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CHARACTERISATION OF NUTRITION RESPONSES IN THREE GENOTYPES OF WHITE CLOVER (*Trifolium repens* L.) SELECTED FOR TOLERANCE TO LOW PHOSPHORUS

A thesis presentation in partial fulfilment of the requirements for the degree of
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ABSTRACT

This study focused on the differences in root system architecture (RSA) of three white clover (*Trifolium repens* L.) genotypes, designated 43-7, 45-14 and 47-9 that were isolated from breeding lines selected to tolerate low phosphorus (P). In this study, genetically identical white clover stolons were cut from stock plants at the fourth node, and primary roots emerged (post excision) from either of the two primordia at nodes three or four. The main objective was to establish if differences in RSA were observed in response to a limited P-supply (10 μM KH_2PO_4) compared with a sufficient P-supply (1 mM KH_2PO_4). Each of the cut stolons had a dominant primary root that was nominated to be used for the analysis of root growth in terms of elongation and lateral root emergence. The analysis was standardised by designating the branching zone as the region of the primary root that contained visible lateral roots. The remaining region was designated the elongation zone, and did not contain visible lateral roots. In P-sufficient media, the branching zone was approximately 50% of the primary root length by 15 days post excision and approximately 80% by 25 days in the three genotypes. After 30 days post excision, the branching zone was maintained between 85% and 90% of the primary root length. The response to low P was measured after the emergence of visible lateral roots in experiment I and prior to the emergence of visible lateral roots in experiment II. A third treatment group with a reduced sulfur supply (in experiment II) tested the specificity of the P-stress response. In summary, the morphological responses to P-stress were characteristic for each genotype; the changes to the primary and/or lateral roots occurred within seven days from the reduction in P-supply; and the timing of the reduction in P-supply influenced the degree of the response that was observed with respect to the branching zone. The responses to P-stress included a stimulation of the primary and lateral roots in 47-9 with a reduced root biomass under S-stress; a decrease in the number of visible lateral roots in 45-14; and the decreased elongation of lateral roots in 43-7, which reduced the initiation of tertiary roots. The branching zone decreased in 45-14 only when the P-supply was changed after lateral root emergence. In contrast, the branching zone increased in 47-9 only when the P-supply was changed before lateral root emergence. The results suggest that the P-stress response in the roots is specific and utilisation of P and S may differ in the three genotypes.

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ABBREVIATIONS

Abbreviation	Description
°C	Degrees Celsius
3PGA	3’ phosphoglycerate
Al	Aluminium
APase	Acid phosphatase
At	*Arabidopsis thaliana*
ADP	Adenosine diphosphate
ATP	Adenosine-5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BL	Breeding line
BZ	Branching zone
Ca	Calcium
Col-1, Col-4	*Arabidopsis* ecotype Columbia
cv	Cultivar
CW	Cell wall fraction of the roots, tested for acid phosphatase activity
DPR	Dominant primary root
DTT	Dithiothreitol
DW	Dry weight
Fe	Iron
FFEL	First fully expanded leaf (also referred to as L1)
FW	Fresh weight
GFP	Green fluorescent protein
GUS	β-glucuronidase
K	Potassium
kDa	Kilodaltons
L1, L2, L3	Leaf 1, leaf 2, leaf 3 (up to leaf 7) numbered from the apex
Low P, –P or P-stress(ed)	Low phosphate media. Hoagland’s media with half strength macronutrients and full strength micronutrients with 10 μM potassium phosphate (K_2PO_4) (Appendix I)
Low S, –S, or S-stress(ed)	Hoagland’s media with half strength macronutrients and full strength micronutrients with 28 μM magnesium sulphate ($MgSO_4$) (Appendix I)
lpi	Low phosphate insensitive mutant
LPR1 and LPR2	Low Phosphate Root 1 and 2
LRD3	Lateral Root Development 3
Mg	Magnesium

Abbreviation	Description
Mo	Molybdenum
MYB	A transcription factor belonging to the **my**elo**b**lastosis family
N	Nitrogen
NZ	New Zealand
P	Phosphorus, Inorganic P (P_i), Organic P (P_o)
p-NPP	*p*-nitrophenol-1-phosphate
P-sufficient, +P or Complete	Hoagland's media with half strength macronutrients and full strength micronutrients (Appendix I)
PAE	Phosphorus Acquisition Efficiency
PAP	Purple Acid Phosphatase
PDR2	Phosphate Deficiency Response 2
PEP	Phosphoenol pyruvate
PHL-1	PHR1-like
PHR1	Phosphate Starvation Response 1
Pht	Phosphate transporter
PNP	*p*-nitrophenol
PUE	Phosphate Use Efficiency
Pv	*Phaseolus vulgaris*
QTL	Quantitative Trait Loci
RGR	Relative Growth Rate
RSA	Root System Architecture
S	Sulfur
SF	Soluble fraction of the representing the cytosol and vacuoles of the roots, tested for acid phosphatase activity
SQD1	UDP-sulfquinovose synthase
SRL	Specific Root Length (cm/mg^{-1})
SUMO	Small ubiquitin-like modifier (E3 ligase siz1)
TPR(s)	Total primary roots representing the dominant primary root and the supporting primary roots

SYMBOLS

Symbol	Description
*	Significant at <0.05 confidence level between P-sufficient and low phosphorus and complete and low sulfur media.
**	Significant at <0.01 confidence level between P-sufficient and low phosphorus (red) and complete media and low sulfur media (olive).
★	Significant at <0.05 confidence level between total relative growth of total primary root length, with significant differences in the dominant primary root indicated by an asterisks (*).
★★	Significant at <0.01 confidence level between total relative growth of primary roots, with significant differences in the dominant primary root indicated by an asterisks (**).
†	Significant at <0.05 confidence level between low phosphorus and low sulfur media.
††	Significant at <0.01 confidence level between low phosphorus and low sulfur media.
3°	Emergence of tertiary roots on the longest lateral root.

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CHAPTER 1. INTRODUCTION

1.1 Overview

There is a world-wide focus on understanding how to breed plants that can tolerate low concentrations of P and utilise the macronutrient efficiently without compromising the nutritional quality or yield of the resulting plant product (Raghothama, 1999; Vance *et al*., 2003; White and Brown, 2010; Veneklaas *et al*., 2012). The macronutrient phosphorus (P) can be a limiting factor for plant growth because the bioavailability properties are low, which is largely due to interactions with other minerals in the soil (Raghothama, 1999).

The importance of Phosphorus Use Efficiency (PUE) in plants is a combination of a global requirement for sustainable food production, the increasing demand for the alternative use of land to grow crops to be used for biofuels, and the decreasing world phosphate rock reserves (Vance *et al*., 2003; Cordell *et al*., 2009; Wang *et al*., 2010b; White and Brown, 2010). The phosphorus efficiency traits in plants span across several biological processes that can be inherited or selected for in plant breeding programmes (Wang *et al*., 2010b; Peret *et al*., 2011; Rouached *et al*., 2011b; Veneklaas *et al*., 2012). In particular, physical traits such as the root system architecture of the plant plays a crucial role in P acquisition, and the utilisation of the available P can influence the health of the plant in terms of biomass (Wang *et al*., 2010b; Peret *et al*., 2011; Veneklaas *et al*., 2012) (refer section 1.3). The signaling processes that are involved in sensing a limited P environment are thought to originate in the roots, and the molecular mechanisms involved in P homeostasis are central to improving P efficiency in plants (Bari *et al*., 2006; Peret *et al*., 2011; Jain *et al*., 2012; Veneklaas *et al*., 2012). A vast majority of the research that connects the role of root system architecture with molecular signaling has been based in the model plant *Arabidopsis*. However, studies in other plant species are demonstrating that the phosphate stress response pathways are conserved (refer section 1.4).

New Zealand (NZ) agriculture is largely based on ruminant cattle grazing pastoral forages, which are essentially converted to milk or meat for human consumption (Chapman and Caradus, 1997; Woodfield and Clark, 2009).

A breeding programme was established in 1986 by Agresearch Grasslands (in NZ) to improve P-efficiency in white clover (*Trifolium repens* L.) (Dunlop *et al*., 1988). The pasture legume provides a good source of protein (for pastures) and fixes nitrogen, which are often limiting in perennial rye grass (*Lolium perenne* L.), and the breeding programme aimed at identifying heritable traits for P efficiency for further characterisation (Dunlop *et al*., 1988; Woodfield and Clark, 2009). In one of the long-term field trials, two cultivars performed particularly well in terms of growth, persistence, and an increase in sheep performance (in terms of growth) was observed (Chapman and Caradus, 1997). The two cultivars (Grasslands Prestige and Grasslands Tahora) were crossed (unpublished) and the progeny were screened for tolerance to low P in four breeding lines (Effendy, 2007).

Effendy (2007) characterised several genotypes for phosphorus efficiency in terms of physical traits and the systemic response to the complete withdrawal of the P-supply. Finally, three genotypes (43-7, 45-14 and 47-9) were selected for this study. The two parameters that were used in the genotype screen for P-starvation responses were the induction of acid phosphatase in the roots, and the decline in the leaf P content of the first fully expanded leaf, located near the apex (Effendy, 2007). One of the genotypes (45-14) showed an increase the root acid phosphatase activity after 14 days from the withdrawal of P, and was considered to be a slow responder, with no response observed in the other two genotypes (43-7 and 47-9) (Effendy, 2007). A decline in P content in the first fully expanded leaf was observed after seven days in 45-14 and 47-9, and were considered to be fast responders, compared with 43-7 that was considered to be a slow responder because the leaf P content declined after 14 days (Effendy, 2007).
The response to low P in terms of the changes to root system architecture were not examined in the original cross between Grasslands Prestige and Grasslands Tahora or the breeding line screens (Chapman and Caradus, 1997; Effendy, 2007). In the genotype screen, the development of the roots occurred in P-sufficient media and the mature plants displayed no differences (in terms of root system architecture) when the P-source was removed (Effendy, 2007).

In contrast, a study by Thibaud *et al*, (2010) using the *Arabidopsis* ecotype Colombia observed that the growth of the primary root in young and mature plants stopped elongating after being exposed P-deficient media, when compared to P-sufficient media. In the same study, the leaves of the P-starved plants were supplied with P, while the roots remained in media without the mineral (Thibaud *et al*., 2010).
The application of P to the leaves did not restore the elongation of the primary root in the P-stressed plants, which confirmed that:

- the roots respond to the local external environment;
- the responses and not the result of systemic signaling from the leaves;
- and occur independently of root age (Thibaud *et al*., 2010).

The short root response phenotype in the *Arabidopsis* ecotype Colombia was shown to be specific to the contact of the root tip to low P media, and previously linked to a quantitative trait loci (QTL) of inbred *Arabidopsis* lines (Reymond *et al*., 2006; Svistoonoff *et al*., 2007). Further studies have characterised the molecular mechanisms that change the root development in the *Arabidopsis* ecotype Colombia in response to low P (refer section 1.3).

In *Arabidopsis,* and white clover, auxin accumulates at root tip in limited P-supply, and it is thought that changes in the sensitivity to the hormone alters the root system architecture (Lopez-Bucio *et al*., 2002; Dinh *et al*., 2012). However, the two plants differ in their root system architecture phenotypes in response to low P (Williamson *et al*., 2001; Dinh *et al*., 2012). In the white clover genotype Challenge 10F, the primary and lateral roots increase in response to low P, and the perception of P-stress is increased in the presence of auxin and ethylene in the growth media (Dinh *et al*., 2012). The different phenotypes in response to P-stress between *Arabidopsis* and white clover suggest that other hormone interactions are involved changing root system architecture in plants (Peret *et al*., 2011; Jain *et al*., 2012). For example in maize, the length of the primary root increases and lateral root emergence is inhibited in limited P-supply (Li *et al*., 2012b).

Microarray analysis of a 1.5 cm region of the root tip revealed that the levels of several hormones including cytokinin, auxin, ethylene and gibberellin were upregulated when compared with maize plants grown in P-sufficient media (Li *et al*., 2012b).

The white clover genotype Challenge 10F was not bred to tolerate low P. Therefore the connection between P efficiency (in terms of the induction of root acid phosphatase and decline in leaf P content) and root development in (terms of primary root elongation and lateral root emergence) in limited P-supply are largely unknown in white clover.

The objectives of this study were:

1. To establish if the root system architecture of the three genotypes differs in a limited P-supply.
2. To establish if the selection process in the genotype screen involved genetic determinants for changes in root system architecture in response to low P-supply specifically, or if the changes to root system architecture were more directed towards a general response to mineral deficiency.
3. To determine if there is a correlation between root system architecture and P homeostasis in the developing roots by the measuring the response of acid phosphatase (in the roots) together with the leaf content of the first fully expanded leaf.

1.2 The importance of phosphorus in plant nutrition

Plant nutrition plays a vital role in the global food chain, and Phosphorus (P) is one of six macronutrients together with nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), and sulfur (S), which are all required for optimum growth (White and Brown, 2010). P is a key component of adenosine-5'-triphosphate (ATP) and adenosine diphosphate (ADP) which are used as an energy source for metabolism and cell cycle function (Raghothama, 1999; Vance *et al.*, 2003; Plaxton and Tran, 2011). P is involved in several other biochemical and physiological processes where it is a substrate for many reactions and is one of the major components of nucleic acids and lipid membranes (Essigmann *et al.*, 1998; Raghothama, 1999; Vance *et al.*, 2003; Plaxton and Tran, 2011).

1.2.1. Phosphate Use Efficiency (PUE) in plants

Phosphorus efficiency in plants is defined as the ability to produce biomass or yield under a given P-supply, and can be enhanced in two ways (Wang *et al.*, 2010b).

1. Phosphorus Acquisition Efficiency (PAE), and refers to the ability to acquire P from the soil (or P source) via the roots.
2. Phosphorus Utilisation Efficiency (PUE), which is the internal use of P (Wang *et al.*, 2010b; Veneklaas *et al.*, 2012).

The concentration of P in soils can be as low as 10 μM and it readily forms complexes with Ca, iron (Fe), and aluminum (Al), which make it insoluble and a limiting factor for plant growth (Raghothama, 1999; White and Brown, 2010). One of the traditional approaches to maintaining high crop yield is by the use of N, P and K fertilisers, and often S, Ca, and Mg are also applied to increase soil fertility. This can be a large expense for pastoral farmers and horticultural growers. Increasing awareness of the decreasing phosphate reserves has prompted the industry to regulate the inputs and minimise waste (Vance *et al.*, 2003; Monaghan *et al.*, 2007; White and Brown, 2010). It is estimated that there is 65,000 metric tonnes of phosphate rock reserves deposited throughout the world with the largest reserves in Morocco, China and America (Cordell *et al.*, 2009).

Mining requires deforestation of vast areas of land, followed by high inputs of fertiliser to raise the soil mineral levels to sustain productive growth of crops (Den Herder *et al*., 2010). Therefore, interest in sustainable food production is currently focusing on improving P-efficiency in plants (Wang *et al*., 2010b; White and Brown, 2010; Veneklaas *et al*., 2012).

The general adaptive response to limited nutrient supply is to divert the resources in favour of root growth, which may result in reduced shoot growth and of crop yield (White and Brown, 2010; Peret *et al*., 2011). The P-stress responses are the result of several post transcriptional signals that contribute to the communication between the roots that sense the external P-supply, and shoots that monitor internal levels to maintain homeostasis (Doerner, 2008; Lin *et al*., 2009; Thibaud *et al*., 2010; Tran *et al*., 2010a; Plaxton and Tran, 2011; Jain *et al*., 2012).

1.2.2. Induction of P-stress response pathways

When limiting P-supply is first sensed by the plant local changes to the roots are induced together with the systemic responses that are involved in maintaining P homeostasis, such as P-recycling, recovery, and transport (Misson *et al*., 2005; Thibaud *et al*., 2010; Peret *et al*., 2011). Several studies have investigated the P-starvation responses or identified plants that can readily adapt to low P. Some of the plants include rice (*Oryza sativa*) (Lian *et al*., 2010; Hu *et al*., 2011); potato (*Solanum tuberosum* L.) (Hammond *et al*., 2011); tobacco (*Nicotiana tabbacum*) (Zamani *et al*., 2012); bean (*Phaseolus vulgaris*) (Hernandez *et al*., 2007; Tian *et al*., 2007); lupin (*Lupinus albus*) (Cheng *et al*., 2011); soybean (*Glycine max* L.) (Qiao *et al*., 2007; Li *et al*., 2012a); maize (*Zea mays*) (Li *et al*., 2012b); rye grass (*Lolium perenne* L.) (Byrne *et al*., 2011) and *Arabidopsis* (Hammond *et al*., 2003; Misson *et al*., 2005; Morcuende *et al*., 2007; Thibaud *et al*., 2010).

In a study by Mission *et al*., (2005) in *Arabidopsis*, 612 genes were induced under P-starvation, and 254 genes were repressed, and the induction and repression of the genes varied with the length of starvation.

The P responsive genes were identified by a whole genome analysis of 22,810 genes using an Affymetrix gene chip from plants under short (up to 12 hours), medium (one to two days) and long term (seven days) P-starvation (Misson *et al*., 2005). In the early stages of P-stress, (after four hours), several genes involved in defense and the cell-cycle and are upregulated during the synthesis phase of DNA replication (S phase), and the genes involved in metabolism are down regulated (Hammond *et al*., 2003). Further microarray studies in *Arabidopsis* have since investigated local and systemic responses to P-supply, and the changes to metabolism (Morcuende *et al*., 2007; Thibaud *et al*., 2010). The early phosphate stress induced responsive genes are regulated by various transcription factors, hormones, microRNAs, sugars and phosphite, to act as local and systemic signals which induce the late responsive genes and maintain homeostasis and alterations to the root system (Doerner, 2008; Lin *et al*., 2009; Hammond and White, 2011; Jain *et al*., 2012).

Many of the phosphate stress induced responsive proteins contain an SPX domain, which is a sequence between 135 and 380 amino acids (Secco *et al*., 2012), and activation of these domains results in negative repression of many phosphate stress induced responsive genes (Nilsson *et al*., 2010). Four classes of proteins containing SPX domains exist, and regulate the phosphate stress induced genes through protein-protein interactions, which contribute to the conservation between the regulatory mechanisms in *Arabidopsis* and rice (Secco *et al*., 2012).

One of the most central regulators of phosphate stress induced responsive genes is Phosphate Starvation Response 1 (PHR1), which is a **my**elo**b**lastosis (MYB) transcription factor (Rubio *et al*., 2001). In low P, an unknown signal induces the sumoylation of PHR1 by the small ubiquitin-like modifier (SUMO) E3 ligase siz1 (Miura et al., 2005), which induces changes to the roots and homeostasis (Nilsson *et al*., 2010). The PB1S sequence (GNATATNC, with N representing a variable nucleotide in the sequence) is a *cis*-regulatory motif that is specific to the P-stress response, and the *Arabidopsis* genome has 3305 putative *PHR1* binding sites (Bustos *et al*., 2010; Rouached *et al*., 2011b).

The role of *PHR1* in the induction of P-stress genes is well conserved between plant species (Rubio *et al*., 2001), with homologs of *AtPHR1* identified in bean (Valdes-Lopez *et al*., 2008), rice (Lian *et al*., 2010), and white lupin (Yamagishi *et al*., 2011). For example, the PB1S motif recognises cytokinin as a negative regulator of P-stress in *Arabidopsis,* rice and bean (Martin *et al*., 2000; Debi *et al*., 2005; Camacho *et al*., 2008; Bustos *et al*., 2010). A recent study examined a PHR1-like (*PHL1*) transcription factor that is thought to be responsible for the repression of genes under P-stress, but is yet to be fully characterised (Bustos *et al*., 2010).

1.3 Root system architecture (RSA)

One of the signature responses of P-starvation is a higher root-to-shoot ratio (when compared to sufficient P-supply) as a result of metabolic changes, recycling and mobilisation of internal P to favour root growth and maximise exploration of new environmental P sources (Peret *et al*., 2011). Until recently, the focus on crop production has been on developing shoot biomass, and as a consequence knowledge of changes that occur in the roots and improvements in P efficiency has been quite limited (Den Herder *et al*., 2010).

The two main roles of an efficient root system are improved structural support, and an increased ability to acquire water and nutrients that are required for plant growth (Peret *et al*., 2011). The root systems of P-efficient crops explore a greater surface area by long taproots, and/or several lateral roots (Crush *et al*., 2008; Wang *et al*., 2010b). The growth of the roots is designed for exploration of the rhizosphere, which includes the top layer of soil that contains organic matter where most of the nutrients are found, and an increased number of root hairs assist with acquisition (Lambers *et al*., 2006; Peret *et al*., 2011). This varies within crop species, and is influenced by environmental conditions (Raghothama, 1999; Veneklaas *et al*., 2012). For example, specialised root structures such as aerenchyma found in maize (*Zea mays*) and bean (*Phaseolus vulgaris*), and clustal roots found in white lupin (*Lupinus albus*) have evolved to enhance acquisition through very different mechanisms (Lambers *et al*., 2006; Cheng *et al*., 2011; Lynch and Postma, 2011).

In addition to the structural traits, P-efficient crops have a greater number of high affinity P-transporters; can form associations with mycorrhizal fungi to enhance acquisition, and can secrete organic acids and acid phosphatases to hydrolyse more P in the rhizosphere (Ramaekers *et al*., 2010).

Arabidopsis is a member of the *Brassicaceae* family and is used as one of the model plants for studying the development and responses to P-supply because it has not evolved specialised root structures and does not form associations with mycorrhizal fungi (Peret *et al*., 2011). The primary roots are broadly divided into three zones and form the basic structure of root system architecture. The zone of cell division includes the root cap, and the elongation zone plus the cells proximal to the root cap up to a region containing root hairs (Malamy and Benfey, 1997). The root apical meristem located at the root tip is involved in sensing nutrients that induce specific changes to root growth, and internal transport to maintain P homeostasis (Schachtman and Shin, 2007; Peret *et al*., 2011). These two regions combined do not contain visible lateral roots, which are in the third region called the branching zone; and is also referred to as the differential zone (Malamy and Benfey, 1997; Williamson *et al*., 2001; Dubrovsky and Forde, 2012). The lateral (secondary) roots provide surface area to the root system architecture to maximise nutrient supply, and are one of the most important parameters for analysis because growth is developmentally regulated and is influenced by the external environment (Williamson *et al*., 2001; Nibau *et al*., 2008; Peret *et al*., 2011; Dubrovsky and Forde, 2012).

