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**Role and function of green manure crops in mobilisation and  
utilisation of soil legacy phosphorus in cropping systems**

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A thesis  
submitted in partial fulfilment  
of the requirements for the Degree of  
Doctor of Philosophy

at  
Lincoln University  
by  
Phuong Van Nguyen

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Lincoln University

2021

Abstract of a thesis submitted in partial fulfilment of the  
requirements for the Degree of Doctor of Philosophy

## **Role and function of green manure crops in mobilisation and utilisation of soil legacy phosphorus in cropping systems**

by

Phuong Van Nguyen

In many agroecosystems, significant quantities of phosphorus (P) applied in mineral and organic forms have accumulated in the soil as “legacy P” which represents a resource that may be used to maintain crop production with reduced P inputs. The objective of this study was to investigate and quantify the impact of various green manure plant species on legacy P mobilisation and utilisation in selected volcanic soils. To achieve this objective, a combination of root study container experiments; short- and long-term glasshouse pot trials; and an analysis of soil from an established field experiment were carried out in this PhD study. A variety of potential green manure species were assessed in volcanic soils derived from pumice and ash. Analyses included plant yield, P uptake, and root organic anion exudation and soil P fractionation, microbial P, and phosphatase enzyme activities. Results showed that plant available inorganic P in excess of agronomic optima prevented the mobilisation of legacy soil P. Lupin (*Lupinus angustifolius*) and pea (*Pisum sativum*) were the best performing green manure species with respect to legacy soil P mobilisation with a rhizosphere P depletion of 8-11%. Lupin was better than pea, which was at least partly attributed to a combination of fine root hairs and organic anion exudation. Inclusion of lupin and pea green manures in the absence of P inputs significantly increased cereal crop yield (27 - 35%) and P uptake (15 - 29%) over two rotations compared with fallow, which was mainly attributed to mobilisation of legacy soil P. However, results from a field trial with continuous P inputs showed that, after 5 years, legume green manure did not improve maize yield and P uptake. This was likely due to a combination of factors including high soil P status via continued P inputs and the fact that green manure was left on the surface rather than incorporated into the soil. The findings of this study clearly demonstrated that legume green manures, when managed properly, have the potential to enhance soil legacy P mobilisation and thus increase P use efficiency in temperate crop systems, which in turn may reduce P inputs required to maintain production.

**Key words:** soil legacy phosphorus, green manure crops, soil P fractionation, low molecular weight organic anions, phosphatase enzyme activities, *Lupinus angustifolius*, *Pisum sativum*.

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# Chapter 1

## Introduction

### 1.1 Soil phosphorus dynamics

Phosphorus (P) plays an indispensable role in maintaining all forms of life as it is a component of every living cell and vital for several physiological and biochemical processes, for example, P constitutes nucleic acids (DNA and RNA), phospholipids, sugar phosphates and ATP (energy-rich pyrophosphate) (Bünemann and Condron, 2007; Syers et al., 2008). Adequate P addition is necessary for the accumulation and release of energy during cellular metabolism and for seed and root formation, crop quality, and strength of straw in cereals (Haygarth et al., 2013). On the contrary, P deficiency causes a wide range of symptoms for plants, for instance, stunted growth, thin stems, bluish-green leaves and stems, delays in maturation, sparse flowering and poor seed quality (Brady and Weil, 2008).

The P cycle in managed agricultural systems (agroecosystems) is mainly controlled by mineral fertilizer and manure inputs (Haygarth et al., 2013). The use efficiency of P inputs is relatively low (only 5-20%) in the year of application, with a vast majority of applied P converted to different inorganic and organic P forms in soils (Loehr, 1974; Sattari et al., 2012; Zhu et al., 2018). Additionally, P loss of 1 - 6 kg P ha<sup>-1</sup> year<sup>-1</sup> occurs via runoff and subsurface flow (Condron, 2004) and even >50 kg P ha<sup>-1</sup> year<sup>-1</sup> due to erosion in some extreme events. These losses can cause significant negative impacts on water quality through accelerated eutrophication (Bolster et al., 2019; Bünemann and Condron, 2007; Condron, 2004).

Most of the P accumulated in soils undergoes a number of transformations, including adsorption-desorption, precipitation-dissolution reactions, and biological mineralisation and immobilisation (Haygarth et al., 2013) (Figure 1.1).

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**Figure 1. 1 An overview of the phosphorus cycle in managed agricultural systems (Haygarth et al., 2013).**

Adsorption-desorption are chemical processes in which soluble P exchanges between the soil solution and solid phases by sorption, where the major P adsorption sites are charged surfaces of iron and aluminium hydrous oxides, clay minerals, and calcium carbonate (calcite) (Frossard et al., 1995). For example, hydrous oxides of iron and aluminium supply reactive surfaces where hydroxyl ions ( $\text{OH}^-$ ) may be exchanged with orthophosphate ions. By contrast, desorption is a release of orthophosphate ions from sorption state in response to reduced inorganic P in soil solution. Desorption may be accelerated by low molecular weight organic anions such as oxalate and citrate exuded by plant roots and associated microbes (Frossard et al., 1995; Hinsinger, 2001; Pierzynski and McDowell, 2005).

Precipitation-dissolution processes between phosphate ions ( $\text{H}_2\text{PO}_4^-$ ,  $\text{HPO}_4^{2-}$ ) and Ca, Al, Fe and Mn ions also control P availability (Haygarth et al., 2013). For example, in neutral to alkaline soils, the precipitation of calcium and phosphate occurs by direct reaction between  $\text{Ca}^{2+}$  and orthophosphate ions and by P adsorption onto calcite which then transforms to calcium phosphate minerals such as hydroxyapatite [ $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ] (Frossard et al., 1995; Hinsinger, 2001). In acid soils, inorganic P ions react with iron (Fe) or aluminium (Al) ions to form minerals such as strengite ( $\text{FePO}_4 \cdot 2\text{H}_2\text{O}$ ) and variscite ( $\text{AlPO}_4 \cdot 2\text{H}_2\text{O}$ ) (Frossard et al., 1995; Hinsinger, 2001). By contrast, dissolution of these minerals is greatest at low soil pH and accelerated by exudation of low molecular weight organic acids by plant roots and microorganisms (Bah et al., 2006; Frossard et al., 1995; Randhawa, 2003; Somado et al., 2003; Vanlauwe et al., 2000).

Mineralization-immobilization are biological processes. Immobilization is the biological conversion of inorganic P to organic P through two principal pathways, namely during decomposition of organic matter by microorganisms to release energy, inorganic P is assimilated from soil solution into microbial biomass and potentially cycled to detritus as organic P, while inorganic P taken up by plants is assimilated into plant tissue in organic forms (Bünemann and Condron, 2007; Haygarth et al., 2013). Mineralization is the process whereby inorganic P is released from organic P compounds, catalysed by the action of phosphatase enzymes (Condron et al., 2005; Richardson et al., 2005; Richardson et al., 2011).

Phosphorus availability in soil is primarily determined by the concentration of inorganic P in the soil solution, which is taken up by plants and microorganisms, although concentrations of inorganic P in soil solution are very low ( $0.1 - 1 \text{ mg P L}^{-1}$ ) (Thomas Sims et al., 2005; Zhu et al., 2018). Inorganic P removed from soil solution by plants and microorganisms has to be continually replenished by mobilization of inorganic and organic P from soil via a combination of dissolution, desorption, and mineralization (Clarholm et al., 2015; Easterwood and Sartain, 1990; Menezes-Blackburn et al., 2017; Ohno and Crannell, 1996; Pypers et al., 2005; Zhu et al., 2018).

However, in high P fertility levels, only available P may sufficiently provide P for the plant uptake due to rapidly soluble P replenishment. However, available P concentration decreased over time and the rate of decrease would be fast in first years and then slow down in following years, which also depended on initial soil P test (Dodd and Mallarino, 2005). Crop yields therefore did not decrease for a number of years with no P application in high available P soil (Dodd and Mallarino, 2005; Gallet et al., 2003; Rowe et al., 2016b). By contrast, although total soil P was significant, available P may be low in soils with high P sorption capacities. The decrease of P availability could be caused by P fixation by active Al and Fe components (Dahlgren et al., 2004; Holford, 1997). Moreover, soluble P depleted by plants is slowly replenished by other forms in high P sorption soils; therefore, plant P

uptake was low in high P sorption soils and vice versa (Holford, 1997). It appeared that organic anions and activity of phosphatase enzymes at root-soil interface may be significantly reduced for P mobilisation by active sorption sites in high P sorptivity.

## **1.2 Legacy soil phosphorus**

In agroecosystems, P use efficiency in soil is relatively low because the majority of applied P complexes with reactive surfaces via ligand exchange between phosphate ions and oxides and hydroxides of Al and Fe on the surface of 1:1 clay minerals, precipitation of Al and Fe phosphates in acid soils, precipitation of Ca-P in alkaline soils, or organically-complexed forms (Coelho et al., 2019; Condrón et al., 2005; Doydora et al., 2020; Menezes-Blackburn et al., 2017). Such complexation reactions are key drivers manipulating the availability of applied P. Therefore, in order to maintain crop production, annual P fertilizer is applied to compensate not only for P removal by crops but also for complexed P in soil (Coelho et al., 2020). Consequently, continued P application has exceeded P crop requirements (Condrón, 2004; Zhu et al., 2018) and accumulated significant amounts of P in soils called “residual P” or “legacy P” (Condrón et al., 2013; Zhu et al., 2018). Even though soil legacy P is present, more external P application may be unavoidable (Doydora et al., 2020). Globally, around 17.5 million tonnes of phosphate rock is used (Lu and Tian, 2017) of which only 5-20% is taken up by plants in the application year (Coelho et al., 2019; Zhu et al., 2018), while P accumulates in soils at a rate of nearly 10 million tons annually (Doydora et al., 2020). According to Sattari et al. (2012), the proportion of accumulated P differed greatly between regions from 1965 to 2007 (Figure 1.2). For example, in Western Europe, cumulative P inputs over 4 decades amounted to an average of 1,115 kg P ha<sup>-1</sup>, while just 32% of this amount was taken up by crops. The P use efficiency was slightly higher in Asia, America and Eastern Europe with half of applied P retained in soil in the same period (Sattari et al., 2012), while nearly 80% of applied P still remained in the soil in Oceania. It is estimated that about 71% arable cropland area had P surpluses (MacDonald et al., 2011).

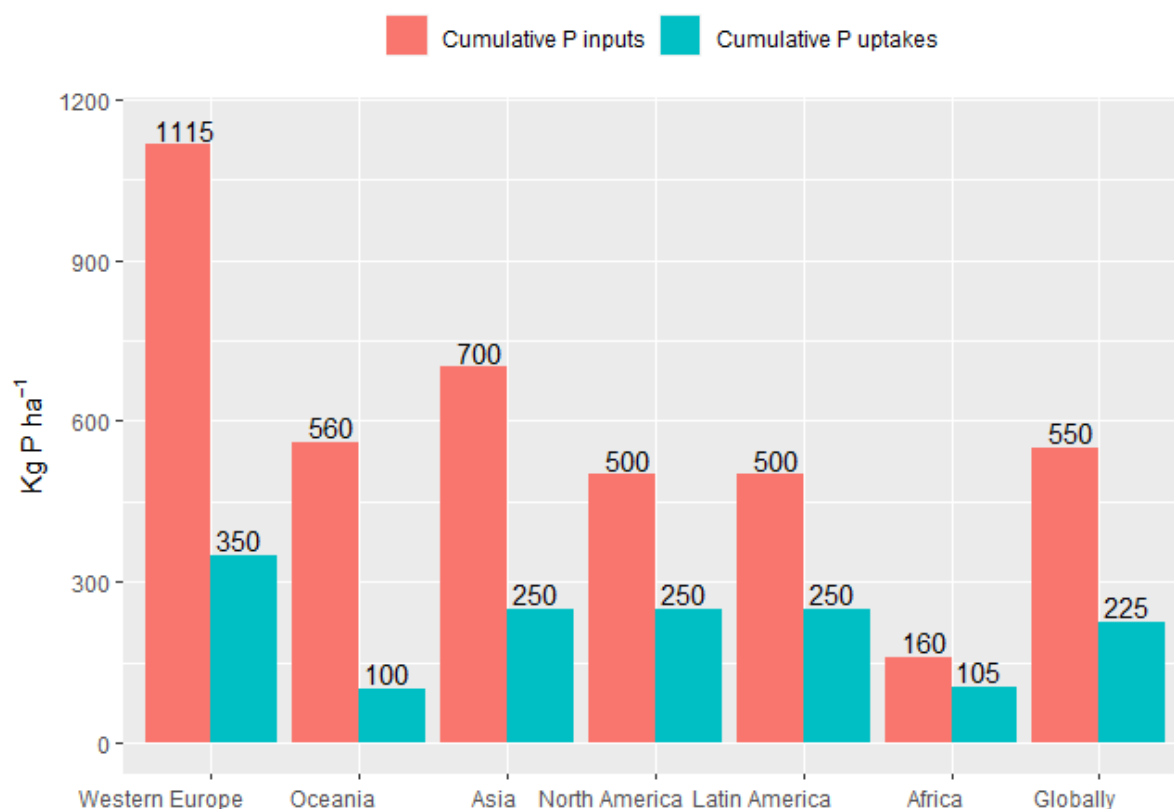


Figure 1. 2 The imbalance between cumulative P inputs and cumulative P uptakes of the world (1965 to 2007) (Data from Sattari et al. (2012)).

The build-up of legacy soil P is a major environmental challenge. Legacy P can eventually be lost via leaching and overland flow for soils containing more labile P (Dodd et al., 2014; McDowell and Sharpley, 2001) and via erosion for soils storing all P forms including less labile pools (Xiong et al., 2021) thus sustaining eutrophication for years to come (Condrón et al., 2013; Haygarth et al., 2013). For example, when concentration of Olsen P in topsoil was above 60 mg P kg<sup>-1</sup>, significant P transfer in drainage water (>150 µg P L<sup>-1</sup>) occurred during winter (Condrón, 2004). At the same time, it is estimated that, in the worst-case scenario, non-renewable phosphate rock may be depleted within 100 years (Dushyantha et al., 2021; Sattari et al., 2012; Van Vuuren et al., 2010; Zhu et al., 2018). Thus, improved utilization of legacy soil P is critical for reducing P inputs as well as minimising environmental risks (Condrón et al., 2013; Stutter et al., 2012).

### 1.3 Reduction and mobilisation of legacy soil phosphorus

Legacy soil P may become available for future crops beyond the year it was first applied and so represents a profound potential for agriculture in the future (Syers et al., 2008). On average, in arable soils and grasslands, total P stocks could potentially provide the equivalent of 352 years of agronomic P use with the orthophosphate pool providing ~201 years and the monoester pool providing ~117 years of crop production (Menezes-Blackburn et al., 2017; Sattari et al., 2016).

Therefore, several options for utilizing soil legacy P have been recommended. Drawing down P to optimum levels in soils with high P status can be simply achieved by ceasing P fertilizer application coupled with repeated biomass removal (Boitt et al., 2017b; Dodd et al., 2013; McDowell et al., 2016; McDowell et al., 2020; Menezes-Blackburn et al., 2016). However, crop production under this approach may be reduced in the long-term if available P drops below optimum levels (Dodd et al., 2012; Menezes-Blackburn et al., 2016).

Other approaches can be used to access soil legacy P when available P stocks are reduced by targeting recalcitrant forms including inorganic and organic P. For example, application of phosphate-solubilizing microorganisms such as bacteria and fungi can mobilise soil P via their release of organic acids and phosphatases (Doydora et al., 2020). In addition, microorganisms such as arbuscular mycorrhizas that have symbiotic relationships with vascular plant roots are able to increase the volume of soil scavenged for P (Smith and Smith, 2011). The development of biofertilizers such as loading phosphatases on clay, nanoclay, nanofertilizers, and inoculation with specific microorganisms (e.g. phosphate solubilising bacteria) can help to mineralize soil organic P (Calabi-Floody et al., 2012; Menezes-Blackburn et al., 2017; Menezes-Blackburn et al., 2011; Raymond et al., 2021). Moreover, plant breeding or genetic modification may lead to genotypes whose roots can release appropriate enzymes and organic anions to mobilise fixed P (Condrón et al., 2013; Menezes-Blackburn et al., 2018; Richardson et al., 2009b; Rowe et al., 2016a) or to genotypes that have more P-efficient root configurations including branched roots, enhanced lateral root length and production, increased root hairs or cluster root formation (Lambers et al., 2006; Lynch, 1995; Lynch and van Beem, 1993).

#### **1.4 Green manures as an approach for mobilising legacy P**

An understudied approach for improving acquisition of soil legacy P is the sowing of green manure crops (cover crops). Following the autumn harvest of crops in temperate agriculture, temperature and light conditions allow some plant growth although insufficient to produce commercial crops (Thorup-Kristensen et al., 2003). Several attempts have been made to use this period to grow plants which can cover land surface, prevent nutrient leaching, and enhance soil fertility. Such crops are often termed cover crops, catch crops or green manures (Thorup-Kristensen et al., 2003). The term “catch crops” applies when cover crops are grown to ‘catch’ available N in the soil, preventing N leaching, while the term “green manure” applies when cover crops are applied mainly to improve the nutrition of the subsequent crops (Fowler et al., 2004; Thorup-Kristensen et al., 2003). Many studies have focused on the effect of cover crops on N leaching reduction (Abdalla et al., 2019; De Notaris et al., 2018; Thorup-Kristensen et al., 2003).

However, green manure plant species are also able to mobilise soil P which may then provide a P source for subsequent crops (Doolette et al., 2019; Hallama et al., 2018; Hassan et al., 2013; Kamh et

al., 1999). After incorporated in soils, green manures can be decomposed and mineralized to release P, directly improving available P (Nziguheba et al., 2000; Randhawa et al., 2005). For example, Randhawa et al. (2005) used an isotopic dilution method to identify organic P flux rates for soils unamended and amended with blue lupin green manure: after 21 days of incubation, daily gross organic P mineralization rates were fivefold higher under amended soil compared with unamended soil. Furthermore, the decomposition of green manures produces organic anions which compete with orthophosphate ions for adsorption sites, thus reducing P sorption of soils (Easterwood and Sartain, 1990; Menezes-Blackburn et al., 2017; Ohno and Crannell, 1996; Pypers et al., 2005). Ohno and Crannell (1996) extracted dissolved organic matter (DOM) from animal manures (cattle and poultry) and green manures [hairy vetch (*Vicia villosa*) and crimson clover (*Trifolium incarnatum*)] and compared their effects with citric acid on soil P sorption. The results illustrated that DOM from green manures and citric acid inhibited P sorption in the order citric acid>clover>vetch, while DOM of animal manures did not inhibit P sorption. Green manure DOM may complex with aluminium (Al) through ligand exchange reactions and so hinder the sorption of orthophosphate ions onto Al minerals, hence increasing P availability.

A combination of green manure and phosphate rocks could be a potential option. The effect of green manures on the solubilisation of phosphate rocks (PRs) indicates a significant efficiency in using PRs in agricultural production. According to Haynes (1992), green manures such as blue lupin often take up more cations than anions so they release a net flux of H<sup>+</sup> ions from roots, causing rhizosphere acidification and thus making PRs as plant-available as monocalcium phosphate. Randhawa (2003) found that after seven weeks of soil-reactive PR contact, 18% of P in reactive PR (RPR) dissolved in bulk soil because of soil acidity, while 31% of RPR dissolved in a blue lupin rhizosphere. Similarly, a combination of PRs and green manures such as Calopogonium (*Calopogonium caeruleum*), Gliricidia (*Gliricidia sepium*), Imperata (*Imperata cylindrical*) significantly increased fertilizer P utilisation of setaria grass (*Setaria sphacelata*) from 3% to 48% (Bah et al., 2006). Soil available P under a combination of green manures and PRs can also increase for subsequent maize crop (Vanlauwe et al., 2000). Likewise, Zaharah and Bah (1997) reported that, compared to compost or farmyard manure, green manures increased the solubility of less reactive PRs (Ohno and Crannell, 1996).

Green manure, a rich C source, increases soil biological activity which is an important property of soil fertility. Biological activity is stimulated, which may lead to, for example, higher phosphatase enzyme production (Alamgir et al., 2012; Cui et al., 2015; Piotrowska-Długosz and Wilczewski, 2014; Pypers et al., 2007) or greater microbial biomass P which is an important pool due to its fast turnover for subsequent crops (Bünemann et al., 2008; Hallama et al., 2018). The quality of green manures may affect their decomposition rates and soil biological activity; the green manure C:N and C:P ratios are likely to be two main parameters to assess quality. Respiration rate typically has a negative

correlation with the C:P ratio (Alamgir et al., 2012) meaning that high C:P ratios may lead to P immobilization (Hassan et al., 2012a; Nziguheba et al., 2000), while low C:P ratios may result in P mineralization from the green manure biomass (Kwabiah et al., 2003; Randhawa et al., 2005). High quality green manures may also be comparable with mineral fertilizers. For example, Nziguheba et al. (2000) suggested that green manure such as *Tithonia diversifolia*, *Sesbania sesban*, *Croton megalocarpus* or *Lantana camara* could be replaceable for inorganic P fertilizer for available P enhancement. In addition, Gachengo et al. (1998) compared treatments of application of *Tithonia diversifolia* and triple superphosphate on maize and noted that P recovery reached the highest in 3 crops (79%) for *Tithonia diversifolia*, while it was 4% for triple superphosphate.

### 1.5 Processes for P mobilisation in the rhizosphere

Processes for P mobilization by plants (green manures) occur in the rhizosphere (root-soil interface) (Figure 1.3) (Richardson et al., 2009a), which is a narrow soil cylinder (between 3 - 5 mm radius) surrounding the root (Chen et al., 2002; Zoysa et al., 1997, 1998b). In the rhizosphere, P solubilization and mineralization occur simultaneously, while plant roots and microorganisms may compete for mobilised P (Marschner et al., 2007).



**Figure 1. 3 Physiological, chemical and biological/biochemical processes that influence P availability and transformation in the rhizosphere (Richardson et al., 2009a).**

Rhizosphere processes that influence P availability and thus plant P uptake mainly involve H<sup>+</sup> ions, organic anions and phosphatase enzymes supplied from roots and microbial activities. A number of studies demonstrate that organic anions change the chemistry of the rhizosphere and solubilize several forms of inorganic and organic P (Clarholm et al., 2015; Richardson et al., 2009a). Under P deficiency, white lupin (*Lupinus albus*), blue lupin, and yellow lupin (*Lupinus luteus*) increased citrate exudation (Egle et al., 2003). Such organic anions can improve soil P solubility by (i) replacing phosphate ions via direct ligand exchange with Al/Fe oxyhydroxides; (ii) complexing with Al/Fe species thus reducing P sorption sites; and by (iii) increasing negative charges of soil particle surfaces through their adsorption (Ae et al., 1990; Bolan et al., 1997; Cu et al., 2005; Egle et al., 2003; Kirk et al., 1999; Otani et al., 1996; Wu et al., 2018).

Organic anions have different capacities in mobilizing soil P. Clarholm et al. (2015) reported that complexes of cations (Ca<sup>2+</sup>, Al<sup>3+</sup> and Fe<sup>3+</sup>) and low molecular mass organic acids increase in stability with the number of carboxylic groups (mono- < di- < tricarboxylates) because di- or tri-carboxylates containing at least two carboxylic groups can chelate with Al/Fe create stable chelation. Moreover, the position of hydroxyl group plays an important role in complex stabilization. For example, citrate with three carboxylic groups and one hydroxyl group in  $\alpha$ -position forms significantly stronger complexes with Al<sup>3+</sup> and Fe<sup>3+</sup> than oxalate. Therefore, in addition to the quantity of organic anions, their quality may be crucial to P mobilization. The H<sup>+</sup> ions released with the organic anions also play an important role in mobilizing soil P. For instance, after 41 days since planting, rape (*Brassica napus* var. Emerald) decreased rhizosphere pH from 6.1 to 5.3, leading to depletion of many inorganic P pools including moderately labile, acid-soluble, and residual P (Hedley et al., 1982). In addition, rhizosphere acidification has been reported for several plant species such as tea (*Camellia sinensis*), calliandra (*Calliandra calothyrsus*), guinea grass (*Panicum maximum*), bean (*Phaseolus vulgaris*) and tithonia (*Tithonia diversifolia*), thus resulting in more phosphate rock dissolution compared with bulk soil (George et al., 2002a; Zoysa et al., 1997, 1998b, 1999).

Clarholm et al. (2015) established an unbutton model with a three-step mechanism for active nutrient acquisition including P from soil organic matter (SOM) as follows: (1) SOM is destabilized by low molecular mass organic anions that are released by roots and fungi, which form stable chemical complexes with the polyvalent SOM-bridging metals (Ca, Al and Fe), (2) exuded phosphatase enzymes break down the destabilized and newly exposed organic compounds, and so release P in bioavailable forms, and (3) local uptake of P by roots and fungi occurs (Figure 1.4).

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**Figure 1. 4 Hypothetical illustration of a plant-induced three-step decomposition procedure of soil organic matter (Clarholm et al., 2015).**

Organic P hydrolysis in the rhizosphere is primarily driven by root acid phosphatase, fungal acid phosphatase, and bacterial alkaline phosphatase enzymes (Chen et al., 2002; Clarholm et al., 2015; Richardson et al., 2001). For example, under sterile conditions, plant roots can secrete extracellular phosphatase enzymes for acquiring more labile organic P (Richardson et al., 2009b). Chen et al. (2002) found that phosphatase enzyme activities increased in the rhizosphere of radiata pine while depletion of organic P (extracted by 0.1 M NaOH) in the rhizosphere of radiata pine significantly correlated with activities of all phosphatase enzymes. Greater rhizosphere phosphatase activity was also observed under several agroforestry species, for instance, tithonia and *Crotalaria* (George et al., 2002b) and cash crops (Hallama et al., 2018; Nuruzzaman et al., 2006). Moreover, it has been demonstrated that organic C derived from roots stimulates microbial activity in the rhizosphere which in turn increases P cycling (Hallama et al., 2018; Hallama et al., 2021).

## **1.6 Knowledge gaps**

Historical P application exceeded plant P demand, accumulated a large P amount in soil as “soil legacy P”. While non-renewable reserves of P would be depleted in 50 – 100 years. Green manure plant species may have ability to mobilise soil legacy P via several mechanisms occurring in the rhizosphere where exudates released by roots such as low molecular weight organic anions and phosphatase enzymes may desorb or mineralise inorganic or organic P forms. Consequently, legacy P

in fixed forms can be unlocked for plant uptake. Included green manures, in turn, are able to return P in available forms for reducing P inputs, while compromising crop productivity. However, very little work has focused on green manures' role for utilising soil P in particular soil legacy P in cropping systems.

## **1.7 Research context, hypothesis, and objectives**

### **1.7.1 Research context**

In many intensively managed agroecosystems, significant quantities of P applied as mineral and organic fertilizers have accumulated in the soil, in both bioavailable and more recalcitrant forms. These reserves of "legacy soil P" represent a valuable resource that could potentially be mobilised to maintain primary production with reduced P fertilizer inputs. Improving overall P use efficiency in this way is desirable since P is a vital, non-renewable resource, and known reserves of readily available phosphate rock are being depleted to meet ever-increasing demand. This ongoing depletion, together with uneven distribution of global phosphate rock reserves, is likely to result in increased volatility in the supply and cost of phosphate rock in the future. Green manures are short-term crops grown between main crops as an alternative to fallow. Green manures are designed to ensure soil cover and enhance nutrient retention and are incorporated into soil at an immature (vegetative) stage. Most studies on the use of green manures in temperate agroecosystems carried out to date have focused on its role in the retention and recycling of nitrate-N. However, there is evidence that specific plant species can mobilise organic, sparingly-soluble, and recalcitrant forms of P in soil, and it may be possible to employ these species as green manures. Enhanced soil biological activity in relation to plant matter decomposition may also enhance P cycling and bioavailability. Volcanic soils cover more than 124 million hectares (1%) and support nearly 10% of the world's population. These soils have a high P retention capacity due to high amorphous oxide content, and therefore represent a significant challenge with respect to legacy P accumulation and mobilization.

### **1.7.2 Main Objective**

To investigate and quantify the impact of selected green manure plant species on legacy P mobilization and utilisation in volcanic soils.

### **1.7.3 Hypothesis**

Selected plant species can be employed as green manures to enhance mobilisation of legacy P and thereby significantly contribute to improved crop P utilisation in P-enriched volcanic soils.

### 1.7.4 Specific Objectives

**Experiment 1:** To assess and quantify the relative ability of a range of potential green manure plant species to access and utilize P in two contrasting volcanic soils that had received significant inputs of phosphorus fertilizer.

**Experiment 2:** To investigate the detailed mechanisms responsible for enhanced mobilization of legacy soil P in the rhizosphere of selected green manure plant species.

**Experiment 3:** To determine the impact of green manure inclusion on soil P dynamics and crop P utilisation in a controlled environment (extended glasshouse experiment).

**Experiment 4:** To assess the immediate impact of different rates of green manure incorporation on soil P availability and crop P utilisation.

**Experiment 5:** To investigate the impact of green manure on soil P dynamics and crop P utilisation in a 5-year field experiment (case study).

