture for Life, Life for Agriculture" Conference Proceedings* (Vol. 1, No. 1, pp. 110-115). Sciendo.

Ryan PR, Delhaize E, Jones DL (2001) Function and mechanism of organic anion exudation from plant roots. Annu. Rev. Plant Physiol. *Mol. Biol*. **52:** 527–560.

Sahu B, Singh J, Shankar G, Pradhan A (2018) *Pseudomonas fluorescens* PGPR bacteria as well as biocontrol agent: A review. *IJCS*, *6*(2), pp.01-07.

Schelud'ko AV, Makrushin KV, Tugarova AV, Krestinenko VA, Panasenko VI, Antonyuk LP, Katsy EI (2009) Changes in motility of the rhizobacterium *Azospirillum brasilense* in the presence of plant lectins. *Microbiol. Res*. 164, 149–156.

Shane MW, De Vos M, De Roock S, Lambers H (2003) Shoot P status regulates cluster-root growth and citrate exudation in *Lupinus albus* grown with a divided root system, *Plant Cell Environ*. **26**: 265–273.

Shen J, Yuan L, Zhang J, Li H, Bai Z, Chen Z, Zhang W, Zhang F (2011) Phosphorus dynamics: from soil to plant. *Plant Physiology*, **156**:997-1005.

Shi SJ, Richardson AE, O'Callaghan M, DeAngelis KM, Jones EE, Stewart A, Firestone MK, Condron LM (2011) Effects of selected root exudate components on soil bacterial communities. FEMS *Microbiol Ecol*. **77**:600-610

Shu L, Shen J, Rengel Z, Tang C, Zhang F, Cawthray GR (2007) Formation of cluster roots and citrate exudation by *Lupinus albus* in response to localized application of different phosphorus sources. *Plant Science*, *172*(5), 1017-1024.

Singh SK, Singh PP, Gupta A, Singh AK Keshri J (2019) Tolerance of Heavy Metal Toxicity Using PGPR Strains of *Pseudomonas* Species. In *PGPR Amelioration in Sustainable Agriculture* (pp. 239-252). Woodhead Publishing.

Smith VH, Tilman GD, Nekola JC (1999) Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environ. Pollut*. 100 (1-3), 179.

Solaiman Z, Marschner P, Wang D, Rengel Z (2007) Growth, P uptake and rhizosphere properties of wheat and canola genotypes in an alkaline soil with low P availability. *Biology and Fertility of Soils*, *44*(1), 143.

Soman C, Li D, Wander MM, Kent AD (2017) Long-term fertilizer and crop-rotation treatments differentially affect soil bacterial community structure. *Plant Soil* 413, 145–159. doi: 10.1007/s11104-016- 3083-y

Song YN, Zhang FS, Marschner P, Fan FL, Gao HM, Bao XG, Sun JH, Li L (2007) Effect of intercropping on crop yield and chemical and microbiological properties in rhizosphere of wheat (*Triticum aestivum* L.), maize (*Zea may*s L.), and faba bean (*Vicia faba* L.). *Biol. Fertil. Soils* **43**: 565–574. doi:http://dx.doi.org/ 10.1007/s00374-006-0139-9.

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.

Spohn M, Kuzyakov Y (2013) Soil Biology & Biochemistry Distribution of microbial- and root-derived phosphatase activities in the rhizosphere depending on P availability and C allocation e Coupling soil zymography with 14 C imaging. *Soil Biology and Biochemistry*. Elsevier Ltd, 67, pp. 106–113. doi: 10.1016/j.soilbio.2013.08.015.

Struik PC, Kuyper TW, Brussaard L, Leeuwis C (2014) Deconstructing and unpacking scientific controversies in intensification and sustainability: why the tensions in concepts and values?. *Current Opinion in Environmental Sustainability*, *8*, 80-88.

Sun C, Chen L, Zhai LM, Liu HB, Zhou HZ, Wang QR, Wang K, Shen, ZY (2018) National-scale evaluation of phosphorus emissions and the related water-quality risk hotspots accompanied by increased agricultural production. *Agriculture, Ecosystems and Environment* **267**: 33–41

Sun B, Gao Y, Wu X, Ma H, Zheng C, Wang X, Zhang H, Li Z, Yang H (2019) The relative contributions of pH, organic anions, and phosphatase to rhizosphere soil phosphorus mobilization and crop phosphorus uptake in maize/alfalfa polyculture. *Plant and Soil*, 1-17. doi: 10.1007/s11104-019-04110-0.

Supit I, van Diepen CA, de Wit AJW, Kabat P, Baruth B, Ludwig F (2010) Recent changes in the climate yield potential of various crops in Europe. *Agricultural Systems*, **103**: 683-694.

Tan H, Barret M, Mooij M, Rice O, Morrissey J, Dobson A, Griffiths B, O'Gara F (2013) Long-term phosphorus fertilisation increased the diversity of the total bacterial community and the *phoD* phosphorus mineraliser group in pasture soils. *Biol Fertil Soils* 49: 661–672.