1.3.1. Relationships between root system architecture and P-efficiency

The genetic inheritance of P efficient root traits has been compared in cultivars that can withstand or tolerate lower levels of P, and forms the basis of further characterisation of specific underlying mechanisms (Tian *et al*., 2007; Crush *et al*., 2008; Wang *et al*., 2010b; Veneklaas *et al*., 2012). The roots adapt to external nutrient supply and endogenous hormone interactions by growth alterations in the primary and/or lateral roots, and the changes vary in different plants and in response to different nutrients (Nibau *et al*., 2008; Rubio *et al*., 2009).

A study by Yang *et al.,* (2010) used scanning technology in combination with measurements of dry weight and root surface area to identify different root phenotypes in *Brassica nupus* (rapeseed). The study identified quantitative trait loci (QTL) and linkage groups from 124 inbred lines from a cross between a P-efficient and P-inefficient genotype (Yang *et al*., 2010). QTL analysis is a method of screening populations that show a particular phenotype, and the linkage groups that are identified are able to locate the genes responsible for that phenotype. Genetic analysis of the inbred lines revealed 62 QTLs for changes to the root system architecture in the inbred recombinant lines that were tested in P-sufficient and a limited P-supply (Yang *et al*., 2010). The 62 QTLs were spread across five traits that included dry weight, root length, root surface area, root volume and P uptake, and the recombinant lines were compared with the P-efficient and P-inefficient parents (Yang *et al*., 2010).

Studies in two bean (*Phaseolus vulgaris* L.) cultivars (G19833, P-efficient and DOR364, P-inefficient) have established connections between the relationship between a high acid phosphatase activity and root system architecture in terms of P-efficiency (Bonser *et al*., 1996; Liang *et al*., 2012b). The on-going characterisation at a molecular level is revealing that the conserved P-stress response pathways in other plants also occur in bean (Tian *et al*., 2007; Valdes-Lopez *et al*., 2008; Liang *et al*., 2012a). The efficient genotype (G19833) forms a shallow root system under limited P-supply and contains more specialised air channels (aerenchyma) that increase metabolism (Lynch and Postma, 2011). The P efficient genotype G19833 also excretes a higher level of organic acids into the low P media compared to the inefficient genotype (DOR364), and responds earlier to P-stress, when grown in a low (5 μM), medium (50 μM and 100 μM), and high (250 μM) concentrations of P_i (Tian *et al*., 2007). The responses include the induction of a Phosphate Starvation induced gene (*PvPS-2*), a Phosphate Transporter gene (*PvPT-1*), and an Iron Deficiency Specific (*PvIDS-4-like*) gene (Tian *et al*., 2007). Together, the expression of these genes heightens perception to external P-supply, and readily restores P to homeostatic levels more efficiently in G19833 compared with DOR364 (Tian *et al*., 2007). Further studies observed higher expression of purple acid phosphatases (PAPs, section 1.4.4) in G19833 (P-efficient) compared with DOR364 (Liang *et al*., 2010; Liang *et al*., 2012b).

1.3.2. The role of the primary root tip in sensing a limited P-supply

It is possible that P homeostasis and root to shoot signaling are integrated with the changes in root system architecture in response to the external P-supply (Bari *et al*., 2006; Bustos *et al*., 2010; Remy *et al*., 2012). The change in sensitivity of hormones differs between plant species, and result in different root phenotypes after sensing a low P-supply (Nibau *et al*., 2008; Lynch and Postma, 2011; Peer *et al*., 2011; Peret *et al*., 2011). For example, the molecular changes that occur in the elongation zone of the primary root in maize were investigated by transcriptional analysis of a region 1.5 cm from the root tips of P-stressed plants (Li *et al*., 2012b). The study indicates a role for the elongation zone (of the primary root) in sensing limited P-supply. The length of the primary root was significantly longer when compared with the P-sufficient plants after eight days in low P and the number of lateral roots was significantly reduced after eight days (Li *et al*., 2012b). The cell length of the P-stressed roots was not different from the P-sufficient plants however; the reduced number of lateral roots originated from less lateral root primordia six days after treatment was started (Li *et al*., 2012b).

The transcriptional analysis revealed that DNA replication; protein synthesis; and genes involved in cell growth were all downregulated. In addition, the P-stressed plants showed differential expression of a number of phytohormones including auxin and cytokinin in the selected region of the elongation zone. In the study, the plants were grown in P-sufficient media (1 mM P) for 11 days before the media was lowered to 5 μM in half of the plants. The transcriptional analysis was carried out after two and eight days treatment to compare the onset of the response with the observed phenotype. The results showed that auxin was synthesised locally in the 1.5 cm region of the root tip and was regulated by the concentration of P in the media. Also, the differential expression of cytokinin and abscisic acid genes was higher after two days compared with auxin, ethylene and gibberellin that were higher after eight days (Li *et al*., 2012b).

The localised induction of ethylene biosynthetic genes are activated when a limited P-supply is sensed, which increases the sensitivity of the roots to auxin (Perez-Torres *et al*., 2008; Thibaud *et al*., 2010; Dinh *et al*., 2012). This is thought to stimulate the initiation of lateral roots before DNA synthesis occurs in the Gap 1/Synthesis phase (G1/S) of the cell cycle (De Smet, 2012).

The induction of ethylene biosynthesis has been proposed to be a target of the transcription factor PHR1 (refer section 1.2.2). The role of local and systemic signaling of ethylene production is a biological target for improving P-efficiency in plants because it regulates the recovery of P in times of low external supply (Bustos *et al*., 2010; Nagarajan *et al*., 2011; Nagarajan and Smith, 2012). For example, ethylene is involved in changes to root system architecture, and regulates the P transporter PHT1;5, (which transfers P from the shoots to the roots), and acid phosphatases (that hydrolyse internal P) (Lei *et al*., 2011; Nagarajan *et al*., 2011; Nagarajan and Smith, 2012).

Root hair development is also regulated by ethylene and by gibberellin, which also plays a role in anthocyanin biosynthesis through the DELLA proteins that are involved in gibberellin signaling (Jain *et al*., 2012). The role of the *HPS4* gene in P starvation that encodes the SABRE allele (At1g58250) and is involved in cell expansion was recently characterised in *Arabidopsis* (Yu *et al*., 2012). The HPS4 gene product is an antagonist to ethylene, and the *hps4* mutant accumulates more auxin at the tip during P-stress, has a higher acid phosphatase activity, *AtPAP10* and *ACP5* expression, a greater reduction in primary root length, a higher expression of the P transporters *PHT1;1* and *PHT1;4*, high expression of *miR399* compared to Colombia (Yu *et al*., 2012). The study highlights the influence of local signals that change the elongation of the primary root in response to the signaling mechanisms of P-stress in *Arabidopsis*.

1.3.3. Natural variation in root responses to P-stress in *Arabidopsis*

Studies in *Arabidopsis* have established that the morphological changes to the root system architecture that occur in response to a low P-supply are genetically inherited (Chevalier *et al*., 2003; Reymond *et al*., 2006; Peret *et al*., 2011). The large resources of mutants and genomic tools in *Arabidopsis* have been used to study the complex genetic and environmental interactions involved in P homeostasis and root development (Lin *et al*., 2009; Den Herder *et al*., 2010; Peret *et al*., 2011). To investigate the natural variation of root responses to a limited P-supply that exist between *Arabidopsis* ecotypes, a large-scale analysis of 73 accessions found that half of the accessions displayed differences in the primary and/or lateral roots in response to a limited P-supply (Chevalier *et al*., 2003).

In the Chevalier *et al.,* (2003) study, a significant inhibition of the primary root and an increased number of lateral roots was displayed in half of the ecotypes. Some of the ecotypes with this response included Columbia (Col-4), Lansberg errecta (Ler-1), Cape Verde Islands (Cvi-4), and Wassilevskaja (Ws-1), and are commonly used as molecular research tools. The other half of the ecotypes in the large-scale analysis displayed changes in either the primary root length (16%) or lateral root number (9%), or were not responsive (25%) (Chevalier *et al*., 2003).

The *Arabidopsis* ecotype Colombia is the most widely studied with respect to the changes in the primary and lateral roots in response to P-supply, and the mechanisms are thought to be inherited (Peret *et al*., 2011). A number of studies have constructed the molecular events that contribute to root development in sufficient P-supply in terms of primary root elongation and lateral root emergence (Laskowski *et al*., 1995; Malamy and Benfey, 1997; Dubrovsky *et al*., 2009; Den Herder *et al*., 2010; Peer *et al*., 2011; De Smet, 2012; Dubrovsky and Forde, 2012). In low P, the primary root of the *Arabidopsis* ecotype Colombia stops elongating, and there is an increase in the number of lateral roots when compared with P-sufficient media (Williamson *et al*., 2001; Chevalier *et al*., 2003; Nacry *et al*., 2005; Svistoonoff *et al*., 2007; Perez-Torres *et al*., 2008; Thibaud *et al*., 2010).

The local perception of external P-supply (at the root tip) that results in the short primary root phenotype was first demonstrated with ten-day-old *Arabidopsis* (Col-0) seedlings that were grown on agar plates containing high P for three days (Svistoonoff *et al*., 2007). The root tips were placed in low P, while the leaves were in contact with high P, and the contact of the root tip to the low P agar media resulted in the inhibition of primary root elongation (Svistoonoff *et al*., 2007). The arrested growth of the primary root was previously mapped by QTL. The two loci were designated Low Phosphate Root 1 (LPR1) and Low Phosphate Root 2 (LPR2), and play a role in maintaining the pluripotent cells in the root meristem (Reymond *et al*., 2006; Svistoonoff *et al*., 2007).

The studies integrate root development with the morphological response to P-stress. In the initial stages of perceiving low P in the environment, *Arabidopsis* strives to maintain the meristem (functionality) for as long as possible before the cells are progressively lost during the progression from P-stress to P-starvation (Sanchez-Calderon *et al*., 2005). In low P, the fate of the existing cells in the meristem is changed from an indeterminate to determinate developmental programme and is not mediated by auxin (Lopez-Bucio *et al*., 2002; Sanchez-Calderon *et al*., 2005). During this time, the quiescent center in *Arabidopsis* is thought to play a role in sensing the environmental supply of P (Sanchez-Calderon *et al*., 2005).

The ability to maintain the meristem during P starvation is a potential target for improvements to the roots as a basis for P efficient plant selection because the progressive loss of meristematic cells is irreversible (Sanchez-Calderon *et al*., 2005; Reymond *et al*., 2006; Sanchez-Calderon *et al*., 2006). The maintenance of the meristem includes the regulation of root patterning by the transcription factor scarecrow (Scr) that interacts with PDR2 and LPR1, and is a cell cycle checkpoint (Cui *et al*., 2007; Ticconi *et al*., 2009). The genes, *LPR1* and *LPR2* (Low Phosphate Root 1 and 2) were identified by QTL analysis and the LPR1 protein was characterised as Multi Copper Oxidase (MCOs) (Reymond *et al*., 2006; Svistoonoff *et al*., 2007).

Further characterisation of the genes *LPR1* and *LPR2* has shown that they are expressed in the root tip, and play a role in iron homeostasis (Ticconi *et al*., 2009). In *Arabidopsis,* LPR2 is located in the endoplasmic reticulum and plays an important role in sensing external P, and encodes a P5 ATPase that assists in the expression of Scr (Ticconi *et al*., 2004; Ticconi *et al*., 2009). The genetic control root apical meristem maintenance and cell division by LPR1 and LPR2 under P-stress (or starvation) is not regulated by the *PHR1* mediated stress response pathway; and therefore separates programmed root development from environmental P-status (Sanchez-Calderon *et al*., 2006; Wang *et al*., 2010a).

The inheritance of the short-primary root phenotype observed in *Arabidopsis* was investigated in Low Phosphorus Insensitive (*lpi*) mutants that did not display short root phenotype in low P (Sanchez-Calderon *et al*., 2006).

The *lpi* mutants and the wild-type plants did not respond to N, S or Fe starvation, and had a reduced primary root length in low K media. The opposite effect occurred in P-deficient media, with no change the primary root length, compared to a 70% reduction in the wild-type plants. The reduction in cell length contributed to the reduction in root length, which is not observed in the *lpi* mutants that are able to maintain the cells in the meristematic state longer than the wild-type plants under P-starvation (Sanchez-Calderon *et al*., 2006). The *lpi1* mutant does not respond to low P by reducing the primary root length and there is no effect on lateral root formation when P is low (Sanchez-Calderon *et al*., 2006). The four *lpi* mutants (*lpi1, lpi2, lpi3* and *lpi4*) segregated (genetically) at a 3:1 ratio, which resulted from a dominant mutation.
The mutant *lpi2* displayed the short-root phenotype when grown without P compared with the long root phenotype observed in the other three mutants (Sanchez-Calderon *et al*., 2006).

The connection between iron and P metabolism and changes to the elongation of the primary root were investigated in two *Arabidopsis* ecotypes (Chevalier and Rossignol, 2011). In the *Arabidopsis* ecotype Colombia (Col-0), the growth of the primary root is reduced in low P; which correlates with elevated levels of iron in the roots and leaves compared with the P-sufficient plants (Williamson *et al*., 2001; Chevalier *et al*., 2003; Misson *et al*., 2005). If the iron concentration in the low P media is reduced, a long-root phenotype is observed compared to the short root phenotype seen when the levels of iron were higher (Ward *et al*., 2008). In a large-scale analysis of the changes to the primary and/or lateral roots during P-stress, the ecotypes Be-0 (German origin) and Ll-0 (Spanish origin) displayed no change to primary root length under P-stress and opposite changes in the lateral root number under P-stress (Chevalier *et al*., 2003).

The proteins from the two ecotypes (grown under P-stress) were separated by using a 2D PAGE gel (Chevalier and Rossignol, 2011). Further analysis by mass spectrometry identified 30 proteins that specifically corresponded to the P-stress response from a total of 465 proteins that were identified. P-stress resulted in less lateral roots for Be-0, and an over accumulation of alcohol dehydrogenase, malic enzyme and aconitrate hydratase 2. In comparison, Ll-0 had an increase lateral roots and decreased accumulation of the three proteins (Chevalier and Rossignol, 2011).

Aconitrate hydratase 2 is thought to play a role in iron homeostasis, and the lower accumulation pattern in the proteomic analysis confirms the tolerance to iron in Ll-0 (Chevalier and Rossignol, 2011). The stimulation of lateral root growth from increased iron concentrations in the *Arabidopsis* ecotype Columbia has recently been attributed to an interaction with auxin (Giehl *et al*., 2012). The study illustrates that signals and proteins influence the root phenotypes that are observed in response to P-stress vary in *Arabidopsis*, and could explain differences between plant species.

1.3.4. Changes to lateral root formation in response to P-supply

In *Arabidopsis,* the formation of lateral roots has four major stages that originate from founder cells in the pericycle, which divide. The first stage of lateral root emergence occurs from a pulse of auxin from the leaves in the basal meristem that stimulates the founder cells to differentiate (Peret *et al*., 2009). The early stages of lateral root emergence occur through a series of asymmetric cell divisions in the pericycle that give rise to the lateral root primordia, which later expand.

The activation of the lateral root primordia stimulates lateral root growth, and is tightly controlled by the polar transport of auxin from the leaves (Malamy and Benfey, 1997; Nibau et al., 2008; Peer et al., 2011; De Smet, 2012). It is thought that cells in the pericycle are primed by a signal from the protoxylem cells (De Smet, 2012) in a pre-initiation phase of lateral root formation that takes place close to the root tip in the basal meristem (Peret *et al*., 2009). Auxin is required for the pericycle cells to progress through the cell cycle; and in *Arabidopsis* seedlings, auxin is synthesised in the leaves and transported to the roots, and promotes lateral root emergence (Peret *et al*., 2009).

When three to five cell layers have formed, lateral root formation becomes autonomous and fails to respond to external auxin (Laskowski *et al*., 1995). This was first demonstrated in cell culture callus that was shown to have two distinct developmental stages of growth, with the first stage of lateral root formation responsive to external auxin (Laskowski *et al*., 1995). After the emergence of the lateral roots, auxin is synthesised to locally in the roots to promote independent growth, which is increased when P is low (Nacry *et al*., 2005; Peret *et al*., 2009).

The polar transport of auxin from the leaves that is required for the initiation of lateral roots in P-sufficient media is changed when the P-supply is low because the sensitivity of auxin is changed by the expression of the auxin Transport Inhibitor Response1 (TIR1) (Perez-Torres *et al*., 2008).

An earlier study by Nacry *et al.,* (2005) established that when a low P is sensed, auxin accumulates at the root tip, which contributes to the decreased elongation of the primary root. It was recently shown that *AtSIZ1* negatively regulates the accumulation of auxin, and induces the *PHR1* regulated P-starvation response pathway (Miura et al., 2005; Miura et al., 2011). A further checkpoint for lateral root formation involves abscisic acid that blocks the activation of the meristem before the emergence of lateral roots in *Arabidopsis* and has a stimulatory effect in legumes (Nibau *et al*., 2008). The inhibition of cell cycle gene expression by abscisic acid is reversible and independent of auxin, but is influenced by nitrate (Nibau *et al*., 2008).

1.4 P-stress response pathways as biological targets for improving P efficiency

Several studies in different plants have over expressed genes that enhance P-acquisition, or genes that enhance internal transport of P and are driven by studies in phosphate perception/response pathways (Ramaekers *et al*., 2010; Peret *et al*., 2011). Some of the biological targets to improve P efficiency in plants include root system architecture, hormones such as ethylene (discussed in section 1.2.2), the three components of the regulatory loop that regulate homeostasis (*PHR1, PHO2* and *miRNA399*), Phosphate 1 (PHO1) which is involved in transferring P to the xylem, high affinity P-transporters, acid phosphatase, phytase, citric or malic acid (Nilsson *et al*., 2007; Ramaekers *et al*., 2010; Rouached *et al*., 2011b).

1.4.1. The role of the roots in P acquisition

Several studies have demonstrated that primary root growth is regulated by the local external P status (Williamson *et al*., 2001; Lopez-Bucio *et al*., 2002; Thibaud *et al*., 2010).

Lateral root development is suggested to be regulated systemically with the assistance of *WRKY75*, and is independent of P status (Devaiah *et al*., 2007a). The transcription factor *WRK75*, is localised in the nucleus and plays a dual role as a negative regulator of root development and a positive regulator of P acquisition during stress (Devaiah *et al*., 2007a). A zinc finger transcription *ZAT6* (zinc finger of *Arabidopsis* 6) is involved in repressing the primary root growth and stimulates lateral root growth together with P acquisition as a part of P homeostasis (Devaiah *et al*., 2007b). When *ZAT6* was over expressed in wild-type *Arabidopsis,* the primary root stopped elongating and the number of lateral roots increased in both P-sufficient and P-deficient media (Devaiah *et al*., 2007b).

Plants take up phosphate from the soil in the form of $H_2PO_4^-$ through a large family of high and low affinity P transporters that are located in different parts of the plant (Mudge *et al*., 2002). In *Arabidopsis,* members of the PHT1 P transport family are largely expressed in the roots and some are upregulated when the plants senses low P (Mudge *et al*., 2002; Bayle *et al*., 2011; Nagarajan *et al*., 2011). Studies in *Arabidopsis* indicate that in low concentrations of P, high affinity transporters are upregulated, and differ from the P transporters expressed in P-sufficient media (Mudge *et al*., 2002; Bayle *et al*., 2011; Remy *et al*., 2012). Some of stress induced P-transporters that are involved in recycling P include *PHT1;1* that mobilises stored P from the vacuole; *PHT1;5* that transfers P from the shoots to roots; and *PHT1;8* and *PHT1;9* that are regulated by P homeostasis (Mudge *et al*., 2002; Bari *et al*., 2006; Doerner, 2008; Bayle *et al*., 2011; Nagarajan *et al*., 2011; Remy *et al*., 2012).

Until recently it was unclear if the responses in the roots (to low P) affects acquisition. It is well established that the primary root stops elongating and the number of lateral roots increase in response to low P in the *Arabidopsis* ecotype Colombia (Williamson *et al*., 2001; Lopez-Bucio *et al*., 2002). Originally, it was thought that the reduction in internal P content of the leaves caused the reduction in primary root length, and the growth of the lateral roots occurred as a secondary response from the recycling of P (Williamson *et al*., 2001). This conclusion was reached by testing the *pho2* mutant, which accumulates high levels of P in the leaves when P is sufficient (Delhaize and Randall, 1995; Williamson *et al*., 2001).

The *pho2* mutant had a longer primary root compared with the wild-type plant (Colombia) in P-sufficient media, and was reduced in low P media (Williamson *et al.*, 2001). If the branching zone is calculated (as a proportion of the primary root length) from the data presented; the branching zone was 36% in the wild-type roots grown in P-sufficient media (Williamson *et al.*, 2001). In low P media, the branching zone proportion increased to 51% of the primary root length, because the number of lateral root was increased. In contrast, the *pho2* mutant had a longer primary root than the wild-type plants with similar branching zones in P-sufficient (49%) and low P media (50%) (Williamson *et al.*, 2001). Therefore, the *pho2* mutant did not respond to the change in P-supply by changing the root system architecture because the perception of P-status is low (irrespective of the external P-supply) (Williamson *et al.*, 2001; Doerner, 2008).

The direct relationship between phosphate transporters and changes to root system architecture has recently been investigated in the *Arabidopsis pht1;9* mutant (Remy *et al.*, 2012). The high affinity P transporter *PHT1;9* mediates P acquisition under low P-supply (Mudge *et al.*, 2002; Doerner, 2008). In low P-supply, the *pht1;9-1* and *pht1;9-2* mutants have shorter primary roots and a higher number of lateral roots that were longer after seven days in low P compared with the wild-type plants when grown in low or high P-supply (Remy *et al.*, 2012). When *PHT1;9* was over expressed in the mutants, the opposite effect was seen in the roots; with a longer primary root in low P media and less lateral roots when compared with the wild-type plants (Remy *et al.*, 2012).
The study concluded that *PHT1;9* does not take up P during P-sufficient conditions and acquisition (during P-stress) is pH dependent. The change in pH is the result of the alkalisation of the media due to the influx of H^+ ions, which has previously been reported (Raghothama, 1999; Remy *et al.*, 2012).

1.4.2. Systemic regulation of P homeostasis

Several similarities in the signaling and regulation of P-stress responses exist between *Arabidopsis* and rice (*Oryza sativa*) (Chen *et al.*, 2009). A recent study in rice identified a mutation that resulted in the leaf tips becoming necrotic in P-sufficient media when compared with wild-type plants (Hu *et al.*, 2011).