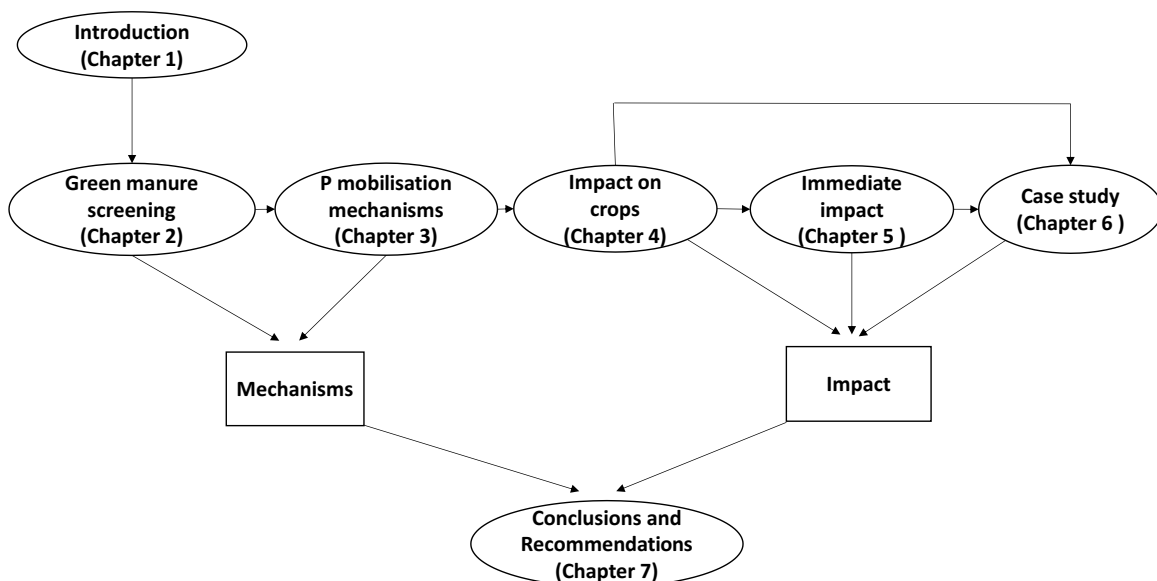


Figure 1. 5 Flow diagram of research program and thesis structure.

## Chapter 2

# Impact of green manure crop species on rhizosphere soil phosphorus

### 2.1 Introduction

Inputs of P are required to enhance and maintain primary production, leading to accumulation of soil legacy P by combination of adsorption, precipitation, and biological immobilization. Enhanced mobilization of soil legacy P could contribute to improving overall P use efficiency in agroecosystems.

Inclusion of green manures or cover crops in crop rotations can significantly enhance biological cycling of P in soil, which may in turn contribute to improving the bioavailability and utilisation of applied P and soil P. However, different plant species may have various impacts on soil P mobilization relating to root morphology and rhizosphere mechanisms.

Very little consideration has been given to the impacts and benefits of green manure crops on dynamics, bioavailability, and utilisation of legacy P in cropping systems. The objectives of this study were to investigate and quantify the short-term mobilisation of immediate P in medium-high fertility soils by a selection of potential green manure crops and determine the relative effect on soil P concentrations and forms of the low molecular weight organic anions in root exudates.

### 2.2 Material and Methods

#### 2.2.1 Green manure species

Based on a review of the literature relating to P demand and acquisition, 11 plant species were selected for preliminary experiment, including *Lupinus angustifolius* (two varieties including early and late flowering varieties) (Egle et al., 2003; Pearse et al., 2006a), *Pisum sativum* (pea) (Nuruzzaman et al., 2006), *Cicer Arietinum* (chickpea) (Veneklaas et al., 2003; Vu et al., 2008; Wouterlood et al., 2004), *Fagopyrum esculentum* (buckwheat) (Possinger et al., 2013; Teboh and Franzen, 2011), *Trifolium repens* (white clover) (Deguchi et al., 2017), *Tithonia speciosa* (tithonia), *Lupinus polyphyllus* (russell lupin) (White et al., 1995), *Brassica napus* (rape) (Hedley et al., 1983; Hoffland, 1992), *Raphanus sativus* (forage radish) (Zhang et al., 1997), and *Brassica juncea* (mustard) (Marschner et al., 2007). The short-term growth and root morphology of these plant species were assessed, and five plant species with high shoot biomass and extensive root systems were selected for further investigation: early- and late-flowering blue lupin, pea, chickpea, and buckwheat.

### 2.2.2 Soils

The two topsoils (0 - 10 cm) selected for this study had been maintained under grazed pasture for over 20 years with annual inputs of P in the form of superphosphate. The soils were derived from different volcanic parent materials, namely pumice (Immature Orthic Pumice (NZ Classification), Typic udvitrand (USDA Classification)) and volcanic ash (Typic Orthic Allophanic (New Zealand Classification); Typic hapludand (USDA Classification)). They were sourced from the Taupo and Taranaki regions of the North Island of New Zealand, respectively. Selected chemical properties of the pumice and volcanic soils are presented in Table 2.1. While the pH and cation exchange capacity of both soils were similar, the Olsen P, total P, and anion storage capacity (P retention) were markedly higher in the volcanic soil compared with the pumice soil. On the other hand, total carbon (C) and nitrogen (N) were higher in the Pumice soil, although corresponding C:N ratios were similar in both soils.

Table 2. 1 Selected chemical properties determined for the pumice and volcanic ash soils.

	Pumice	Volcanic ash
pH <sub>H2O</sub>	5.9	6.4
Total Carbon (%)	8.3	6.5
Total Nitrogen (%)	0.72	0.58
C:N	11.5	11.2
CEC (me 100 g <sup>-1</sup> )	24	22
Total P (mg kg <sup>-1</sup> )	1,123	2,788
Olsen P (mg L <sup>-1</sup> )	21	53
Anion Storage Capacity (%)	39	95

### 2.2.3 Rhizosphere containers

A modified version of the rhizosphere study container system originally developed by Chen et al. (2002) was used to separate and quantify short-term soil P depletion or accretion by the different green manure plant species (Figure 2.1). The study container comprised two 40 mm diameter plastic cylinders with 20 µm nylon mesh (Sefar Nitex, Switzerland) attached to the base of the top 40 mm cylinder to prevent roots from penetrating, other than water, nutrients and root exudates. The mesh created a rhizoplane to the lower cylinder of which the top 5 mm of soil was classified as “rhizosphere” soil, and the remaining 15 mm cylinder represented “non-rhizosphere” or “bulk” soil (sealed with 100

$\mu\text{m}$  nylon mesh). Each container was placed on a layer of cotton felt (5 mm) atop a 25 mm base of acid-washed sand connected to a water reservoir maintained at 0 kPa – allowing water to wick into the container. The pumice and volcanic soils were air-dried, ground  $< 1 \text{ mm}$  and packed into the upper and lower containers at densities of 0.90 and 0.89  $\text{g cm}^{-3}$  based on bulk density, respectively. Due to variability in soil P forms noted by Chen et al. (2003), 8 replicates of each soil-plant combination were included in the experiment. Plants were grown from seed in the upper compartment for 21 days with the addition of nitrogen, sulphur and molybdenum (5 ml  $8.1 \text{ g L}^{-1}$  ammonium sulphate  $((\text{NH}_4)_2\text{SO}_4)$  and 1 ml  $3.6 \text{ g L}^{-1}$  ammonium molybdate  $((\text{NH}_4)_2\text{MoO}_4)$  – equivalent to 360, 390 and 70  $\text{kg ha}^{-1}$  of nitrogen, sulphur, and molybdenum) in a glasshouse maintained at 15 - 23°C. After 21 days, the lower compartment was attached, and the plants were allowed to grow for an additional 40 days prior to plant and soil sampling.

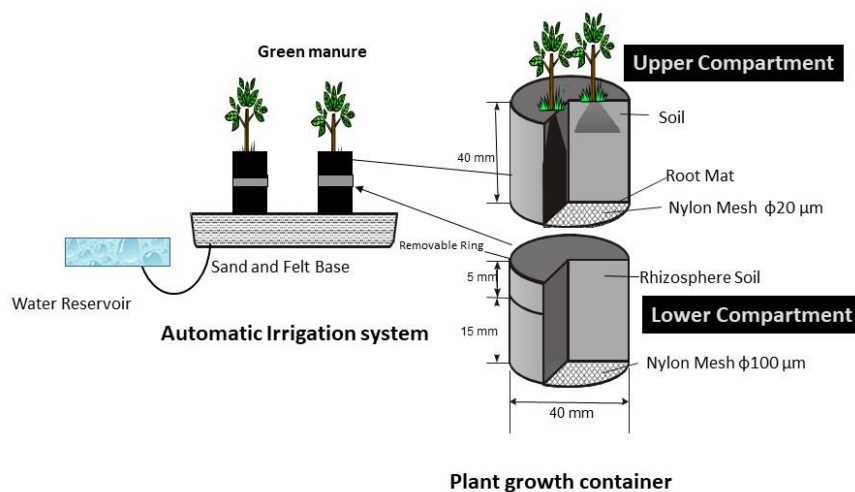


Figure 2.1 Rhizosphere study container and irrigation system.

## 2.2.4 Sampling and analysis

**Plants:** After 61 days (21 + 40) plant shoots were cut at the soil surface, dried at 65°C, weighed, and ground ( $< 1 \text{ mm}$ ) prior to determination of P content by ICP-OES following digestion with nitric acid-hydrogen peroxide (Anderson, 1996; Metson, 1972).

**Soils:** After 40 days, the top 5mm ring of the lower container was carefully separated from the top container mesh and the bulk soil cylinder. Soils from the rhizosphere and bulk soil cylinders were dried at 30°C and sieved  $< 1 \text{ mm}$  prior to P fractionation by sequential extraction. We used the

sequential extraction scheme developed by Boitt et al. (2018b) based on Chen et al. (2000) and Condrón and Newman (2011) (Appendix A1). This involved sequential extraction with 1M ammonium chloride ( $\text{NH}_4\text{Cl}$ ), 0.5 M sodium bicarbonate ( $\text{NaHCO}_3$ ) at pH 8.5, 0.1 M sodium hydroxide ( $\text{NaOH}$ ) ( $\text{NaOH I}$ ), 1 M hydrochloric acid ( $\text{HCl}$ ), 0.1 M  $\text{NaOH}$  ( $\text{NaOH II}$ ), followed by sulphuric acid-hydrogen peroxide digestion of the residue. Inorganic P in the alkaline extracts was determined by colorimetry (Dick and Tabatabai, 1977; He and Honeycutt, 2005) (Appendix A3), and total P was determined by the same method after ammonium persulphate digestion (Eisenreich et al., 1975; Murphy and Riley, 1962) (Appendices A2, A4). The difference between total and inorganic P in the  $\text{NH}_4\text{Cl}$ ,  $\text{NaHCO}_3$ , and both  $\text{NaOH}$  extracts represented organic P. Inorganic and organic P extracted by  $\text{NH}_4\text{Cl}$  and  $\text{NaHCO}_3$  were designated “labile P”, while inorganic and organic P extracted by the first and second  $\text{NaOH}$  extracts were designated “moderately labile P” ( $\text{NaOH I}$ ) and “stable P” ( $\text{NaOH II}$ ), respectively. Inorganic P determined in the  $\text{HCl}$  extract and the final acid digest were designated “acid-soluble-P” (Calcium inorganic P) and “residual P”, respectively (Boitt et al., 2018b).

**Rhizoplane organic anions:** Low molecular weight organic anions (hereafter referred to as organic anions) released by roots in exudates were collected by adsorption onto the anion exchange membrane (No. 55164 2S, BDH Laboratory Supplies, England) at the rhizoplane. Preparation of the membranes involved cutting 2.5 cm x 2.5 cm squares, soaking them in deionized water for 24 hours, and in 0.5 M  $\text{NaHCO}_3$  for 24 hours before being rinsed with deionized water and stored in 0.1 M  $\text{NaCl}$  at 4°C (Cheesman et al., 2010; Schefe et al., 2008; Shi et al., 2011a). After upper and lower cylinders were separated (40 days), the anion exchange membrane squares, backed with moist filter paper (Whatman GFC), were placed under the root mat (rhizoplane) (Figure 2.2) for 2 hours (Shi et al., 2011a). After removal the membranes were rinsed with deionized water and anions desorbed with 4 ml of 0.5 M  $\text{HCl}$  (3 hours at 4°C). Seven organic anions (acetate, citrate, formate, lactate, malate, malonate, and pyruvate) were analysed by High Performance Liquid Chromatography using Shimadzu HPLC with UV-Vis Detector (Shi et al., 2011a).



Figure 2.2 The method of rhizoplane organic anion collection by anion exchange membrane.

### 2.2.5 Statistical analysis

A one-way analysis of variance (ANOVA) was carried out to determine differences in above-ground plant yield and P uptake, total rhizoplane organic anions, depletion of total inorganic P and of total organic P (TukeyHSD;  $\alpha=0.05$ ) between the five plant species for each soil type. One-way ANOVA was also used to investigate differences in the means of each P fraction between rhizosphere and bulk soil for each plant species–soil combination ( $\alpha=0.05$ ). Pearson correlation coefficients ( $r$ ) were used to determine correlations between root citrate exudation and moderately labile and stable inorganic P. The results were calculated and analysed with R statistical software package (version 4.0.2).

## 2.3 Results

Mean data for plant dry matter yield and P uptake for different green manure crops grown in the pumice and volcanic ash soils are shown in Table 2.2. For the pumice soil, plant yield and P uptake were similar for all plant species except early-flowering lupin. On the other hand, for the volcanic soil, yield was significantly higher for pea and buckwheat, and P uptake was significantly greater for buckwheat compared with other species.

Table 2. 2 Mean shoot dry matter yield (DMY) ( $\text{g pot}^{-1}$ ) and P uptake ( $\text{mg P pot}^{-1}$ ) determined for green manure plants grown in the pumice and volcanic ash soils (for each soil type, values for differences without a common letter are significantly different at  $\alpha=0.05$ ). Ef = early flowering; Lf = late flowering.

	Pumice		Volcanic ash	
	DMY ( $\text{g pot}^{-1}$ )	P uptake ( $\text{mg P pot}^{-1}$ )	DMY ( $\text{g pot}^{-1}$ )	P uptake ( $\text{mg P pot}^{-1}$ )
Ef-lupin	1.095 c	1.20 b	1.188 c	1.43 b
Lf-lupin	1.813 a	1.45 ab	1.462 c	1.46 b
Pea	1.465 b	1.61 a	2.350 ab	2.11 b
Chickpea	1.699 ab	1.53 a	1.641 c	1.48 b
Buckwheat	1.650 ab	1.49 a	2.945 a	3.54 a

Boxplots for mean concentrations ( $\text{mg P kg}^{-1}$ ) of labile P, moderately labile P, calcium P, stable P, and residual P determined in the rhizosphere and bulk soils after 40 days growth are presented in Figure 2.3, 2.4, 2.5, 2.6, and 2.7, respectively. Differences in the concentrations of the various soil P fractions determined between rhizosphere and bulk soils were assumed to represent either depletion or accretion of P had occurred in response to 40 days of plant uptake. For the pumice soil, depletion of labile inorganic P occurred for all plant species except pea ( $5 - 8 \text{ mg P kg}^{-1}$ ), while accretion of labile organic P was determined for the same species ( $11 - 28 \text{ mg P kg}^{-1}$ ). For the volcanic ash soil, the only difference was the depletion of inorganic P under blue lupin ( $11 - 13 \text{ mg P kg}^{-1}$ )

(Figure 2.3). Results for moderately labile P showed that for the pumice soil there was a depletion of inorganic P for all plant species (20 - 63 mg P kg<sup>-1</sup>), which was only observed for early-flowering-lupin (101 mg P kg<sup>-1</sup>) in the volcanic ash soil. Moderately labile organic P depletion occurred in the pumice soil for pea and early flowering lupin (45-62 and 45 mg P kg<sup>-1</sup>) (Figure 2.3). Data for acid-soluble inorganic P showed that significant depletion occurred for both lupin species in the pumice soil (15 - 16 mg P kg<sup>-1</sup>) (Figure 2.5). Corresponding results for stable P revealed that depletion of both inorganic P (2 - 7 mg P kg<sup>-1</sup>) and organic P (17 - 33 mg P kg<sup>-1</sup>) occurred in the pumice soil under almost all plant species, while no depletion was observed for the volcanic ash soil (Figure 2.6). Similarly, a reduction for residual P only occurred in the pumice soil for late-flowering lupin, pea, and buckwheat (Figure 2.7).

The impact of the different potential green manure plant species on mobilization of total extracted inorganic and organic P (i.e. sums of extractable P fractions) are presented in Table 2.3. The data clearly showed that in the pumice soil both lupin varieties depleted rhizosphere inorganic P to a much greater extent (88-91 mg P/kg) than the other plant species (16-32 mg P/kg), while significant depletion was only evident for early-flowering lupin in the volcanic ash soil. The corresponding data for organic P revealed that significant depletion only occurred for early-flowering lupin and pea in the pumice soil.

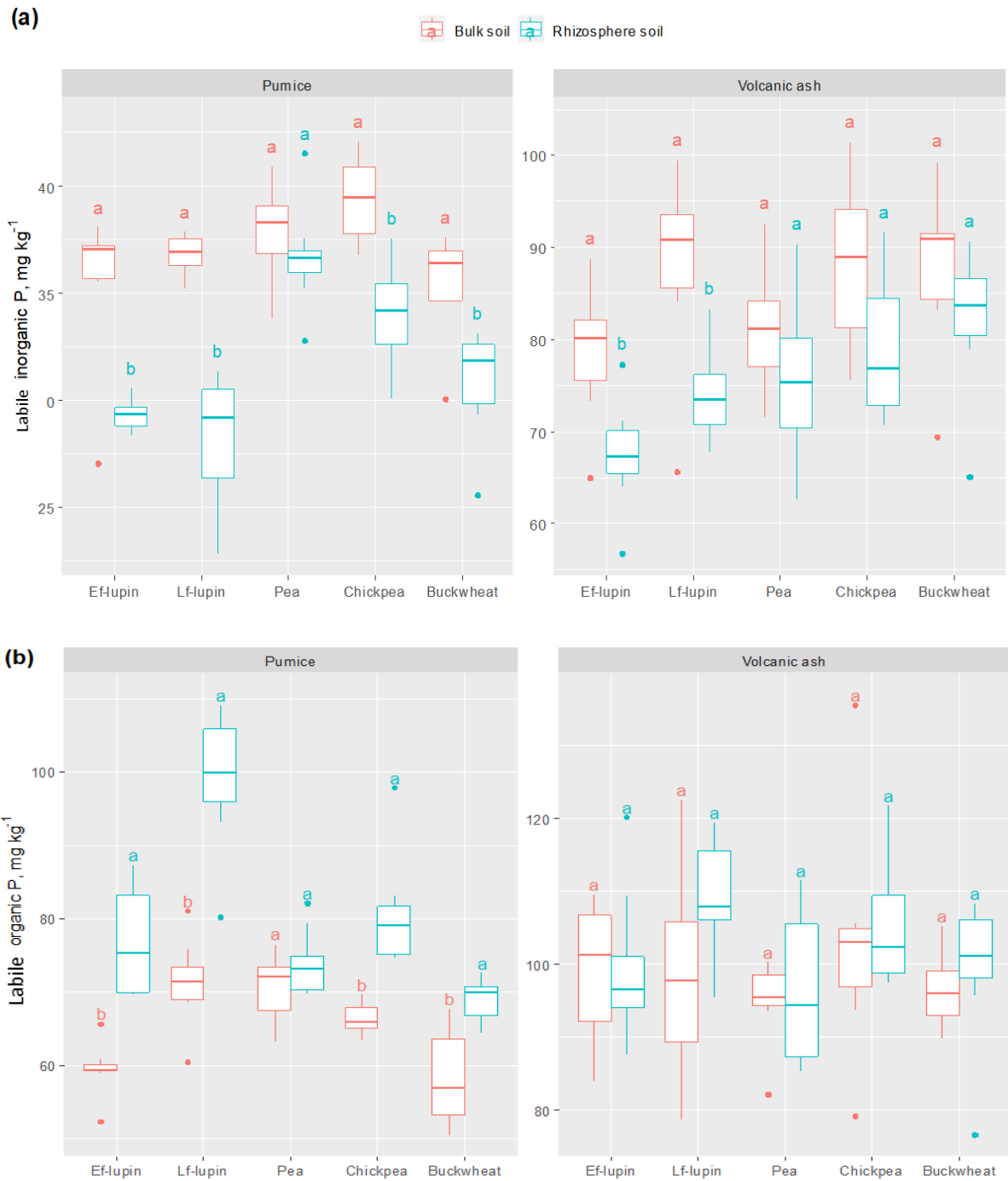
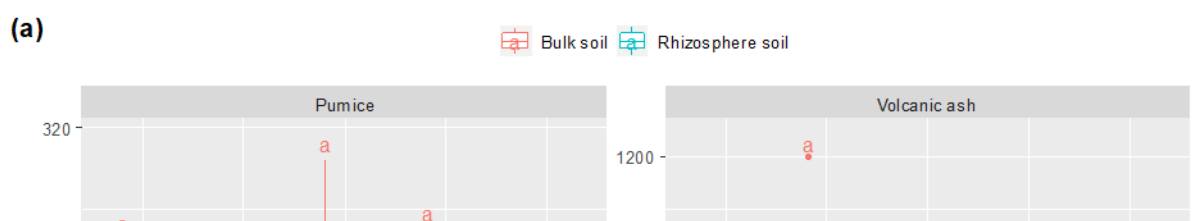


Figure 2. 6 Concentrations ( $\text{mg P kg}^{-1}$ ) of labile inorganic P (a) and organic P (b) determined in rhizosphere and bulk pumice and volcanic ash soils for five green manure plant species after 40 days (for each plant species on a soil type, values for different soil zones without a common letter are significantly different at  $\alpha=0.05$ ; the boxplots describe values including median, two hinges, two whiskers and outliers).



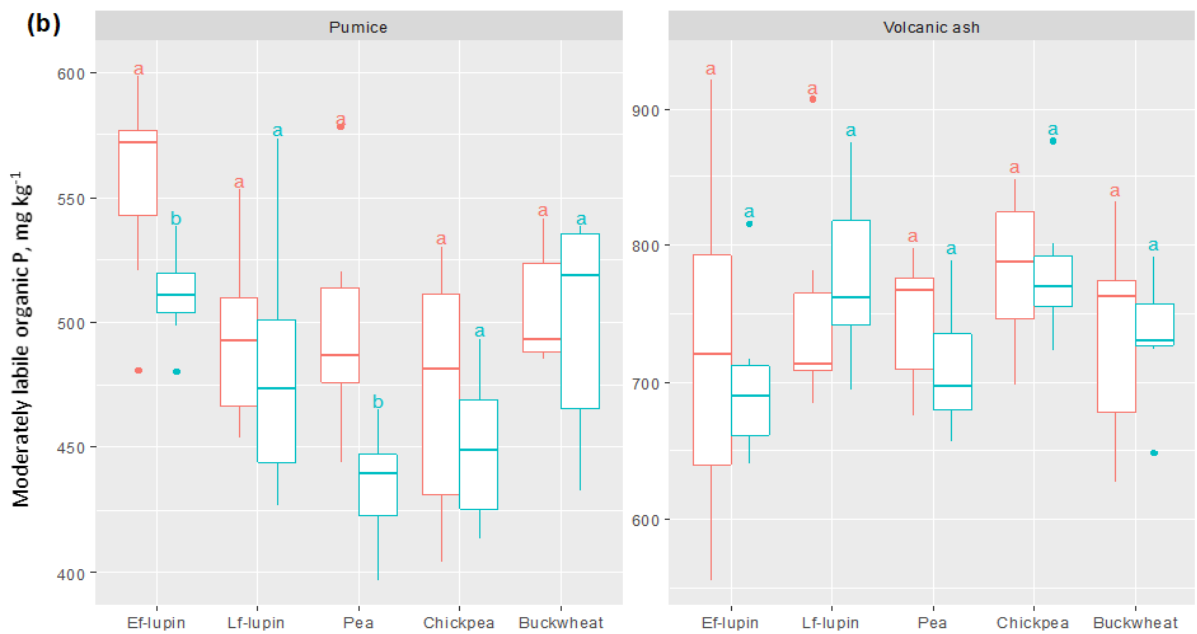


Figure 2. 7 Concentrations ( $\text{mg P kg}^{-1}$ ) of moderately labile inorganic P (a) and organic P (b) determined in rhizosphere and bulk pumice and volcanic ash soils for five green manure plant species after 40 days (for each plant species on a soil type, values for different soil zones without a common letter are significantly different at  $\alpha=0.05$ ; the boxplots describe values including median, two hinges, two whiskers and outliers).  $\alpha=0.05$ ; the boxplots describe values including median, two hinges, two whiskers and outliers).

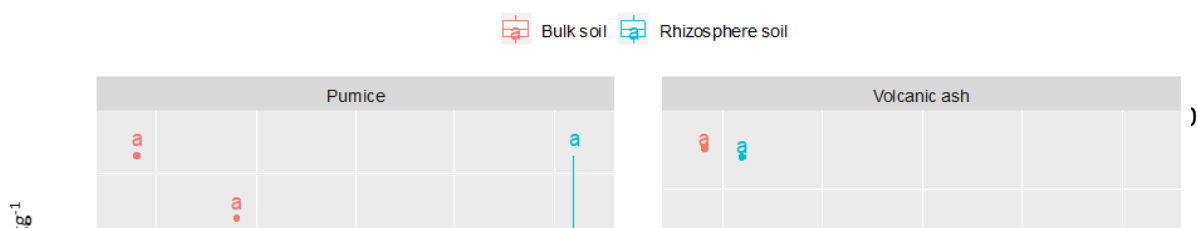
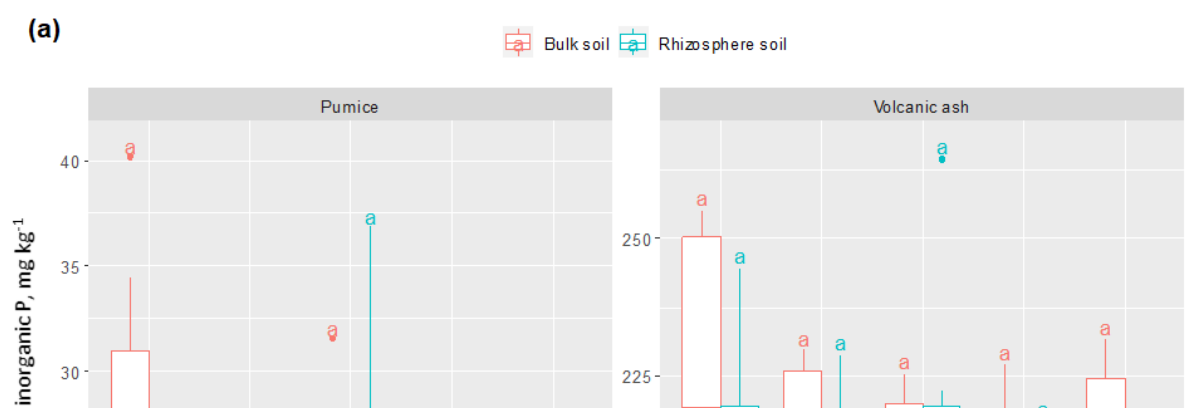


Figure 2.5 Concentrations ( $\text{mg P kg}^{-1}$ ) of calcium inorganic P determined in rhizosphere and bulk pumice and volcanic ash soils for five green manure plant species after 40 days (for each plant species on a soil type, values for different soil zones without a common letter are significantly different at  $\alpha=0.05$ ; the boxplots describe values including median, two hinges, two whiskers and outliers).



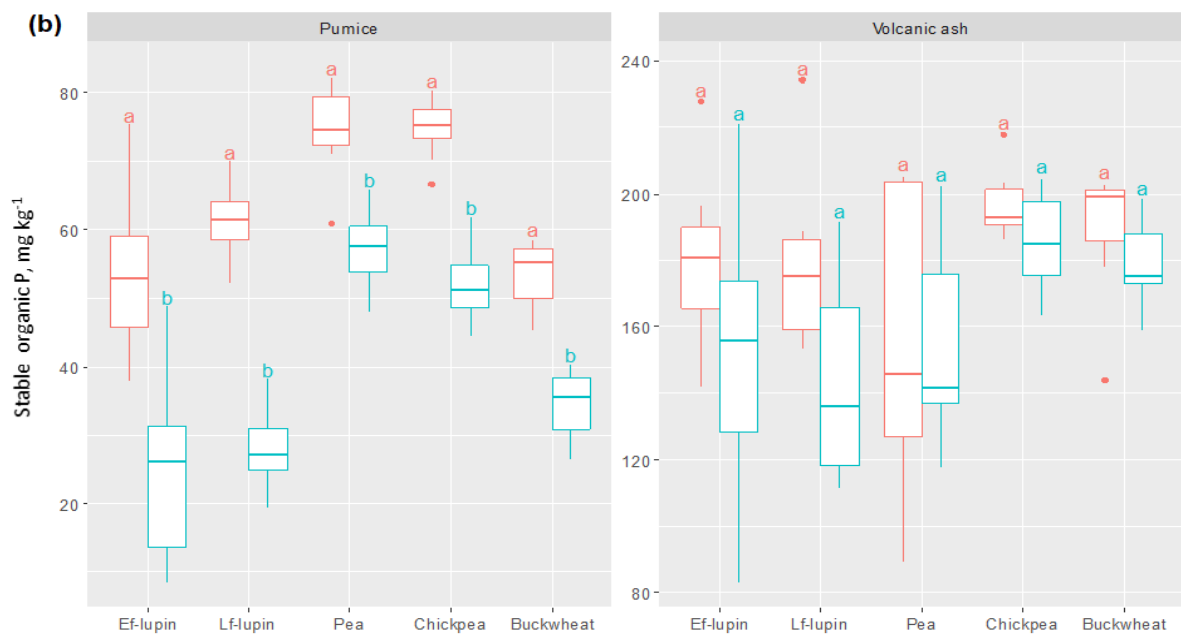


Figure 2. 9 Concentrations (mg P kg<sup>-1</sup>) of stable inorganic P (a) and organic P (b) determined in rhizosphere and bulk pumice and volcanic ash soils for five green manure plant species after 40 days (for each plant species on a soil type, values for different soil zones without a common letter are significantly different at  $\alpha=0.05$ ; the boxplots describe values including median, two hinges, two whiskers and outliers).