Tang X, Bernard L, Brauman A, Daufresne T, Deleporte P, Desclaux D, Souche G, Placella SA, Hinsinger P (2014) Increase in microbial biomass and phosphorus availability in the rhizosphere of intercropped cereal and legumes under field conditions. *Soil Biol. Biochem*. **75**: 86–93. doi:http://dx.doi.org/ 10.1016/j.soilbio.2014.04.001.

Tscharntke T, Klein AM, Kruess A, Steffan-Dewenter I, Thies C (2005). Landscape perspectives on agricultural intensification and biodiversity: ecosystem service management. *Ecology letters*, *8*(8), 857-874.

Tilman D, Balzer C, Hill J, Befort BL (2011) Global food demand and the sustainable intensification of agriculture. *Proc. Natl. Acad. Sci*. U. S. A.

Tilman D, Fargione J, Wolff B, D'antonio C, Dobson A, Howarth R, Schindler D, Schlesinger WH, Simberloff D, Swackhamer D (2001) Forecasting agriculturally driven global environmental change. *Science*, *292*(5515), 281-284.

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. *Nature* **418**:671–677.

Tadano T, Ozawa K, Sakai H, Osaki M, Matsui H (1993) Secretion of acid phosphatase by the roots of crop plants under phosphorus-deficient conditions and some properties of the enzyme secreted by lupin roots. In *Plant Nutrition—from Genetic Engineering to Field Practice* (pp. 99-102). Springer, Dordrecht.

Trivedi P, Sa T (2008) *Pseudomonas corrugata* (NRRL B-30409) mutants increased phosphate solubilization, organic acid production, and plant growth at lower temperatures. *Current microbiology*, *56*(2), pp.140-144.

Turner BL, Papha´zy MJ, Haygarth PM, McKelvie ID (2002) Inositol phosphates in the environment. *Philos Trans R Soc Lond B Biol Sci* **357**: 449–469

Turner BL, Leytem AB (2004) Phosphorus compounds in sequential extracts of animal manures: chemical speciation and a novel fractionation procedure. *Environ Sci Technol* **38**: 6101–6108.

UNEP **(**2016). *A snapshot of the world's water quality: towards a global assessment.* Nairobi, United Nations Environment Programme (UNEP).

Uren NC, Reisenauer HM (1988) The role of root exudation in nutrient acquisition. *Adv. Plant Nutr.* **3**: 79–114.

Vaccari DA (2009) Phosphorus: a looming crisis. *Scientific American*, 300(6), 54-59.

Vacheron J, Renoud S, Muller D, Babalola OO, Prigent-Combaret C (2015) Alleviation of abiotic and biotic stresses in plants by *Azospirillum*. In: Cassán, F. D., Okon, Y., Creus, C.M. (Eds.), Handbook for Azospirillum. *Springer International Publishing*, pp. 333–365.

Valenzuela H (2016) Agroecology: a global paradigm to challenge mainstream industrial agriculture. *Horticulturae*, *2*(1), 2.

Van Kessel C, Singleton PW, Hoben HJ (1985) Enhanced N-transfer from a soybean to maize by vesicular arbuscular mycorrhizal (VAM) fungi. *Plant Physiol*. **79**: 562–563

Vazquez P, Holguin G, Puente M, E Lopez Cortes A, Bashan Y (2000) Phosphate solubilizing microorganisms associated with the rhizosphere of mangroves in a semi arid coastal lagoon. *Biol. Fert. Soils* **30**: 460–468.

Veneklaas EJ, Stevens J, Cawthray GR, Turner S, Grigg AM, Lambers H (2003) Chickpea and white lupin rhizosphere carboxylates vary with soil properties and enhance phosphorus uptake. *Plant Soil* **248**: 187–197.

Vitousek PM, Naylor R, Crews T, David MB, Drinkwater LE, Holland E, Johnes PJ, Katzenberger J, Martinelli LA, Matson PA (2009) Nutrient imbalances in agricultural development. *Science* 324: 1519–1520.

Vyas P, Gulati A (2009) Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent *Pseudomonas*. *BMC microbiology*, *9*(1), p.174.

Wang D, Marschner P, Solaiman Z, Rengel Z (2007) Growth, P uptake and rhizosphere properties of intercropped wheat and chickpea in soil amended with iron phosphate or phytate. *Soil Biol. Biochem*. **39**: 249–256. doi:http://dx. doi.org/10.1016/j.soilbio.2006.07.013.

Wang LJ, Nancollas GH (2008) Calcium orthophosphates: crystallization and dissolution. *Chem Rev* **108**: 4628–4669

Wasaki J, Sakaguchi J, Yamamura T, Ito S, Shinano T, Osaki M, Kandeler E (2018) P and N deficiency change the relative abundance and function of rhizosphere microorganisms during cluster root development of white lupin (*Lupinus albus* L.). *Soil science and plant nutrition*, *64*(6), 686-696. doi: 10.1080/00380768.2018.1536847.