The *LTN1* gene (*Leaf Tip Necrosis1*) (in rice) was identified as the *Arabidopsis AtPHO2* homolog, which plays an important role in signaling under P-starvation (Delhaize and Randall, 1995; Hamburger *et al*., 2002). The *Arabidopsis pho2* and rice *ltn1* mutants both accumulate P in the leaves and are repressed by miRNA399 (Bari *et al*., 2006; Hu *et al*., 2011). The *ltn1* mutant was shorter than the wild-type plants with less tillers and lower fertility in P-sufficient media (Hu *et al*., 2011). The *Arabidopsis pho2* mutants had 50% reduced fresh weight and dark green leaves with purple petioles, which are characteristic of the P-starvation response (Delhaize and Randall, 1995).
The over accumulation of P in the leaves of P-sufficient plants in rice and *Arabidopsis* was the result of an increase in expression of high affinity P transporters that are normally induced when P is limiting (Delhaize and Randall, 1995; Bari *et al*., 2006; Hu *et al*., 2011).

Further characterisation of the *ltn1* mutant revealed it was over sensitive to P starvation compared to the wild-type rice and displayed responses of P-deficiency in P-sufficient media (Hu *et al*., 2011). The *ltn1* mutant had high acid phosphatase and RNase activity in P-sufficient media, and accumulated iron in the roots and a higher proportion of galctolipids compared to phospholipids. In P-deficient media, P was remobilised from the shoots to the roots in the wild-type rice, and was impaired in the *ltn1* mutant.
Also, the primary root length increased in the *ltn1* mutant, and acid phosphatase was induced with the responses higher than the wild-type plants (Hu *et al*., 2011).

A model for the regulation of systemic homeostasis has been proposed from studies in *Arabidopsis* (reviewed by Doerner, 2008). When P is sufficient, the expression of *PHO2* is high, which represses the expression of the high affinity P transporters *PHT1;8* and *PHT1;9*. The *pho2* mutant over accumulates P to toxic levels in the shoot when P is sufficient because the influx of P is not regulated (Delhaize and Randall, 1995).
The low expression of the two high affinity transporters represses the expression of IPS/At4 and high levels of P in the shoot represses miR399 in the phloem.
The homeostatic state of P in *Arabidopsis* involves equal distribution of P in the mature and young leaves (Doerner, 2008). When the leaves sense a low P-supply, the mature leaves divert some P to the younger leaves by the expression of micro RNA399 (miR399), which is upregulated.

The high expression of miR399 represses the expression of PHO2, which promotes the expression of the high affinity transporters *PHT1;8* and *PHT1,9*. As the levels of APS/At4 rise with the increasing P-supply; miR399 is downregulated and P homeostasis is restored (Bari *et al*., 2006; Doerner, 2008). The genetic manipulation of PHO1 during the P-stress response has recently demonstrated that the fresh weight of the *Arabidopsis* plants that under expressed *PHO1* could be maintained to the same level as wild-type with low P content in the vacuole (Rouached *et al*., 2011b). PHO1 is a transmembrane protein that is involved in the transfer of P into the xylem from the root and transferred to the shoot during P-stress (Hamburger *et al*., 2002), and is degraded by PHO2 (an E2 ubiquitin conjugase, UBC24) 24 hours after P is resupplied (Liu *et al*., 2012).

This research has a huge application potential because the genetic control of the cytoplasmic pool of P to be converted into plant biomass is one of the goals in improving P-efficiency in crops (Veneklaas *et al*., 2012). P-efficient plants are thought to have different internal distribution of P compared with P-inefficient plants, and readily utilise and remobilise P under sufficient and deficient P-supply. However, all of these mechanisms and gene networks are unresolved (Veljanovski *et al*., 2006; Chiou and Lin, 2011; Jain *et al*., 2012; Secco *et al*., 2012).

1.4.3. Relationship between P and S through sulfolipid biosynthesis

The early diagnosis of P-deficiency in plants has been developed in *Arabidopsis* using gene expression studies in leaves, with the potential for use in potato (Hammond *et al*., 2003; Hammond *et al*., 2011). A microarray was used to identify P-stress induced genes in the study that were upregulated after the withdrawal of P without compromising the internal levels of the other macro-elements (Hammond *et al*., 2003). The idea of the study was to develop "*smart plants*" that express indicator genes before the onset of P-stress, which trigger a marker dye in the leaves, and then the stress is alleviated with the addition of P fertiliser (Hammond *et al*., 2003). The expression of UDP-sulfquinovose synthase (*SQD1*) occurs four hours after the removal of P and has no influence on the internal levels of other minerals (Hammond *et al*., 2003).

The synthesis of sulfolipids to replace phospholipids is one of the signature metabolic changes in response to P-stress (Plaxton and Tran, 2011). This expression of *SDQ1* serves as an early indicator of the relationship between P and S because it is the first step involved in the biosynthesis of sulfolipids (Essigmann *et al*., 1998; Shimojima, 2011). Two of the high affinity transporters (*SULTR1;1* and *SULTR1;2*) in *Arabidopsis* are highly active under sulfur starvation, and have a PB1S/PHR1 binding domain (as previously described in section 1.2.2), and the two S-transporters are upregulated during P-stress or starvation (Rouached *et al*., 2011a; Takahashi *et al*., 2011).

As the external supply of P decreases in concentration, the phospholipid content decreases, and the sulfolipid and galactolipid content increase in replacement (Essigmann *et al*., 1998). The effect of internal P-supply was tested using a *pho1* mutant that is defective in loading P into the xylem, and the lipid composition compared to wild-type was 50% lower in phospholipids, and 300% higher in sulfolipids (Essigmann *et al*., 1998).

The "*smart plant*" concept was developed in a laboratory setting with *Arabidopsis*, and a recent study by the same group has identified 200 candidate genes in field-grown potatoes (*Solanum tuberosum*) and includes *SQD1* (Hammond *et al*., 2011). In both studies, the plants were grown in hydroponic solution containing a full set of nutrients before the P was removed and re-supplied. The promoter region of the *SQD1* gene was identified and a β-glucuronidase (GUS) reporter gene was used to make a *SQD1::GUS* construct that was induced four hours after the removal of P. The intensity of the reporter gene increased after 28 hours, and was increased further after four and nine days (Hammond *et al*., 2003). The rapid response of the candidate genes in potato and *Arabidopsis*, and has potential for the early diagnosis and correction before the internal supply of P is depleted (Hammond *et al*., 2003; Hammond *et al*., 2011).

1.4.4. Induction of root acid phosphatases (APase)

Acid phosphatases (APases, EC 3.1.3.2) are abundant in plants and their role in P metabolism is to hydrolyse P from orthophosphate monoesters and function by recycling and scavenging P from internal and external sources (Duff *et al*., 1994).

The hydrolysis of inorganic P (P_i) to organic P (P_o) by acid phosphatases in the roots assists the plant to acquire chemically bound forms and have either specific or non-specific substrate specificity, which is important for recycling P (Duff *et al*., 1994). Plants contain a number of intracellular and extracellular acid phosphatases, and increased activity is one of the signature P-stress responses (Duff *et al*., 1994; Tran *et al*., 2010a).

The intracellular phosphatases are localised in the vacuole and cytoplasm (located in the soluble fraction, (SF), and the extracellular acid phosphatases are located in the cell wall (CW). The extracellular acid phosphatases are secreted by the roots under phosphate stress to increase the hydrolysis of esterified P in the rhizosphere. The large family of acid phosphatases function in the recycling and remobilising of P as part of normal P homeostasis (Duff *et al*., 1994; Tran *et al*., 2010a). Other acid phosphatases are specifically induced in response to P starvation, which are regulated by transcription factors such as *PHR1, WKY75* and *ZAT6* (Rubio *et al*., 2001; Devaiah *et al*., 2007a; Devaiah *et al*., 2007b; Tran *et al*., 2010a), and ethylene (Lei *et al*., 2011).

One of the subgroups of induced acid phosphatases, are the purple acid phosphatases (PAPs), which are distinguishable by their pink or purple colour in solution and contain a conserved motif with seven metal ligating residues (Tran *et al*., 2010a). The PAP family contains many members in different plants. For example, there are 29 members in *Arabidopsis* (Li *et al*., 2002), 35 in soybean (Glycine max) (Li *et al*., 2012a) and 26 in rice (*Oryza sativa*) with some that contain PB1S elements in the promoter region (Zhang *et al*., 2011). The different members of the PAP gene family perform different functions in recycling and recovering P, and are a biological target for improving P efficiency (Tran *et al*., 2010a; Wang *et al*., 2010b).

The *Arabidopsis* PAP genes are divided into three groups, and eight subgroups according to the sequences; and characterisation of the different genes is an on-going process (Li *et al*., 2002; Tran *et al*., 2010a). Three (*AtPAP10, AtPAP12* and *AtPAP26*) of the 29 members of PAPs in *Arabidopsis* that are classified into the same group (group 1, subgroup Ia-2) all play an important role in scavenging and recycling P during P-stress and are secreted from the roots (Li *et al*., 2002; Tran *et al*., 2010a; Wang *et al*., 2011).

AtPAP26 is also found in the vacuole, and AtPAP10 and AtPAP12 are located in the cell wall (Hurley *et al*., 2010; Tran *et al*., 2010a).

A recent study with *Arabidopsis* demonstrated that growth of P-starved plants could be restored with ADP supply (Wang *et al*., 2011). Here, wild-type *Arabidopsis* (ecotype Colombia) seedlings and five mutants with no acid phosphatases activity (*nop1-1, nop1-2, nop1-3, nop1-6* and *nop1-10*) were grown for 14 days in agar medium containing a full nutrient supply, or no P-supply which was supplemented with 10 µM, 50 µM or 150 µM of ADP. The shoot and root fresh weight increased in all of the plant lines with increasing ADP, and shoot fresh weight was restored to about 80% of the plants grown on P-sufficient media. Interestingly, the root fresh weight of the wild-type and *nop1-10* plants grown on –P, containing 150 µM of ADP in the media was exceeded the P-sufficient plants. The supply of ADP failed to restore the primary root length of the P-stressed plants. However, the addition of 150 µM ADP to the low P media increased the number of lateral roots in the wild-type plants compared with the P-sufficient plants.

The mutations were located in the *AtPAP10* gene, which is an acid phosphatase that is located at the root surface, and is activated specifically in response to P-stress.
The plants were stained with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), which is cleaved by acid phosphatase to produce a blue colour. There was an increased level of staining in the plants with no P-supply and higher levels of ADP, which confirmed the role of *AtPAP10* in the tolerance to P starvation and the role of ADP in cellular processes (Wang *et al*., 2011).

The significance of the dual role of the *AtPAP26* (At5g34850) gene and its functions in the vacuole are becoming more evident in the adaptation of low P environments, with well-conserved homologs in tomato, soybean, rice and onion (Veljanovski *et al*., 2006; Hurley *et al*., 2010). The AtPAP26 protein is a 55 kDa monomer with two isoforms that are constitutively expressed irrespective of the external nutrient status and further upregulated during P-stress through post-translational glycosylation (Veljanovski *et al*., 2006; Tran *et al*., 2010a; Tran *et al*., 2010b).

The identification of an *atpap26* mutant recently confirmed the specific role in P-stress induced homeostasis by scavenging external P from the environment (Hurley *et al*., 2010). The mutation was the result of a T-DNA insert in the seventh intron of the *AtPAP26* and affected the secretory region of the protein. The *atpap26* mutant had less AtPAP26 activity in the roots and shoots, no expression in suspension cells and impaired development compared to the wild-type plants with no difference in AtPAP12, and AtPAP17 activity (Hurley *et al*., 2010). The shoot fresh weight of the mutant was compared with Colombia under P, N, K and paraquat-mediated oxidative stress, but no differences were observed in any of the other treatments expect for P starvation.
The primary root length of the wild-type and *atpap26* mutants was lower under P starvation as expected, with a further reduction in the *atpap26* mutant. It was concluded that the extra reduction in primary root length was the result of the malfunction of the *AtPAP26* gene to scavenge external P, which makes it a potential target for improving P efficiency in plants (Hurley *et al*., 2010).

The role of P-stress induced PAPs has in scavenging and recycling P is well established, and until recently, there was no link between the changes in root morphology and PAP activity. However, a recent study observed an increase in biomass in tobacco plants transformed with an *Arabidopsis AtPAP18* (Zamani *et al*., 2012). The transgenic lines also had an increased primary root length and more lateral roots than the control plants, and were not affected by low P medium. This suggested that *AtPAP18* plays an indirect role in root modifications. P-acquisition was also enhanced in the transgenic lines, and although the internal levels of P were lower between treatments, they were higher than the wild-type tobacco under P-sufficient and P-deficient conditions (Zamani *et al*., 2012).

1.5 White clover (*Trifolium repens* L.)

White clover (*Trifolium repens*) is found naturally growing amongst grass species and is an important component of agricultural pastures around the world. It is a trifoliate legume in the Fabaceae (Leguminosae) family and is an out breeding allotetraploid species containing two genomes each with 16 chromosomes, to give a total of 32. Molecular analysis suggests that the ancestral lineage occurred from the fusion of *Trifolium occidentale* and *Trifolium pallescens* (Ellison *et al*., 2006). The genetic diversity of *Trifolium repens* has been confirmed by 301 polymorphic markers across 16 accessions, and suggests that *Trifolium nigrescens* also contributed to the genome through the *Trifolium occidentale* lineage (Badr *et al*., 2012).

1.5.1. White clover in New Zealand agricultural pastures

The agricultural industry in New Zealand (NZ) has invested a significant amount of resources into understanding soil structure and the requirements of pasture forages in order to maximise production (pasture and cattle) and reduce fertiliser excess run-off into waterways. The research includes long-term fertiliser and grazing trials with different cultivars (Chapman and Caradus, 1997; Wedderburn *et al*., 2005; McDowell and Condron, 2012). The regulation of the on-farm management practices for fertiliser run-off is an important aspect of managing the environmental effects of P (Wheeler *et al*., 2003; Monaghan *et al*., 2007), and diagnostic tools for the application of fertiliser on different soils have also been addressed (Edmeades *et al*., 2005; Edmeades *et al*., 2006).

The most predominant species in NZ pastures is perennial rye grass (*Lolium perenne* L.), and the white clover component can range from about 10% to 20% in healthy pastures or can be as low a 2% under drought or nutrient stress or poor management (Woodfield and Clark, 2009). The main difference between pasture species such as rye grass and white clover and other crops like brassicas (and *Arabidopsis*) or maize is that they are not replaced annually, and persistence under environmental pressures is important for two reasons. Firstly, white clover is able to fix atmospheric nitrogen, which is a limiting factor for the growth of perennial rye grass.

White clover forms symbiotic associations with *Rhizobium leguminosarum* that allow the atmospheric nitrogen to be fixed through nodules, and requires an optimal P and S supply (Almeida *et al*., 2000; Varin *et al*., 2010). Therefore breeding white clover to withstand grazing pressures and the high use of nitrogen fertilisers is another practical aspect of NZ farming that has taken priority (Woodfield and Clark, 2009). It has been proposed that nitrogen acquisition is down regulated under sulfur starvation, which impacts on the synthesis of N-rich amino acids and a reduction in root biomass in white clover (Varin et al., 2010). Nodule development in soybean (*Glycine max* L.) is also affected by P-deficiency, and recently an acid phosphatase was identified and was shown to play a role in nodulation (Qiao *et al*., 2007; Li *et al*., 2012a). The economic value of having white clover as a component of pastures through annual nitrogen fixation has been estimated at 157 kg N per hectare (Crush *et al*., 2006), with higher rates in summer compared to winter (Chapman and Caradus, 1997).

Secondly, white clover is a good source of protein, which is required by cattle in the production of milk or meat. The nutritional properties that are major contributing factors in live-weight gain or milk production by cattle include dry matter; crude protein; fiber; and metabolisable energy, which are all higher in white clover compared with perennial rye grass (Mackle *et al*., 1996; Harris *et al*., 1998; Crush *et al*., 2006). It is well accepted that cattle prefer to have white clover as a component of their diet; although it is rarely fed to cattle in isolation due to the likelihood of bloat, which is a build-up of gas in the rumen (Chapman *et al*., 2007; Wang *et al*., 2012).

In a feed conversion study, cows were fed unrestricted (*ad lib*) quantities of white clover and rye grass mixed pasture (Harris *et al*., 1998). The clover content varied in the treatment groups with 200 g, 500 g and 800 g of clover per kg of pasture with the balance made up with rye grass. The cows on a diet mix containing 500 g of clover and 500 g of rye grass consumed 1.41 kg (13%) more than the cows with a mix of 200 g of clover and 800 g of rye grass. The preference of the dairy cows for the diet with the higher white clover content consequently produced an additional 3 litres of milk per cow per day when compared to the control group (Harris *et al*., 1998).

1.5.2. Growth, morphology and perception of P-supply

White clover has two distinct morphological phases of growth after seed germination (Brock *et al.*, 2000). The first is the seminal tap rooted stage which lasts for about two years until the taproot dies, and the second is the clonal stage with small plant fragments attached to a main stolon each with their own nodal root system. The clonal stage of white clover persists in the field and allows the legume to spread along the ground (Brock *et al.*, 2000). The stolons are genetically identical to the parental plant, and can be studied in different growth media such as soil, sand or liquid with minimal variation to root system architecture (Crush *et al.*, 2005).

White clover has a diverse range of root morphologies that have been compared in a large-scale study of 386 genotypes (Jahufer et al., 2008). The study identified five distinct cluster groups that separated the traits of the genotypes when grown in sand, and six cluster groups when the genotypes were compared with sand and hydroponic media (Jahufer et al., 2008). The F1 generation plants were grown in P-sufficient conditions and analysed at the whole root level and at a morphological level, with some degree of overlap between the clusters of traits (Jahufer *et al.*, 2008). The traits included root surface area and volume, number of branched forks and root tips, root diameter, root length and specific root length, but were not tested for their response to P-supply (Jahufer *et al.*, 2008).

1.5.3. The white clover P-efficiency breeding programme

The aim of the white clover-breeding programme was to select cultivars that could withstand growth in New Zealand hill country and tolerate low P soil concentrations (Dunlop *et al.*, 1988). The programme was divided into five phases that identified traits for low P tolerance that were heritable, in the field and in the laboratory, together with the characterisation of morphology, physiology, and response to P-supply (Caradus and Snaydon, 1988; Dunlop *et al.*, 1988; Caradus *et al.*, 1991; Chapman and Caradus, 1997).

Phosphorus acquisition

The cation exchange capabilities white clover differ with the available P concentration, and also differ between populations and genotypes (Caradus and Snaydon, 1987a). In addition, the cation exchange capabilities of the leaves is higher compared with the roots (Caradus and Snaydon, 1987a). The acquisition of radioactively labeled ^{32}P was investigated in Grasslands Tahora with the larger leaved genotype Grasslands Kopu with the plants inoculated with *Rhizobium* to fix their own nitrogen during the experiment (Chapman and Hay, 1993).

The results demonstrated that P acquisition occurs from the old and young roots, and is also translocated from the older to the younger roots with no differences between the genotypes (Chapman and Hay, 1993). The 'old' roots were located at node 10 of the stolon compared with the 'young' roots that were located at node three or four. The proportion of ^{32}P taken up (as an estimated percentage) decreased with lower concentrations of P. The roots acquired 26% of the ^{32}P in low 5 μM ^{32}P (155 μg daily) and 30% in the medium 20 μM ^{32}P (620μg daily) concentrations compared with 54% in high 1 mM ^{32}P (31mg daily) concentrations (Chapman and Hay, 1993). Therefore, the P content acquired by the different P-transporters (uncharacterised to date) ranged from 0.04 mg to 16.74 mg daily with no significant differences between the position of the root on the stolon (Chapman and Hay, 1993).

Induction of acid phosphatase in the roots

White clover genotypes from soils containing low P and high P were collected to measure the acid phosphatase activity in the roots as a potential biological marker for P-stress for the selection of P efficiency (Caradus and Snaydon, 1987b). The acid phosphatase activity was similar between the plants from low and high P soils, and lower in the cultivar Maku of *Lotus peduncultus* that is known to tolerate low P soils (Caradus and Snaydon, 1987b). The authors concluded that acid phosphatase activity was not a suitable indicator of P tolerance in plants (Caradus and Snaydon, 1987b).

The relationship between root and shoot communication was investigated by measuring the induction of acid phosphatase in the roots together with the decline in leaf P-content after the P-supply was completely withdrawn from the media (Hunter and McManus, 1999; Zhang and McManus, 2000). Further characterisation of the two white clover P-stress induced acid phosphatases were carried out in the genotype PgH2, which was not selected for tolerance to low P (Zhang and McManus, 2000). A reduction in leaf P-content after two weeks from when the P-source correlated with increased activity of acid phosphatase in the roots after one week (Zhang and McManus, 2000). The acid phosphatases were characterised after three weeks from when they were first induced, and each acid phosphatase contained two isoforms that were named APase I and APase II (Zhang and McManus, 2000).

The two isoforms in APase I (APase IA and IB) were 52 kDa (kilodaltons), and APase II had one isoform that was 113 kDa, and the other was 92 kDa (APase IIA and IIB). The four isoforms had optimum activity in acid conditions and were inhibited by Cu^{2+}, Zn^{2+} and Mo^{2+}. The preferred substrates were ATP followed by PP_i, and the isoforms could not hydrolyse phytate (Zhang and McManus, 2000). The white clover acid phosphatase isoforms were also tested for the specificity to other substrates and included *o*-phospho-L-serine, *o*-phospho-L-tyrosine, phosphoenol pyruvate (PEP) and 3-phosphoglycerate (3PGA), which are integral to the alternative metabolic pathways that are utilised under P-stress (Zhang and McManus, 2000; Plaxton and Tran, 2011).

Perception and phenotypic root responses to a limited P-supply

The variation in root morphology of white clover between long fine roots and short fine roots were compared in two studies with the plants grown in different medium and P concentrations (Crush *et al*., 2005; Crush *et al*., 2008). The first study in 2005 examined the growth of the genotypes in sand and solution with respect to their root morphology traits (Crush *et al*., 2005). The two traits of long fine roots and short thick roots were divided into two categories. The long thin roots were classed as being explorative of the soil, and their root tips covered more surface area (Crush *et al*., 2005). Growth in different concentrations of P-supply produced a higher dry weight and had a greater surface area of the leaves and roots.

The specific root length (a measure of root weight and length) and P uptake were also higher than the short fine roots (Crush *et al*., 2008). The short fine roots were classed as exploitive, with the branching of the lateral roots localised around a small area or central point (Crush *et al*., 2005). The short fine root genotype had less lateral roots than the long thin genotype, and absorbed more P per unit of root length (Crush *et al*., 2008).