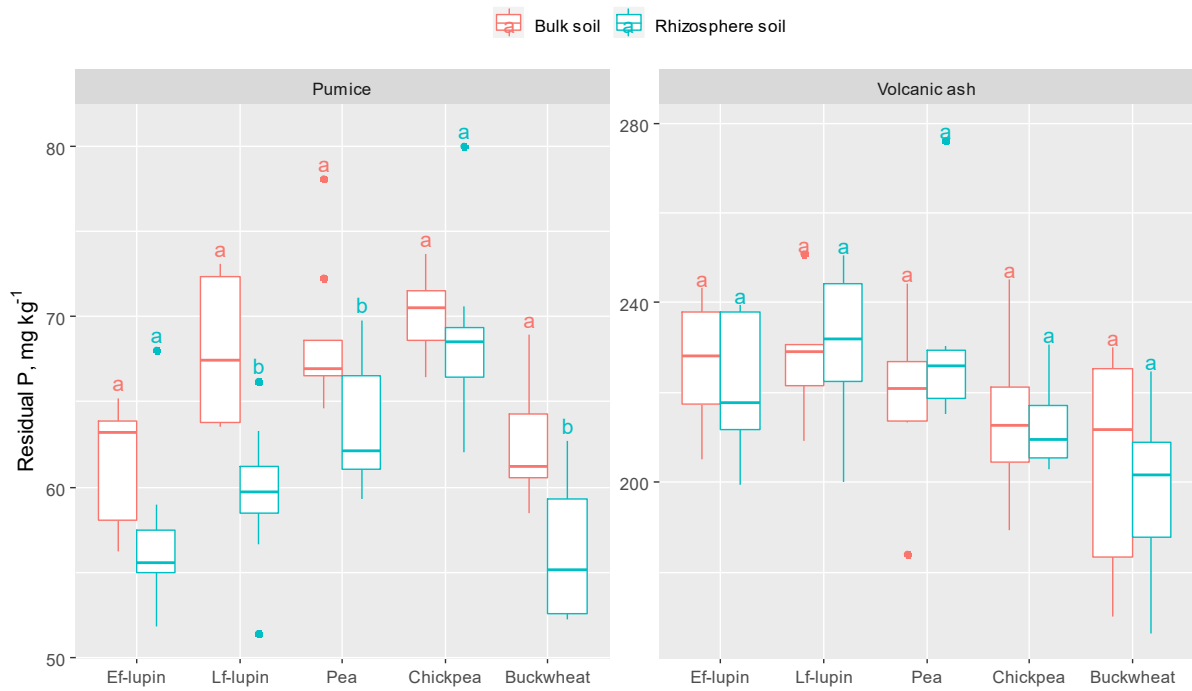


Figure 2.7 Concentrations ( $\text{mg P kg}^{-1}$ ) of residual P determined in rhizosphere and bulk pumice and volcanic ash soils for five green manure plant species after 40 days (for each plant species on a soil type, values for different soil zones without a common letter are significantly different at  $\alpha=0.05$ ; the boxplots describe values including median, two hinges, two whiskers and outliers).

Table 2. 3 Total rhizosphere inorganic P (a) and organic P (b) depletion determined for five green manure species grown in pumice and volcanic ash soils (data presents means within columns for soil zone and within the row for  $\Delta P$  (difference between extracted P in bulk soil and rhizosphere soil), means followed by the same letters are not significantly different at  $\alpha=0.05$  by Tukey's multiple comparison tests). Ef = early flowering; Lf = late flowering.

(a)

Soil zone	Pumice					Volcanic ash				
	Ef-lupin	Lf-lupin	Pea	Chick-pea	Buck-wheat	Ef-lupin	Lf-lupin	Pea	Chick-pea	Buck-wheat
Bulk soil (mg P kg <sup>-1</sup> )	473 a	463 a	472 a	458 a	454 a	1533 a	1554 a	1490 a	1553 a	1494 a
Rhizosphere soil (mg P kg <sup>-1</sup> )	382 b	379 b	449 b	426 b	427 b	1387 b	1503 a	1457 a	1507 a	1461 a
$\Delta P$ (mg P kg <sup>-1</sup> )	91 a	84 a	23 b	32 b	27 b	146	-	-	-	-

(b)

Soil zone	Pumice					Volcanic ash				
	Ef-lupin	Lf-lupin	Pea	Chick-pea	Buck-wheat	Ef-lupin	Lf-lupin	Pea	Chick-pea	Buck-wheat
Bulk soil (mg P kg <sup>-1</sup> )	669 a	626 a	632 a	614 a	618 a	1000 a	1030 a	997 a	1083 a	1018 a
Rhizosphere soil (mg P kg <sup>-1</sup> )	606 b	596 a	565 b	583 a	602 a	950 a	1030 a	963 a	1072 a	1013 a
$\Delta P$ (mg P kg <sup>-1</sup> )	63 a	-	67 a	-	-	-	-	-	-	-

Concentrations of total exuded organic anions (sum of acetate, citrate, formate, lactate, maleate, malonate, and pyruvate) determined at the rhizoplane after 40 days are presented in Figure 2.7. These data revealed considerable variability in total organic anion exudation from the various plant species (0.010-0.100  $\mu\text{mol cm}^{-2} \text{h}^{-1}$ ), and rates were highest for chickpea and late-flowering lupin in both soils. However, these differences were only significant between chickpea and other plant species for the pumice soil, and between chickpea and buckwheat for the volcanic ash soil. The relative proportions of different organic anions are presented in Table 2.4 and show that lactate (9-78%, average 45%), citrate (5-27%, average 13%), and malate (3-31%, average 14%) were the most abundant anions detected. The combination of lactate, citrate, and malate accounted for over 70% of total organic anions found in exudates from early- and late-flowering lupin and buckwheat for both soils, while relatively high amounts of malonate (20-52%) were present in chickpea exudates. There were no significant correlations observed between total organic anion exudate concentrations at the rhizoplane and total soil P depletion in the rhizosphere across all soil-plant combinations. However, significant correlations were observed between concentrations of malate in exudates and depletion of moderately labile inorganic P ( $p=0.012$ ) and stable inorganic P ( $p=0.007$ ) (Figure 2.8).

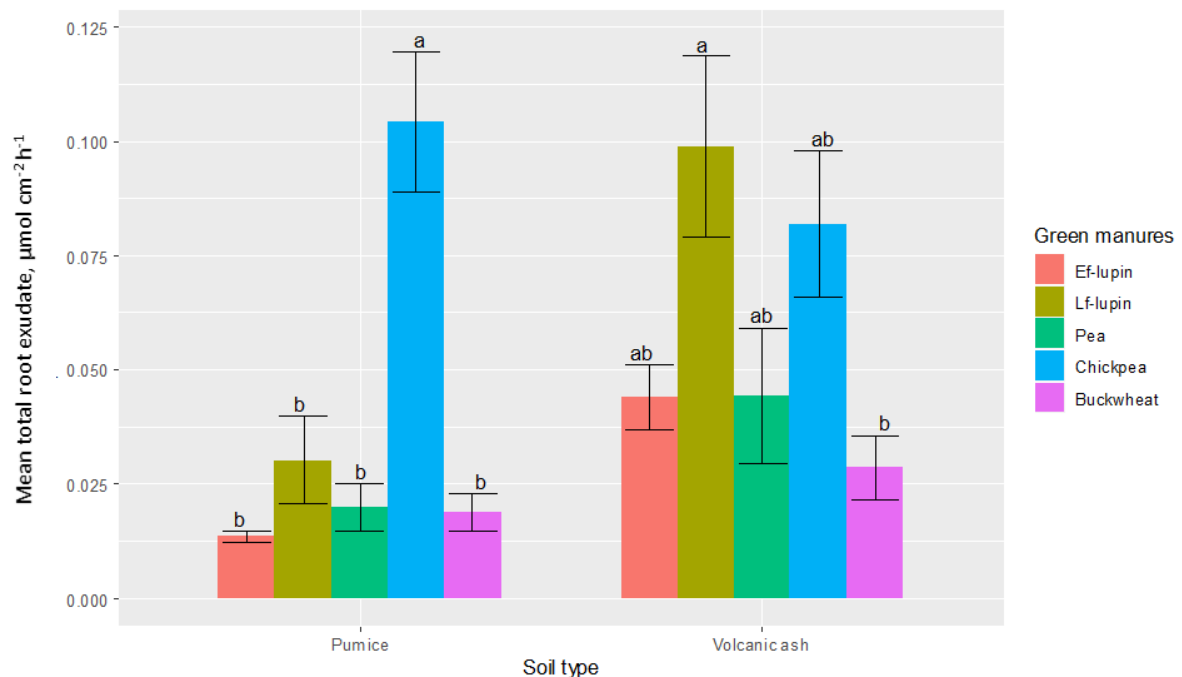


Figure 2. 10 Mean total root organic anion exudation ( $\mu\text{mol cm}^{-2} \text{h}^{-1}$ ) determined for five green manure plant species at the rhizoplane in pumice and volcanic soils (For each soil type, values for different plant species without a common letter are significantly different at  $\alpha=0.05$ ). Ef = early flowering; Lf = late flowering.

Table 2. 4 Relative proportions of different organic anions (% of total) detected in rhizoplane exudates for green manure plant species grown in pumice and volcanic ash soils after 40 days (nd = none detected). Ef = early flowering; Lf = late flowering.

		Acetate	Citrate	Formate	Lactate	Malate	Malonate	Pyruvate
Pumice	Ef-lupin	3	10	14	41	29	1	2
	Lf-lupin	3	12	16	39	27	2	1
	Pea	14	11	4	41	15	nd	15
	Chickpea	5	24	1	9	8	52	1
	Buckwheat	12	5	8	65	5	nd	5
Volcanic ash	Ef-lupin	2	27	13	43	12	1	2
	Lf-lupin	7	12	8	34	31	4	4
	Pea	2	6	5	78	3	nd	6
	Chickpea	2	15	4	50	5	20	4
	Buckwheat	13	11	5	54	8	4	5

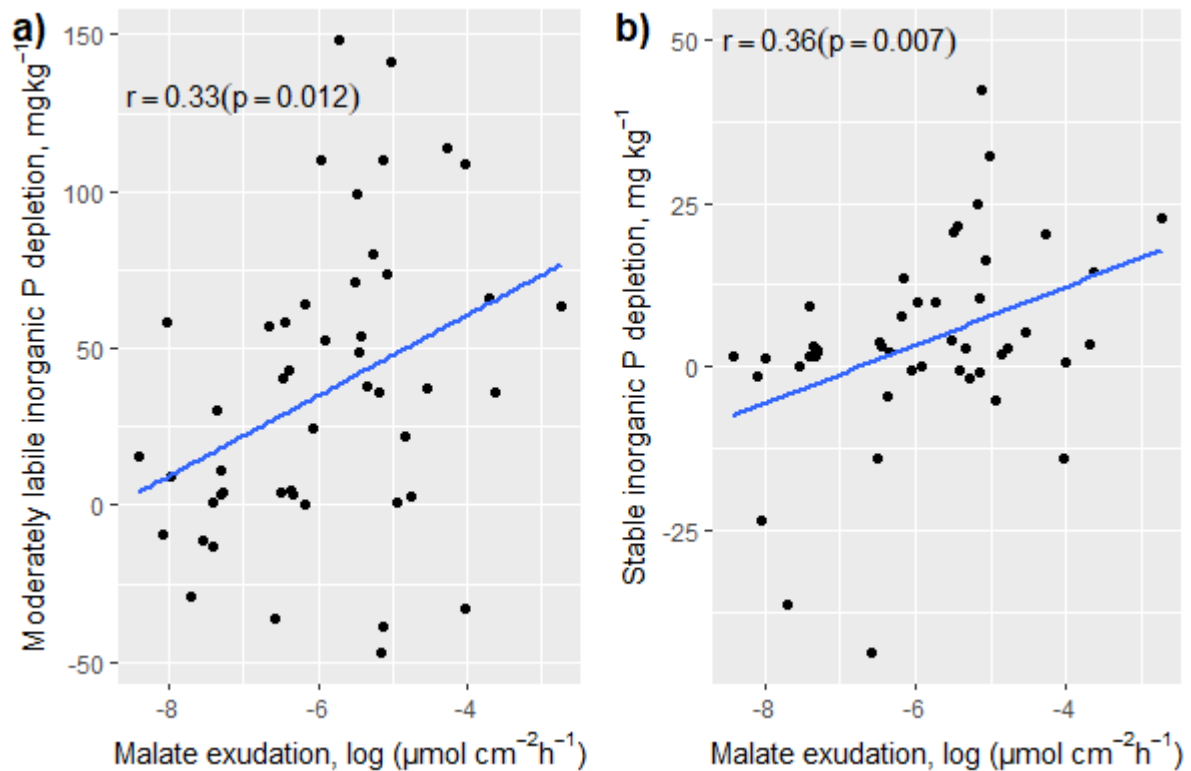


Figure 2.9 Relationship between depletion of moderately labile inorganic P (a) and stable inorganic P (b) in rhizosphere soil and malate concentration determined in rhizoplane root exudates for all soil-plant combinations.

## 2.4 Discussion

It has been acknowledged that the inclusion of green manures in crop rotations has the potential to alter P cycling, improve P use efficiency, and thereby reduce the quantity of P inputs required to maintain production (Condrón et al., 2013; Hallama et al., 2018). It is therefore important to investigate and quantify the impact of different green manure plant species on the acquisition of P from soil and subsequent crop growth and P uptake (Hallama et al., 2018; Mat Hassan et al., 2012; Pearse et al., 2006a), including soils that contain significant quantities of legacy P.

More consistent and significant depletion of P by all five plant species was determined in the medium P status pumice soil compared with the higher P status volcanic ash soil. Initial P concentration and anion storage capacity of the soil may relate to P depletion by plants (Dodd et al., 2012; McDowell and Condrón, 2004). In high P status soil levels of bioavailable P need to be substantially reduced by repeated biomass removal with no P inputs (Boitt et al., 2018a; Dodd et al., 2012, 2013; McDowell et al., 2020) to reach agronomic optimum P concentrations (McDowell et al., 2020). Crop yield may not decrease with no P addition until soil test P dropped below critical

agronomic levels (Rowe et al., 2016b). This may also be confirmed by the results of the present study (Table 2.2) that even though green manure plant species (except Ef-lupin) did not significantly depleted rhizosphere P, shoot P uptake was similar or higher under all plant species in the volcanic ash soil compared with the pumice soil (although anion storage capacity was high in the volcanic ash soil, it may not significantly negatively affect P uptake by crops at high available P levels). It appeared that the high level of available P in the volcanic ash soil supplied sufficiently P for the plants via rapid P replenishment for soil solution. While in the pumice soil with medium P concentration the plant species depleted not only labile P but also less labile P meaning that depletion of soluble P may need to be replenished by many different P pools. Therefore, at optimum P levels, the use of alternative plant species to further utilise legacy P and reduce risk of P loss in drainage can be expected to be a viable option. At the same time, in many different soils with high labile P, crop yields did not decrease for a number of years with no P application and 60-70% of labile P decrease was due to crop offtake (Rowe et al., 2016b). For example, Dodd and Mallarino (2005) demonstrated that with no P application it took up to 20 years for crop yields' response in soils containing soil test P 43 - 96 mg P kg<sup>-1</sup>.

The enhanced mobilization of inorganic P compared with organic P is consistent with findings from other soil P depletion experiments (Boitt et al., 2017a; Chen et al., 2019; McDowell et al., 2016; Perrott, 1992). The observed depletion of P in both soils was not related to plant yield and P uptake, which was as expected given that impact assessment was based on differences in soil P determined at and adjacent to the rhizoplane created by the nylon mesh (Chen et al., 2002). The enhanced depletion of extractable inorganic P by both lupin varieties were mainly due to substantial decreases in the moderately labile fraction, which was also observed by Boitt et al. (2017a) and Chen et al. (2019). Likewise, Chen et al. (2002) confirmed that inorganic P depletion was greater in the *Lolium perenne* (ryegrass) than *Pinus radiata* (radiata pine). The corresponding depletion of organic P by early flowering lupin and pea were due to decreases in moderately labile and stable P, although for lupin this was countered by a concomitant increase in labile organic P. The latter is consistent with findings from previous studies and can be related to a combination of factors. Firstly, labile organic P accretion could be attributed to the immobilization of inorganic P into organic P forms by microbes in the rhizosphere in response to enhanced exudation of readily available carbon by early flowering lupin (Armstrong and Helyar, 1992; Grayston et al., 1997; Mat Hassan et al., 2012). For example, Chen et al. (2002) found that greater labile organic P in the rhizosphere under radiata pine was related to increased microbial biomass carbon compared with perennial ryegrass. Secondly, increases in labile organic P could have been derived from breakdown of more stable forms of organic P linked to a combination of increased organic anions and enhanced microbial activity in the

rhizosphere. The absence of labile organic P accretion in the rhizosphere of pea may indicate enhanced mineralization of this form of organic P compared with early flowering lupin (Armstrong and Helyar, 1992). The contrasting extent of inorganic P depletion and pattern of organic P mobilization suggests that the mechanisms involved were different for lupin compared with pea. Moreover, the difference regarding to P mobilization could owe partly to the symbiosis of different mycorrhizae associated with roots (Chen et al., 2002).

Consideration of the potential role of root exudate low molecular weight organic anions in rhizosphere P dynamics and bioavailability was included in the current study to investigate the “unbutton” model of soil P acquisition proposed by Clarholm et al. (2015). According to this model, organic anions produced by plant roots and microorganisms play a crucial role in the release of inorganic and organic P from mineral surfaces and organic matter by chelation of binding metal ions such as iron and aluminium. Investigating the role and function of organic anions is acknowledged to be extremely challenging due to a combination of factors including separating roots from soil, spatial and temporal variations in exudate production, and the labile nature of these compounds in the rhizosphere environment (Jones et al., 2004; Oburger and Jones, 2018; Shi et al., 2013). Nonetheless, it is possible to capture exudate organic anions at the plant-soil interface using an anion exchange membrane (Scheffe et al., 2008; Shi et al., 2011a), and the composition of organic anions present has been shown to have a major impact on microbial activity and diversity (Shi et al., 2011b).

The mass and morphology of the root mat formed at the nylon mesh in the root study container would vary between plant species and possibly soil type, which in turn may influence organic anion capture by the attached exchange membrane. This may at least partly account for the high degree of variability in the total concentrations of organic anions determined for the different plant species in both soils. Rhizosphere P mobilization occurred over a period of 40 days prior to the sampling of organic anions at the rhizoplane, which would influence the nature of any relationship between P depletion and the release of exudates from roots. Nonetheless, comparison between the relative proportions of the seven organic anions detected at the rhizoplane of both lupin varieties and pea in the pumice soil revealed that formate and malate were greater for lupin compared with acetate and pyruvate for pea. Given that individual organic anions influenced the activity of specific soil bacterial communities Shi et al. (2013), it is possible that different organic anions may be involved in the release of P from different metal complexes in soil. Thus malate, a dicarboxylate may create more stable chelation with soil cations compared with monocarboxylate, such as acetate (Clarholm et al., 2015) because dicarboxylic groups may chelate with polyvalent cations (Fe, Al) and form more stable chelation. As a result, more P was released into soil solution for plant uptake compared with

monocarboxylate. Accordingly, exudation of malate by lupin may have played a role in mobilizing soil inorganic P. This, in turn, could at least partly account for the significant correlation observed between depletion of moderately labile and stable forms of inorganic P and the concentration of malate in root exudates.

Further detailed investigation of how organic anions are involved in the mobilization of inorganic and organic P in the rhizosphere of lupin and pea is warranted, including the complementary role of phosphatase enzyme activity.

## **2.5 Conclusions**

The short-term study of various green manure species demonstrated no significant relationship was observed between P uptake and rhizosphere P depletion. More consistent and greater P depletion was found in pumice soil with lower soil P content and lower anion storage capacity compared with the high P, high anion storage capacity volcanic ash soil. Depletion of inorganic P was greater than organic P in the rhizosphere. However, there was some indication of mobilisation of organic P from the pumice soil under early flowering lupin and pea. High variability in total organic anion exudation at rhizoplane was found between plant species in both soil types. However, the quality and proportion of organic anions (e.g. malate) could play an important role in P mobilization. For early flowering blue lupin and pea, there was appeared to be a difference in P mobilization mechanisms. A further study is, therefore, required to investigate detailed mechanisms responsible for enhanced soil P depletion.

## Chapter 3

# Mobilisation and acquisition of soil phosphorus in the rhizosphere of lupin (*Lupinus angustifolius*) and pea (*Pisum sativum*)

### 3.1 Introduction

Results presented in Chapter 2 revealed that early flowering blue lupin (lupin) (*Lupinus angustifolius*) and pea (*Pisum sativum*) were superior to other selected plant species at mobilizing and acquiring P from a medium fertility pumice soil. This indicated that both these species may be suitable for use as green manures in temperate cropping systems on soils with accumulated legacy P. Results also indicated that lupin was capable of preferentially mobilizing inorganic forms of soil P compared with pea, and there was evidence that malate released to soil in root exudates played a role in mobilizing inorganic P.

More detailed investigation of the soil-plant mechanisms responsible for enhanced mobilisation of soil P by lupin and pea at interface (rhizoplane-rhizosphere) is given here. In addition to measuring organic anions in root exudation, the role of phosphatase enzymes produced by plant roots and microorganisms was also assessed for organic P mineralisation, together with differences between soils derived from pumice and volcanic ash in this study.

The main objective of the research presented in this chapter was to assess and quantify the rhizosphere properties and processes involved in the mobilisation and acquisition of P from volcanic soils by lupin and pea.

### 3.2 Material and Methods

#### 3.2.1 Soils

Two topsoils (0-10 cm) used in this study were derived from different volcanic materials, including pumice soil (same as in Chapter 2) and volcanic ash soil which was different from the soil in Chapter 2. No P depletion occurred in the volcanic ash soil containing high available P under across the plant species, thus low P availability of the volcanic ash soil was replaced in this study. The soils were ground to pass through a 1 mm stainless steel sieve. Soil chemical properties are presented in Table 3.1. Both soils were acidic with similar CEC. Total C and N were at moderate and high levels, respectively, and corresponding C:N ratios were the same for both soils. Even though total P is similar in both soils, Olsen P is medium for the pumice soil and low for the volcanic ash soil (21 and 6

mg P L<sup>-1</sup>, respectively). Moreover, there was a contrasting anion storage capacity: 39% for pumice soil and 95% for volcanic ash soil.

Table 3. 1 Chemical and physical properties of the pumice and volcanic ash soils.

Analysis	Units	Pumice soil	Volcanic ash soil
pH <sub>H2O</sub>	-	5.9	5.5
Total C	%	8.3	9.7
Total N	%	0.72	0.84
C:N ratio	%	11.5	11.5
CEC	me/100g	24	23
Total P	mg/kg	1,123	1,046
Olsen P	mg/L	21	6
Anion Storage Capacity	%	39	95

### 3.2.2 Rhizosphere study technique

Rhizosphere containers were similar to those used in Chapter 2, although more detailed investigation of rhizosphere P dynamics involved analysis of soil at 0-2, 2-4, 4-6 and 6-20 mm from the rhizoplane. The experiment included 4 replicates for each soil-plant combination, and each replicate comprised of 4 containers to obtain sufficient soil for analysis.

### 3.2.3 Anion exchange membrane preparation and organic anion collection

The procedure of anion exchange membrane preparation and organic anion collection was performed as in Chapter 2.

### 3.2.4 Sampling and analysis

**Plants:** Plant shoots were harvested by cutting at the soil surface. Roots were separated from soil and washed. Shoots and roots of the 4 identical containers for each replicate were bulked, dried, and analysed for P as described in Chapter 2.

**Soils:** The top 6 mm ring of the lower container was carefully separated from the top container mesh and the bulk soil cylinder and the lower container was then frozen at -20°C. The soil was sliced with a Lipshaw 45 Rotary Microtome (Lipshaw Mfg. Co, Detroit, Michigan, USA) into different sections: 0 - 2; 2 - 4; 4 - 6; 6 - 20 mm from the rhizoplane (Figure 3.1). The same sections from 4 identical containers were bulked into one sample for analysis.

Acid and alkaline phosphatase enzyme activity were determined based on the release of *p*-nitrophenol after 1 hour of incubation of the soil with *p*-nitrophenyl phosphate at 37°C (Tabatabai, 1994) (Appendix A.7). Microbial P was measured following fumigation by liquid chloroform for 24 hours and 0.5 M NaHCO<sub>3</sub> extraction (McLaughlin et al., 1986) (Appendix A.6).

Phosphorus fractionation was performed as described in Chapter 2.

**Rhizoplane organic anions:** Rhizoplane organic anion collection and analysis were determined using the same Chapter 2.



Figure 3. 1 Rotary microtome.

### 3.2.5 Statistical analysis

Differences between the two plant species including dry matter yield, P uptake and root organic anion exudation were analysed with the t-test at  $\alpha=0.05$ . Phosphorus fractions, phosphatase enzyme activity and microbial P between soil sections were compared between distances from the rhizoplane within a plant species via one-way ANOVA followed by Tukey's honest significant difference method ( $\alpha=0.05$ ). Correlation coefficient ( $r$ ) was calculated to determine correlation between moderately labile inorganic P and root citrate exudation. The results were calculated and analysed with R statistical software package (version 4.0.2.).

### 3.3 Results

Mean values for plant dry matter yield and P uptake for early flowering blue lupin (lupin) and pea grown in the pumice and volcanic ash soils are presented in Table 3.2. Plant yield and shoot P uptake were higher in the pumice soil relative to the volcanic ash soil. Shoot yields were significantly higher (68% and 61%), while root yields were significantly lower (37% and 9%) for pea compared with lupin in the pumice and volcanic ash soils, respectively. Overall, yields of whole pea plants were higher than those of lupin by 33% in the pumice soil, and 39% in the volcanic ash soil. Similarly, pea shoot P uptake was 89% and 84% higher than lupin in the pumice and volcanic ash soils, respectively.

Table 3. 2 Mean data for shoot and root dry matter yield (g pot<sup>-1</sup>) and shoot P uptake (mg P pot<sup>-1</sup>) determined for green manure plants grown in the pumice and volcanic ash soils. Values represent the mean. Significant differences between plant species detected using t-test at  $\alpha=0.05$ .

Plant species	Pumice soil				Volcanic ash soil			
	Shoot	Root	Shoot+root	Shoot P uptake	Shoot	Root	Shoot+root	Shoot P uptake
Lupin	1.045	0.525	1.570	0.836	0.725	0.338	1.063	0.508
Pea	1.755	0.332	2.087	1.580	1.170	0.308	1.476	0.936
	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

Mean concentrations of P fractions determined in different distances of 0-2, 2-4, 4-6 and 6-20 mm from the rhizoplane for the trend of detailed P dynamics after 40 days of growth for the two green manure plant species are shown in Figures 3.2 and 3.3, respectively. Differences in the concentrations of the various soil P fractions occurring over distance from the rhizoplane were interpreted here as either depletion or accumulation of P relative to bulk soil following 40 days of plant growth and P uptake. For the pumice soil, significant depletion of labile inorganic P only

occurred within 2 mm from the rhizoplane for both plant species (8 - 9 mg P kg<sup>-1</sup>). Conversely, increases in labile organic P of 19 and 13 mg P kg<sup>-1</sup> over 2 mm from rhizoplane were observed in the rhizosphere of lupin and pea, respectively. Furthermore, significant accumulation of labile organic P extended to 6 mm under pea. Significant depletion of moderately labile inorganic P occurred in 0 - 2 mm from the rhizoplane of the pumice soil (35 mg P kg<sup>-1</sup>) under lupin compared with only 7 mg P kg<sup>-1</sup> for pea. Under lupin, moderately labile inorganic P depleted up to 4 mm from the rhizoplane. Significant depletion of moderately labile organic P occurred within 0 - 2 mm under only lupin in the pumice (31 mg P kg<sup>-1</sup>), while significant accumulation of 30 and 38 mg P kg<sup>-1</sup> in the 0 - 2 mm section was observed for both species in the volcanic ash soil. Data for acid-soluble and stable inorganic P indicate that there were no significant changes in these P pools in the rhizosphere regardless of plant species or soil type. On the other hand, significant depletion of stable organic P was observed for both plants in the pumice soil of which the depletion was 19 - 14 mg P kg<sup>-1</sup> under lupin and 8-19 mg P kg<sup>-1</sup> under pea over distances of 2-6 mm from the rhizoplane. Significant depletion of residual P was evident in the 0 - 2 mm rhizosphere zone of lupin and pea in both the pumice (8 - 9 mg P kg<sup>-1</sup>) and volcanic ash (17 mg P kg<sup>-1</sup>) soils.