Webber H, Ewert F, Olesen JE, Müller C, Fronzek S, Ruane AC, Bourgault M, Martre P, Ababaei B, Bindi M, Ferrise R, Finger R, Fodor N, Gabaldón-Leal C, Gaiser T, Jabloun M, Kersebaum KC, Lizaso JI, Lorite IJ, Manceau L, Moriondo M, Nendel C, Rodríguez A, Ruiz-Ramos M, Semenov MA, Siebert S, Stella T, Stratonovitch P, Trombi G, Wallach D (2018) Diverging importance of drought stress for maize and winter wheat in Europe. *Nat Commun*.; **9**: 4249.

Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y, Birmingham A, Hyde ER (2017) Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*, **5**(1), p.27.

Westerman RL (1990) Soil testing and plant analysis, 3rd edn. American Society of Agronomy and Soil Science Society of America, Madison, Wisconsin.

Wichern F, Mayer J, Joergensen RG, Müller T (2007) Release of C and N from roots of peas and oats and their availability to soil microorganisms. *Soil Biol. Biochem.* **39**: 2829–2839. doi:http://dx.doi.org/10.1016/j.soilbio.2007.06.006

Wieland G, Neumann R, Backhaus H (2001) Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. *Appl. Environ. Microbiol*. **67**: 5849–5854. doi:http://dx.doi.org/ 10.1128/AEM.67.12.5849-5854.2001.

Wik M, Pingali P, Broca S (2008) Global Agricultural Performance: Past Trends and Future Prospects. Background Paper for the World Development Report (World Bank, Washington, DC).

Willey RW (1990) Resource use in intercropping systems. Agric. Water Manage. Irrig. Sugarcane Assoc. Crops **17**: 215–231. doi:http://dx.doi.org/10.1016/0378- 3774(90)90069-B.

Wouterlood M, Cawthray GR, Scanlon TT, Lambers H, Veneklaas EJ (2004a) Carboxylate concentrations in the rhizosphere of lateral roots ofchickpea (*Cicer arietinum*) increase during plant development, but are not correlated with phosphorus status of soil or plants. *New Phytologist*. **162**: 745–753.

Wouterlood M, Cawthray GR, Turner S, Lambers H, Veneklaas EJ (2004b) Rhizosphere carboxylate concentrations of chickpea are affected by genotype and soil type. *Plant and Soil*. **261**:1–10.

Wouterlood M, Lambers H, Veneklaas EJ (2005) Plant phosphorus status has a limited influence on the concentration of phosphorus-mobilising carboxylates in the rhizosphere of chickpea. *Functional Plant Biology*. **32**: 153–159.

Xue Y, Xia H, Christie P, Zhang Z, Li L, Tang C (2016) Crop acquisition of phosphorus, iron and zinc from soil in cereal/ legume intercropping systems: a critical review. *Ann Bot* **117**:363–377

Yadav RS, Tarafdar JC (2001) Influence of organic and inorganic phosphorus supply on the maximum secretion of acid phosphatase by plants. *Biol Fert Soils.* **34**: 140–143.

Zabihi HR, Savaghebi GR, Khavazi K, Ganjali A, Miransari M (2011) *Pseudomonas* bacteria and phosphorous fertilization, affecting wheat (*Triticum aestivum* L.) yield and P uptake under greenhouse and field conditions. *Acta physiologiae plantarum*. *33*(1), pp.145-152.

Zak DR, Holmes WE, White DC, Peacock AD, Tilman D (2003) Plant diversity, soil microbial communities, and ecosystem function: are there any links? *Ecology* **84**: 2042–2050.

Zarei T, Moradi A, Kazemeini SA, Farajee H, Yadavi A (2019) Improving sweet corn (*Zea mays* L. var *saccharata*) growth and yield using *Pseudomonas fluorescens* inoculation under varied watering regimes. *Agricultural Water Management*, *226*, p.105757.

Zhalnina K, Louie KB, Hao Z, Mansoori N, da Rocha UN, Shi S, Cho H, Karaoz U, Loqué D, Bowen BP, Firestone MK, Northen TR, Firestone MK (2018) Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nature microbiology*, *3*(4), 470. doi: 10.1038/s41564-018-0129-3.

Zheng BX, Ibrahim M, Zhang DP, Bi QF, Li HZ, Zhou GW, Ding K, Penuelas J, Zhu YG, Yang, XR (2018) Identification and characterization of inorganic-phosphate-solubilizing bacteria from agricultural fields with a rapid isolation method. *AMB express*, *8*(1), 47. doi: 10.1186/s13568-018-0575-6.

Zhou D, Huang XF, Chaparro JM, Badri DV, Manter DK, Vivanco JM, Guo J (2015) Root and bacterial secretions regulate the interaction between plants and PGPR leading to distinct plant growth promotion effects. *Plant Soil* **401**: 259– 272. doi:http://dx.doi.org/10.1007/s11104-015-2743-7.

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