The phenotypic response to a limited P-supply differs between the *Arabidopsis* ecotype Colombia and the white clover genotype Challenge (10F) (Williamson *et al*., 2001; Dinh, 2009; Dinh *et al*., 2012). However, the two plant species could perceive a limited P-supply through a similar mechanism, which results in the accumulation of auxin at the root tip (Nacry *et al*., 2005; Perez-Torres *et al*., 2008; Dinh, 2009; Dinh *et al*., 2012). In the genotype Grasslands Challenge (10F), the primary roots are longer with an increased number of lateral roots in response to a limited P-supply (10 μM KH_2PO_4) when compared with P-sufficient media with 1 mM KH_2PO_4 (Dinh, 2009; Dinh *et al*., 2012). The response was visualised at the root tip using *DR5::GUS* transformants in the P-stressed plants, and was also induced in the P-sufficient plants when ethylene (1 μM ACC) was added. The response to ethylene indicates that the hormone is involved in the P-stress response pathway, which results in changes to root system architecture. The ethylene-induced stress response in the roots in P-sufficient media and the activation of the *DR5::GUS* reporter construct by auxin in low P media is consistent with the increased sensitivity observed in the *Arabidopsis* root tip (Nacry *et al*., 2005; Dinh, 2009; Dinh *et al*., 2012). It is thought that difference in auxin sensitivity results in the different phenotypes observed in the two plant species in response to P-stress (Dinh *et al*., 2012).

Origins of selected genotypes in this study

In one of the field trials of the white clover P-efficiency breeding programme, two cultivars (*cv*. Grasslands Tahora and *cv*. Grasslands Prestige) performed well in the hill country trial in terms of persistence, nitrogen fixation, growth and weight gain in grazing sheep (Chapman and Caradus, 1997). Further field studies were carried out and a series of crosses were made between Grasslands Tahora and Grasslands Prestige based on their performance (unpublished).

The crosses generated several breeding lines and four (BL43, BL45, BL47 and BL49) were selected together with seven genotypes during three preliminary screenings (Effendy, 2007). The plant material from the breeding line screen was investigated further together with a genotype screen. At the conclusion of the study, three genotypes (43-7, 45-14, and 47-9) were selected by Effendy (2007) for this project.

The evaluation 43-7, 45-14 and 47-9 by Effendy, (2007) established the growth of the of the white clover genotypes in P-sufficient media for eight weeks followed by the withdrawal of the external P-supply for 21 days. The genotype 43-7 was considered the least efficient of the seven genotypes due to a low fresh weight yield, high root-to shoot ratio, smallest leaf size, area, and weight. The most efficient genotype from the screen was 45-14 (Effendy, 2007). The third genotype (47-9) was selected from breeding line 47 (BL47) and this breeding line displayed high performance in high and low fertility in the field and liquid media, and the root fresh weight was not affected by P-starvation. The medium leaf size and root-to-shoot ratios were similar in 47-9 and 45-14 (Effendy, 2007).

The whole-plant approach used for the genotype screen performed by Effendy (2007) was based on reduction in biomass, increased root-to-shoot ratio, decline internal P content, and the induction of acid phosphatases that have previously been studied as biological markers for assessing the level of P-stress (Duff *et al*., 1994; Hammond *et al*., 2003; Zamani *et al*., 2012). The genotype screen performed by Effendy (2007) did not include any assessment of root system architecture in the criteria.

1.6 Thesis objectives

The main focus of this study, is to extend the evaluation of three genotypes of white clover (43-7, 45-14 and 47-9) by characterising the root system architecture in terms of primary root elongation and lateral root emergence (and elongation) to determine if any differences are observed when the genotypes are grown with a limited P-supply.
The extensive characterisation of root development in P-sufficient and limited P-supply carried out in *Arabidopsis* (section 1.3) suggest that the elongation of the primary root and initiation of the lateral roots occur through a programmed developmental pattern that is influenced by the environmental P-supply (Peret *et al*., 2011). It is proposed that the PHR1 mediated stress response pathway regulates P homeostasis and P-acquisition through interactions with PHO2 and miR399 in *Arabidopsis* but does not influence the changes to the root system architecture (Bari *et al*., 2006; Perez-Torres *et al*., 2008; Miura *et al*., 2011). Alternatively, the changes to primary and/or lateral roots in response to low P occur through changes in hormone sensitivity (Perez-Torres *et al*., 2008; Jain *et al*., 2012; Nagarajan and Smith, 2012).

The study carried out by Dinh *et al.,* (2012) observed the accumulation of auxin at the root tip of white clover in response to a limited P-supply resulted in changes to the primary root length and lateral roots however; the leaf P content and acid phosphatase activity were not tested. The study by Effendy (2007) essentially observed the response to re-establish P homeostasis in terms of induction of root acid phosphatase and the decline in leaf P content in the three white clover genotypes following the complete withdrawal of external P-supply. The induction of root cell wall activity in 45-14 was observed after 14 days from the withdrawal of the P-supply; which was classed as a slow response (Effendy, 2007). There was no significant difference in the cell wall acid phosphatase activity in 43-7 and 47-9; and these two genotypes were classed as having no response (Effendy, 2007).

In 45-14 and 47-9, a decline in leaf P-content was observed after seven days and was considered to be a fast response. In contrast, the leaf P content was the highest in 43-7 in P-sufficient media and was dramatically reduced after 14 days P-starvation; and was considered to be a slow response (Effendy, 2007).

The connection between the accumulation of auxin at the root tip, the resulting changes to the primary and lateral roots, the induction of root acid phosphatase and decline in P content that correlate with P-efficiency are not known in white clover. In order to resolve this question, the changes to the primary and lateral roots in response to low P needs to be characterised in 43-7, 45-14 and 47-9.

Hypothesis

"*If changes in root system architecture occur in response to the reduction in the external P-supply after the emergence of visible lateral roots, then those changes are also expected to occur in the primary and/or lateral roots if the external P-supply is reduced before the emergence of visible lateral roots in the respective genotypes*".

Objectives

1. Characterise the morphology of the primary roots in the three genotypes;
    - by defining the branching zone as a proportion of the primary root length containing lateral roots,
    - by counting the newly emerged lateral roots shorter than 10 mm separately from the lateral roots that are longer than 10 mm in length.
2. Determine if any changes that occur in the primary and/or lateral roots are specific to the reduction in P-supply;
    - by reducing the P-supply before and after the emergence of visible lateral roots,
    - by reducing the sulfur supply (as another macro-element).
3. To observe the relationship between root system architecture and P-efficiency by assessing the genotypes for overall P-stress in the whole plant, roots and leaves from the plant material collected from one final destructive harvest;
    - by measuring fresh weight root-to-shoot ratios and specific root length.
    - by measuring the leaf fresh weight and P content and root acid phosphatase activity.

The basis for standardising the analysis of the primary root in terms of the branching zone in this study originates from previous observations in white clover. The results of the study by Dinh *et al.,* (2012) suggest that the increase in the number of lateral roots is proportional to the length of the primary root; however the branching zone was not measured. It is possible that stimulation of lateral roots occurred because there was physically more space on the longer primary root to accommodate lateral roots, and did not appear to change the lateral root density (Dinh *et al*., 2012). The application of exogenous ACC (ethylene precursor) and auxin (NAA) did appear to have an effect on the lateral root density (in terms of the number of lateral roots with respect to primary root length) in the P-sufficient plants.

When 100 nM ACC was applied, the primary root length appeared to increase and the number of lateral roots did not appear to increase in branching zone as proportion of the primary root length, which possibly decreased the lateral root density. In the low P plants, the application of ACC appeared to increase the lateral root density, by increasing the primary root length and number of emerged lateral roots.
When 5 nM NAA was applied to P-sufficient plants, the lateral root density appeared to increase because the number of lateral roots increased in the branching zone. In the other three treatment groups the number of lateral roots in the branching zone increased as a proportion of primary root length, with no apparent change to the lateral root density (Dinh *et al*., 2012).

Secondly, Dinh (2009) reported that the number of lateral roots became too numerous to physically count after 24 days post excision, so by observing the changes in the branching zone, the analysis of primary root elongation and lateral root emergence (and elongation) can be extended.

CHAPTER 2. MATERIALS AND METHODS

2.1 Plant material

The stock plants of the three genotypes (43-7, 45-14, and 47-9) originated from a breeding programme to select germplasm for tolerance to low phosphorus at Agresearch Grasslands in Palmerston North, New Zealand (Dunlop *et al*., 1988; Effendy, 2007).

Maintenance of stock plants

The plants were maintained in the Massey University Ecology glasshouse, Palmerston North, New Zealand [(Latitude -40.35 (40°21'00"S), Longitude +175.61 (175°36'36"E), Altitude ~30 m] and watered daily. Fertiliser (Yates Thrive®) was applied weekly at one third of the manufactures recommendation and regular trimming was carried out to remove dead matter and to prevent over growing. The source of lighting and temperature inside the glasshouse were dependent on seasonal fluctuations.
Air conditioning prevented the temperature from exceeding 25°C (degrees Celsius), particularly during late spring, summer and early autumn (November to March).
Insects such as thrips, were treated with Pyrethrum® as required and slug bait was applied approximately every four months.

Sample collection and storage

All of the experiments were terminated by one destructive harvest. The plants were removed from the media, dried with paper towels, weighed and dissected with a sterile blade by removing the roots and leaves (separately) and stored at -80°C for further analysis. In experiment III, the roots from three plants in each treatment group were pooled for quantitative polymerase reaction (qPCR) analysis (refer section 2.5).
The acid phosphatase activity and leaf P content analysed individually (refer section 2.4). Samples of liquid media were taken and the pH was recorded (data not shown), before freezing the tubes with the intention of analysing it for acid phosphatase activity and mineral content. Preliminary analysis revealed that the acid phosphatase activity was very low in these samples, and required purification and so this was not pursued as part of this investigation.

2.2 Experimental design and schedule

Five experiments with the three white clover genotypes were carried out in this study in hydroponic media. Two preliminary experiments were carried out from June to September 2010 to observe the development of the stolons in P-sufficient (+P) hydroponic media (Table 4), and formed the basis of the P-stress response experiments.

The experimental schedule consisted of a pre-treatment phase where the stolons were cut from the stock plants and grown in vermiculite for one week before transferring to P-sufficient media until the experimental phase of the experiment, where the media was changed to compare the response to the change in P-supply. All of the experiments were carried out in a Contherm controlled climate chamber at the Plant Growth Unit (PGU), Massey University in Palmerston North (Figure 2.1c).

The stolons were left in the glass house for four to five days to recover from the shock of cutting and then transferred to the growth controlled climate growth chamber. The night (dark) conditions were set at 0 µM m^{-2} s^{-1} of light intensity at 14°C for eight hours. The growth chamber was set to long-day conditions with 16 hours of light with an intensity of 300 µM m^{-2} s^{-1} at 22°C (degrees Celsius). The humidity remained constant throughout the experiments at 70%. Lesley Taylor at the PGU monitored the internal temperature of the chamber and the data was downloaded weekly, with no reports of abnormal fluctuations over the course of the experiments.

In this study, the term “post excision” refers to roots that emerge from the cut stolon, and is equivalent to the number of days (growth) after the stolons were cut from the stock plant. The term “treatment” refers to the number of days growth in a limited nutrient supply (P or S).

2.2.1. Pre-treatment phase of experiments

At the beginning of the pre-treatment phase of the experiments, between 50 and 55 white clover stolons were cut from the stock plants and the fresh weight was recorded. A sterile dissection blade was used to cut the stolons from the stock plants proximal to the fourth node from the apex, with up a maximum of 1 mm of the internode remaining to prevent adventitious roots forming at the wound site.

The stolons were buried in inside 50 mL falcon tubes containing vermiculite above node three and moistened with 30 mL of half strength Hoagland's media to initiate primary roots from each of the two primordia at nodes three and four (Figure 2.1a). The falcon tubes were lined with a black polyurethane insert to prevented breakage of the roots during transfer to the hydroponic solution (Figure 2.1a).

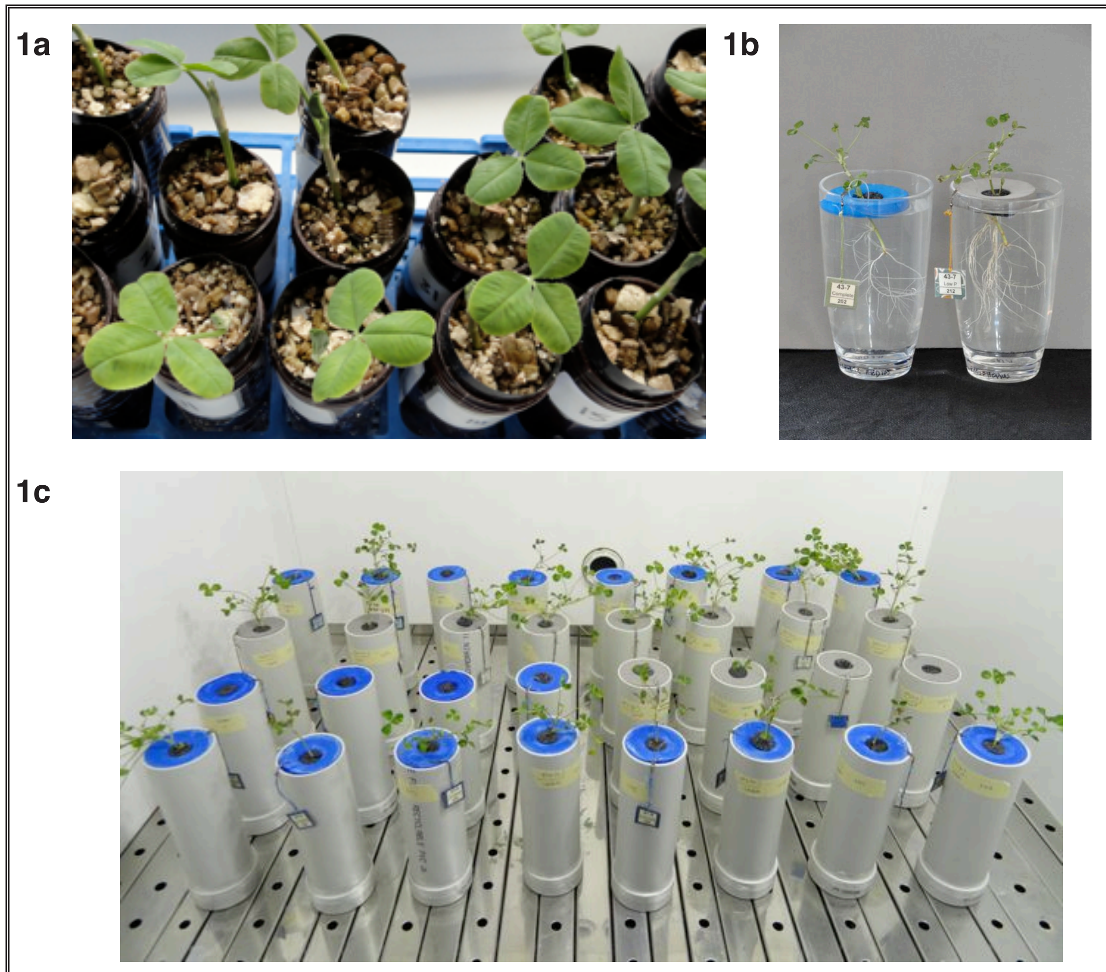


Figure 2.1. Primary root initiation and growth in liquid media

The stolons were cut and grown in vermiculite inside 50 mL falcon tubes with a black polyurethane insert for one week and transferred to liquid media for up to three days. **b.** The lids containing the plants were removed for the root measurements and placed in fresh treatment media to prevent drying out while the lateral roots were counted and the media was replaced. **c.** The stolons were transferred to 600 mL PVC pipes with half strength Hoagland's media for the experiments. The plants with P-sufficient media had blue caps (left) and the grey caps (right) were used for the plants in P-deficient media.

When the cut stolons were transferred to the controlled climate growth chamber, the stolon cuttings were watered daily with 3 mL of half strength Hoagland's solution.
The fresh weight of the cut stolons ranged from 140 mg to 600 mg (Table 1).
After approximately one week the percentage of stolons with primary roots ranged from 52% to 73% (Table 1).

Table 1. Selection of stolons for final data set

Between 50 and 55 stolons were cut from the stock plants and weighed after cutting.
The initiation of primary roots in vermiculite was recorded in each experiment and varied between genotypes and experiments. Eight and six stolons were grown for each treatment group and genotype in experiments I (A) and II (B) respectively. The final number of plants used in each data set was determined by removing plants that had a harvested root-to shoot-ratio outside the range of the group due to the late initiation of primary roots and poor growth.
Three plants were tested at each time point of experiment III (C). The roots were pooled for analysis and the leaves were tested individually.

Experiment			43-7	45-14	47-9
A.	I	Mean weight	209 mg	319 mg	348 mg
		Weight range	170 mg – 250 mg	200 mg – 450 mg	250 mg – 530 mg
		Primary root initiation	54% in 9 days	58% in 9 days	57% in 9 days
Plants in dataset		+P	8	8	6
		-P	8	8	7
B.	II	Mean weight	176 mg	206 mg	302 mg
		Weight range	140 mg – 260mg	170 mg – 250mg	210 mg – 420mg
		Primary root initiation	62% in 7 days	73% in 6 days	67% in 7 days
Plants in dataset		+P	6	6	5
		-P	6	6	5
		-S	5	6	6
C.	III	Mean weight	Not tested	332mg	260mg
		Weight range	-	260 mg – 420mg	160 mg – 430mg
		Primary root initiation	-	72.5% in 7 days	58.8% in 8 days
Plants in dataset		+P	-	3 per day	3 per day
		-P	-	3 per day	3 per day

2.2.2. The hydroponic growing system

The stolons were removed from the vermiculite and placed in the 50 mL falcon tubes (as an intermediary step) with half strength Hoagland's solution for up to three days before transfer to the 600 mL polyvinyl chloride (PVC) pipes containing hydroponic P-sufficient media. The intermediary step was necessary because more primary roots were initiated on the stolons without primary roots (seven days post excision) during this step compared with leaving the stolons in vermiculite. The stolons were grown in P-sufficient media in the 600 mL PVC pipes until treatment was started.

The hydroponic growing system consisted of 60 mm diameter PVC pipes (donated by Iplex pipelines, New Zealand) that were cut to 200 mm lengths with fitted caps at the base and secured with Parafilm® to prevent leakages (Figure 2.1c). The experimental system excluded light; was water-tight; and filled to 600 mL with a modified recipe of Hoagland's liquid media (Gibeaut *et al*., 1997). A lid was cut to fit the top of the pipe from waterproof foam and a 2.5 cm hole was cut in the top where a sponge was placed which held the plant in place (Figure 2.1b and c). A nylon handle was threaded through the lid so it could be easily removed from the pipe (Figure 2.1b).

The P-sufficient media contained half-strength macronutrients (1 mM KH_2PO_4, 2.25 mM $Ca(NO_3)_2$, 1.8 mM KNO_3, 1.125 mM $MgSO_4$) and full strength micronutrients at pH 6.0 (Appendix I). The media for the P-stressed plants (low P) contained 10 µM KH_2PO_4 and 3.36 mM KNO_3 was used maintain the potassium levels equivalent to the nutrient sufficient media. A sulfur depleted media (low S) was used in experiment II, and contained 28 µM $MgSO_4$, and 1.097 mM $Mg(NO_3)_2$ maintained the magnesium levels. New media and pipes were replaced at the start of the experiments (Day 0) for all of the treatment groups. The Hoaglands solution was not buffered, so the pH was monitored throughout the experiments in separate pipes that did not contain plants. Fluctuations in pH were recorded between 5.5 and 6.8 in the low P media, and between 5.9 and 6.1 in the complete or low S media (data not shown). The pH of the plants was also monitored for experiments I and II (data not shown).

In the preliminary experiments, the 600 mL of media contained in the PVC pipes was enough to sustain the plants for five days without compromising plant growth.

In experiments I, II and III, (P-stress response experiments, refer section 2.2.3) the nutrient level was kept constant by removing the equivalent of 100 mL of media per day and replaced with fresh media adjusted to pH 6.0 with KOH. After 30 days post excision, the amount of replacement media was increased to the equivalent of 150 mL per day. The term, 'equivalent' refers to 200 mL every second day, up to 30 days post excision or 300 mL every second day from 30 days post excision.

2.2.3. Experimental schedule for P-stress response experiments

The experimental design was based around the timing of lateral root formation. Three experiments were carried out to characterise the development of the primary roots and measure the responses (in terms of primary root elongation and lateral root emergence and elongation) of the three genotypes under limited P-supply (Table 2).

Experiment I (section 3.2)

The criteria for experiment I, was to start the treatment two days after the initiation of lateral roots in P-sufficient media (pre-treatment phase) and to harvest the plants between five and six weeks post excision when the elongation of the primary roots decreased in P-sufficient media. The treatment regime was carried out according to the plan for 43-7 and 45-14 and was started 15 days post excision. The stolons were harvested 35 days post excision (43-7) and 36 days post excision for 45-14.

The treatment for 47-9 was delayed by one week due to insufficient numbers of stolons with equivalent primary root length due to the variation in primary root elongation. Therefore, the treatment started 23 days post excision for 47-9, and the plants were harvested 40 days post excision, which included 17 days treatment.

The treatment schedule differed in 47-9 by three days compared with 43-7 and four days compared with 45-14, which was equivalent to an additional five (43-7) or six days (45-14) post excision (Table 2A). In experiment I, there were eight plants used in the analysis of 43-7 and 45-14 for both treatment groups (+P and –P). For 47-9, there were six plants used in the final data set for +P, and seven for the –P group (refer Table 1).

Experiment II (section 3.3)

In experiment II, the lateral roots were initiated in P-sufficient (+P and +S) or a limited nutrient supply (-P or -S), and the plants were harvested when the lateral roots became too numerous to count (Table 2B). The S–stressed plants were used to compare the responses or the roots and distribution of leaf P content in terms of a general mineral starvation response, or specific to limited P-supply. In experiment II, all of the genotypes and treatment groups contained six plants except for 43-7 (-S), 47-9 (+P) and 47-9 (-P) that had five plants analysed in the final data set (refer Table 1).