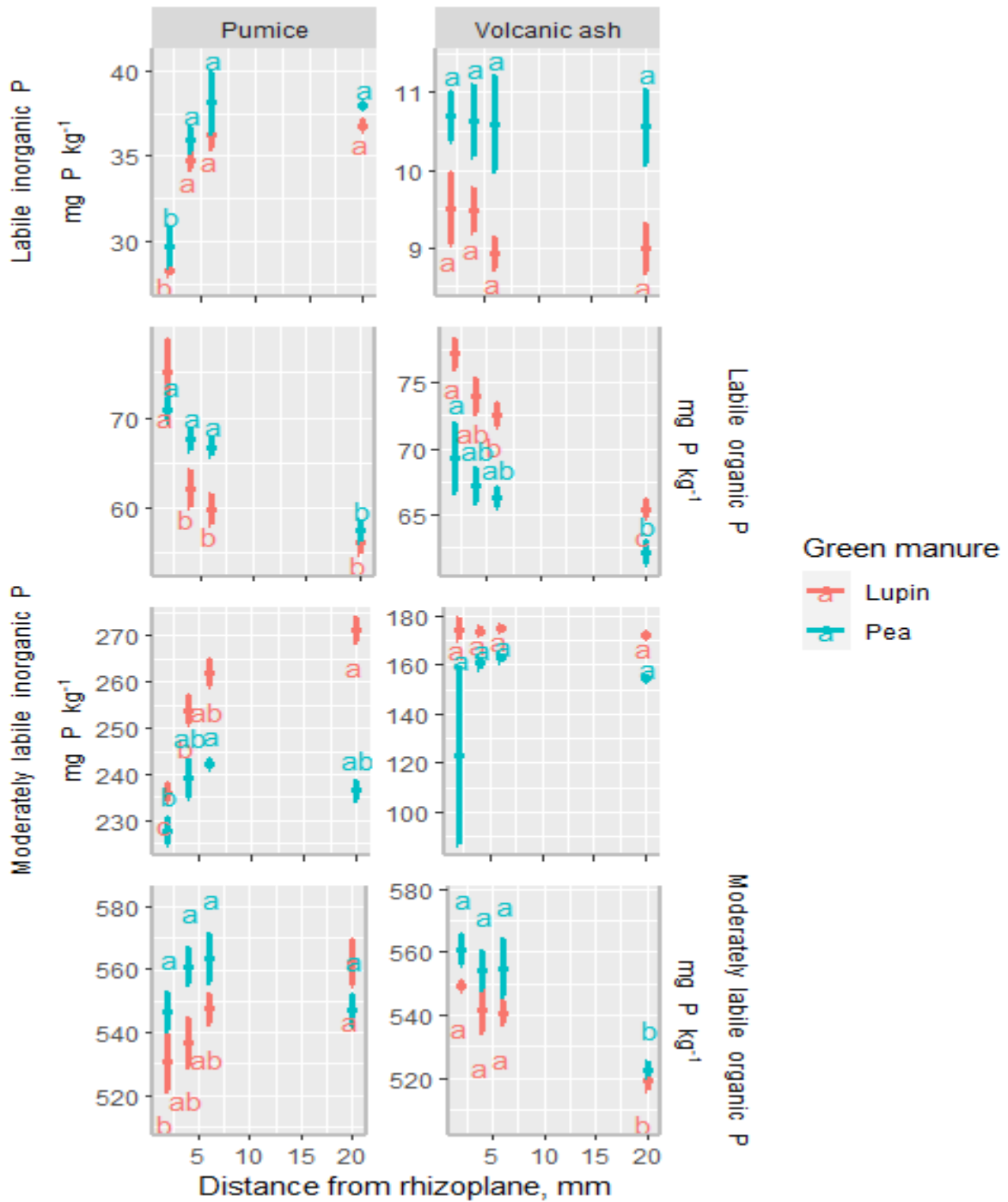


Figure 3. 2 Concentrations ( $\text{mg P kg}^{-1}$ ) of labile and moderately labile P fractions extracted by  $0.5\text{M NaHCO}_3$  and  $0.1\text{M NaOH}$  ( $\text{NaOH1}$ ), respectively, determined for four soil zones from rhizoplane (0-2, 2-4, 4-6, 6-20 mm) of the pumice and volcanic ash soils for lupin and pea after 40 days. Dot plots represent the mean and standard errors. Within a plant species for a soil type, different lowercase letters denote significant differences between soil zones detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

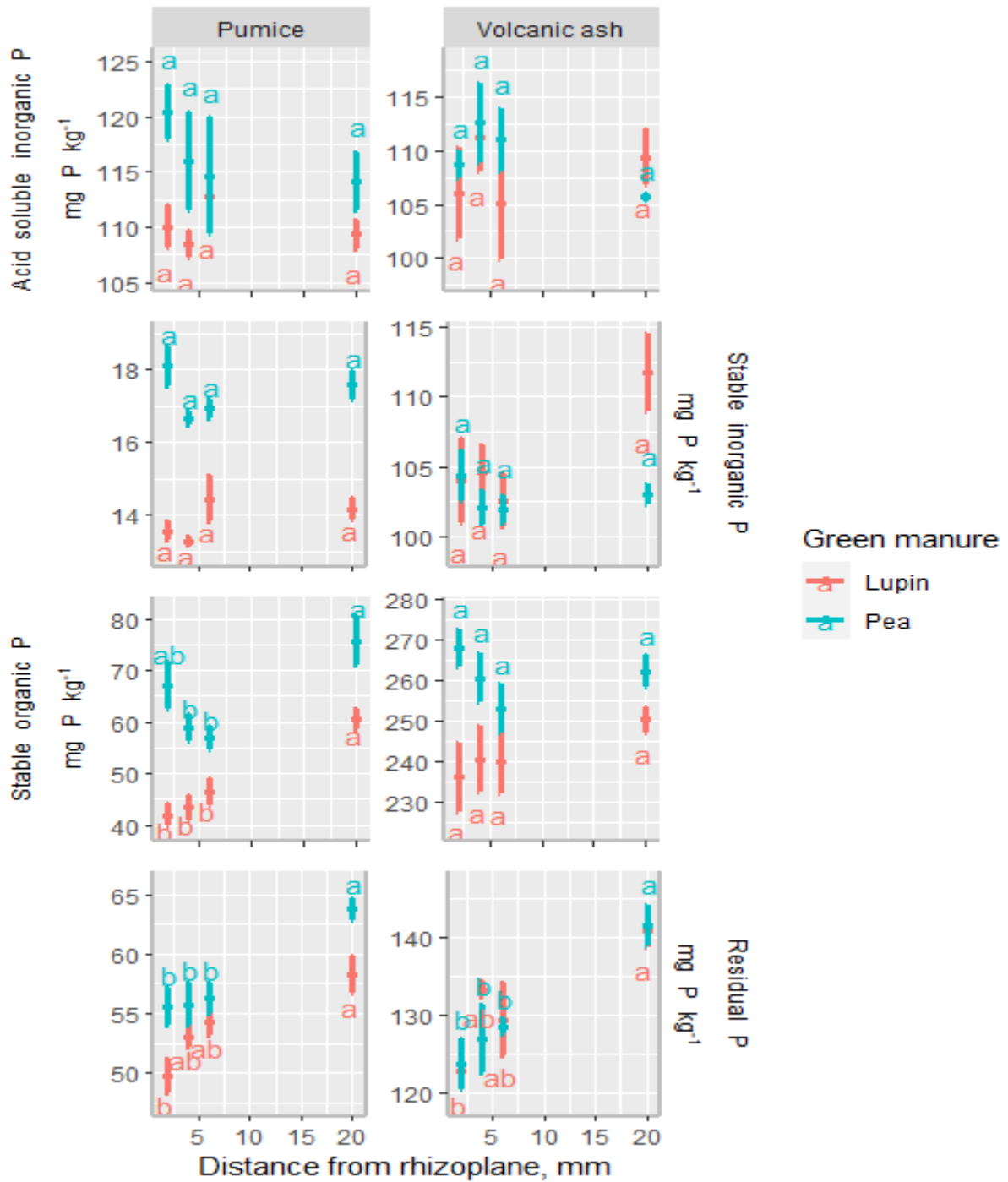


Figure 3. 3 Concentrations (mg P kg<sup>-1</sup>) of acid-soluble (1M HCl), stable (0.1M NaOH [NaOH<sub>2</sub>]) and residual P fractions determined for four soil zones from rhizoplane (0-2, 2-4, 4-6, 6-20 mm) of the pumice and volcanic ash soils for lupin and pea after 40 days. Dot plots represent the mean and standard errors. Within a plant species for a soil type, different lowercase letters denote significant differences between soil zones detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

Microbial P was highly variable and no significant differences were observed between the rhizosphere and bulk soil under either lupin or pea in both soils (Figure 3.4). Acid phosphatase

activity was greater than alkaline phosphatase activity across plant species and soil types (Figure 3.5). For both plant species in the pumice soil, there was a significant increase in both acid and alkaline phosphatase activities in the rhizosphere (0 - 2 mm) compared with bulk soil. In the volcanic ash soil, significantly higher acid phosphatase activities persisted up to 4 mm and 6 mm under lupin and pea, respectively, while higher activities of alkaline phosphatases extended to 6mm for lupin and 4 mm for pea.

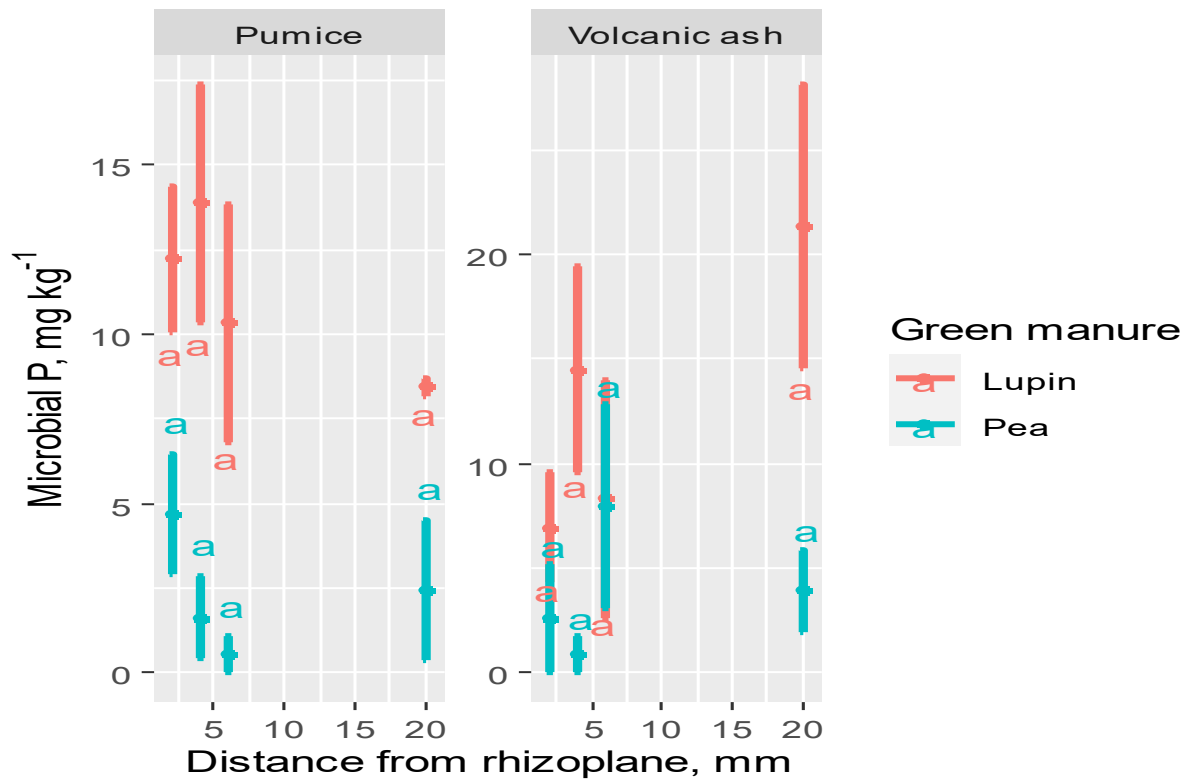


Figure 3. 4 Concentrations (mg P kg<sup>-1</sup>) of microbial P determined for four soil zones from rhizoplane (0-2, 2-4, 4-6, 6-20 mm) of the pumice and volcanic ash soils for lupin and pea after 40 days. Dot plots represent the mean and standard errors. Within a plant species for a soil type, different lowercase letters denote significant differences between soil zones detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

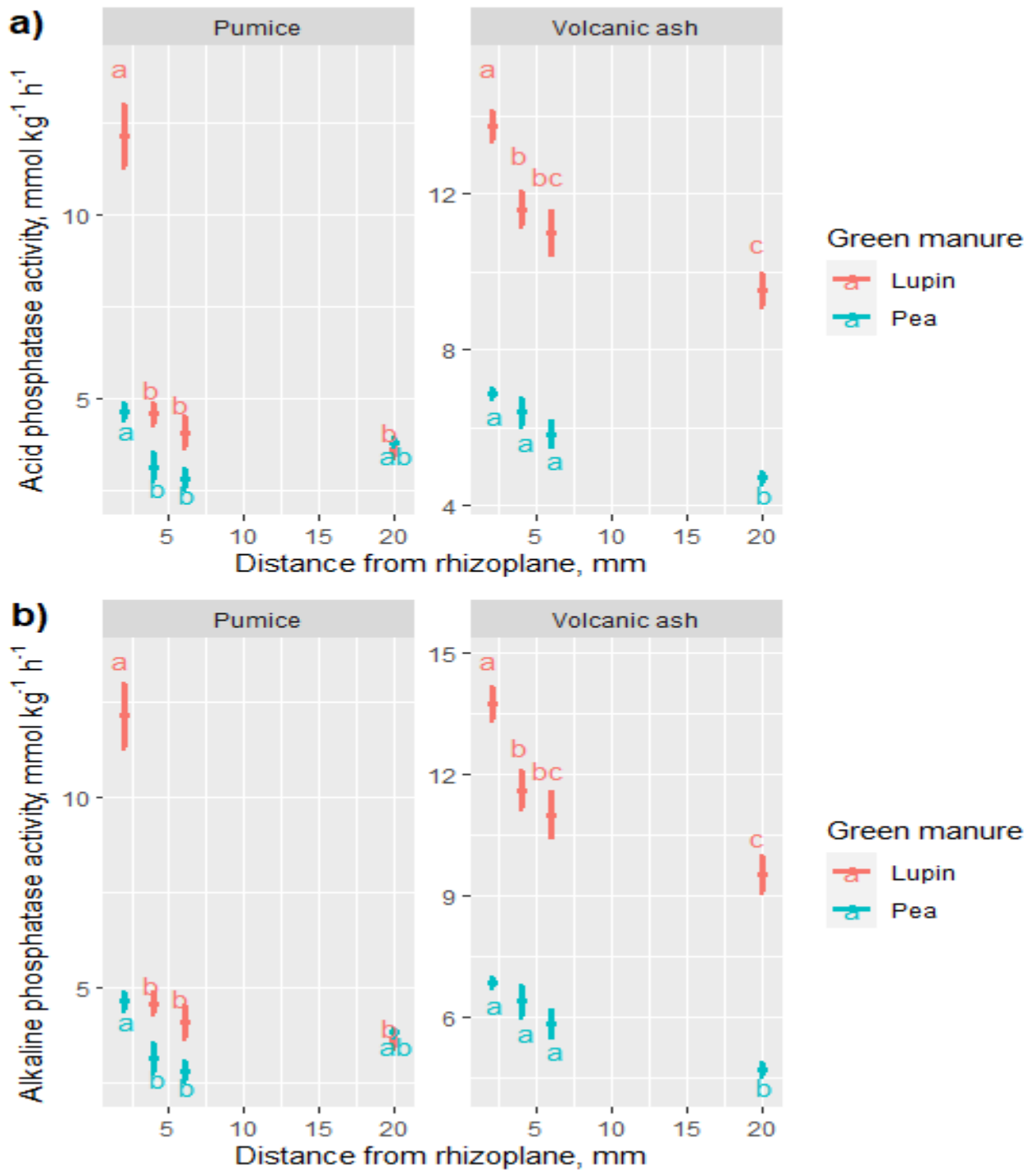


Figure 3. 5 Acid phosphatase (a) and alkaline phosphatase (b) activity ( $\text{mmol p-nitrophenol kg}^{-1} \text{h}^{-1}$ ) determined for four soil zones from rhizoplane (0-2, 2-4, 4-6, 6-20 mm) of the pumice and volcanic ash soils for lupin and pea after 40 days. Dot plots represent the mean and standard errors. Within a plant species for a soil type, different lowercase letters denote significant differences between soil zones detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

Total concentrations and the relative proportions of seven root organic anions, namely acetate, citrate, formate, lactate, malate, malonate and pyruvate determined at the rhizoplane after 40 days growth are shown in Tables 3.3 and 3.4. Total organic anion concentrations were higher in the pumice soil compared with volcanic ash soil for both lupin and pea. For the pumice soil, total organic anion concentrations were similar for lupin and pea, although total organic anion concentrations were significantly higher for lupin compared with pea in the volcanic ash soil. The primary organic anions detected at the rhizoplane in the pumice soil were citrate (42%), formate (19%), malate (14%), and lactate (11%) for lupin, compared with lactate (43%), formate (18%), malate (18%) and acetate (13%) for pea. Dominant organic anions for both plant species in the volcanic ash soil were monocarboxylates (acetate, formate, lactate) which accounted for 91% and 95% of total anions detected for under lupin and pea, respectively. No significant correlations were determined between total root exudate organic anion concentrations and the rhizosphere P depletion, except for citrate which was significantly correlated with moderately labile inorganic depletion (within 2 mm from the rhizoplane) in the pumice soil (Figure 3.6).

Table 3. 3 Total root organic anion concentrations ( $\mu\text{mol cm}^{-2} \text{h}^{-1}$ ) determined for lupin and pea on the pumice and volcanic ash soils after 40 days. Values represent the mean. Significant differences between species within a soil type detected using t-test at  $\alpha=0.05$ . ns: not significant.

	Total organic anion concentration ( $\mu\text{mol cm}^{-2} \text{h}^{-1}$ )	
	Pumice	Volcanic ash
Lupin	0.037	0.016
Pea	0.028	0.010
p-value	ns	<0.001

Table 3. 4 Relative proportions of different organic anions (% of total) detected in rhizoplane exudation for lupin and pea grown in pumice and volcanic ash soils (nd = none detected).

Soil type	Plant species	Relative proportions of different organic anions (% of total)						
		Acetate	Citrate	Formate	Lactate	Malate	Malonate	Pyruvate
Pumice	Lupin	8	42	19	11	14	3	3
	Pea	13	4	18	43	18	nd	4
Volcanic ash	Lupin	13	2	38	40	1	3	3
	Pea	16	3	33	46	nd	nd	2

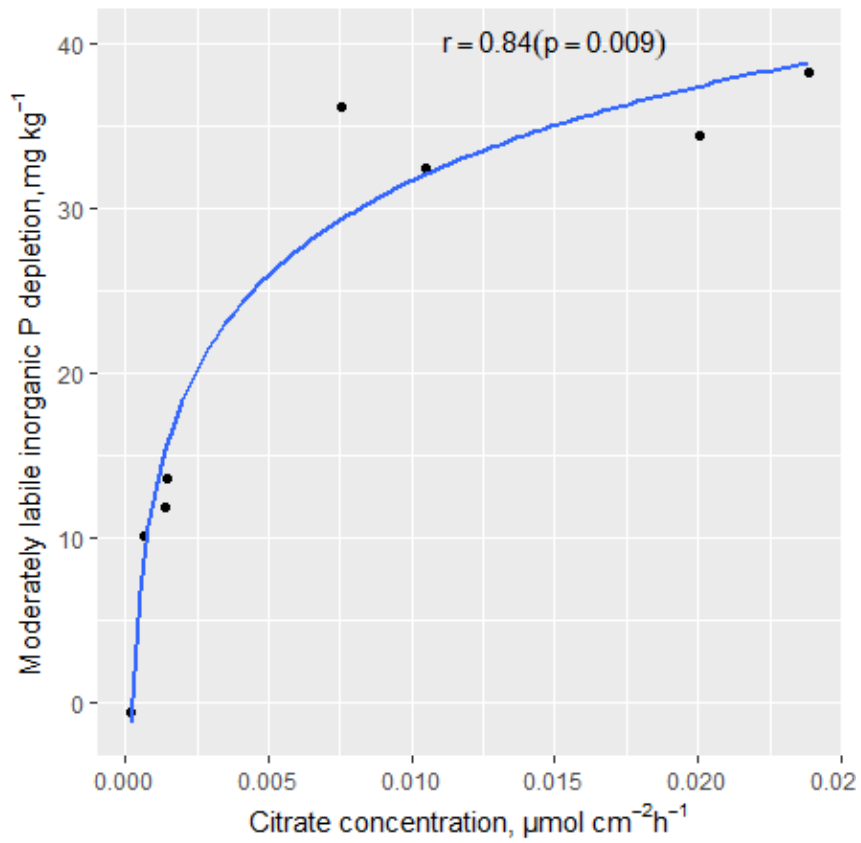


Figure 3. 6 Relationship between citrate concentration ( $\mu\text{mol cm}^{-2} \text{h}^{-1}$ ) and depletion of moderately labile inorganic P ( $\text{mg P kg}^{-1}$ ) in 0 - 2 mm section determined for pumice soil under the two species.



Figure 3. 7 Different root structures of lupin (left) and pea (right).

### 3.4 Discussion

The balance between root and shoot growth for lupin and pea was very different, which could reflect differences in root morphology (Figure 3.7). Pea had denser and longer secondary roots which significantly increased contact areas between root surface and soil in the upper container, probably leading to enhanced nutrient uptake including P (Zoysa et al., 1998a). While lupin developed the taproot much faster than secondary roots, created a thick root mat above the mesh and reduced root-soil contact area in the upper cylinder, thus decreased shoot yield and shoot P uptake compared with pea.

Lupin and pea depleted more soil P in the pumice soil than in the volcanic ash soil. Low P availability and high anion storage capacity of the latter may provide the primary limitations to plant growth (Dahlgren et al., 2004). For the pumice soil, the increased inorganic P mobilisation relative to organic P and lack of relationship between P depletion and either plant yield or P uptake were in agreement with results presented in Chapter 2. Inorganic P depletion by lupin and pea in the pumice soil was primarily due to decreases in labile and moderately labile inorganic P, which was also observed by Boitt et al. (2017a) and Chen et al. (2002).

Labile inorganic P depletion could be mainly attributed to plant P uptake and immobilisation (Cross and Schlesinger, 1995; Li et al., 2008; Zoysa et al., 1998b, 1999), which was similar for lupin and pea. However, it was evident that fine root hairs of lupin had in fact penetrated the 20  $\mu\text{m}$  mesh, which was not observed for pea (Figure 3.8, and 3.9). This may at least partly account for the enhanced mobilisation of moderately labile inorganic P in the rhizosphere by lupin compared with pea. The impact of fine root hairs on P mobilisation has been demonstrated in several previous studies (Chen et al., 2002; Gahoonia et al., 1997; Gahoonia and Nielsen, 1998). For example, Gahoonia et al. (1997) suggested that selection of wheat or barley cultivars with denser and longer root hairs may increase soil inorganic P acquisition. Also, Chen et al. (2002) found greater depletion and extension of moderately labile inorganic P under ryegrass (*Lolium perenne*) compared with radiata pine (*Pinus radiata*), which was partly attributed to differences in root hair density and length. The difference in inorganic P depletion between plant species could also be due to root surface area and root mass above the mesh (Chen et al., 2002; Zoysa et al., 1998a), however concentrations of organic anions were similar between lupin and pea, hence mass of root was not investigated in the present study.

In contrast to the pumice soil, the lower P fertility and higher anion storage capacity of the volcanic ash soil limited the availability of inorganic P to lupin and pea. Hence, lupin and pea did not deplete inorganic P pools in the rhizosphere in this soil.

For both plant species, labile organic P increased in the rhizosphere of pumice soil, while both labile and moderately labile organic P increased in the rhizosphere of the volcanic ash soil. Increases in organic P in the rhizosphere may reflect immobilisation of inorganic P by microorganisms in response to root exudates (Boitt et al., 2017a; Chen et al., 2002; Hassan et al., 2012b; Li et al., 2008; Zoysa et al., 1997, 1998b). Moreover, increased labile organic P could be due to the conversion of less labile forms of organic P (moderately labile, stable and residual organic P pools), while accumulation of moderately labile organic P may be attributed to the transformation of stable and residual organic P which are destabilized by organic anion exudation from roots and microorganisms. In the pumice soil, moderately labile organic P remained unchanged in the rhizosphere of pea, yet significantly decreased (by 31 mg P kg<sup>-1</sup>) in the rhizosphere of lupin. The difference was probably due to density and length of fine root hairs between lupin and pea.

According to Ma et al. (2018) higher enzyme activity occurred under dense and long root hairs compared with short and sparse root hairs. In the present study, a significant but small increase of acid phosphatase activity (by 0.8 mmol *p*-nitrophenol kg<sup>-1</sup>) in the rhizosphere of pea with shorter and sparser fine root hairs, whilst there was a substantial and significant enhancement of acid phosphatase activity (by 8.5 mmol *p*-nitrophenol kg<sup>-1</sup>) in the rhizosphere of lupin with longer and denser fine root hairs. Similarly, alkaline phosphatase enzyme activity in the rhizosphere of lupin was fivefold higher compared with pea indicating that higher microbial activity occurred in the rhizosphere of lupin with the improved organic compound secretion by long and dense root hairs (Chen et al., 2019; Nannipieri et al., 2011; Nguyen, 2003). The low phosphatase activity under pea may not have been sufficient to promote mineralization of moderately labile organic P.

Stable organic P and residual P were depleted in the pumice soil, while residual P depletion was observed in the volcanic ash soil under both lupin and pea. Depletion of these P pools, which were similar between both plant species across the soils, may not relate to differences in fine root hairs. The similarity of the depletion was mainly likely to be due to similar organic anion exudation in both soils despite a significant but small difference occurred in volcanic ash soil. Fixed organic P forms would not be mineralized by phosphatase enzymes (George et al., 2002b) until they are desorbed by organic anions released by roots and microorganisms (Clarholm et al., 2015; Richardson et al., 2009b). Therefore, according to the unbutton model suggested by Clarholm et al. (2015) there may be two steps to convert fixed organic P to available inorganic P pools: (1) organic anions secreted by plant roots and microorganisms destabilise soil organic matter including organic P by forming stable complexes with polyvalent metal cations particularly Ca<sup>2+</sup>, Al(OH)<sub>n</sub><sup>(3-n)+</sup>, Fe(OH)<sub>n</sub><sup>(3-n)+</sup> which form bridges with the organic compounds, (2) phosphatase enzymes released by roots and microbes can

hydrolyse destabilised organic P to available inorganic P. However, some destabilised organic P may not be mineralised, resulting in a build-up of P in labile or moderately labile organic P pools as mentioned above, consistent with findings of several studies (Gardner et al., 1983; Negassa and Leinweber, 2009; Saleque and Kirk, 1995).

Compared with the volcanic ash soil, the greater total organic anion concentrations determined in the pumice soil for the two plant species could be due to the higher mass of the root mat formed at the nylon mesh in the experimental container. For the pumice soil, total organic anions did not differ between lupin and pea, which was consistent with results for Chapter 2, but there was a difference in the relative proportions of individual organic anions. Different organic anion composition is likely to be involved in the liberation of P from different metal complexes in soil (Richardson et al., 2009a). Citrate, a tricarboxylate, probably reacts differently with soil polyvalent cations compared with monocarboxylates such as lactate (Clarholm et al., 2015). Accordingly, exudation of citrate by lupin (42% of total) may have played a role in depleting soil inorganic P. This, in turn, could be partly responsible for the significant correlation between depletion of moderately labile inorganic P and root exudate citrate concentration ( $r = 0.84$ ,  $p=0.009$ ).



Figure 3. 8 Root hairs clearly captured for lupin (left), but unclear for pea (right).

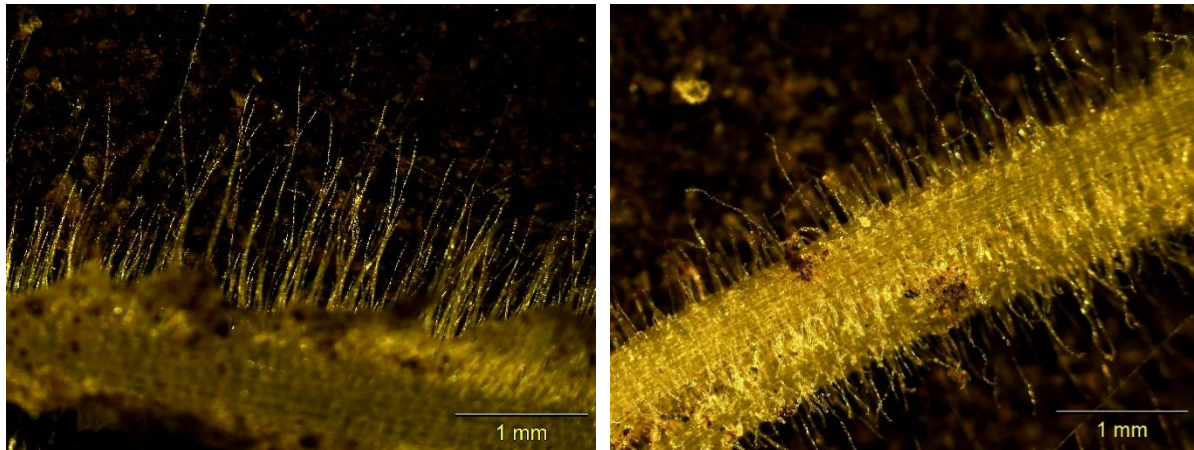


Figure 3. 9 Root hair length determined for lupin (left) and pea (right) by microscope.

### 3.5 Supplementary Experiment

#### Rationale

The reason for conducting this supplementary experiment was based on the observation that fine root hairs had penetrated the 20  $\mu\text{m}$  mesh under lupin, which were clearly visible. Therefore, it was necessary to further investigate the role of fine root hairs by using a much finer mesh (1  $\mu\text{m}$ ) to create a rhizoplane for the pumice soil with lupin and pea.

#### Materials and Methods

Soil: Topsoil (0 -10 cm) used in this study was another pumice soil (which is different from the soil in Chapter 3.2 due to lack of sufficient mass of the previous soil) and its properties are presented in Table 3.5. The soil was acidic with medium CEC and total carbon. Total nitrogen was at a low level and the C:N ratio was 16.4. Despite low total P, Olsen P was at a medium level (24 mg P  $\text{kg}^{-1}$ ). Anion storage capacity of the soil was 46%.