Table 2. Experimental schedule summary

Schedule for P-stress response experiments. **A.** Experiment I - initiated lateral roots at the start of treatment and harvested after the primary roots decreased elongation. **B.** Experiment II – no lateral roots initiated at the start of the experiment and harvested when the lateral roots became too numerous to count. **C.** Seven-day time course between 45-14 and 47-9 with treatment starting 21 days post excision with preliminary growth in P-sufficient media. The number of days in the pre-treatment phase is made up of root initiation in vermiculite (^{a}) and acclimatisation in P-sufficient media (^{b}). The number of days spent in each stage were based on development of the primary roots, and differed between genotypes.

A.	**Experiment I – October to December 2010**			
	Schedule	**43-7**	**45-14**	**47-9**
	Pre treatment	15 (9^{a} + 6^{b})	15 (9^{a} + 6^{b})	23 (9^{a} + 14^{b})
	Days treated	20	21	17
	Total days of experiment	35	36	40
B.	**Experiment II – February to March 2011**			
	Schedule	**43-7**	**45-14**	**47-9**
	Pre treatment	9 (6^{a} + 3^{b})	9 (6^{a} + 3^{b})	12 (7^{a} + 5^{b})
	Days treated	15	16	15
	Total days of experiment	24	25	27
C.	**Experiment III – November 2011**			
	Schedule	**43-7**	**45-14**	**47-9**
	Pre treatment	Not tested	20 (7^{a} + 13^{b})	21 (8^{a} + 13^{b})
	Days treated	Not tested	1,2,3,5,7	1,2,3,5,7
	Total days of experiment	Not tested	27	28

Experiment III (section 3.4.3)

Experiment III was a seven-day time course designed to observe when the branching zone approached 80% of the primary root length. This was between 24 and 25 days post excision and common to the three genotypes (refer Table 6). The stolons were buried in vermiculite for seven days (45-14) or eight days (47-9) to initiate primary roots then the stolons were transferred to P-sufficient media on the same day (Table 2C).
The treatment was started 21 days post excision for 45-14 and 22 days post excision for 47-9. The timing of treatment for experiment III was a more refined repeat of experiment I for 47-9 with plants harvested on day 0 (before treatment) and one, two, three, five and seven days after treatment. For experiment III, each of the time points contained three plants and there were no plants excluded from the analysis.

2.2.4. Criteria for selection of final data sets and statistical analysis

At the conclusion of the pre-treatment phase, the stolons were selected for the experiments by matching groups of two (experiment I) or three (experiment II) stolons with similar primary root length (total) and fresh weight (FW). The stolons were divided to make equal sets of stolons for each treatment group (data not shown).
In experiment I, the two treatment groups had eight plants. In experiment II each of the three treatment groups had six plants. In experiment III, each treatment group had three plants for the six time different points (0, 1, 2, 3, 5 and 7 days). The final data set excluded plants that had a harvested root-to-shoot ratio that was inconsistent with the rest of the treatment group due to poor recovery post excision, such as damaged or broken roots or wilted stolons (Table 1).

Statistical analysis

All of the analyses were carried out using Microsoft Excel, and the mean values of each of the parameters (roots, petiole length, leaf, root acid phosphatase activity) were plotted on the graphs. The error bars were calculated from the standard error of eight, six or three individual plants from each treatment group (refer Table 1). The statistical significance to $P < 0.05$ (*) and $P < 0.01$ (**) between treatment groups was analysed using the student's T-test.

The differences between complete (+PS) and P-deficient (-P) media are indicated by a red astricks (* or **). The differences between complete and S-deficient (-S) media are indicated by an olive green astricks (* or **). The differences between –P and –S are indicated by an olive green cross (✝). Significant differences between the total (⋆) and dominant primary root (*) were analysed separately in Figure 3.5.

The samples for the qPCR analysis of *PAP26, PHT1* and *PHT5* from Experiment III were pooled from three individual plants at each time point (by Susanna Leung and Afsana Islam). Statistical analysis was carried out on three technical replicates for *PAP26* and there was no statistical analysis carried out on the relative expression of the phosphate transporters *PHT1* and *PHT5*.

2.3 Analysis of root system architecture and leaf growth

The petiole and root measurements were made in mm during the course of the experiments with a Celco 30 cm hardened stainless steel ruler because it is labeled from the edge. The measurements were started at 10 am, which was equivalent to four hours after the beginning of the photoperiod (6 am), and the plants were measured in sequential order each day. The roots were out of liquid media for a maximum of five minutes while the primary root lengths were measured and the number of lateral roots was counted. The photographs presented were taken with a hand-held Sony DSC-H55 digital camera. The settings were determined automatically by the camera and are recorded in Appendix VI. The whole plant fresh weight was recorded after the final destructive harvest, and the roots and upper plant parts were weighed separately, to calculate the root to shoot ratios and estimated specific root length (SRL) (refer Table 3).

Leaf measurements and terminology

The newly emerged leaves on the main stolon were counted as they cleared the sheath that surrounds the unemerged leaves at the stolon apex (A) as a measure of leaf emergence and development (Figure 2.2a).

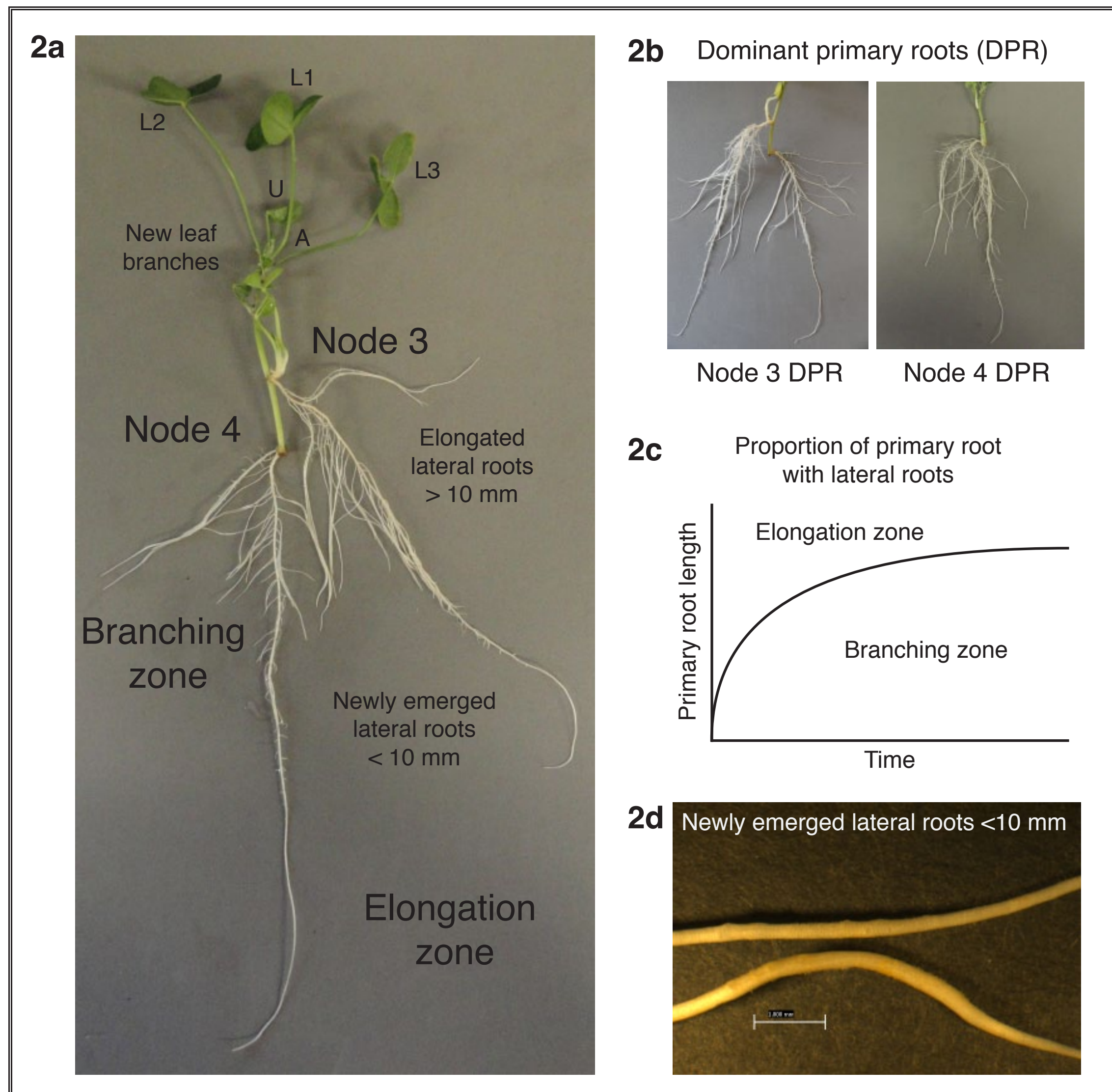


Figure 2.2. Petiole length and root system architecture measurements

a. Image of 45-14 grown in P-sufficient media for 23 days (post excision). The leaves are labeled from the apex (A). L1 is the first fully expended leaf (FFEL), and L3 is the oldest leaf. The unfolded leaf (U) was not used in the analysis. The primary roots emerge from either node three or node four. The lateral roots are visible in the branching zone. The newly emerged lateral roots (> 10 mm) were counted separately from the elongated lateral roots (> 10 mm). **b.** Stolons with the dominant primary root at node 3 (left) or node 4 (right). **c.** Schematic diagram of the branching zone that contains visible lateral roots, and the elongation zone without visible lateral roots as a proportion of the primary root length. **d.** Microscopic image showing the emerging lateral roots (top) and the region containing root hairs which is visible to the naked eye. The scale bar represents 1 μM. There were no adventitious roots formed from wounding, due to the straight cut with the dissection blade below the 4th node.

The leaves were labeled relative to the position from the apex at harvest (Figure 2.2a). The youngest (first) fully expanded leaf grown on the main stolon is designated L1 and leaf 7 (L7) the oldest (Figure 2.2a). The difference in petiole length between treatment groups was calculated and is shown as Figure 3.4 for experiment I together with the final petiole length at harvest. In Figure 3.4, (b, d and f) a positive value represents a longer petiole in the P-sufficient group when compared with the P-stressed group and a negative value represents a longer petiole in the P-stressed plants. The data for petiole lengths in experiments II and III is not shown. The leaves on the lateral branches extending from the main stolon were only measured as part of the final fresh weight during the destructive harvest at the end of each experiment.

Root measurements and terminology

The lengths of the primary roots that grew from either node three or four were measured and the number of lateral roots on each of the primary roots was counted during the course of the experiment. The total primary root length was calculated for each day that the plants were measured by adding the lengths of all of the primary roots. The relative growth rate of the primary root length was calculated during the course of the experiment by dividing the difference in primary root length (growth between days) by the primary root length on the current day of measurement (Table 3). The estimated specific root length (SRL) was calculated after the final destructive harvest by dividing the harvested fresh weight by the total primary root length at harvest (Table 3).
The specific root length is only estimation because the length of the lateral roots was not taken into account, but contributed to the total fresh weight.

All of the genotypes had a dominant primary root, which was used to represent lateral root development in response to limited P or S-supply (Figure 2.2b). The other primary roots are referred to as the “supporting primary roots” and contributed to the entire root system architecture (RSA) of the plant. The data for the number of lateral roots on the supporting primary roots is not shown. The lateral roots are in the designated branching zone, and absent from the lateral root formation zone designated ‘elongation zone’ in this study (Dubrovsky and Forde, 2012). An enlarged area between the branching and elongation zones was visible to the naked eye (because it contains a clump of root hairs), and was used to define the boundary between the two zones (Figure 2.2c).

The branching zone was determined by subtracting the elongation zone length from the primary root length and expressed as a percentage (of the primary root length).
The number of visible lateral roots less than 10 mm were counted separately from the number of lateral roots greater than 10 mm, and the total for the two parameters were added together to give the total. The length of 10 mm was chosen arbitrarily for this study to assist with quantifying lateral root emergence (<10 mm) and elongation (>10 mm). In experiment II, the longest lateral root was also measured and the emergence of the tertiary roots was recorded (refer Figure 3.17). The lateral root density was calculated for the dominant primary root (Table 3), where larger value represents less lateral roots in the branching zone compared with a smaller value that represents a greater number of lateral roots in the branching zone (refer Figure 3.12).

Table 3. Measurements and calculations for root system architecture

Abbreviations: Primary root (PR), lateral root (LR), fresh weight (FW).
#The term 'growth of primary root' refers to the primary root length on current day of measurement minus the previous day's primary root length.

Measurement	Calculation
Total primary root length	Length of dominant and supporting primary roots
Relative growth rate of PR length (%)	#Growth of PR (mm)/PR length (mm)
Branching zone (%)	PR length (mm) – elongation zone (mm)
LR density (LR/mm)	PR length (mm) -elongation zone (mm)/total LRs
Estimation of specific root length ($cm.mg^{-1}$)	Harvested root FW (mg)/total PR length (cm)

2.4 Biochemical analysis

The leaf phosphate content and the root acid phosphatase activity were determined from the individual plant material from the final destructive harvest of each experiment.

2.4.1. Quantification of leaf phosphate

A standard curve containing 0 to 200 μM KH_2PO_4 (MW 136.1 g/mol) was used to quantify the level of phosphorus in the leaves (Figure 2.3). The optical density was read at 620nm in a 96-well microtitre plate reader (Bio Strategy) and the concentration of KH_2PO_4 was calculated from the standard curve and expressed as % $P.g^{-1}$ FW.

The frozen pre-weighted leaves were ground in three volumes of 5 M H_2SO_4 in a 1.5 mL eppendorf® tube with an eppendorf® grinder. The mixture was vortexed, and then centrifuged at 13,000*g* for 10 minutes (min). The supernatant was diluted by removing 100 μL of liquid and added to 1 mL of MilliQ water. A stock solution of concentrated phosphate reagent containing 16 mM $(NH_4)6(Mo_7O_2)_4$, 0.15 mM antimony potassium tartrate oxide, and 2.25 mM H_2SO_4 was made to use for the phosphate reaction mix. Immediately prior to testing, the phosphate reaction mix for 50 reactions was made and contained 1.25 mL concentrated phosphate reagent, 5 mL of 197.58 mM ascorbic acid and 3.75 mL MilliQ water in a total of 10 mL. To test the leaf P content, duplicate assays of each leaf contained 10 μL of the diluted sample, 40 μL of MilliQ water, and 200 μL of phosphate reaction mix in a total volume of 250 μL.

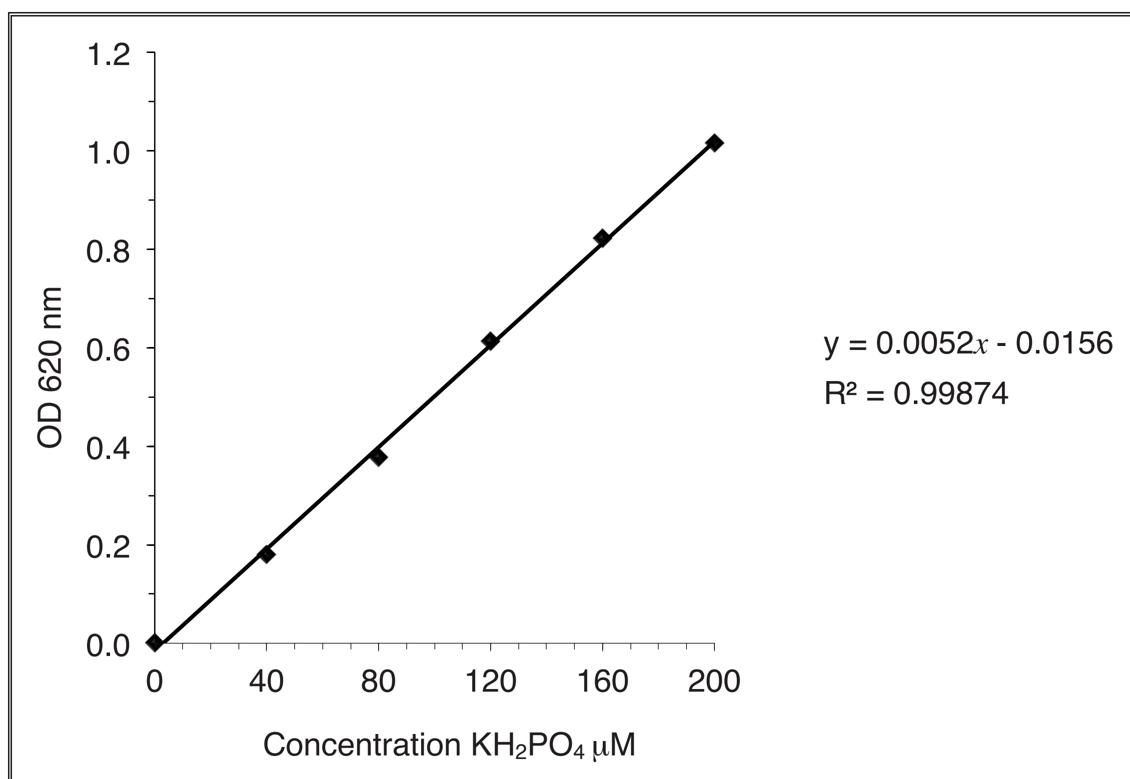


Figure 2.3. Standard curve for 200 mM KH_2PO_4

The concentration of phosphate in the leaves was determined by a standard curve of KH_2PO_4 ranging from to 200 μM in a total volume of 250 μL.

2.4.2. Acid phosphatase activity of root soluble and cell wall fractions

Extraction procedure

All of the frozen root material was ground in liquid nitrogen and a sample of approximately 300 μg was transferred and weighed into an eppendorf® tube. The root samples were resuspended in three volumes of 1 mM dithiothreitol (DTT), and then centrifuged at 6,000*g* for 10min. The supernatant was removed and designated the soluble fraction, and stored at 4°C overnight for analysis of acid phosphatase activity the following day. The pellet was washed with 100 μL of 1 mM DTT and centrifuged at 6,000*g* for five min, the supernatant discarded and the pellet washes were repeated another two times, with the last wash centrifuged at 13,000*g* for five min. The pellet was then washed with 100 μL of MilliQ water three times and centrifuged at 13,000*g* for five min each time, before being extracted overnight at 4°C with 1 M NaCl.
The amount of NaCl was approximately 50 μL and was just enough to cover the surface of the pellet. The next day, the suspended pellet was centrifuged at 13,000*g* for five min and the supernatant was collected and designated the cell wall fraction. The pellet was then resuspended in 1 M NaCl and incubated at 37°C for one hour. The suspended pellet was then centrifuged at 13,000*g* for five min and the supernatant was collected and combined with the cell wall fraction that was previously collected.

Enzyme activity

The acid phosphatase assay was modified from the method used by Zhang and McManus (2000). The 5.4 mM *p*-nitrophenyl phosphate (*p*NPP) substrate buffer was freshly made by dissolving one 5 mg tablet of *p*NPP in 2.5 mL of 0.1 M sodium citrate buffer (pH 5.6). The acid phosphatase activity was measured in duplicate by adding 20 μL of the soluble and cell wall fractions to 80 μL of MilliQ water and 100 μL of substrate buffer. The samples were incubated at 37°C, the absorbance read at 405 nm every min for a total of 30 min. The activity of acid phosphatase was calculated from the amount of *p*-nitrophenol (*p*NP) formed in 30 min from a standard curve ranging from 0 μM to 500 μM *p*NP (Figure 2.4). The soluble protein was determined (Figure 2.5), and the acid phosphatase activity was expressed as $mmol^{-1}$ min^{-1} mg.

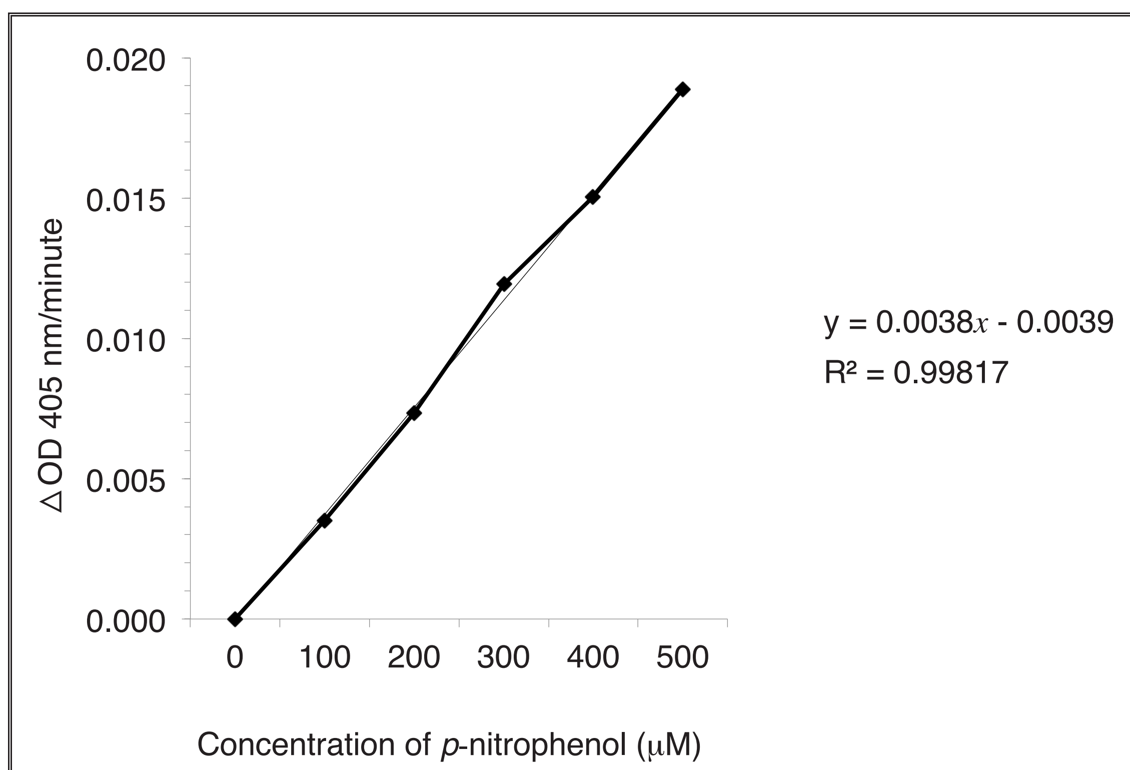


Figure 2.4. Standard curve for p-nitrophenol

Standard curve for *p*-nitrophenol. The enzyme assay was measured over 30 min at 405 nm and the change was calculated to give a rate per minute.