Table 3. 5 Chemical properties of the pumice soil.

Analysis	Units	Level
pH <sub>H2O</sub>	-	5.8
Total Carbon	%	4.6
Total Nitrogen	%	0.28
C:N	-	16.4
CEC	me/100g	15
Total P	mg/kg	645
Olsen P	mg/L	24
Anion Storage Capacity	%	46

All experimental steps were similar to Chapter 3.2, except that a 1  $\mu\text{m}$  nylon mesh with doubled layers was applied on the bottom of the upper container. With the absence of fine root hairs, the rhizosphere zone was expected to be reduced. Thus soil was sliced in thinner sections (unlike 2 mm for a section in Chapter 3.2): 0 - 1; 1 - 2; 2 - 3; 3 - 4 mm from the rhizoplane and 4 - 20 mm was considered bulk soil.

The procedures used for sampling and analysis of soil and root exudates were similar to those described in Chapter 3.2. No changes of microbial biomass P were observed in the 20  $\mu\text{m}$  nylon mesh experiment, while alkaline phosphatase activity was much lower compared with acid phosphatase activity; hence, microbial biomass P and alkaline phosphatase activities were not determined in this experiment.

## Results

Mean concentrations of P fractions and acid phosphatase activities determined in sections 0 - 1; 1 - 2; 2 - 3; 3 - 4 mm and 4 - 20 mm from the rhizoplane after 40 days growth by the two green manure plant species are shown in Table 3.6 and Figure 3.10, respectively. No depletion or accumulation of P was found in the rhizosphere regardless of plant species except acid-soluble inorganic P which increased by 16 and 9 mg P kg<sup>-1</sup> under lupin and pea, respectively. Acid phosphatase activity increased by 1.29 and 1.07 mmol *p*-nitrophenol kg<sup>-1</sup> h<sup>-1</sup> within 0-1 mm from the rhizoplane of lupin and pea, respectively. The enhanced acid phosphatase activity extended to 2 mm for lupin and 3 mm for pea.

Table 3. 6 Concentrations (mg P kg<sup>-1</sup>) of P fractions determined for five soil zones from rhizoplane (0-1, 1-2, 2-3, 3-4, 4-20 mm) of the pumice soil for lupin and pea after 40 days. Values represent the mean. Within a plant species, different lowercase letters in the same row denote significant differences between soil zones detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

P fractions	Lupin					Pea				
	0-1 mm	1-2 mm	2-3 mm	3-4 mm	4-20 mm	0-1 mm	1-2 mm	2-3 mm	3-4 mm	4-20 mm
Labile inorganic P	40 a	39 a	38 a	40 a	40 a	39 a	38 a	37 a	40 a	40 a
Labile organic P	43 a	46 a	44 a	42 a	37 a	43 a	46 a	45 a	43 a	39 b
Moderately labile inorganic P	255 a	260 a	266 a	264 a	266 a	267 a	268 a	273 a	273 a	275 a
Moderately labile organic P	299 a	313 a	317 a	311 a	303 a	322 a	326 a	328 a	326 a	304 a
Acid soluble inorganic P	93 ab	96 a	92 ab	87 ab	80 b	91 a	90 ab	90 a	84 ab	82 b
Stable inorganic P	21 a	21 a	20 a	21 a	19 a	20 a	21 a	21 a	21 a	19 a
Stable organic P	10 a	11 a	11 a	12 a	14 a	10 a	10 a	10 a	11 a	13 a
Residual P	33 a	33 a	30 a	31 a	29 a	27 a	30 a	29 a	30 a	32 a
Total inorganic P	409 a	415 a	417 a	412 a	406 a	417 a	416 a	422 a	417 a	416 a
Total organic P	352 a	370 a	372 a	364 a	354 a	375 a	382 a	383 a	380 a	355 a

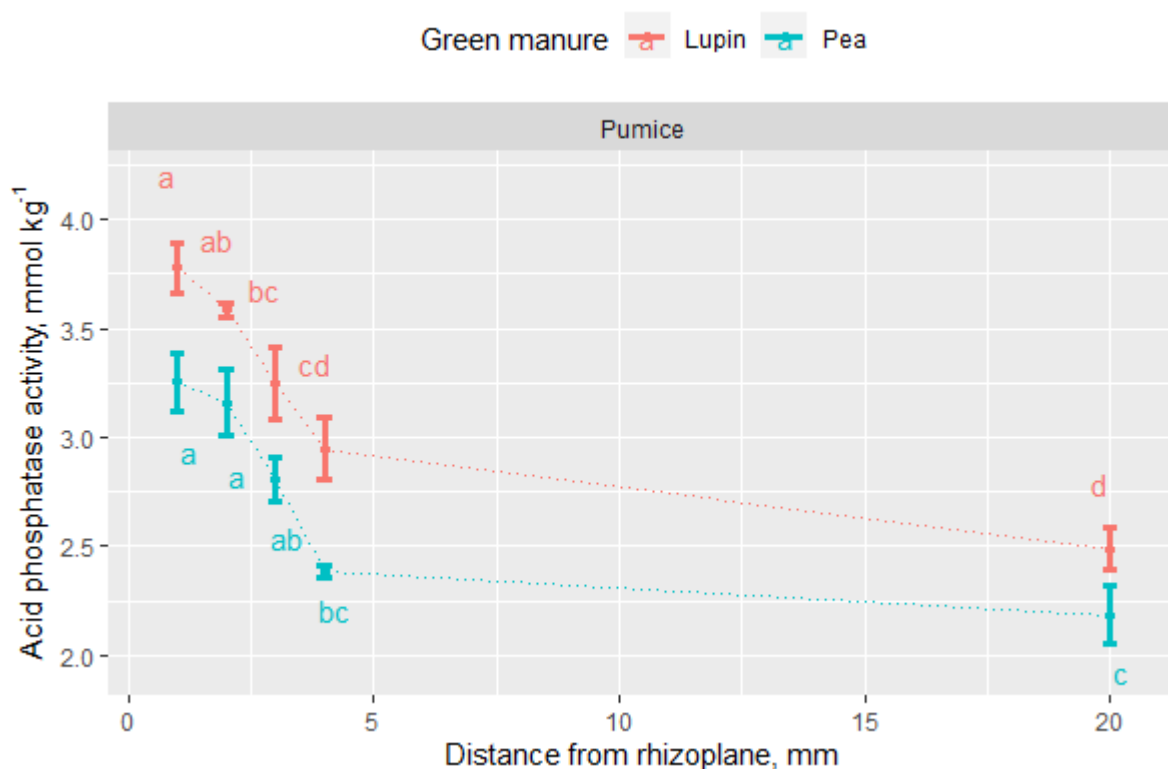


Figure 3. 10 Acid phosphatase activity (mmol p-nitrophenol kg<sup>-1</sup> h<sup>-1</sup>) of determined for five soil zones from rhizoplane (0-1, 1-2, 2-3, 3-4, 4-20 mm) of the pumice soil for lupin and pea after 40 days. Graphs represent the mean and standard errors. Within a plant species, different lowercase letters denote significant differences between soil zones detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

Total concentrations and the relative percentages of five root organic anions including acetate, citrate, formate, lactate, and pyruvate after 40 days growth are shown in Tables 3.7 and 3.8. Total organic anion concentration was significantly greater under pea compared with lupin. The major proportions of organic anions detected were formate (42%), and lactate (43%) for lupin (constituting 85% of total organic anions), while formate (20%) and lactate (69%) were the predominant organic anions for pea (constituting 89% of total organic anions).

Table 3. 7 Rhizoplane organic anion concentration ( $\mu\text{mol cm}^{-2} \text{h}^{-1}$ ) determined after 40 days growth of lupin and pea. Values represent the mean. Significant differences between treatments detected using t-test at  $\alpha=0.05$ .

Lupin	Pea	P value
0.012	0.019	<0.001

Table 3. 8 Relative proportions of different organic anions (% of total) detected in rhizoplane exudation for lupin and pea grown in pumice and volcanic ash soils.

Species	Acetate (%)	Citrate (%)	Formate (%)	Lactate (%)	Pyruvate (%)
Lupin	10	1	42	43	4
Pea	7	1	20	69	3

## Discussion

Visual assessment confirmed that the rhizoplane created by the 1  $\mu\text{m}$  nylon mesh prevented the penetration of fine root hairs to the rhizosphere, causing a clear difference in soil P mobilisation compared with that created by the 20  $\mu\text{m}$  nylon mesh in Chapter 3. Accordingly, no significant P depletion occurred in the rhizosphere of both lupin and pea in this experiment. The doubled layers of nylon mesh likely impeded the upward diffusive movement of P from the lower container soil, although diffusion rates of P in soil are commonly low (Richardson et al., 2009b). Contrary to the two previous experiments (Chapters 2 and 3.3), pea released more total organic anions than lupin over both times of collection in this experiment. The two plant species consistently and predominantly exuded monocarboxylates, especially formate and lactate, which accounted for more than 80% of total organic anions. This possibly led to diminished P desorption compared to the previous experiments due to the lower organic anion quality: these organic anions have only one carboxylic group (Clarholm et al., 2015; Richardson et al., 2009a). Furthermore, only five organic anion species were detected in this experiment compared with seven in the 20  $\mu\text{m}$  nylon mesh experiments. Obviously, the quality and composition of organic anions decreased significantly in the absence of root hair penetration into the rhizosphere. Although the importance of root organic anions in mobilizing soil P has been acknowledged (Richardson et al., 2009a; Wang and Lambers, 2020), the development of root hairs and their access to soil (among other root characteristics) should also be considered. Root hairs play an important role in rhizosphere P mobilisation, and are perhaps even more important than root exudates in some settings (Gahoonia et al., 1997). Moreover, although rhizosphere acid phosphatase activities were greater for lupin and pea by 1.29 and 1.07 mmol *p*-nitrophenol  $\text{kg}^{-1} \text{h}^{-1}$ , respectively, this difference in activity may still be too low to affect rates of organic P mineralization. In contrast, a considerable difference of 8.0 mmol *p*-nitrophenol  $\text{kg}^{-1} \text{h}^{-1}$  for acid phosphatase activity under lupin likely caused a significant mineralization of moderately labile organic P in the rhizosphere in the 20  $\mu\text{m}$  nylon mesh experiment.

## 3.6 Conclusions

Lupin and pea significantly depleted many different P pools in both inorganic and organic P forms in the pumice soil, while they did not deplete P in the volcanic ash soil. Moreover, Lupin and pea

released higher organic anions in the pumice soil compared with the volcanic ash soil. With the presence of fine root hairs (<20  $\mu\text{m}$ ) in the rhizosphere, lupin depleted higher P and released more phosphatase enzymes than pea in the pumice soil. The relative proportions of organic anions was different between the two plant species of which lupin released the greatest amount of citrate, while lactate was the most predominant under pea in the pumice soil. The difference in rhizoplane organic anion exudation was at least partly involved in different P depletion between the two green manures. The absence of root hairs in the 1  $\mu\text{m}$  nylon mesh experiment prevented P depletion under both lupin and pea, while more monocarboxylates were determined at the rhizoplane of both plant species. Although there was a significant increase of acid phosphatase activity, organic P mineralization was not detected for this experiment. Overall, in addition to root organic anions and phosphatase enzyme activity, the density and length of fine root hairs in the rhizosphere may be important drivers for different P mobilisation between lupin and pea.

## Chapter 4

# Influence of green manure inclusion and legume intercropping on crop yield, nutrient uptake, and soil phosphorus dynamics and bioavailability

### 4.1 Introduction

The inclusion of a green manure or cover crop has the potential to enhance the bioavailability and utilisation of legacy soil P in cropping systems. Results presented in Chapter 2 clearly demonstrated that blue lupin (*Lupinus angustifolius*) and pea (*Pisum sativum*) showed the most promise as green manure crop species and confirmed that significant mobilisation of legacy soil P was more likely to occur in a medium P fertility soil than in high P fertility soil. In addition to investigating the mechanisms responsible for soil P mobilisation by these legumes (Chapter 3), it is important to investigate and quantify the impact of the inclusion of these two green manures on subsequent crop performance, the associated change in soil P forms and concentrations, as well as compare the impacts of legume vs non-legume green manure.

It was not feasible to carry out this investigation in the field which would have taken 2-3 years. Accordingly, an extended pot experiment was employed to assess the impact of repeated green manure crop inclusion on yield and P uptake by successive cereal crops. The specific objective of the study presented in this chapter was to determine the impact of legume and non-legume green manure crops on crop growth, nutrient uptake, and soil P dynamics.

### 4.2 Materials and Methods

#### 4.2.1 Soil

The experiments were carried out in a glasshouse under controlled environmental conditions (10-25°C) over a period of 12 months using a Pumice soil. The soil was the same as that used in supplementary study, collected from the top 10 cm at a site in the Central of North Island, New Zealand. Key properties of the pumice soil used in this experiment are presented in Table 4.1. The soil was from a permanent grazed grassland site that had received moderate inputs of P fertiliser over many years. The soil was air dried and sieved < 4 mm prior to use.

Table 4. 1 Chemical properties determined for the pumice soil.

Analysis	Units	Level
pH <sub>H2O</sub>	-	5.8
Total Carbon	%	4.6
Total Nitrogen	%	0.28
C:N	-	16.4
CEC	me 100g <sup>-1</sup>	15
Total P	mg kg <sup>-1</sup>	645
Olsen P	mg L <sup>-1</sup>	24
Anion Storage Capacity	%	46

#### 4.2.2 Crop rotation

Green manure inclusion involved growing and incorporating early flowering blue lupin, pea and cereal [wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*)] together with a no plant fallow treatment between two successive main crops of wheat (Graham) and barley (Cask) which were grown to the maximum vegetative stage. Seeds of lupin and pea were pre-germinated for 4 days, while wheat and barley were pre-germinated for 2 days in petri dishes lined with wet filter papers. After germination, seeds were sown into polyethylene pots (width x length x depth = 6.5 cm x 6.5 cm x 16 cm), filled with 420 g of soil (pre-incubated). The soil moisture was maintained at 70% field capacity (Randhawa et al., 2005) by weighing pots and watering daily. There were 3 green manure treatments and a fallow control with 8 replicates per treatment. The green manures (or fallow) and main crops were rotated as described in Table 4.2.

Table 4. 2 Selected green manures rotated with successive crops.

Treatments	1 <sup>st</sup> green manure	Barley	2 <sup>nd</sup> green manure	Wheat
Fallow	Fallow	Barley	Fallow	Wheat
Cereal	Wheat	Barley	Barley	Wheat
Lupin	Lupin	Barley	Lupin	Wheat
Pea	Pea	Barley	Pea	Wheat

After 40 days of growth the green manure plants were harvested (before flowering). Prior to harvest the soil was allowed to dry to 40% of field capacity to minimize the soil adhering to roots, while avoiding the wilting point. Soil adhering to roots was removed by shaking the roots. The shoots and roots were separated, weighed and then chopped into 0.5 cm long pieces (Figure 4.1).



Figure 4. 1 Incorporation of green manures into soil.

All pieces of green manures were incorporated into the soil, mixed thoroughly, transferred back to the pots, and incubated at 10 - 25°C and 70 % field capacity for 30 days to allow for decomposition

of the plant matter. After 1 month of incubation of the first green manure or fallow, barley was sown at a density of 4 plants pot<sup>-1</sup> and grown for 60 days. 10 mL of a nutrient solution was added to minimize nutrient deficiency (except for P). These solutions included: (1) 4.8 g NH<sub>4</sub>Cl and 2.8 g Na<sub>2</sub>SO<sub>4</sub> were dissolved in 1 litre of water; (2) 2.5 g MgCl<sub>2</sub> and 0.2 g ZnCl<sub>2</sub> in 1 litre of water; (3) 0.02 g (NH<sub>4</sub>)<sub>2</sub>MoO<sub>4</sub> and 0.07 g H<sub>3</sub>BO<sub>3</sub> in 1 litre of water. Basal nutrient concentrations added to soil were equivalent to 30 mg N, 15 mg S, 15 mg Mg, 2 mg Zn, 0.2 mg Mo and 0.3 mg B per kg soil.

Before the second green manure crop was sown, 1.435 g CaCO<sub>3</sub> was applied for each pot (equal to 3.5g CaCO<sub>3</sub> kg<sup>-1</sup> soil) to increase soil pH and supply more calcium to the soil. The soil was then moistened to 70% field capacity and incubated for one week before being sown with the second green manure. The second green manure was grown, harvested and incubated in the soil under the same conditions as the first green manure treatment.

Following the second green manure incorporation, wheat was grown under the same conditions with the same basal fertilizer application as used for the barley crop.

#### 4.2.3 Sampling and analysis

**Plants:** After 60 days, plant shoots were cut at the soil surface. Roots were separated from soil and washed. Shoot and root samples were dried at 65°C for 48 hours for dry matter yield determination, ground to < 1 mm and analysed for total N by Dumas combustion method, and total P as described in Chapter 2.

Plant P (or N) uptake was calculated as:

$$P \text{ (or N) uptake (mg pot}^{-1}\text{)} = \text{Dry matter yield (g pot}^{-1}\text{)} \times P \text{ (or N) concentration (\%)} \times 100.$$

**Soils:** Following the barley crop, 10 g soil per pot was collected (soil from a pot was mixed thoroughly and spread on a tray, and collected 5 points along diagonal lines as a sample) for analysis of pH<sub>H2O</sub> (Blakemore et al., 1987). After the wheat crop, fresh soil samples were taken for acid and alkaline phosphatase activity and microbial P analysis as described in Chapter 3. Subsamples of dry soils were used for P fractionation as described in Chapter 2 and for total carbon and nitrogen determination using an Elementar Vario Max CN Elemental Analyser.

#### 4.2.3 Statistical analysis

Statistical analysis of the data was carried out with the R statistical software package (version 4.0.2). Differences between multiple green manure treatments were estimated by one-way analysis of

variance (ANOVA), including green manure biomass, crop yields and P uptake, N uptake, soil C, N and C:N ratios; soil P fractions, microbial P and phosphatase enzyme activities (Tukey's HSD;  $\alpha=0.05$ ).

### 4.3. Results

Data for fresh green manure yields are presented in Figure 4.2. There was a significant and consistent difference in biomass between three green manure species over two rotations. Overall, lupin produced the greatest biomass ( $73 \text{ g pot}^{-1}$ ), followed by pea ( $48 \text{ g pot}^{-1}$ ), and cereal ( $22 \text{ g pot}^{-1}$ ).

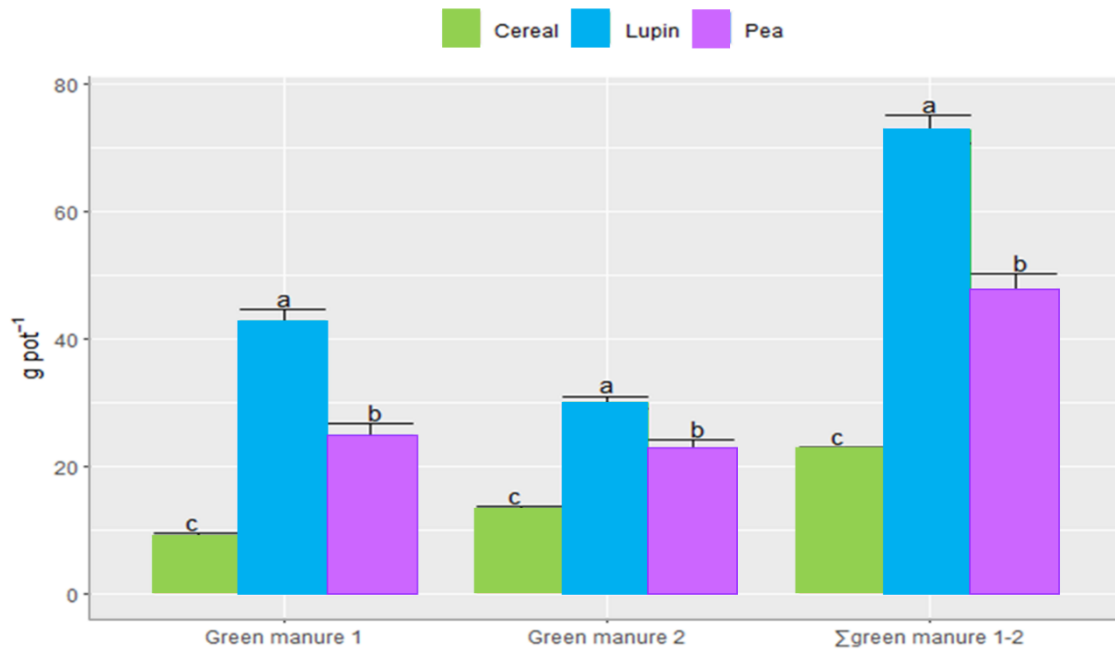


Figure 4. 2 Mean data for fresh matter yield (shoot+root) ( $\text{g pot}^{-1}$ ) determined for the first and second green manures and their sum. Bars represent the mean and standard errors. Within a green manure crop different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .  $\Sigma$  crop 1-2: sum of green manure yield for each treatment.

Mean dry matter data for crop yield of shoots and roots are shown in Figure 4.3. Crop yields in the cereal green manure treatment were significantly lower (33% barley roots, 32% wheat shoots, 24% wheat roots) compared with fallow, while legume green manures (lupin and pea) significantly increased yields of barley (30 - 42% shoots, 30 - 48% roots) and wheat (31- 41% shoots, 18 - 19% roots). Total cumulative barley and wheat yields were significantly lower (13%) under cereal green manure compared with fallow, while significant increases occurred under legumes (35% for lupin, 27% for pea).

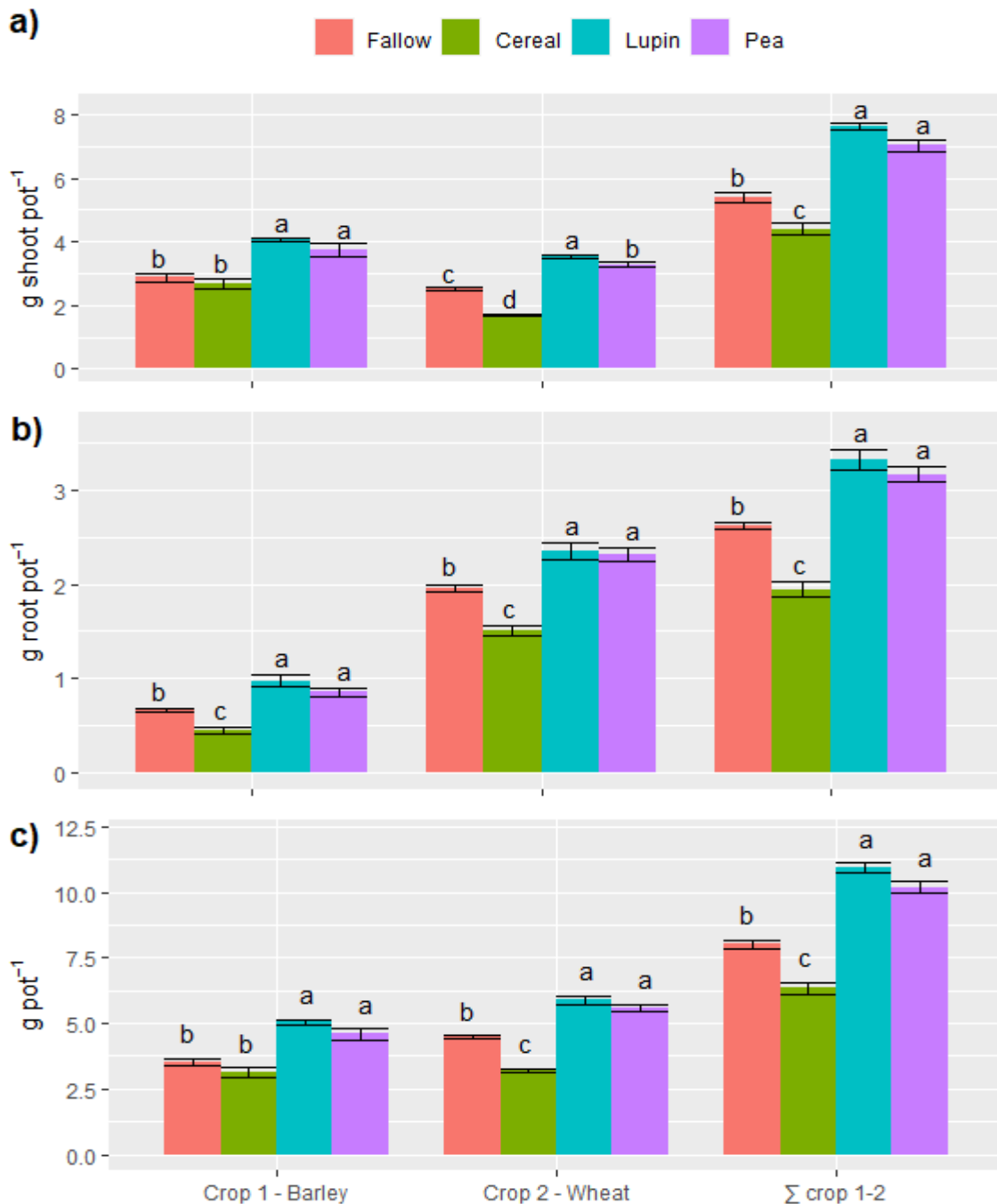


Figure 4. 3 Mean data for dry matter yield (g pot<sup>-1</sup>) for shoot (a), root (b) and shoot+root (c) determined for two successive crops (barley and wheat) maintained under fallow or green manures (cereal, lupin, pea). Bars represent the mean and standard errors. Within a crop different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .  $\Sigma$  crop 1-2: sum of dry matter yield for two crops for each treatment.

Figure 4.4 shows P uptake by barley and wheat crops. The cereal green manure significantly decreased barley root P uptake by 28% compared with fallow, while there were significant increases in P uptake under both legume green manures (shoots 14 - 33%, roots 21 - 37%). For wheat, the

cereal green manure reduced root and shoot P uptake by 18% and 32%, respectively, compared with fallow. Corresponding data for P uptake showed that legume green manures significantly increased by 15 - 33% and 9 - 11% for shoots and roots, respectively. Overall crop P uptake decreased by 19% under the cereal green manure and increased by 29% and 15% under the lupin and pea green manures, respectively. Compared with the pattern of P uptake, N uptake by subsequent crops impacted by green manures was similar (Figure 4.5). Inclusion of a cereal green manure significantly decreased total N uptake by 25%, while the lupin and pea green manures significantly increased total N uptake by 63% and 21%, respectively, compared with fallow.

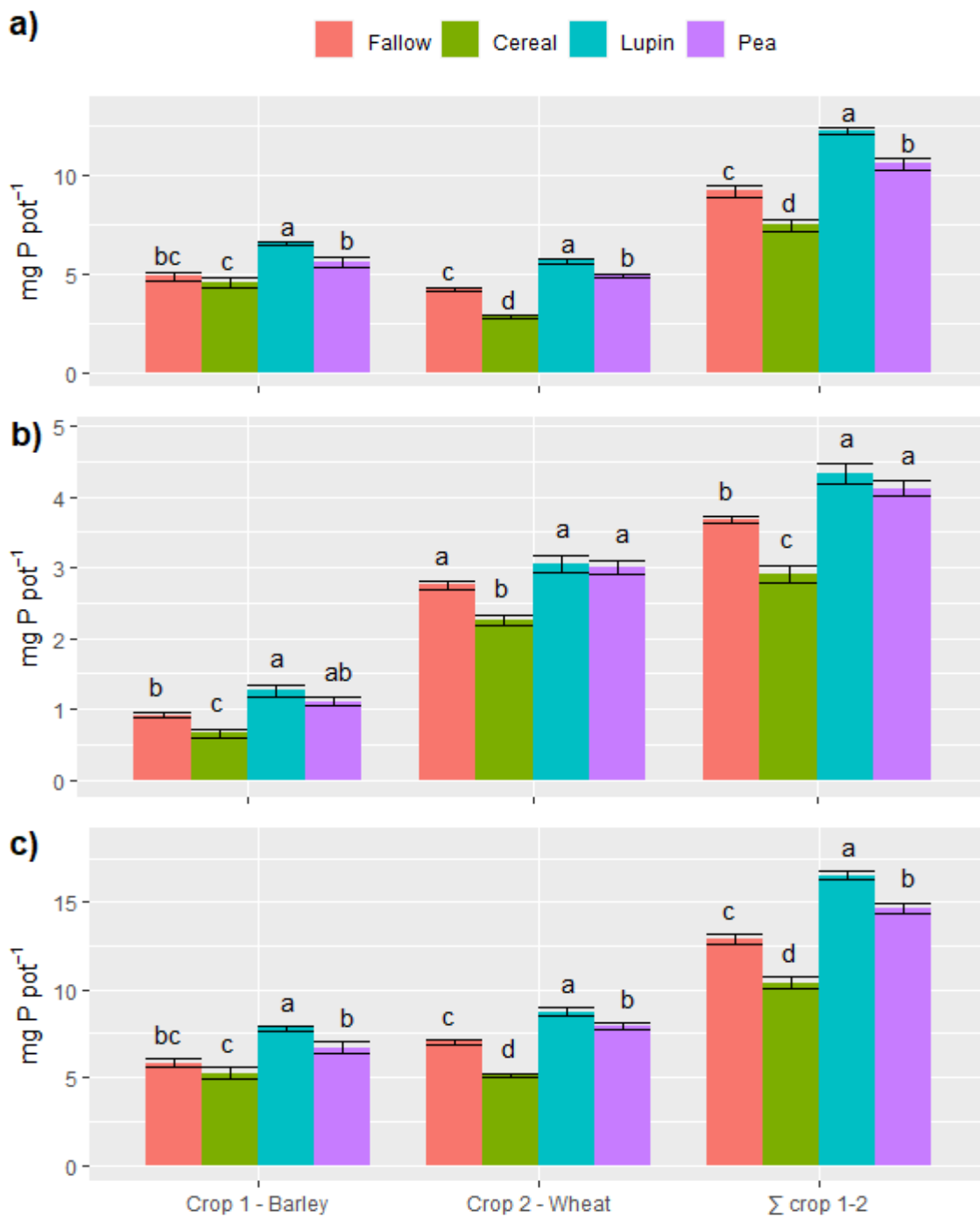


Figure 4. 4 Mean data for crop P uptake (mg P pot<sup>-1</sup>) for shoot (a), root (b) and shoot+root (c) determined for two successive crops (barley and wheat) maintained under fallow or green manures (cereal, lupin, pea). Bars represent the mean and standard errors. Within a crop different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .  $\Sigma$  crop 1-2: sum of P uptake for two crops for each treatment.

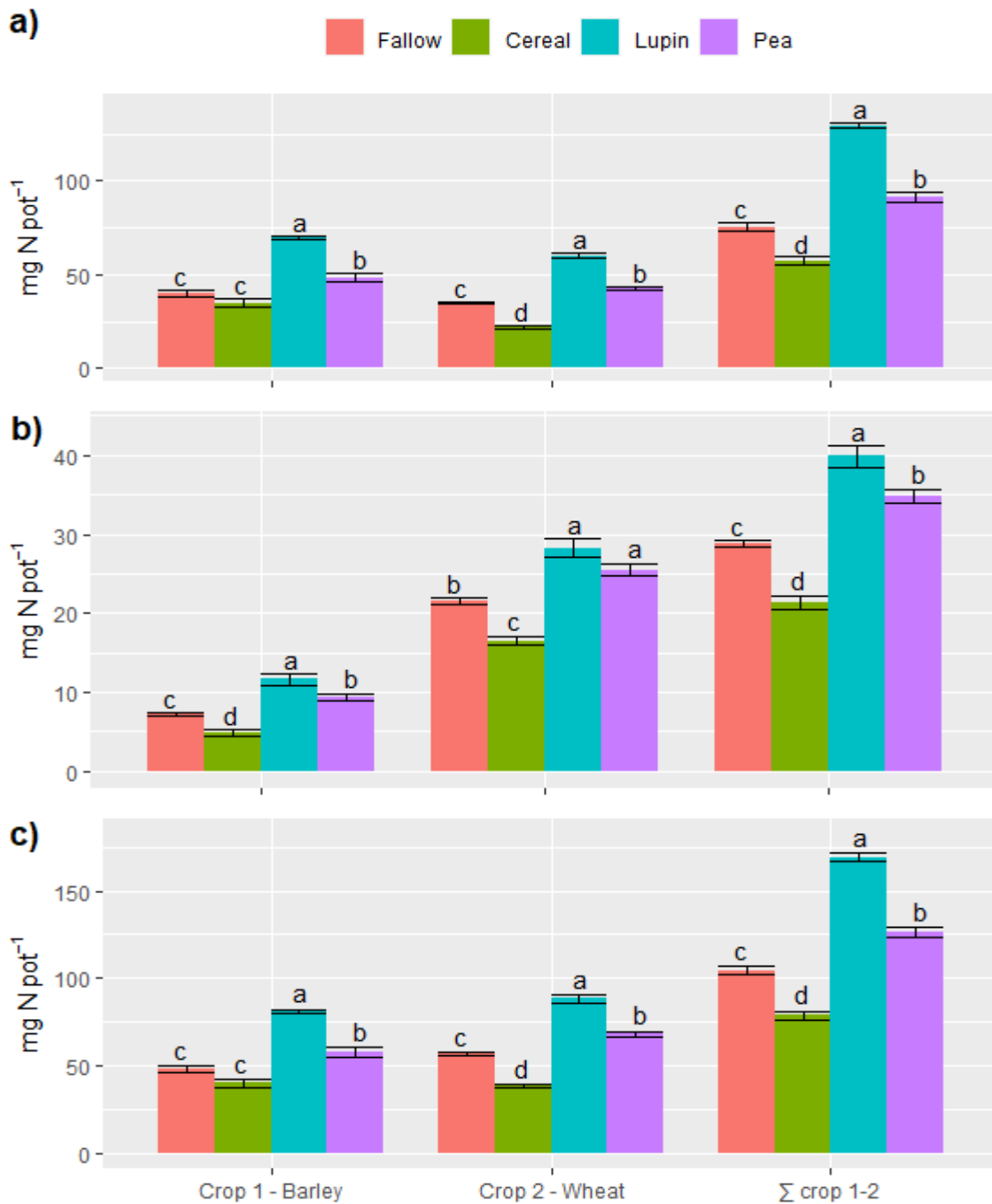


Figure 4.5 Mean data for crop N uptake (mg N pot<sup>-1</sup>) for shoot (a), root (b) and shoot+root (c) determined for two successive crops (barley and wheat) maintained under fallow or green manures (cereal, lupin, pea). Bars represent the mean and standard errors. Within a crop different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .  $\Sigma$  crop 1-2: sum of N uptake for two crops for each treatment.

Data for soil P fractions and microbial P determined at the conclusion of the experiment are shown in Table 4.3. No significant differences were observed between fallow and green manures for soluble inorganic P, acid-soluble inorganic P, and residual P. Inclusion of all three green manures significantly

increased microbial P and labile organic P, and significantly decreased labile inorganic P compared with fallow, although the magnitude of the respective changes was small. Moderately labile inorganic P significantly decreased by legume green manures (12 - 33 mg P kg<sup>-1</sup>) compared with fallow but increased under cereal green manure (13 mg P kg<sup>-1</sup>) compared to fallow. There was also a significant increase in moderately labile organic P observed under pea (25 mg P kg<sup>-1</sup>) and cereal (56 mg P kg<sup>-1</sup>). Only lupin caused a small but significant decrease in stable inorganic P, while organic P significantly increased under the cereal green manure (10 mg P kg<sup>-1</sup>) compared with fallow. Inclusion of the lupin green manure resulted in a significant decrease in total inorganic P (35 mg P kg<sup>-1</sup>), while a significant increase occurred under the cereal green manure (15 mg P kg<sup>-1</sup>). Total organic P increased by 71 and 36 mg P kg<sup>-1</sup> under cereal and pea, respectively, compared with fallow.

Table 4. 3 Mean data for P fractions and microbial P (mg P kg<sup>-1</sup>) determined for the soil following wheat crop. Values represent the mean. Within a row different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ . Total inorganic P: sum of inorganic P pools. Total organic P: sum of organic P pools.

P fractions	Fallow	Cereal	Lupin	Pea
Microbial P	7 b	10 a	11 a	10 a
Soluble inorganic P	0.40 ab	0.41 ab	0.38 b	0.46 a
Labile inorganic P	28 a	26 b	24 c	26 b
Labile organic P	23 c	28 ab	27 b	30 a
Moderately labile inorganic P	234 b	247 a	201 d	222 c
Moderately labile organic P	202 c	258 a	209 c	227 b
Acid soluble inorganic P	83 a	86 a	85 a	87 a
Stable inorganic P	15 a	16 a	14 b	16 a
Stable organic P	25 b	35 a	25 b	29 b
Residual P	41 a	42 a	41 a	42 a
Total inorganic P	360 b	375 a	325 c	352 b
Total organic P	250 c	321 a	260 c	286 b

With respect to phosphatase activity (Figure 4.6), acid phosphatase activity was greater than alkaline phosphatase. Acid phosphatase activity was significantly greater for the lupin (5.24 mmol *p*-nitrophenol kg<sup>-1</sup>) and pea (5.02 mmol *p*-nitrophenol kg<sup>-1</sup>) green manure treatments compared with fallow (3.82 mmol *p*-nitrophenol kg<sup>-1</sup>). Similarly, alkaline phosphatase activity increased by 0.52, 0.19 and 0.32 mmol *p*-nitrophenol kg<sup>-1</sup> in response to the lupin, pea, and cereal green manures, respectively, compared with fallow.

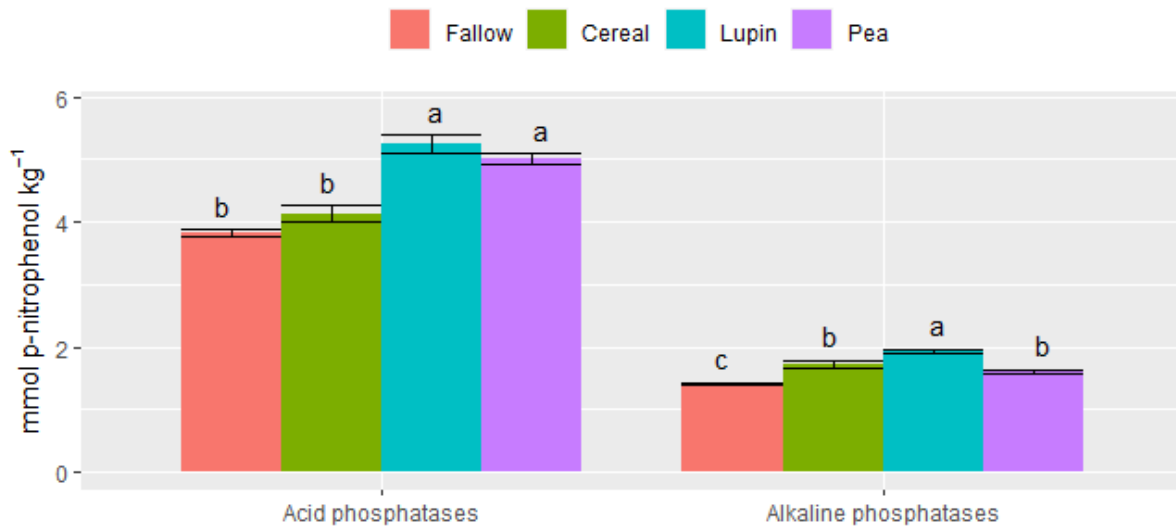


Figure 4. 6 Mean data for phosphatase enzyme activity ( $\text{mmol } p\text{-nitrophenol kg}^{-1} \text{ h}^{-1}$ ) determined for the soil following wheat crop. Bars represent the mean and standard errors. Within a phosphatase group different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

Mean data for soil C, N, and C:N ratio determined at the conclusion of the experiment are shown in Table 4.4. Soil C was significantly lower under lupin and pea compared with cereal, while N was significantly lower under fallow relative to green manures, except for pea. The C:N ratio was significantly lower for legume green manure treatments compared with fallow and cereal green manure.

Table 4. 4 Mean data for total carbon, nitrogen, and C:N ratio determined for the soil following wheat crop. Values represent the mean. Within a column different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

Treatment	C %	N %	C:N
Fallow	4.03 ab	0.25 b	16 a
Cereal	4.41 a	0.28 a	16 a
Lupin	3.85 b	0.29 a	13 b
Pea	3.82 b	0.27 ab	14 b

### 4.3. Discussion

The fact that the legume green manures produced significantly greater biomass compared with the cereal green manure may be attributed to legumes having a higher demand for P than cereals together with biological N fixation. To utilise the extra N resources, additional P is required leading to the enhanced mobilisation of soil P. These factors may also account for the consistently higher yield observed for lupin and pea compared with non-legume.

The relative agronomic performance of the different green manures was also reflected in the individual and cumulative yield, P uptake, and N uptake by subsequent cereal crops. In fact, inclusion of legume green manures significantly increased yield, P uptake, and N uptake compared to the fallow treatment, while the cereal green manure caused a significant decrease of these parameters. Improved or reduced P uptake and N uptake by subsequent crops following different green manures could be partly due to the mineralisation or immobilisation of P and N released from green manures, during decomposition (Alamgir et al., 2012; Randhawa et al., 2005).

Soil analyses carried out at the conclusion of the experiment clearly showed that inclusion of legume green manures resulted in significant depletion of moderately labile inorganic P compared with fallow, indicating mobilisation of legacy soil P in the absence of P inputs. This could be attributed to enhanced exudation of low molecular weight organic anions by legume roots (Hallama et al., 2018; Hocking and Randall, 2001; Ryan and Delhaize, 2001), which may in turn be partly related to the release of organic anions by rhizobia bacteria responsible for biological N fixation in legumes (Gyaneshwar et al., 2002; Halder and Chakrabartty, 1993). This is also consistent with results described in Chapters 2 and 3. Furthermore, incorporation of immature legume plant material into the soil as green manure has been shown to significantly enhance the rate of key biological processes, including gross organic P mineralization (Randhawa et al., 2005). The stable and residual P pools were expected to decrease in the rotation system under lupin. However, this did not show in the system. It appeared that lupin depleted the P pools, but a small amount because lupin root systems could not create rhizosphere in whole soil pot over two rotations. Therefore, dilution may cause an insignificance between lupin green manure treatment and fallow. It was expected that more rotations may result in significant depletion of the stable and residual P. Higher biological activity with green manure incorporation was clearly demonstrated by small but significant increases in soil microbial P, labile organic P, and acid and alkaline phosphatase enzyme activities for the legume green manures compared with fallow. Increased biological activity may cause a low organic C concentration as a consequence of more decomposition of C-C bonds in organic matter from green manure biomass, while greater N was imported from legume N fixation. The significant decrease in

the C:N ratio for legume green manures is consistent with this theory and has also been observed by Randhawa et al. (2005). Enhanced biological activity may also partly explain the significant increase in moderately labile organic P that occurred for the pea green manure which was not observed in the lupin green manure treatment. This indicates a clear difference between the impact of pea and lupin plant material decomposition on soil P dynamics. Greater lupin green manure biomass may supply more available organic C and stimulate microbial activity (Chen et al., 2002), which in turn produces more phosphatases especially alkaline phosphatases for improved mineralization relative to pea (Chen et al., 2019; Nannipieri et al., 2011). Moreover, slower pea decomposition was likely due to lower shoot P concentration of pea compared with lupin (Pearse et al., 2006b). Since legumes can fix N via symbiotic rhizobia (Liu et al., 2011; Thorup-Kristensen et al., 2003), even though there was a mineral N addition for subsequent crops, total N significantly increased under legume green manures particularly lupin relative to fallow.

It is clear that the cereal green manure treatment did not increase mobilisation of moderately labile soil inorganic P compared with legumes, which may be at least partly attributed to reduced exudation of organic anions (Pearse et al., 2006a; Pearse et al., 2006b). Improved biological activity, stimulated through the release of C, N and P during decomposition, may have led to a significant increase in organic P for cereal green manure compared with fallow. The high C:P ratio of the cereal green manure probably favoured microbial immobilisation during decomposition, thus delaying residue mineralization and reducing bioavailable P (Alamgir et al., 2012; Hassan et al., 2012a; Maltais-Landry et al., 2014). Moreover, acid phosphatase enzyme activity was low under cereal green manure and unamended soil, which may have promoted greater organic P, especially moderately labile and stable organic P, after the two crop rotations. In addition to P immobilisation, cereal green manure with high C:N ratio also resulted in N immobilisation by microbial activity (Hassan et al., 2012a; Jin et al., 2008).

#### **4.4 Conclusions**

The findings of this controlled environment study clearly demonstrated that the inclusion of blue lupin or pea as manure crops significantly increased cereal crop biomass, P uptake, and N uptake over two rotations compared with a fallow treatment, and the overall impact was greatest for blue lupin. This was attributed to a combination of factors including enhanced mobilisation and acquisition of moderately labile forms of legacy soil P and increased biological cycling of P in soil, while increased biological N fixation also contributed, despite the uniform addition of N fertiliser. On the other hand, inclusion of a cereal green manure significantly decreased cereal crop biomass, P uptake, and N uptake over two rotations compared with fallow, which was mainly attributed to

enhanced net immobilisation of P during plant matter decomposition. These results showed that in the absence of continued P inputs, inclusion of a legume manure increased crop biomass and P availability in a soil containing significant quantities of legacy P. However, these findings need to be further investigated under field conditions, including an assessment of the impact of P inputs on legume green manure performance. In this regard legume green manure may be an effective means of maintaining crop yield with reduced P inputs.

## Chapter 5

# Immediate impact of green manure quantity and quality on soil phosphorus bioavailability and crop utilisation

### 5.1 Introduction

Results presented in Chapter 4 demonstrated that inclusion of legume green manures (lupin (*Lupinus angustifolius*) and pea (*Pisum sativum*)) significantly improved cereal crop yield and P uptake, which were attributed to enhanced access and utilisation of soil P. The findings of this study also revealed differences in the fate and bioavailability of P added in lupin compared with pea green manure. Relative to one another, lupin depleted greater inorganic P while pea accumulated greater organic P. In temperate cropping systems green manures are grown during autumn-winter when environmental conditions, especially temperature, are likely to affect yield and therefore the potential impact on subsequent crop growth and P uptake.

The main objective of the experiment described in this chapter was to investigate and quantify how different quantities and combinations of lupin and pea green manure influence cereal crop growth, nutrient uptake, and the dynamics and bioavailability of P in soil. It was hypothesised that the agronomic benefits of both green manure crops would be directly related to the quantity incorporated, and that lupin would be superior to pea.

### 5.2 Materials and Methods

#### 5.2.1 Green manure preparation and incubation

Lupin and pea were grown in a medium fertility Templeton silt loam soil (Typic immature Pallic (NZ classification) or Udic Haplustept (USDA classification)), shoots were harvested after 40 days growth (before flowering), cut into 0.5 cm pieces, thoroughly mixed with 400 g dry pumice soil (its properties were described in Chapter 4) at 50 and 100 g fresh biomass kg<sup>-1</sup> soil (equivalent to 45 and 90 tonnes fresh biomass ha<sup>-1</sup>); for comparison, the rate derived from the results of Chapter 4 was 50 g kg<sup>-1</sup>. Dry matter rates were 6.8 and 13.6 tonnes ha<sup>-1</sup> for lupin, 5.9 and 11.8 tonnes ha<sup>-1</sup> for pea, and 6.3 and 12.6 tonnes ha<sup>-1</sup> for the mixtures (Table 5.1). Combinations of soil and green manures were filled in polyethylene pots (width x length x depth = 6.5 cm x 6.5 cm x 16 cm) with 4 replicates per treatment and incubated for a month at 70% water holding capacity.

Table 5. 1 Rates of fresh matter, dry matter, N, and P addition for the different green manures treatments.

Treatment	Fresh matter (g kg <sup>-1</sup> )	N (mg kg <sup>-1</sup> )	P (mg kg <sup>-1</sup> )	Fresh matter (tonnes ha <sup>-1</sup> )	Dry matter (tonnes ha <sup>-1</sup> )	N (kg ha <sup>-1</sup> )	P (kg ha <sup>-1</sup> )
Control (Nil)	0	0	0	0	0	0	0
Lupin 50	50	294	13	45	6.8	265	12.9
Lupin 100	100	588	26	90	13.6	530	25.8
Pea 50	50	215	12	45	5.9	195	10.0
Pea 100	100	430	24	90	11.8	390	20.0
Lupin-pea 50	50	253	13	45	6.3	229	11.3
Lupin-pea 100	100	506	26	90	12.6	458	22.6

### 5.2.2 Barley crop

After the green manures were incubated for one month, barley was grown at a density of 4 plants pot<sup>-1</sup> in a glasshouse under controlled environment conditions (10-25°C) to maximum yield stage. Basal nutrients as described in Chapter 4 were applied at the beginning of the experiment and repeated after one month due to crop leaf chlorosis (Figure 5.1).



Figure 5. 1 Nitrogen deficiency symptom after 30 days of barley growth (No.1: control; No.5: lupin 50; No.9: lupin 100).

### 5.2.3 Soil and plant sampling and analysis

**Soils:** A month after green manure incorporation, subsamples of fresh soil were analysed for microbial P and phosphatase activity as described in Chapter 3; microbial biomass-C, and -N methods are described in Appendix A.9. At the completion of the experiment (barley harvest), fresh soils were analysed for soluble inorganic P and labile inorganic P as described in Chapter 4.

**Plants:** Barley shoots were harvested and dried for dry matter yield. Subsamples of green manures were dried as described in Chapter 2 for dry matter ratio. All samples were analysed for total P and N as described in Chapter 4.

### 5.2.4 Statistical analysis

Statistical analysis of the data was carried out with the R statistical software package (version 4.0.2). Differences between green manure treatments were analysed using one-way analysis of variance (ANOVA, TukeyHSD;  $\alpha=0.05$ ), including crop yield, P uptake, N uptake, phosphatase enzyme activities, microbial biomass CNP, and soluble and labile inorganic P.

### 5.3 Results

Mean data for phosphatase enzyme activities and microbial biomass C, N, and P for the different treatments after one month of green manure incubation are presented in Figures 5.2 and 5.3, respectively. Acid phosphatase enzyme activity was consistently higher than alkaline phosphatase for all treatments. Green manure addition significantly increased both acid and alkaline phosphatase enzyme activities compared with the control (except lupin-pea 50), and these activities were significantly higher for the high rate of addition compared with low rate (except acid phosphatase activity in the pea treatment). Concentrations of microbial biomass C, N, and P were highly variable (438 - 584 mg C kg<sup>-1</sup>, 57 - 405.0 mg N kg<sup>-1</sup>, 4 - 9 mg P kg<sup>-1</sup>) and no significant differences were observed between treatments, other than microbial N which was significantly lower for lupin-50 and pea-100 compared with lupin-pea-50 treatments. Nonetheless, concentrations of microbial biomass C and P were consistently lower in the green manure treatments compared with the control.

Mean data for barley shoot yield, P, and N uptake are shown in Figure 5.4. Addition of green manure significantly increased crop yield, except for lupin 100, and the highest yields were observed for the combined lupin-pea treatments (c. 34% compared with 21-28% for other treatments). Similarly, crop N uptake was also significantly increased by green manure addition, and the magnitude of the increases were similar for the lupin, pea, and lupin-pea treatments. Unlike yield, N uptake increases were consistently and significantly higher for the 100g addition rate (430-588 mg N) compared with 50g rate (215-294 mg N). Consistent with yield and N uptake, crop P uptake was significantly increased by green manure addition across all treatments compared with the control. However, in contrast to N, P uptake was consistently lower for the high addition rate compared with the low rate, and the differences were significant for the lupin and pea treatments.

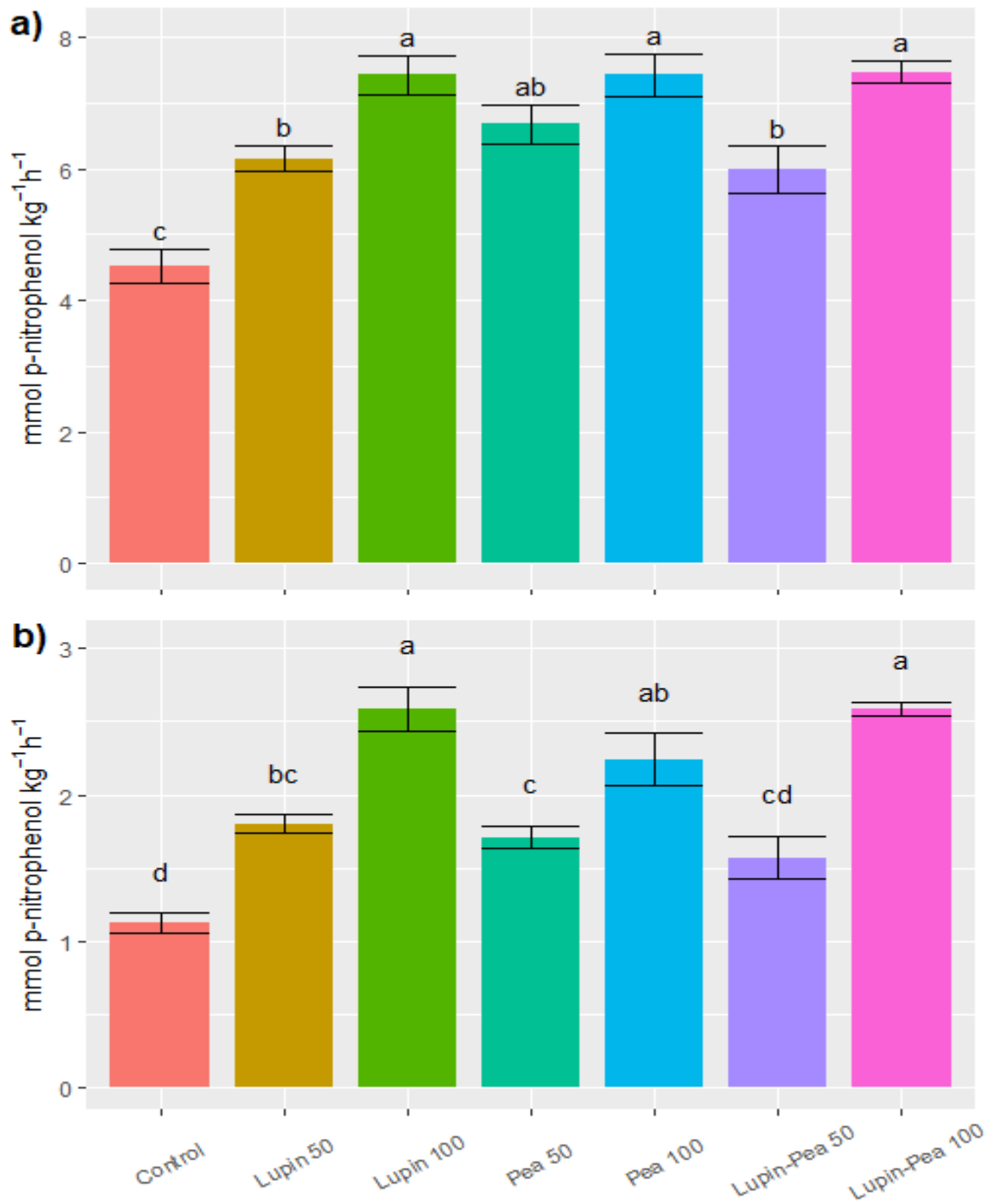


Figure 5. 2 Mean data for soil acid (a) and alkaline (b) phosphatase activity determined following green manure incubation. Bars represent the mean and standard errors. Different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

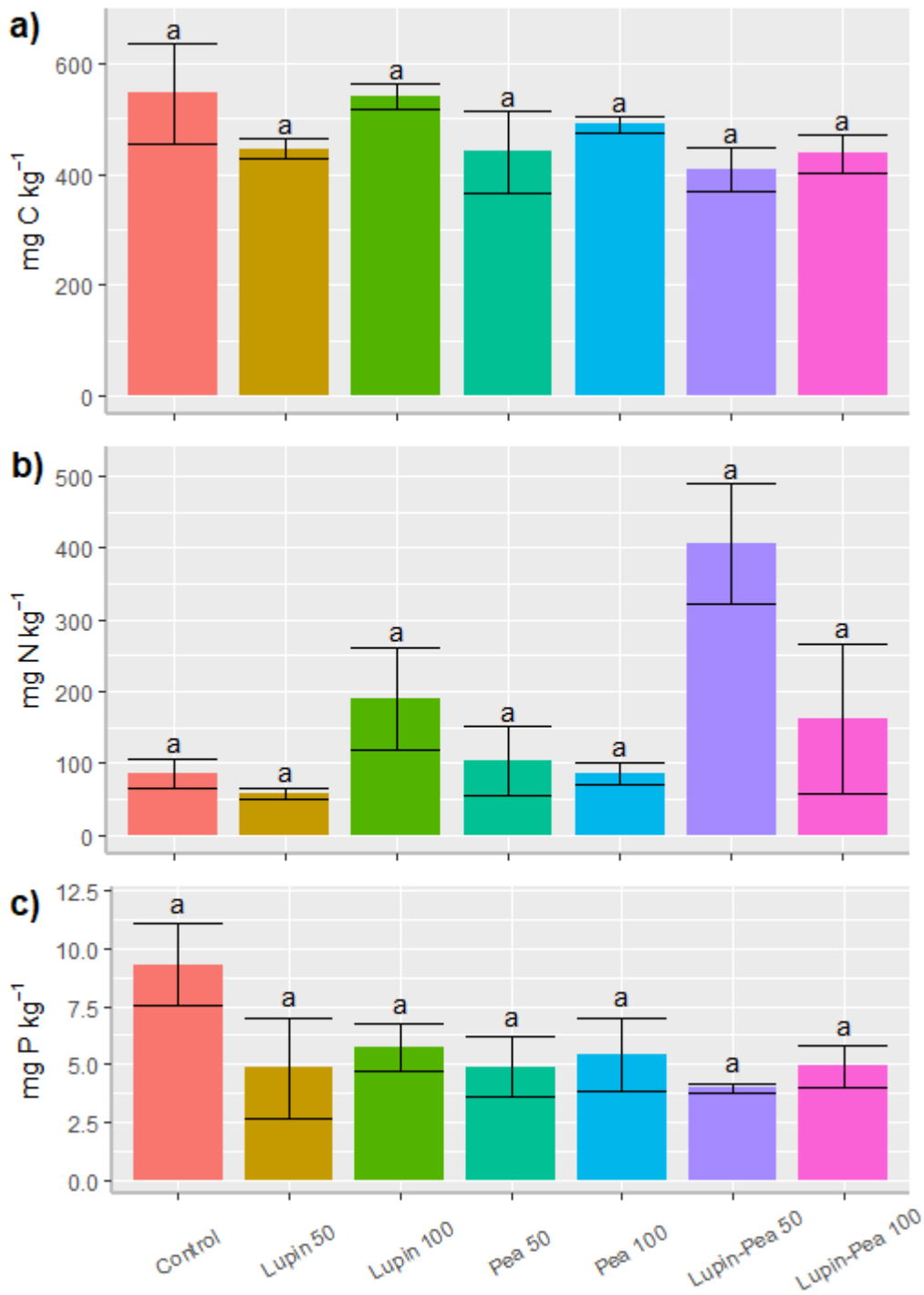


Figure 5. 3 Mean data for soil microbial biomass C, N, and P determined following green manure incubation. Bars represent the mean and standard errors. Different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey’s honest significant difference method at  $\alpha=0.05$ .