2.4.3. Quantification of soluble protein

A standard curve was used to quantify the amount of soluble protein in 1 mg to 5 mg of bovine serum albumin (BSA) (Figure 2.5). The microtitre plates contained the various amounts of BSA in 160 μL with 40 μL of Bio-Rad protein dye then added to give a final volume of 200 μL and the absorbance read at 595 nm in a 96-well Bio Strategy microtitre plate reader at room temperature. The quantity of soluble protein in the soluble and cell wall fractions of the roots used to determine the acid phosphatase activity described in section 2.4.2 was then determined. The assay contained 10 μL of protein extract with 150 μL Milli-Q water and then 40 μL of Bio-Rad protein dye.

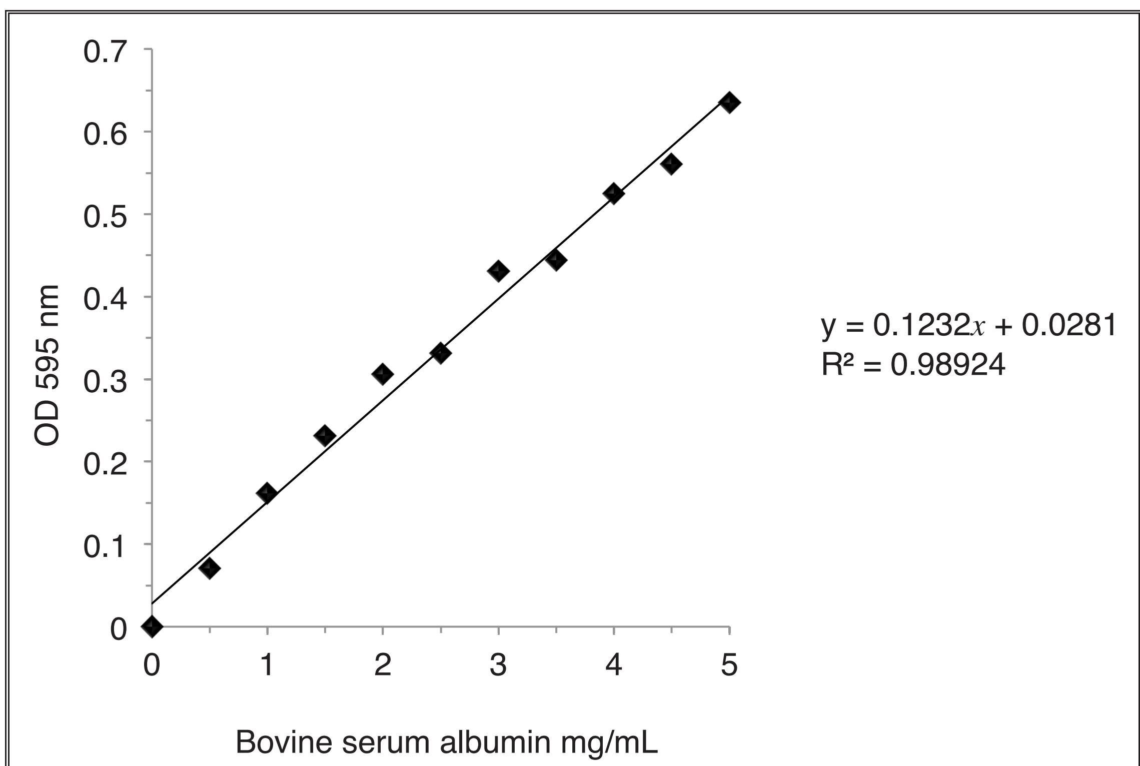


Figure 2.5. Standard curve for 1 mg/mL to 5 mg/mL bovine serum albumin

A standard curve of Bovine Serum Albumin (BSA) ranging from 1 mg/mL-5 mg/mL to quantify the amount of soluble protein in 200 μL assay containing 40 μL and 160 μL protein.
The absorbance was read at 595 nm in a 96-well Bio Strategy microtitre plate reader at room temperature and the curve was constructed.

2.5 Expression of *PAP26* by quantitative PCR

The methods were provided by Susanna Leung who performed the quantitative polymerase chain reaction (qPCR) analysis of the *PAP26* gene on the plant material from experiment III.

2.5.1. Preparation of chemicals and materials for RNA extraction

The glassware, which included Schott® bottles, beakers, measuring cylinders, mortars, pestles and spatulas were wrapped individually in foil and baked at 180°C overnight. Di-ethyl pyrocarbonate (DEPC) treated water was prepared by adding 1 mL of DEPC into 1 L of Milli Q water in the fume hood, and then stirred overnight at room temperature. It was then autoclaved before it was used. The plastic ware and stirring bars were sterilised by treating with 3% (v/v) hydrogen peroxide (H_2O_2) overnight and rinsed the next day with DEPC-treated water. The 30% (v/v) H_2O_2 stock was stored in the cold room (4°C). The disposable tips, falcon tubes and microtubes were handled with gloves, and not treated with 3% (v/v) H_2O_2.

Hot borate buffer was used for the RNA extraction. The chemicals in the extraction buffer were dissolved in pre-warmed DEPC-treated water, and the pH was adjusted to pH 9 with 5 M NaOH, and then it was autoclaved (Appendix II). The 10 mM DTT was prepared from 1 M stock solution with DEPC-treated water, which was stored at -20°C.

2.5.2. RNA Extraction and DNase I treatment

The RNA was extracted using the Hot Borate Method followed by DNase I treatment. The extraction buffer was pre-warmed to 80°C and 1.0 mL was added to the ground tissue in a 1.7 mL microtube and resuspended by vortexing followed by the addition of 7.5 μL Proteinase K and incubated at 42°C for 1.5 hours with continuous shaking. After the incubation, 0.08 volumes (80 μL) of 2M KCl was a added and swirled gently on ice for 30 min, and centrifuged at 14,000 rpm for 20 min at 4°C. The supernatant was transferred into a fresh 2 mL centrifuge tube, and 800 μL (0.8 x volumes) of cold 4 M LiCl was added. The RNA samples were left overnight at 4°C to precipitate.
The following day, the precipitate was centrifuged at 14,000 rpm at 4°C for 30 min, and the supernatant was discarded.

The pellet was resuspended in 200 μL of DEPC-treated water, 20 μL of 3 M sodium acetate (pH 5.2), and 200 μL of chloroform/isoamyl alcohol, (24:1) were then added and samples mixed by vortexing and centrifuged at 14,000 rpm for 5 min at 4°C.
The aqueous phase (top layer) was transferred to a fresh 1.7 mL microtube, and the chloroform/isoamyl alcohol step was repeated by adding 200 μL chloroform/isoamyl alcohol (24:1) mixing by vortex before centrifuging at 14,000 rpm for 5 min at 4°C.
The aqueous phase (top layer) was transferred to a fresh 1.7 mL microtube and 200 μL (1 volume) of isopropanol was added and incubated on ice for 1 hour followed by centrifugation at 14,000 rpm for 30 min at 4°C. The pellet was washed with 80% (v/v) ethanol and centrifuged at 14,000 rpm for 10 min. The supernatant was decanted and the pellet was air-dried for 10 min. The RNA was resuspended in 500 μL of DEPC-treated water with 400 μL (0.8 x volume) of cold 4 M LiCl, added, and the RNA left to precipitate overnight at 4°C. The following day, the precipitate was collected after centrifugation at 14,000 rpm at 4°C for 30 min, and the supernatant was discarded.
The pellet was washed with 2 M LiCl, and centrifuged at 14,000 rpm for 10 min, and the supernatant was discarded. The pellet was washed with 80% (v/v) ethanol and centrifuged at 14,000 rpm for 10 min and air dried for 10 min and the supernatant was discarded. The pellet was washed with 80% (v/v) ethanol and centrifuged at 14,000 rpm for 10 min and air dried for 10 min. The RNA was resuspended in 20 μL of DEPC-treated water, and then quantified. For the DNase treatment, the RNA was adjusted to 45 μL with DEPC-treated water and 5 μL of 10-fold DNase reaction buffer and 1 μL of DNase I (10 U Roche) added and the reaction incubated at 37°C for 20 min. The DNase reaction was stopped by adding 2 μL of 0.2M EDTA (pH 8.0) followed by heat inactivation at 75°C for 5 min. An extra 450 μL of DEPC-treated water was added to the DNase-treated RNA samples, and they were precipitated with 400 μL (0.8 times volumes) of 4M LiCl added and the RNA left to precipitate at 4°C overnight.
The following day, the samples were centrifuged at 14,000 rpm for 30 min at 4°C and the supernatant was discarded. The pellet was washed with 80% (v/v) ethanol and centrifuged at 14,000 rpm for 10 min. The pellet was air-dried for 10 min, and the RNA was re-suspended in 20 μL of DEPC-treated water and stored at minus 80°C.

CHAPTER 3. RESULTS

3.1 Preliminary experiments to optimise root analysis

The root system architecture (RSA) of white clover stolons that is based on growth post excision from the main plant differs from the single taproot found in *Arabidopsis* that are typically grown as seedlings for experiments. Two preliminary experiments were carried out to determine the timing of primary root and lateral root initiation during development (Table 4a). The preliminary experiments were also used to test the hydroponic PVC pipe system for limitations such as root length and nutrient supply before the phosphorus (P) experiments were started. The PVC pipes conferred no limitations on primary root length when the plants were grown for up to 42 days (Figure 3.1 right).

3.1.1. Primary root initiation and timing of lateral root development

The number of stolons that initiated primary roots differed between the genotypes, and 47-9 was the most variable. In 47-9 the majority of primary roots were less than 10 mm after seven days, and ranged from 3 mm to 20 mm. All of the stolons were left in vermiculite for a further three days to allow 47-9 to elongate the primary roots before being transferred to liquid media containing 1 mM KH_2PO_4. The number of stolons cut was increased to between 50 and 55 for the P-stress response experiments to allow for the slower elongation of initiated primary roots in 47-9 (refer Table 1). The primary roots of all three of the genotypes responded well to the liquid by rapidly elongating before initiating lateral roots after approximately 13 days post excision (Table 4a). The initiation of tertiary roots (3° roots) and growth of secondary stolons occurred between 19 to 27 days post excision. After 24 days post excision, the number of lateral roots became numerous to count, and branching zone was approximately 80% of the primary root length (Table 4a). The branching zone increased to between 85% and 90% of the primary root length between 25 and 30 days post excision, and was maintained when the plants were grown for six weeks (data not shown). The growth of the stolons increased between 30 and 42 days post excision, which is apparent from the photograph plates in Figure 3.1 that compare the growth 28 days post excision (left) with 42 days post excision (middle and right).

In the second preliminary experiment, the plants were harvested 28 days post excision, which included 17 days in liquid media. The heaviest of the three genotypes was 45-14, that also had significantly longer primary roots than 43-7 and 47-9 (Table 4b).

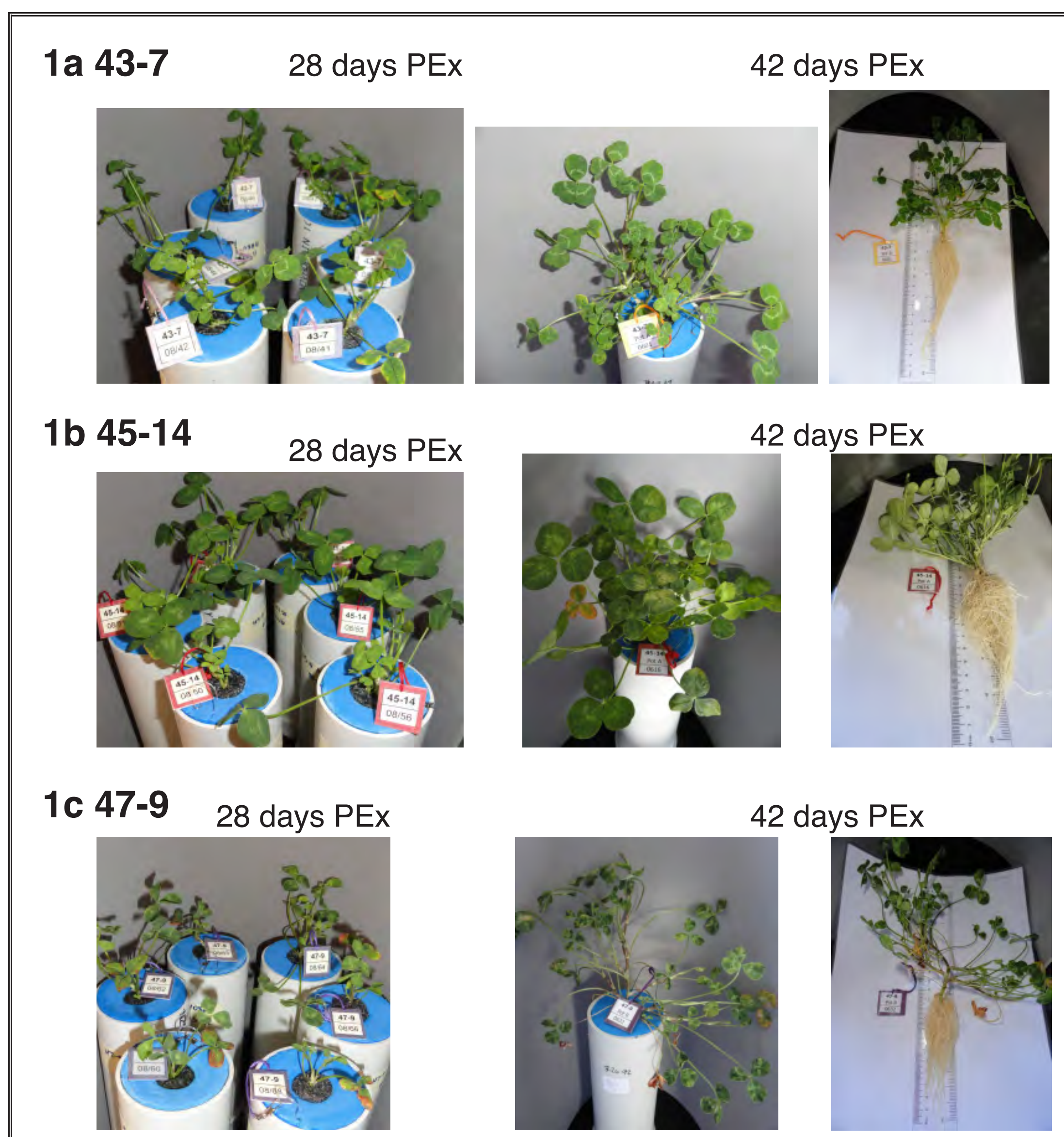


Figure 3.1. Growth in P-sufficient after 28 and 42 days

Preliminary experiments in 600 mL of P-sufficient media for 28 and 42 days post excision (PEx). **a.** 43-7 Small leaves with multiple branches off the main stolon and long roots **b.** 45-14 Medium sized leaves with long roots **c.** 47-9 Medium sized leaves with low branching off the main stolon, short dense roots and high variability between stolons. Six plants were grown in each of the experiments with one third (200 mL) of the media changed every second day up to 30 days and then increased to half (300 mL) every second day.

The harvested fresh weight of 47-9 ranged between 0.72 g and 2.3 g (Table 4b), with a significantly lower proportion of roots (and higher shoots) compared with 45-14.
The analysis was based on six plants for each genotype except 47-9 that had two plants excluded from the data set due to poor growth, which was defined by the harvested root-to-shoot ratio well outside the range of the group. This exclusion criterion for the data sets was also applied to the P-stress response experiments.

Table 4. Summary of development in P-sufficient hydroponic media

A. Observations of stolon development from preliminary experiments of the three genotypes in P-sufficient media after post excision. Day 0 represents excision from the stock plant. Branching zone (BZ). 2° stolons refers to lateral stolons extending from the main stolon. **B.** Final harvested fresh weight (FW), root biomass ratio and dominant primary root length from the second preliminary experiment after 28 days post excision. Calculations based on six plants for 43-7 and 45-14, and four for 47-9. There were no significant differences between 43-7 and 47-9 using the student's T-test. The differences between 43-7 and 45-14 are indicated with an asterisks (*P<0.05, **P< 0.01 and the differences between 45-14 and 47-9 are indicated with a star (★P<0.05, ★★P<0.01).

A. Days post excision	Growth of roots	Number of emerged leaves
0	3rd and 4th nodes cut from main stolon buried in vermiculite	1 unfolded leaf
5 to 8	Primary root initiation in vermiculite	1
9 to 12	Primary root elongation (liquid media)	2
13 to 14	Initiation of lateral roots	3
16 to 18	Initiation of tertiary roots	4 start of 2° stolons
24 to 25	BZ approximately 80% of primary root length	5 start of 2° stolons
26 to 30	BZ between 80% and 85% of primary root length	6 with 2° stolons
35 to 40	Relative growth of primary roots maintained below 8%	7 with 2° stolons

B. Growth after 27 days post excision	43-7	45-14	47-9
Fresh weight (FW)	1.64 g**	2.63 g**★	1.25 g★
Range in biomass	1.07 g to 2.19 g	1.89 g to 3.14 g	0.72 g to 2.3 g
% of root biomass to total FW	25%	26%★	18%★
Dominant primary root length	138 mm*	172 mm*★★	98 mm★★

3.2 Experiment I – Reduced P-supply after lateral root emergence

The treatment regime for experiment I was to reduce the P-supply two days (decided arbitrarily) after the emergence of visible lateral roots, which was 15 days post excision. The treatment regime was carried out for 43-7 and 45-14 and the plants were harvested after 20 and 21 days treatment respectively (Figure 3.2a, b, d and e). In 47-9, the emergence of the primary roots at nine days post excision was comparable with the other two genotypes, at 57% (Table 1A). However, at 15 days post excision, only 20 of the stolons survived (40%) and the primary root length ranged from 9 mm to 78 mm, with a mean primary root length of 41 mm (data not shown). Therefore, the 20 stolons were left in P-sufficient media for an extra seven days (23 days post excision) to allow selection of stolons with primary root over 10 mm. At 23 days post excision, the primary root lengths ranged from 16 mm to 95 mm. The plants were harvested after 17 days treatment, (40 days post excision). The initial slower elongation of the primary roots in 47-9 did not differ from the developmental pattern of the primary roots that was observed in the other two genotypes, and will be discussed in section 3.4.2 (Table 6). The treatment regime for 47-9 in experiment I was addressed again in experiment III (presented in section 3.4.3).

3.2.1. Final effects of P-stress

The plant material from the final destructive harvest was used to determine the fresh weight, root-to-shoot ratio, acid phosphatase activity, leaf fresh weight and leaf P content to assess the degree of P-stress in the three white clover genotypes (Figure 3.2 and Figure 3.3). A significant reduction in biomass was observed between treatment groups for 43-7 and 45-14, and was not observed in 47-9 (Figure 3.2g). The mean fresh weight of P-sufficient plants ranged from 3.3 g (47-9) to 6.0 g (45-14), and fresh weight of the P-stressed plants ranged from 1.4 g (43-7) to 3.4 g (47-9). The difference in total fresh weight biomass between the treatment groups was 58% for 43-7 and the difference was 59% for 45-14 (Figure 3.2g). There were no significant differences in the harvested fresh weights of the treatment groups in 47-9 (Figure 3.2g).

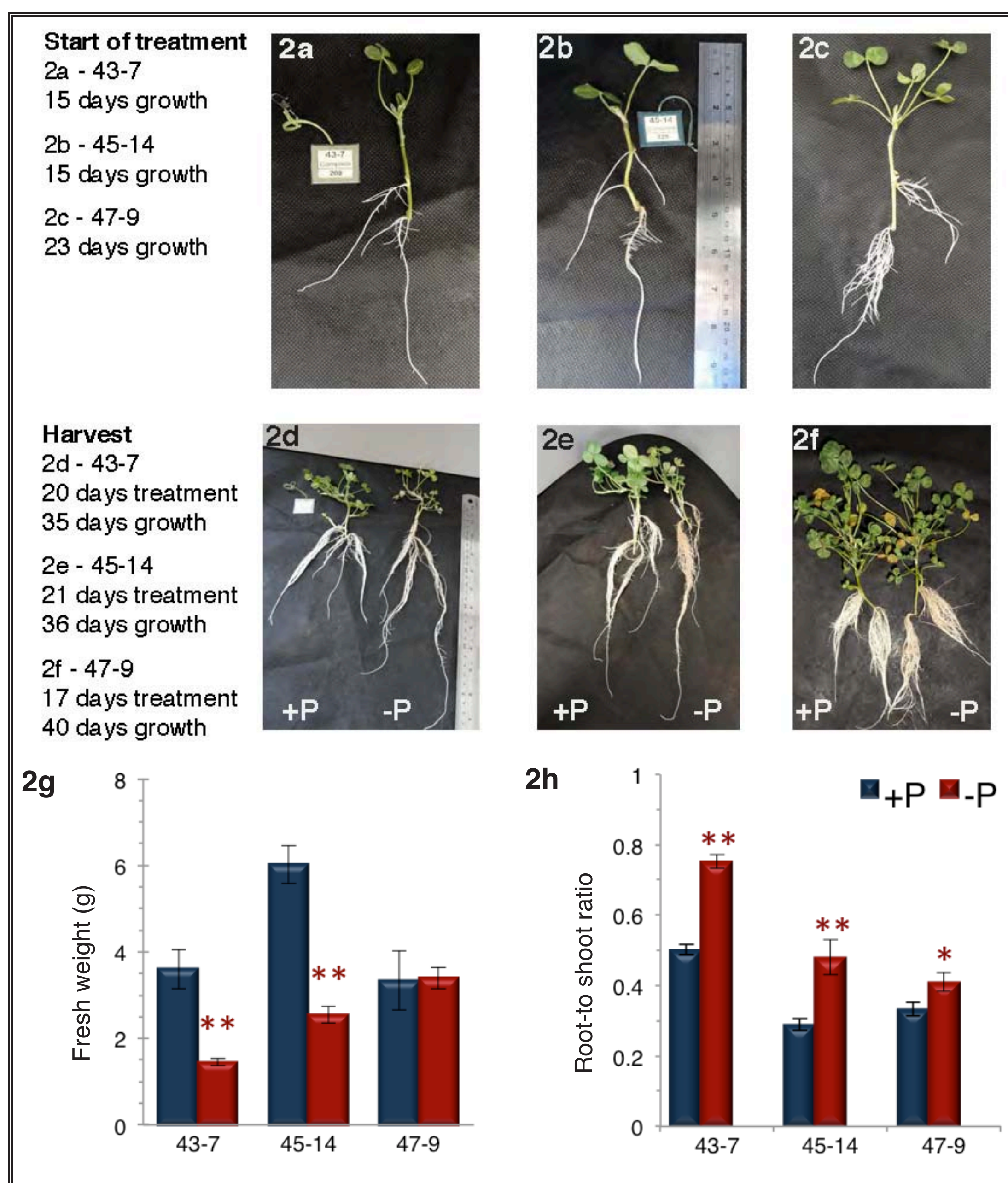


Figure 3.2. Growth of 43-7, 45-14 and 47-9 in limited P-supply

a. 43-7, **b.** 45-14, **c.** 47-9. Genotypes at the start of treatment. **d.** 43-7, **e.** 45-14, **f.** 47-9. Harvested plants with the number of days treatment and age post excision (growth) indicated. The P-sufficient plants (+P, left in photographs, and blue in graphs) were grown in full media containing 1 mM KH_2PO_4, and the P-stressed plants (-P, right in photographs, red in graphs) were grown in 10 μM KH_2PO_4. **g.** Mean fresh weight of harvested plants. **h.** Root-to-shoot ratios. Significant differences P <0.05 (*) P <0.01 (**), and were analysed using the student's t-test from a minimum of six plants. The camera settings for the photographs are recorded in Appendix V.