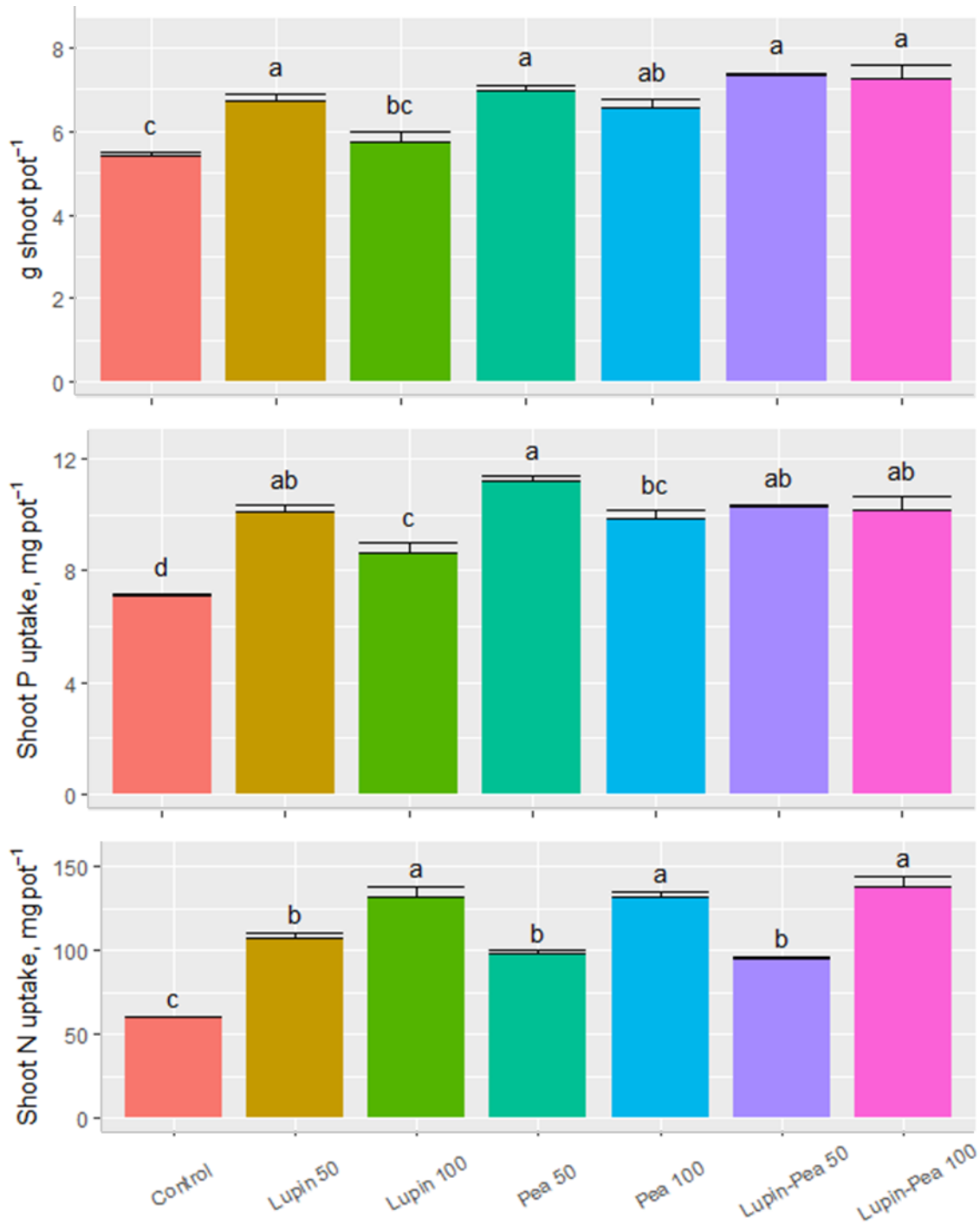


Figure 5. 4 Mean data for barley shoot dry matter yield (g pot<sup>-1</sup>), P uptake (mg P pot<sup>-1</sup>), and N uptake (mg N pot<sup>-1</sup>) determined for barley following different green manure types and doses. Bars represent the mean and standard errors. Different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

After the barley harvest, no significant differences in soluble inorganic P were observed between the green manure treatments and the control, except for lupin 50 which was significantly lower than the lupin 100, pea 100, and lupin-pea 50 treatments. Concentrations of labile inorganic P were lower in

the green manure treatments (34-36 mg kg<sup>-1</sup>) compared with the control (37 mg kg<sup>-1</sup>), although the differences were only significant for the lupin 50 and lupin-pea 50 treatments (Table 5.2).

Table 5. 2 Mean data for soluble and labile inorganic P (mg P kg<sup>-1</sup>) determined for soil sampled after barley harvest at different green manure treatments. Within a row, different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's honest significant difference method at  $\alpha=0.05$ .

	Control	Lupin 50	Lupin 100	Pea 50	Pea 100	Lupin-pea 50	Lupin-pea 100
Soluble inorganic P	0.54 ab	0.42 b	0.68 a	0.61 ab	0.64 a	0.64 a	0.60 ab
Labile inorganic P	37 a	34 b	35 ab	35 ab	36 ab	34 b	35 ab

## 5.4 Discussion

Green manure addition increased crop yield and P and N uptake compared with control. The extent of N uptake increase was directly related to the quantity of green manure incorporated. For example, the rate of 100 g green manure kg<sup>-1</sup> significantly increased N uptake relative to 50 g green manure kg<sup>-1</sup>. However, this was not the case for P uptake and yield. For lupin and pea green manures, P uptake was actually significantly lower for the high rate compared with the low rate. Lupin-pea treatments increased crop yield to a greater extent than lupin or pea alone, but this was not reflected in N and P uptake.

Based on data from Chapter 4, it is reasonable to assume that barley is poor at mobilizing soil P and so the agronomic and soil impacts observed can be attributed to a combination of nutrients added in green manure and the effect these additions had on P dynamics and availability, mainly P release from decomposing plant matter. The fact that P was added to soil in green manure may account for increased yield and P uptake. If this was the case it would be expected that soluble and labile P would have increased, but after two months cropping there was no difference between treatments for soluble P and labile P was only slightly lower in green manure treatments compared with control, while there were no differences observed between addition levels (50 and 100 g kg<sup>-1</sup>). It appeared that available inorganic P released from green manure biomass could be only partly taken up by the barley crop, while the remaining P may be rapidly immobilised by microorganisms or fixed by soil.

Green manure addition consistently increased phosphatase enzyme activities compared with control, and in most treatments 100 g kg<sup>-1</sup> was greater than 50 g kg<sup>-1</sup>. These differences were all statistically significant for alkaline phosphatase. However, microbial C, -N and P did not significantly

differ between treatments and were very variable, indicating that microbial activity was still high after one month of green manure decomposition. At a longer time (81 days) of incubation, Stark et al. (2008) found that a high rate of lupin resulted in significantly higher microbial biomass C and N compared with a low rate. It appears that a one month period was not sufficient for green manure decomposition. The fact that, for lupin and pea, P uptake was actually significantly lower for the high rate compared with the low rate strongly indicates that the higher rate of green manure incorporation significantly enhanced P immobilisation due to higher microbial activity at the expense of P uptake (Dijkstra et al., 2015). It is likely that the high green manure rates supplied more C (energy) for microbial activity, thereby increasing microbial P immobilisation (Joergensen et al., 1995; Sorkau et al., 2018; Stutter et al., 2015). Enhanced P immobilisation under lupin at the high rate led to significantly lower yield compared with lupin at the low rate. Furthermore, mineral N addition (10 - 28% relative to that in green manures) may have increased the overall N:P ratio, leading to more P immobilisation for the high green manure addition rate. The higher green manure rate increased N uptake while decreases in the C:N ratio increased mineralization rates of organic N materials (Enwezor, 1976; Reddy et al., 2008).

Unlike lupin or pea alone, two rates of lupin-pea treatments had similar crop yield and P uptake. It appears that there was a complementarity between the two green manure species during decomposition due to chemical diversity which resulted in changes of microbial community composition (Chapman and Newman, 2010). Hence, nutrients in lupin-pea green manures may be released consistently for crop uptake.

## **5.5 Conclusions**

The findings of this controlled environment study confirmed that the quantity and composition of legume green manures incorporated into the soil has the potential to significantly affect P availability for the succeeding crop. For lupin in particular, crop yield and P uptake were significantly reduced for the high rate of above-ground green manure incorporation compared with the low rate, which was attributed to enhanced microbial P immobilisation during residue decomposition. However, the observation that this impact did not occur for the combination of lupin and pea may reflect differences in the chemical nature of organic carbon in lupin and pea which influenced microbial decomposition and P release. It was also evident from this study that legume green manure incorporation has the potential to adversely affect N availability for the succeeding crop at high rates of incorporation, which may require targeted inputs of N fertilizer.

## Chapter 6

### Case Study: Cumulative effects of different green manure crops on maize yield, phosphorus uptake, and soil phosphorus after five years

#### 6.1 Introduction

As indicated in the introduction to Chapter 4, due to time constraints it was not feasible to conduct a field experiment to complement the controlled environment studies included in this PhD research programme. However, recent and ongoing research carried out in New Zealand on cover crops/green manures has focused on their use in reducing N loss by leaching over winter from various crop systems, including winter forage crops (Carey et al., 2016; Malcolm et al., 2021; Malcolm et al., 2020). Accordingly, extensive enquires were directed to various New Zealand research organisations regarding the status and availability of any field experiments on cover crops/green manures that could be used to investigate impacts on soil P dynamics and bioavailability. Fortunately, we discovered that the Foundation for Arable Research (FAR) had been conducting a fully-replicated green manure field experiment on a volcanic/allophanic soil in Waikato on no-till continuous maize (*Zea mays*) for 5 years (2016-2021). The trial was primarily designed to investigate the impact of different green manure crops in combination with various herbicide regimens on weed suppression and maize grain yield compared with fallow. The trial design and operation did not include any specific consideration or analysis of treatments impacts on soil properties and processes. The trial was completed following maize harvesting in autumn 2021, and we were allowed to sample soil from replicate plots of all the fallow/green manure treatments immediately following maize harvesting in early June 2021. In addition, we obtained data for 2020 green manure yields, together with 2021 maize grain yield data and samples for nutrient analysis.

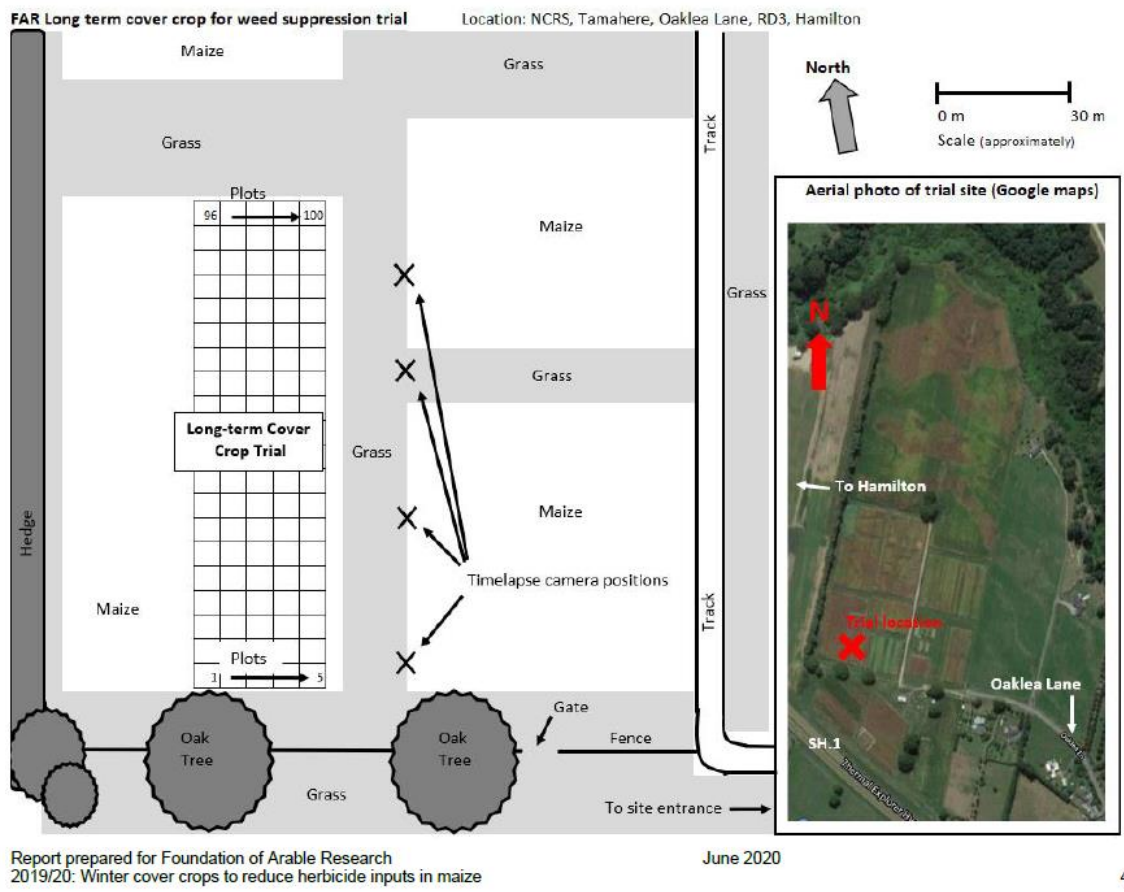
The main objective of the research described in this chapter was to investigate and quantify the cumulative effect of 5 years of inclusion of different green manure crops on maize grain yield, nutrient uptake (P, N), and soil P dynamics and bioavailability. We hypothesised that the inclusion of green manure crops over the previous 5 years would significantly increase maize grain yield compared with fallow, and that the legume green manures would perform better than non-legume green manures. Furthermore, differences in the agronomic impact of green manure inclusion would be reflected in changes in the nature and bioavailability of P in topsoil.

## 6.2 Materials and Methods

### 6.2.1 Field Trial

The field trial was established in 2016 at the FAR Northern Crop Research Site at Tamehere (Waikato) on Allophanic slit loam soils (Horitiu/Bruntwood series - Typic Orthic Allophanic Soil (New Zealand Soil Classification)) (McKay, 2020). The main objective of the trial was to investigate the use of cover crops/green manure crops to suppress weeds and reduce the use of herbicides to lessen the chances of herbicide resistance emerging (<https://www.far.org.nz/assets/files/blog/files//b316e4bf-2b9e-59d4-b24d-dbf729bb837.pdf>). The trial comprised four winter green manure crop treatments, namely faba bean (*Vicia faba* c.v. Wizard), oats (*Avena sativa* c.v. Milton), annual ryegrass (*Lolium multiflorum*, 2020/21 changed to blue lupin (*Lupinus angustifolius* c.v. unknown) and mustard (*Brassica rapa* c.v. unknown)), gland clover (*Trifolium glanduliferum*, 2020/21 changed to woolly pod vetch (*Vicia villosa* c.v. RM4)), and a fallow treatment. Five herbicide regimes were also assessed in the trial, including a dual post-emergence treatment (Callisto<sup>®</sup> mix = Callisto (a.i. 480 gm/L mesotrione) @ 200 mL ha<sup>-1</sup> + Atrflow @ 1L ha<sup>-1</sup> applied with Synoil™ 1% v/v early, plus Astound<sup>®</sup> Ultra (a.i. 40 g L<sup>-1</sup> nicosulfuron) @ 1.5 L ha<sup>-1</sup> + Synoil™ 1% v/v). The trial comprised four replicate plots of each fallow/green manure and herbicide combination, arranged in semi-randomised blocks (Figure 6.1).

The green manures were direct drilled into maize stubble after maize harvest in May each year and terminated by crimp rolling and sprayed with glyphosate in October. Maize (hybrid PAC343 (105 CRM)) was planted by direct drill in late October @ 95,000 ha<sup>-1</sup>, and base (300 kg ha<sup>-1</sup> muriate of potash (K 50), 150 kg ha<sup>-1</sup> Ammo 36N (N 36, S 9), 150 kg ha<sup>-1</sup> granulated CalMag (Mg 38, Ca 2)), starter (200 kg ha<sup>-1</sup> YaraMila ACTYVA S (N 15, P 7, K 13)) and side-dress (275 kg ha<sup>-1</sup> Sustain) fertilisers were applied each year.



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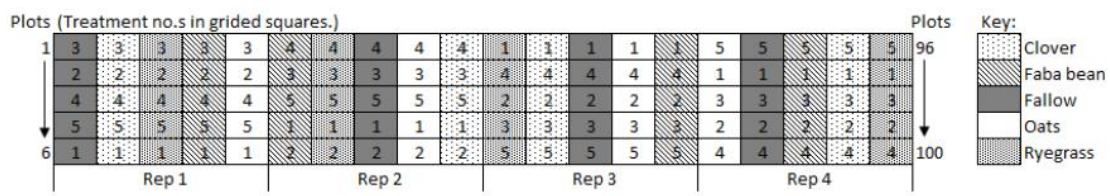


Figure 6. 1 The green manure field trial located at the FAR Northern Crop Research Site, Tamehere, Waikato.

### 6.2.2 Soil sampling and analyses

Topsoil (0-10cm) soil samples were taken from replicate plots of the 5 fallow/green manure treatments that had received dual post emergence herbicide in early June 2021 immediately following the final maize harvest (20 samples from plots 4, 9, 14, 19, 24, 28, 33, 38, 43, 48, 55, 60, 65, 70, 75, 76, 81, 86, 91, 96). Soils were sieved through 2 mm and any plant material removed prior to analysis. Microbial biomass C, N, and P, together with acid/alkaline phosphatase enzyme activities (as described in Chapter 4 and 5) and dehydrogenase enzyme activity (Appendix A.9) and were determined on fresh soil, while pH, total C, total N, and P fractionation analyses were carried out on air dried soil (as described in Chapter 4).

### 6.2.3 Plant analyses

FAR provided dry matter yield data for the fallow/green manure treatments determined at termination in October 2020, and for the maize grain harvested in May 2021. Samples of maize grain from the plots sampled in June 2021 were analysed for total N and P (as described in Chapter 4).

### 6.2.4 Statistical analysis

Statistical analysis of the data was carried out by using R statistical software package (version 4.0.2). Differences between multiple treatments were detected using one-way analysis of variance (ANOVA; TukeyHSD;  $\alpha=0.05$ ), including plant yields and N and P uptake, soil microbial biomass C, N, and P, dehydrogenase enzyme activity, acid/alkaline phosphatase enzyme activities, pH, total C, total N, and P fractionation.

## 6.3 Results

Mean data for green manure yields determined in October 2020 are shown in Table 6.1, and revealed that lupin-mustard green manure yield at  $5.34 \text{ t ha}^{-1}$  was markedly higher than the other green manure treatments ( $2.13\text{-}3.72 \text{ t ha}^{-1}$ ), although the difference was only significant between lupin-mustard and vetch.

Table 6. 1 Mean dry matter yields ( $\text{t ha}^{-1}$ ) determined for the green manure treatments in October 2020. Within a row different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's HSD multiple comparison at  $\alpha=0.05$ .

Vetch	Faba bean	Oat	Lupin-Mustard
2.76 b	2.13 bc	3.72 ab	5.34 a

Yield and nutrient uptake data for the subsequent maize crop are presented in Table 6.2. While all measured differences between treatments were not statistically significant, means for yield, N uptake, and P uptake were consistently higher for the vetch green manure treatment and lowest for the fallow treatment. Similarly, data for soil pH, total C, and total N (Table 6.3) and most P fractions revealed no significant differences between treatments (Table 6.4). Concentrations of moderately labile organic P, and hence total organic P, were significantly higher for the vetch green manure compared with the lupin-mustard green manure. Corresponding data for soil microbial biomass and extracellular enzyme activities are shown in Table 6.5. Although most differences between treatments were once again not statistically significant, microbial biomass C was lower under fallow

compared with the green manures, while microbial biomass P concentrations were substantially and significantly higher for the fallow and vetch treatments (17-18 mg kg<sup>-1</sup>) compared with the lupin-mustard green manure (2 mg kg<sup>-1</sup>). All enzyme activities were consistently lowest in soil maintained under fallow compared with green manure, although differences between treatments were not significant.

Table 6. 2 Mean maize grain dry matter yields (t ha<sup>-1</sup>), and nutrient uptake (kg ha<sup>-1</sup>) determined for the fallow/green manure treatments in May 2021. Within a row different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's HSD multiple comparison at  $\alpha=0.05$ .

	Fallow	Vetch	Faba bean	Oat	Lupin-Mustard
Yield	9.51 a	10.7 a	10.3 a	10.0 a	10.6 a
N uptake	124 a	147 a	138 a	128 a	140 a
P uptake	25.8 a	29.4 a	26.7 a	26.3 a	27.6 a

Table 6. 3 Mean soil pH, total C, total N, and C:N ratio data determined for the fallow/green manure treatments in May 2021. Within a row different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's HSD multiple comparison at  $\alpha=0.05$ .

	Fallow	Vetch	Faba bean	Oat	Lupin-Mustard
pH <sub>H2O</sub>	6.33 a	6.30 a	6.02 a	6.22 a	6.08 a
C (%)	5.68 a	5.78 a	6.25 a	5.40 a	5.52 a
N (%)	0.610 a	0.612 a	0.640 a	0.622 a	0.590 a
C:N ratio	9.3 a	9.4 a	9.7 a	8.8 a	9.4 a

Table 6. 4 Mean data for soil P fractions (mg P kg<sup>-1</sup>) determined for the fallow/green manure treatments in May 2021. Within a row different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's HSD multiple comparison at  $\alpha=0.05$ . Total inorganic P: sum of inorganic P fractions. Total organic P: sum of organic P fractions.

P fractions	Fallow	Vetch	Faba bean	Oat	Lupin- Mustard
Soluble inorganic P	1.01 a	1.13 a	1.03 a	1.03 a	0.89 a
Labile inorganic P	94 a	85 a	73 a	81 a	74 a
Labile organic P	24 a	34 a	28 a	29 a	24 a
Moderately labile inorganic P	1181 a	1209 a	1066 a	1154 a	1066 a
Moderately labile organic P	705 ab	736 a	698 ab	714 ab	671 b
Acid soluble inorganic P	614 a	612 a	563 a	592 a	555 a
Stable inorganic P	158 a	163 a	148 a	164 a	155 a
Stable organic P	258 a	270 a	242 a	259 a	242 a
Residual P	102 a	105 a	99 a	102 a	99 a
Total inorganic P	2048.01 a	2070.13 a	1851 a	1992.03 a	1850.89 a
Total organic P	987 ab	1040 a	968 ab	1002 ab	937 b

Table 6. 5 Mean data for soil microbial parameters ( $\text{mg kg}^{-1}$ ), activities of phosphatase enzymes ( $\text{mmol } p\text{-nitrophenol kg}^{-1} \text{ h}^{-1}$ ) and dehydrogenases ( $\text{mg 1, 3, 5-triphenyltetrazolium formazan kg}^{-1} \text{ h}^{-1}$ ) determined for the fallow/green manure treatments in May 2021. Within a row different lowercase letters denote significant differences between treatments detected using one-way ANOVA followed by Tukey's HSD multiple comparison at  $\alpha=0.05$ .

	Fallow	Vetch	Faba bean	Oat	Lupin- mustard
Microbial C	2174 a	2412 a	2772 a	2611 a	2336 a
Microbial N	62 a	52 a	53 a	77 a	42 a
Microbial P	17 a	18 a	5 ab	8 ab	2 b
Acid phosphatases	4.23 a	4.30 a	4.43 a	4.69 a	4.29 a
Alkaline phosphatases	2.18 a	2.47 a	2.66 a	2.70 a	2.40 a
Dehydrogenases	0.13 a	0.20 a	0.18 a	0.20 a	0.15 a

## 6.4 Discussion

The findings of this case study revealed that the inclusion of a variety of green manure crops over a 5 year period did not significantly increase maize grain yield or P uptake. Similarly, with the exception of vetch, the green manures had no significant impacts on the nature, dynamics, and bioavailability of P in the topsoil. The absence of response was surprising given the substantial quantities of plant biomass contained in the green manure crops (e.g. 2-5 t ha<sup>-1</sup> in 2020). However, the fact that the green manure biomass was left on the soil surface rather than being incorporated and mixed into the soil may have contributed to the lack of any significant impacts on soil organic matter and fertility. Moreover, mature green manures (5 months of growth) are unlikely to decompose much further and contribute to successive crops. The surface deposition of the green manures was entirely consistent with the main objective of the field trial which was to investigate the impact of different green manure/cover crops on the effectiveness of various combinations of herbicides in controlling weeds. It has been shown that continued return of cut plant biomass to the soil surface in a permanent grassland over 20 years resulted in significant increases in topsoil organic matter and biological activity associated with P cycling compared with biomass removal (Boitt et al., 2017a; Boitt et al., 2018a). This was mainly attributed to continuous incorporation of plant biomass into the soil by the actions of a large earthworm population. It is possible that earthworm numbers and activity in the current field trial were limited due to the history of intensive cultivation at the site prior to trial establishment. Haynes and Tregurtha (1999) showed that long-term intensive cultivation dramatically reduced earthworm populations compared with permanent pasture in volcanic soil in

South Auckland (Pukekohe), and that earthworm numbers recovered slowly when cultivated land was converted to permanent pasture.

The P fertility of the soil was very high (bioavailable (labile) inorganic P 73-94 mg kg<sup>-1</sup>), and soluble P fertiliser was applied to the trial annually (14 kg P ha<sup>-1</sup>). Both these factors may at least partly explain why inclusion of green manure crops did not significantly alter the forms and bioavailability of P in soil after 5 years. This is consistent with the findings reported in Chapter 2 of this thesis which showed that a variety of green manure legumes had limited impact on P cycling and bioavailability in a high P fertility soil compared with a medium fertility soil. Nonetheless, there was evidence that the legume vetch green manure increased concentrations of soil organic P and microbial biomass P which may reflect enhanced biological activity. This was also consistent with the fact that maize crop P uptake, soil microbial biomass C, and soil phosphatase enzyme activities were consistently lower under fallow compared the green manure treatments.

## 6.5 Conclusions

While somewhat disappointing, the findings of this case study highlights the need for the establishment and maintenance of medium to long-term field experiments in New Zealand specifically designed to investigate and quantify the specific impacts of legume green manure crops on P (and N) dynamics and bioavailability, together with nutrient use efficiency and crop performance. It is clear from this study that old green manures placed on soil surface in combination with high available P level and annual P application did not cause a difference in P utilisation and crop yield compared with fallow.

## Chapter 7

### General discussion and recommendations for future research

The findings of this research programme demonstrated that legume green manures, as opposed to non-legumes, have the potential to enhance mobilization of legacy P in volcanic soils and thereby contribute to improving P utilisation in temperate cropping systems. Results confirmed that significant mobilization of legacy P by legume green manures is not effective in soils that contain levels of plant-available inorganic P in excess of the appropriate agronomic optimum designed to support 75 - 90% of maximum yield. In these soils, available P may rapidly transform into soluble P to meet plants' requirement. Therefore, P inputs should be discontinued for an appropriate period while P drawdown occurs. For agricultural soils in New Zealand, it may take up to 12 years to lower plant-available inorganic P to agronomic optimum levels depending on current soil P level and soil characteristics. Once below these soil P levels, management can then prioritise increasing P use efficiency under low P inputs.

For volcanic soils without excess P, legume green manures, as opposed to non-legumes, had the greatest potential to mobilise legacy P. Two legumes, lupin and pea, outperformed a variety of green manure species in Chapter 2 in mobilising soil P, although they were less effective in a volcanic ash soil. The other species tested showed little capacity for mobilising legacy P, meaning that, although they may provide other functions, some green manure plant species will have little impact on P mobilisation.

In the absence of P inputs, lupin was superior to pea at mobilising legacy P and therefore increasing crop P uptake, which was at least partly due to a combination of factors including more fine root hairs, organic anions, and phosphatases. For lupin, the presence of longer and denser fine root hairs together with elevated concentrations of citrate in root exudates contributed to greater mobilisation of moderately labile inorganic P compared with pea (Chapters 3 and 4). Citrate is a tricarboxylate which may form stronger ligand complexes with polyvalent metal cations compared to other organic anions. In addition, higher acid and alkaline phosphatase enzyme activities in the rhizosphere of lupin likely increased the mineralisation of moderately labile and stable organic P compared with pea (Chapter 3).

Since the green manure effect on crop P use efficiency relies on decomposition, the potential benefits of including a legume green manure in a crop rotation may be dependent upon incorporation and mixing with soil (i.e., tillage) rather than leaving the green manure crop on the soil

surface. Furthermore, the availability of P in green manure plant tissue following incorporation, and hence its impact on the P nutrition of the succeeding crop (determined by yield), was shown to be influenced by the quantity of green manure incorporated together with residue quality (tissue C:N and C:P) as determined by legume species (Chapters 5 and 6).

Based on the findings of this study, green manures may be possible to enhance utilisation of legacy soil P in temperate crop systems where plant available inorganic P is at or below agronomic optimum levels. The enhancement of legacy soil P utilisation would enable maximum yield to be maintained with reduced P inputs. Or the green manure may maintain acceptable crop production in practices with a large reduction of P inputs or no P inputs. With continued P inputs, the inclusion of legume green manure may reduce the rate of legacy P accumulation in the soil, while in the absence of continued P inputs, green manure inclusion may result in a reduction in legacy soil P. These should be further investigated in cropping systems in a combination of green manures with different rates of P input.

Legacy P utilisation may also depend on the vegetative production of green manure which is influenced by many factors such as rainfall and temperature during winter. Therefore, assessing different green manure biomass on subsequent crop performance should be essential. High yields and incorporation of green manures may slow their decomposition rates, thus potentially lowering P uptake and yield of following crops (Chapter 5). The cycling of plant biomass as mediated by soil biological activity is critical for both P use efficiency and for soil fertility. Therefore, the biomass yield of a green manure and potential decomposition rates should be accounted for when scheduling the starting date of the successive crop.

The findings below in this study have contributed to an improved understanding of the rhizosphere P dynamics and P cycling for crop systems that include green manures:

- Blue lupin is the best green manure plant species for mobilising soil legacy P
- Blue lupin green manure inclusion significantly increased crop plant biomass and P uptake in a simulated crop rotation compared with fallow
- Blue lupin mobilised more soil legacy P due to longer fine root hairs, higher root citrate concentration and phosphatase enzyme activity
- Mobilisation of soil legacy P was influenced negatively by concentration of plant available P and P sorption (volcanic ash soil vs pumice soil)

- Evidence that the agronomic impact of legume green manure was influenced by the quantity of green manure incorporated and method of addition to soil

However, future work is needed in the following general areas:

- i) In order to investigate the impact of rhizoplane organic anions with minimal interferences of fine root hairs of legumes on P mobilisation, alternative experimental methods are needed. For example, a fine nylon mesh (< 1  $\mu\text{m}$ ) was used to create a vertical rhizoplane which can reduce effect of gravity on P movement.
- ii) Long-term and short-term field experiments should be carried out for blue lupin in soils with medium P levels and different P sorption capacities.
- iii) A combination of green manure inclusion and different P inputs should be investigated in rotation systems
- iv) Based on exudation rates and composition of root organic anions, similar artificial solutions could be made to assess their ability to desorb soil unavailable P in controlled experiments. These experiments could isolate the root exudate characteristics most conducive to mobilising legacy P.
- v) Green manures did not well perform in the volcanic ash soil with low and high available P status, how the plant species work in the soil containing medium P availability should be assessed.
- vi) A glasshouse experiment (Chapter 4) showed positive impacts of green manures on P uptake and yield of successive crops in the pumice soil but this experiment only ran for 2 rotations. Therefore, long-term field trials should be established to determine the performance of green manures such as lupin and pea in mobilizing soil legacy P and increasing P cycling. Monitoring yields, P use efficiencies, and soil P levels on decadal scales would be very insightful for how management can improve P sustainability. In addition, a combination of green manure inclusion rates with different levels of P inputs could be investigated.
- vii) Investigation of the impact of different methods of returning green manures to soil such as inclusion vs surface deposition on soil P dynamics should be carried out. Different tillage practices and 'kill' methods (e.g., crimp roller and glyphosate in Chapter 6) will likely influence the cycling of green manure biomass P.

- viii) Different growth stages of green manures may have various decomposition rates after inclusion in soil. This should be investigated to assess how management can improve the timing of finishing green manures and starting main crops to optimise P utilisation.
- ix) More green manure plant species should be studied for P mobilisation. Due to time constraints, this research only examined 11 species but this was not exhaustive. Many green manure (cover crop) species are used worldwide. The species used and the environment where they are grown will likely impact P use efficiency.

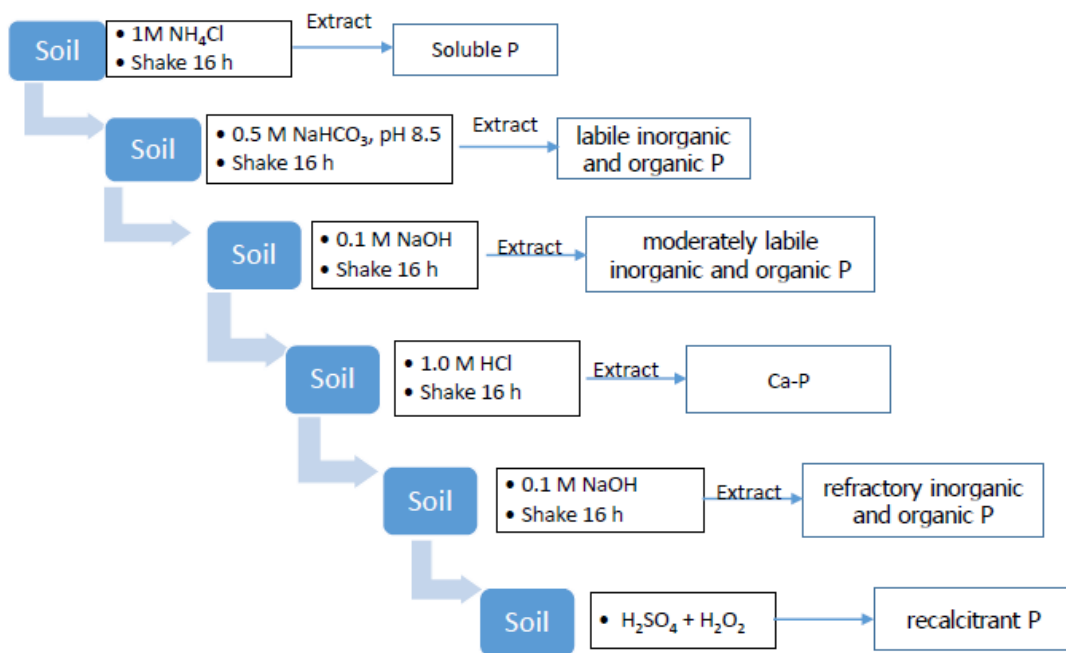
More technical recommendations for future research based on the findings of this study are:

- i) While the results presented in this thesis demonstrated that inclusion of legume green manures in temperate cropping systems has the potential to enhance overall P use efficiency by mobilizing legacy soil P, this needs to be verified in the field with various combinations of green manure and main crops. This would require the establishment and maintenance of appropriate long-term field trials and the research should include investigation of properties and processes that influence green manure decomposition and P dynamics, including the impact of reduced- or no-till compared with conventional cultivation.
- ii) Further detailed research is required to assess and quantify the individual and combined roles and functions of root and mycorrhizal exudates and phosphatase enzymes in soil P acquisition by different plant species.

## Appendix A

### Analytical Methods for Soil Phosphorus, Carbon, Nitrogen and Enzyme Activities

A.1 Soil phosphorus fractionation scheme compiled from Boitt et al. (2018b) based on Chen et al. (2000) and Condon and Newman (2011).



#### Reagents and solutions:

1 M NH<sub>4</sub>Cl: dissolve 53.5 g of NH<sub>4</sub>Cl with 800 ml deionized water (DI H<sub>2</sub>O) in a 1000 ml beaker and transfer to a 1000 ml volumetric flask and make up to 1000 ml with DI H<sub>2</sub>O

0.5 M NaHCO<sub>3</sub>: dissolve 42.00 g of NaHCO<sub>3</sub> and 5 pellets NaOH with 900 ml DI H<sub>2</sub>O in a 1000 ml beaker. Adjust pH to 8.5 with 6 M NaOH and HCl 6 M solutions. Transfer the solution to a 1000 ml volumetric flask and make up to 1000 ml with DI H<sub>2</sub>O. Freshly prepare the solution.

0.5 M NaCl: dissolve 29.25 g of NaCl with 800 ml DI H<sub>2</sub>O in a 1000 ml beaker and transfer to a 1000 ml volumetric flask and complete the volume with DI H<sub>2</sub>O.

0.1 M NaOH: Dissolve 4.00 g of NaOH with 800 ml DI H<sub>2</sub>O in a 1000 ml beaker. Transfer the solution into a 1000 ml volumetric flask and make up to 1000 ml with H<sub>2</sub>O.

1 M HCl: Add 84 ml of concentrated HCl (37%) in a 1000 ml beaker containing roughly 700 ml DI H<sub>2</sub>O. Transfer to a 1000 ml volumetric flask and make up the volume with DI H<sub>2</sub>O.

**Procedure:**

Weigh 0.5 g of soil into 15 ml centrifuge tube;

**Add 10 ml of 1 M NH<sub>4</sub>Cl;**

Shake for 16 hours end-over-end (60 rpm);

Centrifuge at 3500 rpm for 15 minutes; reserve the extract for P analysis (Appendix A.2).

**Add 10 ml 0.5 M NaHCO<sub>3</sub>, pH 8.5;**

Shake for 16 hours end-over-end (60 rpm);

Centrifuge at 3500 rpm for 15 minutes; reserve the extract for analysis: inorganic P (Appendix A.3) and total P (Appendix A.4);

Add 10 ml 0.5 M NaCl, shake the tube;

Centrifuge at 3500 rpm for 10 minutes, get rid of the supernatant as much as possible;

**Add 10 ml of 0.1 M NaOH;**

Shake for 16 hours end-over-end (60 rpm);

Centrifuge at 3500 rpm for 15 minutes; reserve the extract for analysis: inorganic P (Appendix A.3) and total P (Appendix A.4);

Add 10 ml 0.5 M NaCl, shake the tube;

Centrifuge at 3500 rpm for 10 minutes, get rid of the supernatant as much as possible;

**Add 10 ml of 1 M HCl;**

Shake for 16 hours end-over-end (60 rpm);

Centrifuge at 3500 rpm for 15 minutes; reserve the extract for inorganic P analysis (Appendix A.2);

Add 10 ml 0.5 M NaCl, shake the tube;

Centrifuge at 3500 rpm for 10 minutes, get rid of the supernatant as much as possible;

**Add 10 ml of 0.1 M NaOH;**

Shake for 16 hours end-over-end (60 rpm);

Centrifuge at 3500 rpm for 15 minutes; reserve the supernatant for analysis of inorganic P (Appendix A.3) and total P (Appendix A.4);

Add 10 ml 0.5 M NaCl, shake the tube;

Centrifuge at 3500 rpm for 10 minutes, get rid of the supernatant as much as possible;

Dry residual soil samples in drying oven at 40°C and digest a subsample (0.1 g) for residual P analysis (Appendix A.5).

## **A.2 Determination of phosphorus in acid soil extracts according Murphy and Riley (1962) and Eisenreich et al. (1975)**

### **Reagents and solutions:**

Solution A: dissolve 15.35 g  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 24\text{H}_2\text{O}$  with approximately 200 ml of DI  $\text{H}_2\text{O}$  in a 500 ml beaker. Dissolve 0.2743 g  $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$  with approximately 100 ml of DI  $\text{H}_2\text{O}$  in a 250 ml beaker. In a 1000 ml beaker containing roughly 300 ml of DI  $\text{H}_2\text{O}$ , very slowly add 178 ml of concentrated  $\text{H}_2\text{SO}_4$  (95-98%). After this solution has cooled down, transfer to a 1000 ml volumetric flask, add the solutions of  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 24\text{H}_2\text{O}$  and  $\text{K}(\text{SbO})\text{C}_4\text{H}_4\text{O}_6$  and make up to 1000 ml with DI  $\text{H}_2\text{O}$ .

Solution B: dissolve 1.356 g  $\text{C}_6\text{H}_8\text{O}_6$  with 100 ml of solution A in a 100 ml volumetric flask. This solution must be prepared freshly before use.

10 M NaOH: dissolve 400 g of NaOH in a 1000 ml beaker containing roughly 600 ml of DI  $\text{H}_2\text{O}$ . After cooling down, transfer to a 1000 ml volumetric flask and adjust the volume with DI  $\text{H}_2\text{O}$ . Store in a plastic bottle.

*p*-nitrophenol 0.25% (w/v): weigh 0.25 g of *p*-nitrophenol (MW 139.11 g) and dissolve in 100 ml of DI  $\text{H}_2\text{O}$ . Store in an amber or dark bottle and preserve in fridge.

### **Procedure:**

Pipette a proper aliquot of the extract to be analysed into a 35 ml vial;

Add DI H<sub>2</sub>O in order to achieve a final volume of 3 ml;

Add one drop of *p*-nitrophenol 0.25%;

Neutralize the solution with 10 M NaOH added drop-wise until the solution becomes yellow;

Add 0.5 ml of solution B, leave for 30 minutes;

Read the absorbance using UV-Vis spectrophotometer at the wavelength 882 nm. The color is stable for 3-4 hours;

The method and spectrophotometer need to be calibrated by preparing a calibration curve with P standard solutions in the matrix to be analysed.

#### **Calculations:**

The concentration of P in each fraction is calculated using the following equation:

P concentration (mg/kg) =

[Conc. of P(mg/L)]x[Dilution of extract]x[Volume of extractant (ml)] ÷ [Weight of soil(g)]

### **A.3. Determination of inorganic phosphorus in alkaline soil extracts according to Dick and Tabatabai (1977) with modification by He and Honeycutt (2005)**

#### **Solutions:**

Solutions A: dissolve 8.80 g C<sub>6</sub>H<sub>8</sub>O<sub>6</sub> (Ascorbic acid, MW 176.12 g) and 41.00 g of Cl<sub>3</sub>CCOOH (Trichloroacetic acid, MW 163.39 g) with approximately 400 ml of DI H<sub>2</sub>O in a 500 ml beaker. Transfer to a 500 ml volumetric flask and adjust the volume with DI H<sub>2</sub>O. This solution must be prepared freshly before analysis.

Solution B: dissolve 6.20 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O with approximately 400 ml DI H<sub>2</sub>O in a 500 ml beaker, transfer to a 500 ml volumetric flask and make up to 500 ml with DI H<sub>2</sub>O.

Solution C: dissolve 29.90 g of Tri-Sodium citrate (MW 294.1 g) and 26.00 g of Sodium arsenite (MW 129.91 g) in a 1000 ml beaker containing roughly 800 ml DI H<sub>2</sub>O. Add 50 ml glacial acetic acid (99%, d=1.05 g/ml). Transfer the mixed solution in the beaker to a 1000 ml volumetric flask and adjust the volume with DI H<sub>2</sub>O.

#### **Procedure:**

Pipette 2 ml of the alkaline extract into a 35 ml vial. The extract should be previously diluted as required;

Add 2.5 ml of solution A and 0.5 ml of solution B at the same time;

Add 1.25 ml of solution C after a consistent length of time less than 2 minutes (e.g. 70 seconds). This step is crucial to ensure the reproducibility and sensitivity of the method;

Read the absorbance using a UV-Vis spectrophotometer at 850 nm (He and Honeycutt, 2005);

The method and spectrophotometer need to be calibrated by preparing a calibration curve with P standard solutions in the matrix to be analysed.

#### **Calculations:**

The concentration of P in each fraction is calculated using the following equation:

P concentration (mg/kg) =

$[\text{Conc. of P(mg/L)}] \times [\text{Dilution of extract}] \times [\text{Volume of extractant (ml)}] \div [\text{Weight of soil(g)}]$

#### **A.4. Auto-clave digestion of alkaline soil extracts (NaHCO<sub>3</sub> and NaOH extractants) for total phosphorus analysis according to USEPA (1983) with recommendations from Do Nascimento et al. (2015)**

##### **Solutions:**

Sulfuric acid 1:1: Add slowly 500 ml of concentrated H<sub>2</sub>SO<sub>4</sub> (95-98%) in approximately 450 ml DI H<sub>2</sub>O. Wait for it to cool down, transfer to a 1000 ml volumetric flask and make up to 1000 ml with DI H<sub>2</sub>O.

Ammonium persulfate 7.5% (w/v): dissolve 75 g of (NH<sub>4</sub>)<sub>2</sub>S<sub>2</sub>O<sub>8</sub> in 800 ml of DI H<sub>2</sub>O. Transfer to a 1000 ml volumetric flask and complete the volume with DI H<sub>2</sub>O. Prepare fresh before use.

##### **Procedure:**

Pipette an appropriate aliquot of the alkaline soil extract into a 50 ml centrifuge tube with screw cap (volume of aliquot depends on P concentration and humic acid in the extract);

Add 5 ml of ammonium persulfate 7.5% and 1 ml of 1:1 H<sub>2</sub>SO<sub>4</sub>;

Loosely screw the cap onto the tube;

Autoclave at 121°C and 103 kPa for 240 minutes ;

Cool down the centrifuge tube, then make up to 50 ml with DI H<sub>2</sub>O;

Determine phosphorus concentration according to Appendix A.2.

**Calculations:**

The concentration of total P is calculated using the following equation:

P concentration (mg/kg) =

$$[\text{Conc. of P (mg/L)}] \times [\text{Dilution of extract}] \times 50 \times 10 \div [\text{ml extract for autoclave} \times \text{Weight of soil(g)}]$$

**A.5. Block digestion of residual soil for total phosphorus analysis (Olsen and Sommers, 1982)**

**Equipment:**

Aluminium digestion block with adjustable temperature;

Glass tube diameter: 25 mm; height: 250 mm;

Glass funnels: 25-35 mm diameter.

**Reagents:**

Concentrated H<sub>2</sub>SO<sub>4</sub> (95-98%);

Concentrated H<sub>2</sub>O<sub>2</sub> (30% v/v).

**Procedure:**

Weigh 0.1 g of finely ground soil into a glass digestion tube;

Add 1 ml concentrated H<sub>2</sub>SO<sub>4</sub>;

Place a funnel on the top of the tube;

Gradually raise the temperature to 225°C (5°C per minute), hold this temperature for 1 hour;

Remove the tube from the block and let it cool down to room temperature;

Add 2 ml of concentrated H<sub>2</sub>O<sub>2</sub>;

Gradually raise the temperature to 135°C (5°C per minute), hold this temperature for 1 hour;

If the sample is not clear. It is due to organic matter which is not digested completely, repeat the steps “e” to “g”, but add 1 ml of concentrated H<sub>2</sub>O<sub>2</sub> at this time. Steps “e” to “g” can be repeated until the solution become clear;

To completely remove H<sub>2</sub>O<sub>2</sub>, gradually raise temperature to 150°C and hold for 30 minutes (the leftover of H<sub>2</sub>O<sub>2</sub> may interfere colorimetric methods, see Olsen and Sommers, 1982)

Remove the tube from the block and let it cool down to room temperature;

Transfer the extract to a 50 ml falcon tube, make up to 50 ml;

Determine phosphorus according to Appendix A.2.

#### **Calculations:**

The concentration of P is calculated using the following equation:

P concentration (mg/kg) =

[Conc. of P(mg/L)]x[final dilution volume] ÷ [Weight of soil(g)]

### **A.6 Soil microbial phosphorus (Brookes et al., 1982; Hedley and Stewart, 1982; McLaughlin et al., 1986)**

#### **Solutions**

M NaHCO<sub>3</sub>, pH of 8.5: Weigh 42.0 g of NaHCO<sub>3</sub> and 5 NaOH pellets, and dissolve in approximately 900 mL DI H<sub>2</sub>O in a 1000 mL beaker, adjust pH to 8.5 by 6 M NaOH solution, make to volume in 1 L volumetric flask

M NaHCO<sub>3</sub>, pH of 8.5, spiked to 1.67 mg P L<sup>-1</sup>: Prepare solution as above, but add 1.67 mL of a 1000 mg P L<sup>-1</sup> stock solution, make to volume in the 1 L flask.

#### **Procedure**

Weigh an appropriate weight of fresh soil (equivalent to 0.5 g dry soil) in triplicate (A, B, C) into 15 mL centrifuge tubes;

Add 0.25 mL chloroform to the set of samples A, cap closely, fumigate for 24 hours; meanwhile, sets B and C remain capped and stored in the dark at room temperature;

Remove the caps on the set of samples A and put in the fumigator for evacuating chloroform several times in the vacuum box using electric pump.

Add 7.5 ml 0.5M NaHCO<sub>3</sub> (pH 8.5) to sets of samples A and B; add 7.5 ml 0.5M NaHCO<sub>3</sub> (pH 8.5) containing 1.67 mg P L<sup>-1</sup> (to make a spike of 25 mg P kg<sup>-1</sup> soil) to the set C, shake end-over-end (60 rpm) for 30 minutes;

Centrifuge at 3,500 rpm for 10 minutes, collect the supernatant;

Analyse inorganic P of all sets of samples by method of Dick and Tabatabai (1977) (Appendix A.3).

Microbial biomass P (mg P kg<sup>-1</sup>) is determined by:

$$\text{Microbial P} = \frac{(P_A - P_B)}{K_p} \times \frac{1}{\frac{(P_C - P_B)}{P_{\text{spike}}}}$$

where K<sub>p</sub> is a conversion efficiency coefficient for how much of the microbial biomass P can be converted to NaHCO<sub>3</sub>-extractable P via CHCl<sub>3</sub>. K<sub>p</sub> is 0.4, based on measurements of P extractions from lab-grown microbes added to soils before measurement (Brookes et al., 1982; Hedley & Stewart, 1982; McLaughlin et al., 1986).

P<sub>spike</sub> is the concentration (mg P kg<sup>-1</sup>) of the spike added (25 mg P kg<sup>-1</sup>).

## **A.7 Acid and alkaline phosphatase enzyme assay (Tabatabai, 1994)**

### **Reagents and solutions**

Toluene.

Sodium hydroxide (NaOH) 1 N: Dissolve 40 g NaOH in 800 ml deionized (DI) H<sub>2</sub>O in 1 L beaker, then transfer into 1000 ml volumetric flask, adjust volume to 1 L by DI H<sub>2</sub>O.

Modified universal buffer (MUB) stock solution: Dissolve 12.1 g of tris(hydroxymethyl)aminomethane (THAM), 11.6 g of maleic acid, 14.0 g of citric acid and 6.3 g of boric acid in 488 ml 1 N NaOH and dilute the solution to 1 L with DI H<sub>2</sub>O. Store it at 4°C in a fridge.

Modified universal buffer, pH 6.5 and 11: Place 200 mL of MUB stock solution in a 500 mL beaker containing a magnetic stirring bar and place the beaker on a magnetic stirrer. Titrate the solution to pH 6.5 by using 1 M HCl with pH meter Mettler Toledo and dilute to 1 L with DI H<sub>2</sub>O. Titrate another 200 mL of the MUB stock solution to pH 11 by 1 M NaOH and adjust the volume to 1 L with DI water.

*p*-nitrophenyl phosphate solution (0.05 M): Dissolve 0.84 g of disodium *p*-nitrophenyl phosphate tetrahydrate (Sigma – Aldrich, Co., St. Louis, MO) in 50 mL of MUB pH 6.5 for assay of acid phosphatase and pH 11 for assay of alkaline phosphatase. Store the solutions in a fridge.

Calcium chloride (CaCl<sub>2</sub>) 0.5 M: Dissolve 73.5 g of CaCl<sub>2</sub>.H<sub>2</sub>O in approximately 800 mL DI H<sub>2</sub>O, and adjust the volume to 1 L with water.

NaOH 0.5 M: Dilute 500 mL 1 N NaOH with 500 mL DI water by using a 500 mL volumetric flask.

Standard *p*-nitrophenol stock solution (1 g/L): Dissolve 1 g of *p*-nitrophenol in about 800 mL of water and dilute the solution to 1 L with water. Store the solution in an amber or dark glass bottle in a fridge.

## Procedure

Place 0.5 g of fresh soil in a 35 mL vial;

Add 0.2 mL toluene, 4 ml MUB pH 6.5 for acid phosphatase (or pH 11 for alkaline phosphatase), swirl the vial few seconds to disperse thoroughly the soil in the suspension;

Add 1mL *p*-nitrophenyl phosphate, stopper vials and swirl for a few seconds;

Place it in an incubator at 37 °C for 1 hour;

Remove the stopper, add 1 ml 0.5 M CaCl<sub>2</sub> and 4 mL 0.5 M NaOH, swirl for a few seconds;

Filter the soil suspension through Whatman no. 2 v-folded filter paper. If the extract has precipitates then filter again with a cellulose acetate syringe filter (pore size 45 μm);

To prepare standard solutions, dilute 1 mL of the standard *p*-nitrophenol stock solution to 100 mL with DI H<sub>2</sub>O in a 100 mL volumetric flask to get 10 mg *p*-nitrophenol L<sup>-1</sup>. Pipette 0, 1, 2, 3, 4, 5 ml aliquots of the diluted standard solution into 35 mL vials, adjust volume to 5 mL by DI H<sub>2</sub>O, add 1 mL 0.5 M CaCl<sub>2</sub> and 4 mL 0.5 M NaOH, mix and filter to achieve standard concentrations: 0, 1, 2, 3, 4, 5 mg *p*-nitrophenol L<sup>-1</sup>;

Measure absorbance of *p*-nitrophenol in samples and standard solutions at a wavelength of 420 nm;

If samples exceed 5 mg *p*-nitrophenol L<sup>-1</sup>, they need to be diluted with DI H<sub>2</sub>O.

## **A.8 Soil microbial biomass carbon and nitrogen (Brookes et al., 1982; Scott-Denton et al., 2006; Vance et al., 1987)**

Ethanol-free chloroform.

0.5M K<sub>2</sub>SO<sub>4</sub>: Weigh 87.125 g of K<sub>2</sub>SO<sub>4</sub> into a 1 L beaker. Add 800 roughly 800 mL of deionized water. Add a stir bar to the beaker, place on the magnetic stirrer, set temperature at 40°C and stir until completely dissolved. Transfer the solution to the 1L volumetric flask and make up to the mark with deionized water.

### **Procedure**

Pass the moist soil samples through 4 mm;

Weigh a moist soil amount (equivalent to 5 g dry soil), place into centrifuge tubes (50 ml);

Directly add 2.5 ml ethanol-free chloroform, close the tubes, and incubate for 24 hours at room temperature;

After incubation, evacuate chloroform several times in the vacuum box using electric pump;

Shake the samples with 20 mL 0.5M K<sub>2</sub>SO<sub>4</sub> for 30 minutes and centrifuge at 3000 rpm for 10 minutes;

Collect the supernatant for analysis via the TOC analyser.

### **Calculation**

#### ***Biomass C:***

$$\text{mg C kg}^{-1} (\text{dry soil}) = [(a - \text{blank}) * 24 * 100] / (b * \text{DM})$$

Where:

a = soil solution TOC in mg L<sup>-1</sup>

b = moist soil weight

DM = dry matter in %

$$\text{Biomass C} = (B_c, \text{mg C kg}^{-1}) = 2.22 * (F - \text{NF})$$

Where:

NF = the mean value for the non-fumigated replicate samples

F = the mean value for the fumigated replicate samples

2.22: correction factor

**Biomass N:** The calculation is the same as C

## A.9 Dehydrogenase enzyme assay

### Reagents:

*TRIS buffer:* Weigh 12.11 g of TRIS into a 1 litre beaker. Add approximately 800 mL of deionized water. Add a stir bar to the beaker, place on the magnetic stirrer and stir until completely dissolved. Adjust the pH by adding drop-wise amounts of 1 M HCl to 7.6 (soil pH was between 6.0 and 7.0). Remove stir bar and transfer to the 1 litre volumetric flask and make up to the mark with deionized water.

*Substrate solution (1% w/v TTC - 2, 3, 5-triphenyltetrazolium chloride):* Weigh 1.00 g of TTC into a 100 mL beaker and dissolve with 80 mL of TRIS buffer. Transfer to a 100 mL volumetric flask and make up to the mark with TRIS buffer.

*1, 3, 5-triphenyltetrazolium formazan standards (TPF):*

*Stock solution:* Weigh 0.0500 g of TPF into a 100 mL beaker and add 80 mL methanol and dissolve. Transfer to a 100 mL volumetric flask and make up to the mark with methanol. Prepare a set of working standards (0 – 40 mg TPF L<sup>-1</sup>) with methanol.

### Procedure:

Homogenise and grind the field moist samples, pass through a 2 mm sieve. Store the samples in a fridge.

Weigh 2.00 g of field moist soil into 35 mL vial. Add 2 mL of substrate solution to the vial and cap. Add 2 mL of TRIS buffer solution to 2 empty vials to use as reagent blanks. Shake by hand to thoroughly mix the sample and solutions. Put into incubator set at 25°C for 24 hours.

Remove from the incubator and add 10.0 mL of methanol. Shake the vials. Transfer the liquid phase into 15 mL centrifuge tubes and centrifuge for 10 minutes at 3000 rpm. Determine the absorbance of standards, blanks, and samples at 485 nm on the spectrophotometer.

**Calculation:**

$$\text{DHA (mg kg}^{-1} \text{ dry soil.h}^{-1}) = [(a-b)*MF]/[24*m]$$

Where: a = TPF concentration of sample in mg L<sup>-1</sup>

b = average of blanks in mg L<sup>-1</sup>

m = weight of sample

MF = moisture factor

**A. 10 pH<sub>H2O</sub>**

Weigh 10 g of air dry soil into 70 mL vial. Add 25 mL of deionised water and shake the samples end-over-end for 30 minutes. Leave to stand for 4 hours the stability before measuring by calibrated pH meter.

**A.11 Total carbon and nitrogen**

Total soil carbon and nitrogen were determined by Dumas combustion method. The sample is combusted at 900°C in an oxygen atmosphere. Carbon and nitrogen are converted into CO<sub>2</sub> and N<sub>2</sub>, and these gases are then passed through a thermal conductivity cell for measurement.

**A.12 Cation exchange capacity (CEC)**

CEC is determined by summation of extractable cations (K, Ca, Mg and Na) and acidity using 1M neutral ammonium acetate followed by ICP-OES.

**A.13 Total phosphorus**

Soil samples are digested by nitric and hydrochloric acids based on US EPA 200.2 followed by ICP-OES.

### **A.14 Olsen phosphorus**

Weigh 3.0 g of soil in a 50 mL centrifuge tube. Add 30 mL of 0.5 M of  $\text{NaHCO}_3$  (pH: 8.5) and then shake 30 minutes on an end-over-end shaker. Centrifuge at 3500 rpm for 15 minutes. Determine inorganic P according to Appendix A.3.

### **A.15 Anion storage capacity**

Anion storage capacity is determined by near infra-red spectroscopy (NIRs), calibration based on; equilibration with  $1000 \text{ mg L}^{-1}$  P solution followed by colorimetric analysis.

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