The proportion of root biomass in the P-sufficient plants was 33% in 43-7 compared with the lower proportions of 25%, 23% observed in 45-14 and 47-9 (data compared for experiment II and III in Appendix III). The P-stressed plants had significantly higher root biomass compared with the P-sufficient plants in all three of the genotypes, with the differences of 33% (43-7), 40% (45-14) and 19% in 47-9 (Figure 3.2h). The higher root biomass in the P-stressed plants contributed to the increased root-to-shoot ratio, and indicates that limited P-supply affected the genotypes at a whole plant level, and is a signature response to P-stress (Figure 3.2g, h).

The acid phosphatase activity in the roots and the reduction in leaf P content also indicate local responses to P-stress in the respective plant parts (Figure 3.3). The acid phosphatase activity in the soluble fraction (SF) represents the P pool in the vacuole, and it was significantly increased in the P-stressed roots of all three genotypes (Figure 3.3a). The acid phosphatase activity in the cell wall fractions of the P-stressed plants was increased in 43-7 and 45-14, but an increase in the cell wall (CW) fraction was not observed in 47-9 (Figure 3.3a).

Leaf P content and fresh weight

The leaves were removed at harvest, and the fresh weight and P content were measured (Figure 3.3b, c). Leaf 1 (designated L1), and leaf 3 (designated L3), were initiated in the respective treatment media. The age of the leaf at harvest was determined by recording the emergence during the course of the experiment as an indication of when P became a limiting factor for growth (Figure 3.4b, d, f). There was a large range of P-content in the P-sufficient leaves, and it was higher in L1 compared with L3 in all three of the genotypes (Figure 3.3b). The minimum P content in the P-stressed leaves of all three of the genotypes ranged from 0.03g to 0.05g 100g FW^{-1}. In 43-7, the fresh weight (FW) of L1 and L3 were significantly reduced in the P-stressed plants, which correlated with a lower P content when compared with the P-sufficient leaves (Figure 3.3b, c, left).
In 45-14, there was a significant reduction in fresh weight in L3 that had similar P-content in both treatment groups, and in L1 the P-content was reduced but the fresh weights were similar (Figure 3.3b, c, middle). In 47-9, L1 and L3 had a higher fresh weight in the P-stressed treatment group and a significantly lower P content when compared to the P-sufficient leaves (Figure 3.3b and c, right).

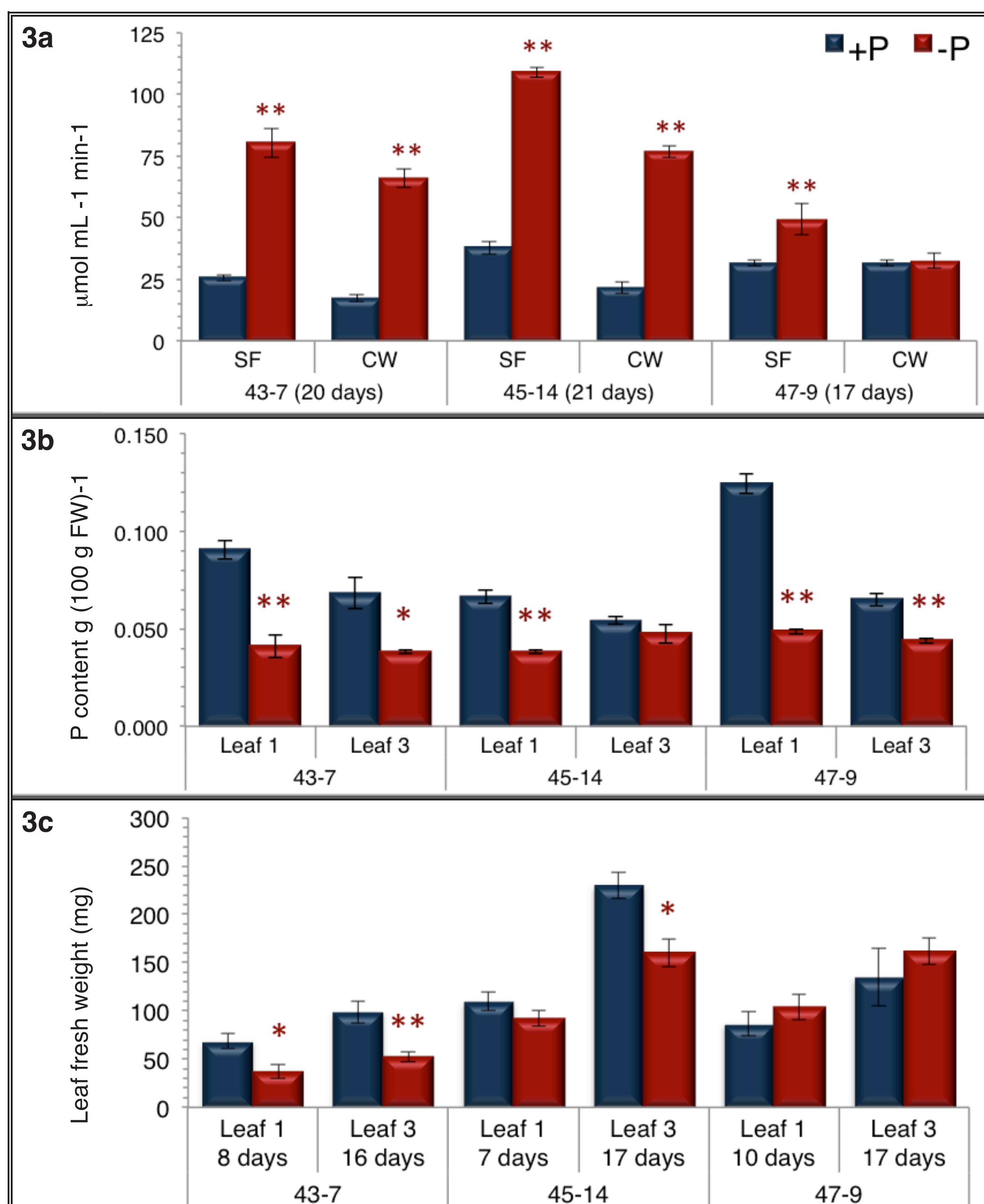
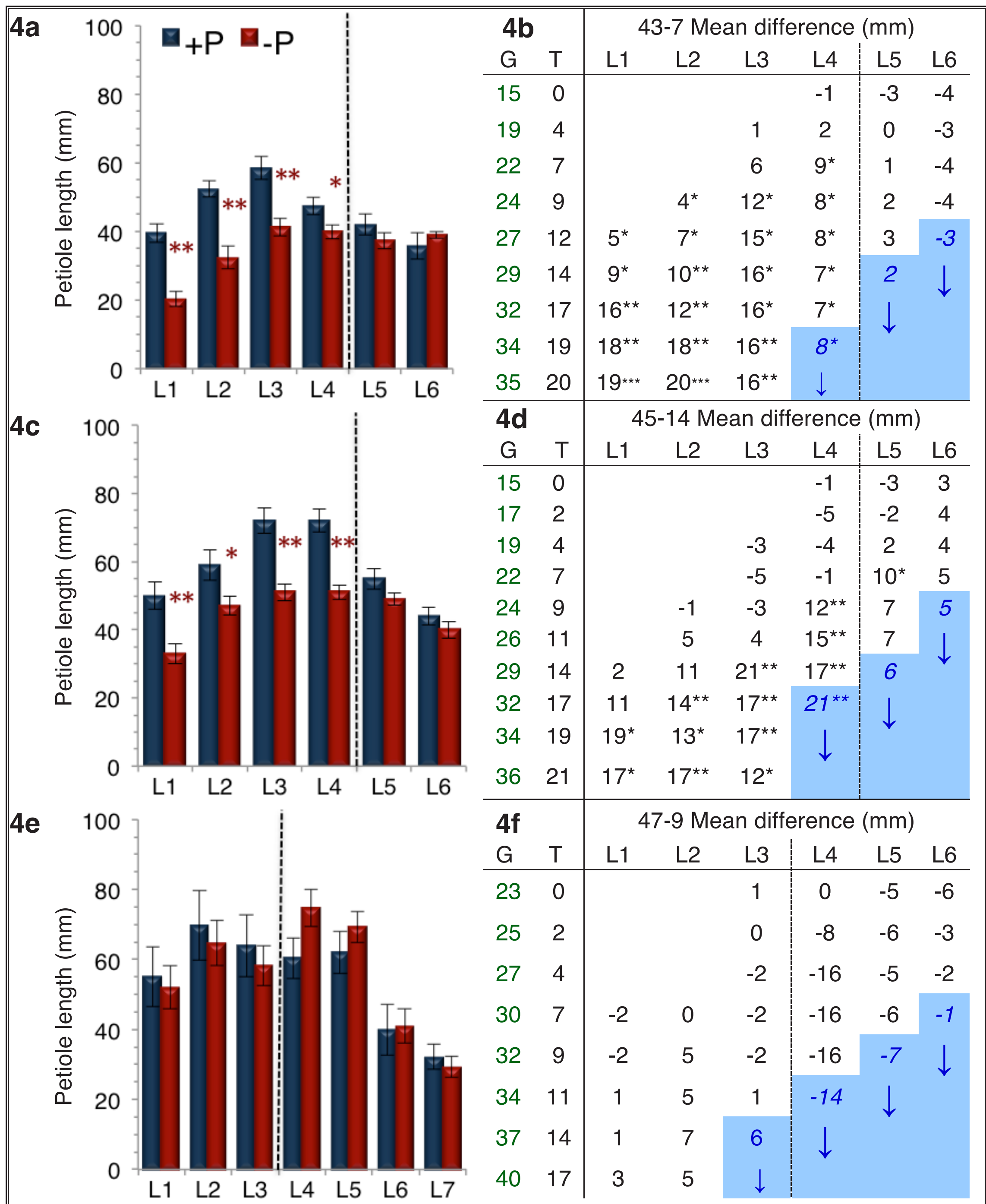


Figure 3.3. Root acid phosphatase activity and leaf phosphate content

a. Acid phosphatase activity in the soluble (SF) and cell wall (CW) fractions of the harvested roots after 20 (43-7), 21 (45-14), and 17 (47-9) days treatment. **b.** Phosphate content in L1 and L3. **c**. Fresh weight of Leaf 1 (L1) and Leaf 3 (L3). Both of the leaves harvested were grown in the respective treatment media in all three of the genotypes, with the age on the day of harvesting indicated. The significant differences and standard errors P <0.05 (*), P<0.01 (**) were analysed using the student's t-test from a minimum of six plants.



4b

G	T	43-7 Mean difference (mm) L1	L2	L3	L4	L5	L6
15	0				-1	-3	-4
19	4			1	2	0	-3
22	7			6	9*	1	-4
24	9		4*	12*	8*	2	-4
27	12	5*	7*	15*	8*	3	-3
29	14	9*	10**	16*	7*	2	↓
32	17	16**	12**	16*	7*	↓	
34	19	18**	18**	16**	8*		
35	20	19***	20***	16**	↓		

4d

G	T	45-14 Mean difference (mm) L1	L2	L3	L4	L5	L6
15	0				-1	-3	3
17	2				-5	-2	4
19	4			-3	-4	2	4
22	7			-5	-1	10*	5
24	9		-1	-3	12**	7	5
26	11		5	4	15**	7	↓
29	14	2	11	21**	17**	6	
32	17	11	14**	17**	21**	↓	
34	19	19*	13*	17**	↓		
36	21	17*	17**	12*			

4f

G	T	47-9 Mean difference (mm) L1	L2	L3	L4	L5	L6
23	0			1	0	-5	-6
25	2			0	-8	-6	-3
27	4			-2	-16	-5	-2
30	7	-2	0	-2	-16	-6	-1
32	9	-2	5	-2	-16	-7	↓
34	11	1	5	1	-14	↓	
37	14	1	7	6	↓		
40	17	3	5	↓			

Figure 3.4. Final petiole lengths and difference between treatments

a, b 43-7, **c, d.** 45-14, and **e**, **f.** 47-9. Final length (left). Mean difference in petiole length (right). The dotted line represents the start of treatment, and leaves to the left were initiated in the treatment media. Longer petioles in +P are indicated by a positive number. The blue shaded region indicates when the petiole stopped growing. Abbreviations: G (days growth post excision, green). T (days treated). Significant differences (*P<0.05, **P<0.01) and standard errors were analysed using the student's t-test from a minimum of six plants.

3.2.2. Leaf development

As the leaves emerged, the lengths of the petioles were measured as an indication of active growth, and the final lengths from the harvested plants were plotted (Figure 3.4). The tables in Figure 3.4 (b, d and f) show the difference in petiole lengths between the two treatments during the course of the experiment. Each of the genotypes had six leaves on the main growing stolon, except 47-9, which had seven leaves. The first recorded difference in petiole length of the respective leaves indicate when that leaf emerged from the leaf sheath in the P-sufficient treatment group, but was delayed in the P-stress plants. For example in 43-7, L3 emerged 19 days post excision (4 days treatment) and the petioles were 1 mm longer in the P-sufficient plants (Figure 3.4b). The leaves (L5 and L6 for 43-7 and 45-14, and L4 to L7 for 47-9) to the right of the tables in Figure 3.4 were initiated in P-sufficient media (pre-treatment), and the leaves to the left of the dotted line were initiated in the treatment media (Figure 3.4b, d, f). The petiole of the first leaf that was initiated post excision, and stopped elongating from 24 days (45-14) post excision (Figure 3.4d) and are represented by the blue shaded region. L7 stopped growing two days after treatment began (25 days post excision), so the growth data is not shown. In 43-7 and 45-14, the final lengths of L1, L2, L3 and L4 petioles were significantly reduced in the P-stressed (Figure 3.4a and c). The differences in petiole length can be traced to day seven (22 days post excision) for both genotypes (indicated below the horizontal dotted line Figure 3.4b and d).

The emergence of new leaves (L4, L3, L2 and L1) in limited P-supply was delayed in 43-7, which reduced the petiole length, fresh weight and P-content at harvest (Figure 3.4a, b). The reduced petiole length of the P-stressed plants in 45-14 was due to the slower elongation and not from the delayed emergence that was observed in 43-7 (Figure 3.4d). The limited P-supply stimulated the elongation of the petioles in 47-9 (L5 and L4), and the negative values on day seven represent the reduced petiole length of the P-sufficient plants (Figure 3.4f).

The difference in petiole length between treatment groups occurred before the first signs of necrosis were observed in the P-stressed leaves. In 45-14, the first signs of necrosis were observed after nine days treatment and after 11 days treatment for 43-7 and 47-9.

This was respectively equivalent to 24, 26 and 34 days post excision for 43-7, 45-14 and 47-9 (data not shown).

The timing of secondary stolons and leaves that grow laterally from the main stolon differed between the genotypes (through genotypic variation) and treatment groups (data not shown). In 43-7, the secondary stolons were observed 19 days post excision (+P) and were delayed by eight days (27 days post excision) in the P-stressed plants (data not shown). The development of the secondary stolons occurred 24 days post excision in 45-14, and was delayed by five days under limited P-supply (29 days post excision). In 47-9, the development of secondary stolons occurred 27 days post excision and (this branching) was not affected by the limited P-supply (data not shown).
The secondary stolons and leaves contributed to the leaf fresh weight biomass that was observed at harvest (Figure 3.2g).

3.2.3. Root system architecture

The relative growth rate of the dominant primary root length followed the same trend as the total primary root length for all three genotypes (Figure 3.5d, e, and f).
The treatment media had no effect on the emergence of supporting primary roots during the course of the experiments. The relative growth rate of the primary root length was consistent with the preliminary experiments, which confirmed that the dominant primary root alone was suitable for representing the morphology of each genotype when grown in limited P-supply. In Figure 3.5a, b and c, the dark bars represent the dominant primary root length and the opaque bars represent the total primary root length.

The newly emerged lateral roots that were less than 10 mm were counted separately from the elongated lateral roots that were greater than 10 mm in length until they were too numerous to count (Figure 3.7, left and middle). This was after nine days of treatment for 43-7 and 45-14, (24 days post excision, Figure 3.7a, b), and four days of treatment (27 days post excision) for 47-9 (Figure 3.7c). The length of 10 mm was chosen arbitrarily to capture the three-dimensional nature of lateral root development together with the branching zone (refer Figure 2.2). The growth in the branching zone represents dominant primary root elongation and lateral root emergence after the lateral roots could be physically counted.

Two observations were consistent in the roots of the three genotypes. The first observation was that all of the genotypes displayed a response to the reduced P-supply within seven days from the start of treatment. This is important because the reduced P-supply was started 23 days post excision for 47-9, compared with 15 days post excision for the other two genotypes (Figure 3.2c). The second observation was that at 24 to 25 days post excision, the branching zone represented 80% of the dominant primary root length in the three genotypes (indicated in blue on the x-axis of Figure 3.6a, b and c). For 43-7 and 45-14, 25 days post excision is equivalent to nine days treatment, and two days treatment for 47-9. It is also important to note that the dominant primary root continued to elongate until harvested because the branching zone was maintained between 80% and 85% of the dominant primary root length from 30 days post excision in all of the genotypes. This is is equivalent to 15 days treatment for 43-7 and 45-14 compared with seven days treatment for 47-9 (Figure 3.6d, e, f). The consistency of the proportions of the branching zone as a percentage of the primary root length with respect to time indicate that a common developmental pattern is shared that is independent of P-supply.

A detailed description of the growth of the roots (and differences between treatment groups) in terms of primary root elongation and lateral root emergence is described for each of the genotypes later in this section. In summary, the genotypes differed in the contribution of the dominant primary root to the entire root system architecture of the plant with 43-7 having more supporting primary roots, compared with 47-9. In 47-9, the primary roots elongated in response to the limited P-supply with no differences in the branching zone or number of lateral roots during the time they were able to be counted, which was 27 days post excision (Figure 3.5c, Figure 3.6c, f, Figure 3.7c). The relative growth rate of the primary roots increased in 43-7 and 45-14 in response to the limited P-supply, and resulted in longer primary roots in 43-7, but not in 45-14 (Figure 3.5a, b, c, d). The limited P-supply reduced the emergence of visible lateral roots and reduced the branching zone as a proportion of the primary root length in 45-14 (Figure 3.6e and Figure 3.7b). In 43-7, the number of lateral roots greater that 10 mm was decreased on day four of treatment, with no further differences between treatment groups during the course of the experiment (Figure 3.7a).

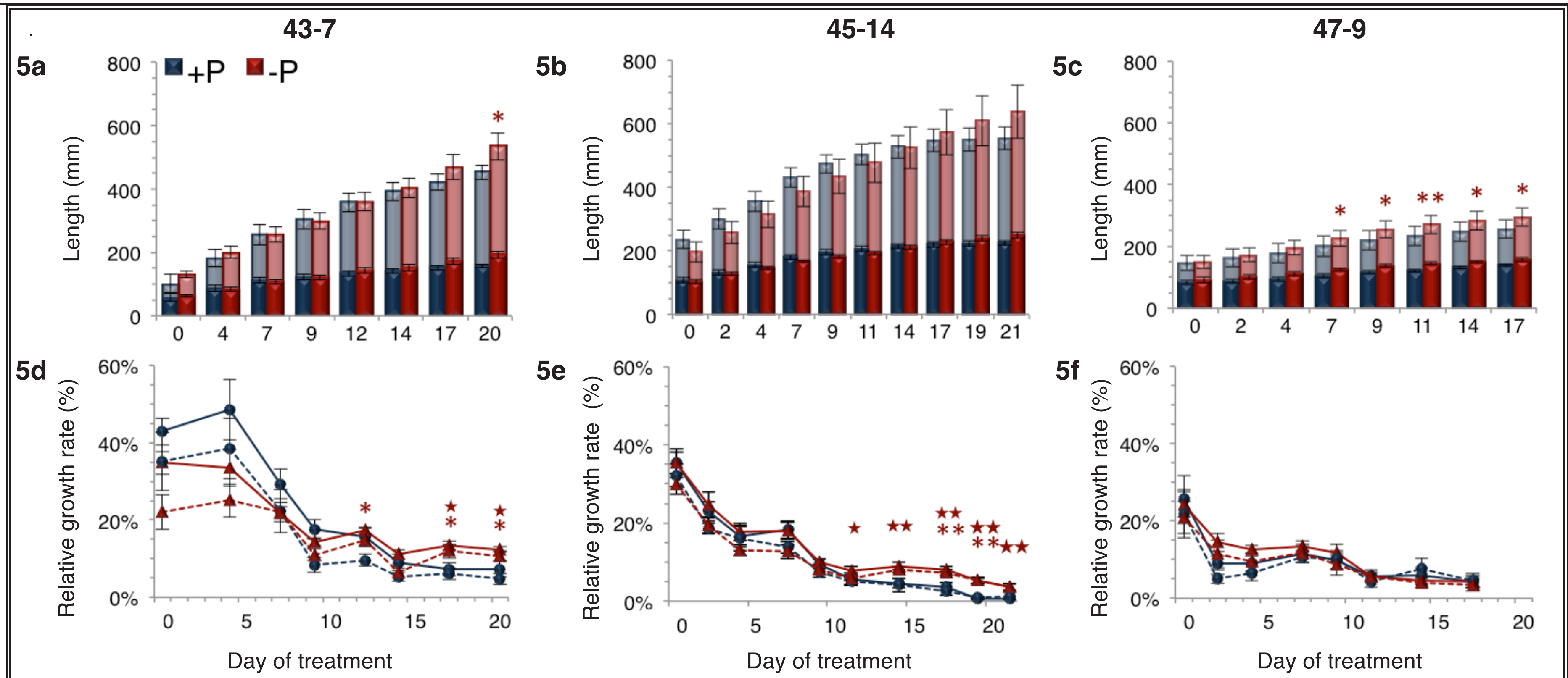


Figure 3.5. Primary root length and relative growth rate

a. 43-7, **b.** 45-14, **c.** 47-9. Dominant primary root length (dark) as a proportion of the total primary root length (transparent) **d.** 43-7, **e.** 45-14, **f.** 47-9. Relative growth rate of total length (solid line —), and dominant primary root length (dashed line ---). The significant differences and standard errors for total length P< 0.05 (★) P<0.01 (★★) and dominant primary root length P < 0.05 (*), P<0.01 (**) were analysed using the student's T-test from a minimum of six plants.

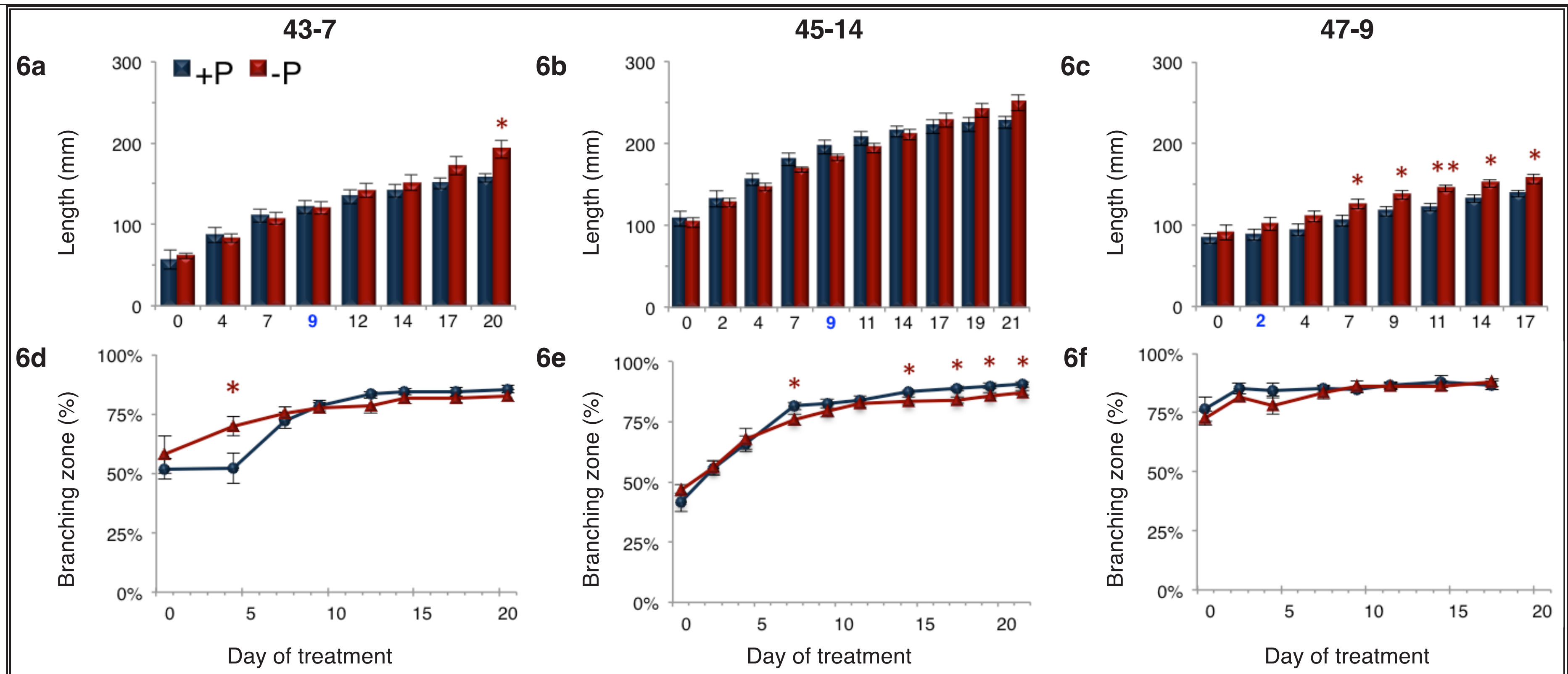


Figure 3.6. Dominant primary root length and branching zone

a. 43-7, **b**. 45-14, **c**. 47-9. Length of dominant primary root. The day (of treatment) that the branching zone is 80% (9 days or 2 days for 47-9) of the primary root length is marked in blue on the x axis. **d**. 43-7, **e**. 45-14, **f**. 47-9. Proportion of visible lateral roots in the branching zone of the dominant primary root. The significant differences and standard errors $P < 0.05$ (*), $P<0.01$ (**) were analysed using the student's T-test from a minimum of six plants.

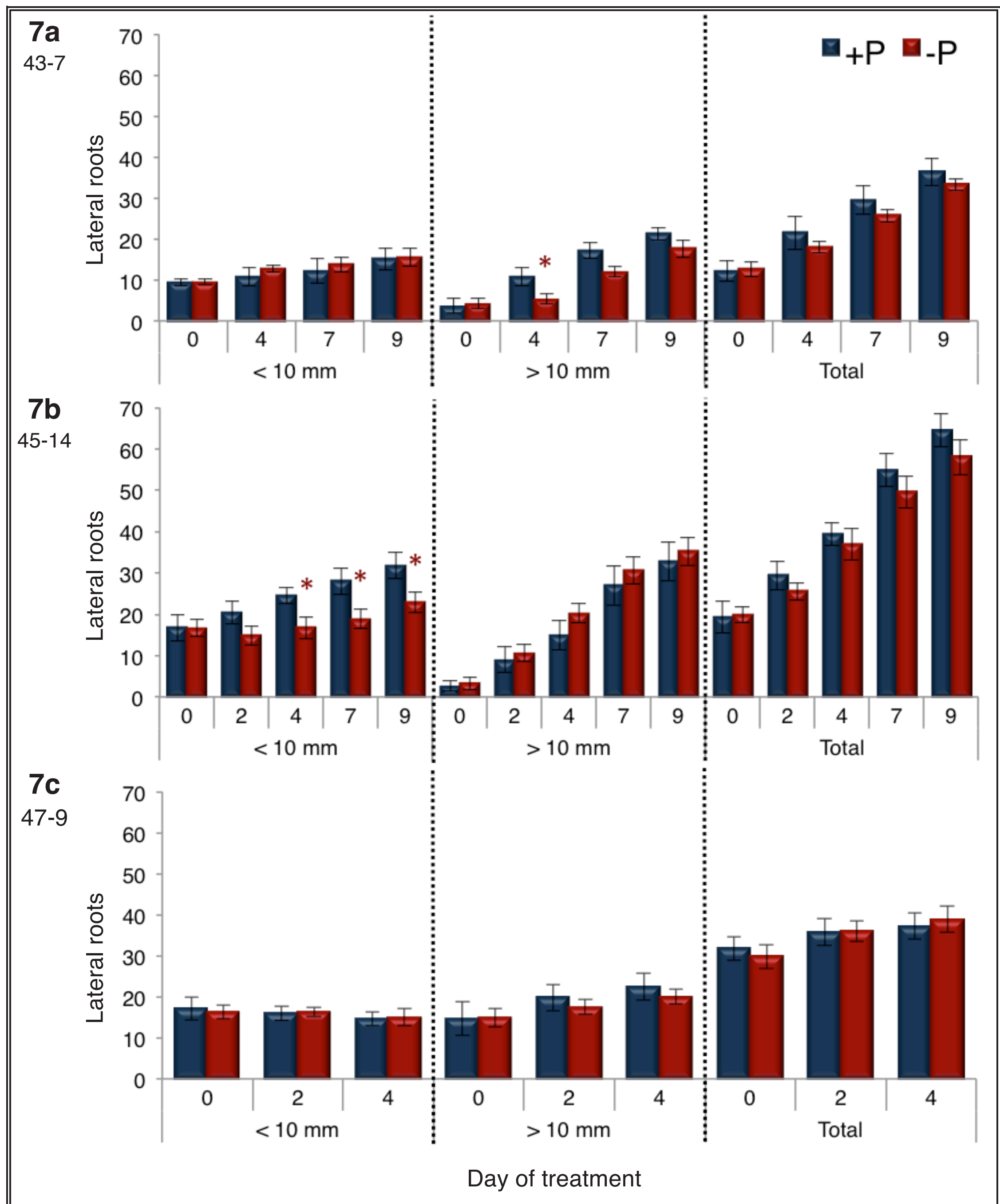


Figure 3.7. Number of lateral roots less and greater that 10 mm

The number of newly emerged lateral roots (<10 mm) were counted separately from the elongated lateral roots (>10 mm) and combined to give the total number of visible lateral roots **a** 43-7, **b** 45-14, **c** 47-9. Treatment was started after 15 days post excision for 43-7 and 45-14 and after 23 days post excision for 47-9. The significant differences and standard error P <0.05 (*), were analysed with the student's t-test from a minimum of six plants.

Root system architecture in 43-7

The dominant primary root in 43-7 contributed the lowest proportion of the total primary root length across the three genotypes. The mean length of the dominant primary root was 59 mm at the start of treatment (15 days post excision) and contributed 56%, of the total primary root length (Figure 3.5a). At harvest (35 days post excision), the dominant primary root contributed to 35% (+P) and 37% (-P) of the total primary root length, with the supporting primary roots contributing to the remainder of the primary root length (Figure 3.5a). The primary roots were similar in length in both treatment groups until day 12 of treatment when the relative growth rate of the primary root length in the P-stressed roots increased, with further increases after 17 and 20 days in limited P-supply (Figure 3.5d). The increase in relative growth rate of the primary root length resulted a significantly longer dominant primary root (18%) in the P-stressed treatment group at harvest, and although the total primary root length was also increased, the differences were not significant (Figure 3.5a, d). The dominant primary root was similar between treatment groups up to day nine of treatment (24 days post excision, indicated in blue in Figure 3.6a).

The number of newly initiated lateral roots (< 10 mm) was slightly stimulated in the P-stressed plants between four and seven days after the P-supply was reduced (Figure 3.7a). The elongation of the existing lateral roots in the P-stressed plants was decreased during the same period, together with the elongation of the primary root; which resulted in the reduction in the total number of lateral roots after nine days of treatment (Figure 3.7a and Figure 3.6a). The increase in the number of newly initiated lateral roots in the P-stressed plants between days four and seven temporarily increased the proportion of lateral roots in the branching zone of the dominant primary root (Figure 3.7d).
The branching zone in the P-sufficient plants was 79% on day nine of treatment and the branching zone was 85% of the primary root length when the plants were harvested. The branching zone was 78% in the P-stressed on day nine of treatment and slightly less at harvest, which is possibly due to the significantly longer primary root and decreased lateral root emergence (Figure 3.6a, d).

Root system architecture in 45-14

In 45-14, the dominant primary root length was 105 mm at the start of treatment (15 days post excision) and contributed 57% of the total primary root length. At harvest (36 days post excision), the dominant primary root contributed 42% in the P-sufficient plants and 44% of the total primary root length in the P-stressed plants due to the emergence of supporting primary roots in the respective treatment media (Figure 3.5b).

The mean total primary root length was longer in the P-sufficient plants until day 11 of treatment (26 days post excision) (Figure 3.5b). An increase in the relative growth rate of the primary root length in the P-stressed plants resulted in longer primary roots compared with the P-sufficient group at harvest, although the differences were not significant (Figure 3.5b, e). In the dominant primary root, the increase in the relative growth rate of the primary root length in the P-stressed plants occurred later on day 17 of treatment, which also resulted in a slightly longer dominant primary root when compared to the P-sufficient treatment group (Figure 3.5b, e).

In 45-14, the change in P-supply inhibited the number of newly initiated lateral roots (<10 mm) and continued to decrease the growth of the new lateral roots throughout the experiment (Figure 3.7b). There were less lateral roots in the branching zone of the P-stressed treatment group from day seven (76%) compared with 82% in the P-sufficient plants because the newly initiated lateral roots were slower to emerge (Figure 3.6e and Figure 3.7c). By day 14 of treatment, the branching zone was 91% of the primary root length in the P-sufficient treatment group compared with the branching zone that was 87%, in the P-stressed plants. The significant difference in branching zones between treatment groups was maintained until the plants were harvested after 21 days in low P media (Figure 3.6e). The number of newly initiated lateral roots was lower in the P-stressed plants from day four of treatment (17, 19 and 23) compared with the P-sufficient plants (25, 28 and 32) from day four to nine (Figure 3.7c). The elongation of the existing lateral roots was unaffected by low P, and continued to grow consistently with the P-sufficient treatment group (Figure 3.7b).

Root system architecture in 47-9

The root system architecture of 47-9 was mostly made up of the dominant primary root, and the majority of supporting primary roots were initiated before the start of treatment (23 days post excision). The mean dominant primary root length was 87 mm and contributed 63% of the total primary root length at the start of treatment, and 59% in the P-sufficient plants (Figure 3.5c). In the P-stressed plants, the dominant primary root contributed 57% of the total primary root length at harvest (40 days post excision) (Figure 3.5c). The elongation of the primary roots was stimulated in the P-stressed plants from day seven of treatment (Figure 3.5c). The difference between the two treatment groups ranged from 12% and 16% from day seven till they were harvested on day 17 of treatment (40 days post excision).

In 47-9, the number of newly elongated lateral roots (< 10 mm) were similar between the two treatment groups four days after the P-supply was lowered to 10 µm KH_2PO_4 (Figure 3.7c). In addition, the number of elongated lateral roots was slightly higher (but not significant) in the P-sufficient plants (Figure 3.7c). The primary roots were longer in the P-stressed roots of from day seven of treatment until harvest, and the number of lateral roots could not be physically counted after four days treatment (27 days post excision). In 47-9, branching zone remained constant between 84% and 88% of the primary root length from day two of treatment and was not affected by the increase in primary root length in the P-stressed roots (Figure 3.6c, f). This suggests that the initiation of lateral roots was not reduced by the change in P-supply because the number of newly initiated lateral roots increased proportionally with the elongation of the primary root.

3.3 Experiment II – Reduction of P-supply prior to lateral root emergence

In experiment II, treatment began prior to the emergence of visible lateral roots and the plants were harvested when the number of lateral roots could not be physically counted (Figure 3.8). A third group of plants were subjected to low sulfur (S) media that contained 28 μM $MgSO_4$ compared with 1.125 mM $MgSO_4$, and the magnesium was replaced by 1.097 mM Mg $(NO_3)_2$ (refer Appendix I). This was to test that the responses observed in the primary and/or lateral roots were specific to the reduction in P-supply, and not a general mineral stress response (refer section 1.6).

In experiment I, the stolons were in vermiculite for nine days and the percentage of stolons that initiated primary roots was low, which had implications for the treatment regime for 47-9 because the elongation of the primary roots were slow (discussed in section 3.2). The roots responded very well to the P-sufficient liquid media during the acclimatisation process after being removed from the vermiculite in experiment I, and resulted in fast elongation of the primary root in all three of the genotypes. Therefore, in experiment II, the primary roots in all of the genotypes were initiated in vermiculite for six (43-7 and 45-14) or seven (47-9) days and transferred to P-sufficient media to acclimatise; which also resulted in a slightly higher percentage of initiated primary roots (refer Table 1B and Table 2B). The primary roots in 47-9 were transferred into P-sufficient media for five days to acclimatise and elongate to at least 10 mm before the start of treatment, compared with three days for 43-7 and 45-14 (Figure 3.8c).

Treatment was started nine days post excision for 43-7 and 45-14, and 12 days post excision for 47-9 (Figure 3.8a, b, c). Two of the genotypes (43-7 and 47-9) were harvested after 15 days treatment and 45-14 was harvested after 16 days treatment, which was equivalent to 24, 25 and 27 days post excision respectively (Figure 3.8c, d, e). The extra day of treatment for 45-14 was to observe if the leaves developed necrosis (Figure 3.9b), because there were no symptoms on day 15 of treatment.
In experiment I, 45-14 showed the first signs of necrosis on day nine of treatment, which was equivalent to 24 days post excision (discussed in section 3.2.2).

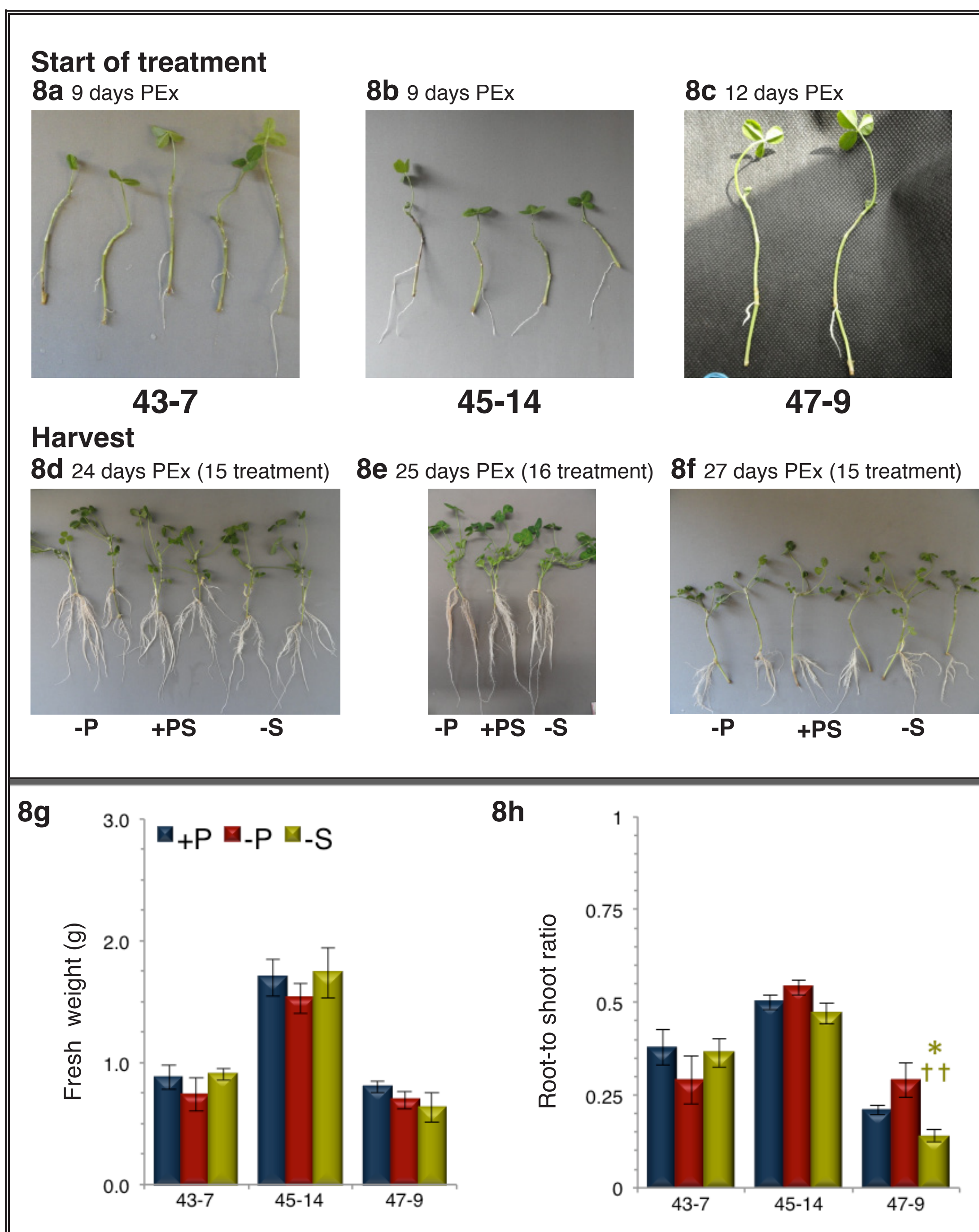


Figure 3.8. Fresh weight after growth period in limited P or S-supply

Plants at the start of and end of treatment period **a, d.** 43-7, **b, e** 45-14, **c, f** 47-9. **g.** Fresh weight of plants at harvest. **h.** Root-to-shoot ratios of plants at harvest. The mean values and standard errors were calculated from a minimum of five plants in each treatment group (refer Table 1). The significant differences between +P and –P (* P<0.05) and –P and -S (†† P<0.01) were calculated using the student t-test.

Harvested fresh weight and root-to-shoot ratios

The final harvested fresh weight of all of the genotypes was less than 2 grams (Figure 3.8a), which was considerably less than experiment I (Figure 3.2g). The fresh weight of 45-14 was the highest of the three genotypes (for all treatment groups), and 47-9 was the lowest (Figure 3.8a). The fresh weight of the P-stressed plants was 16%, 10% and 13% lower than the P-sufficient plants for 43-7, 45-14 and 47-9 respectively, although not significant (Figure 3.8g).

All three of the genotypes had one leaf at the start of treatment, which is designated leaf 4, L4 (oldest) at harvest (indicated on the right of the dotted lines in Figure 3.10). At harvest, L4 was between 24 (43-7) and 27 days old (47-9), and there were no significant differences between treatment groups or genotypes (Figure 3.10). At the end of the experiment, the plants had five actively growing leaves (including the apex), which were harvested individually (Figure 3.10), and the leaf P content was measured (Figure 3.11). Leaf three (L3) was unfolded at the start of treatment in all three of the genotypes therefore, most of the development of L3 occurred in the treatment media together with leaf 2 (L2), leaf 1 (L1) and the apex (indicated to the left of the dotted lines in Figure 3.10).

Acid phosphatase activity and leaf necrosis in 45-14

In experiment II, 45-14 was the only genotype to have elevated acid phosphatase activity in the soluble and cell wall fractions of the P-stressed roots, and 45-14 was also the only genotype to show necrosis in both treatment groups (Figure 3.9a, middle and b). The acid phosphatase activity in the soluble fraction (SF) of the harvested roots was also higher in the S-stressed plants compared to the P-sufficient plants, and indicates that P metabolism is compensating for S-stress. A further observation that supports the level of nutrient stress in 45-14 was from the early signs of necrosis in L2 of both treatment groups (Figure 3.9b). Sulfur necrosis occurred in the middle region of the leaf compared with the edge of the leaf in the P-stressed plants (Figure 3.9b, right).

In 43-7, the acid phosphatase activity was reduced in soluble fraction (SF) of the P-stressed roots, and there was no difference in the cell wall (CW) fraction (Figure 3.9a, left). In 47-9, the acid phosphatase activity was similar between treatment the groups in both of the soluble and cell wall fractions (Figure 3.9a, right).

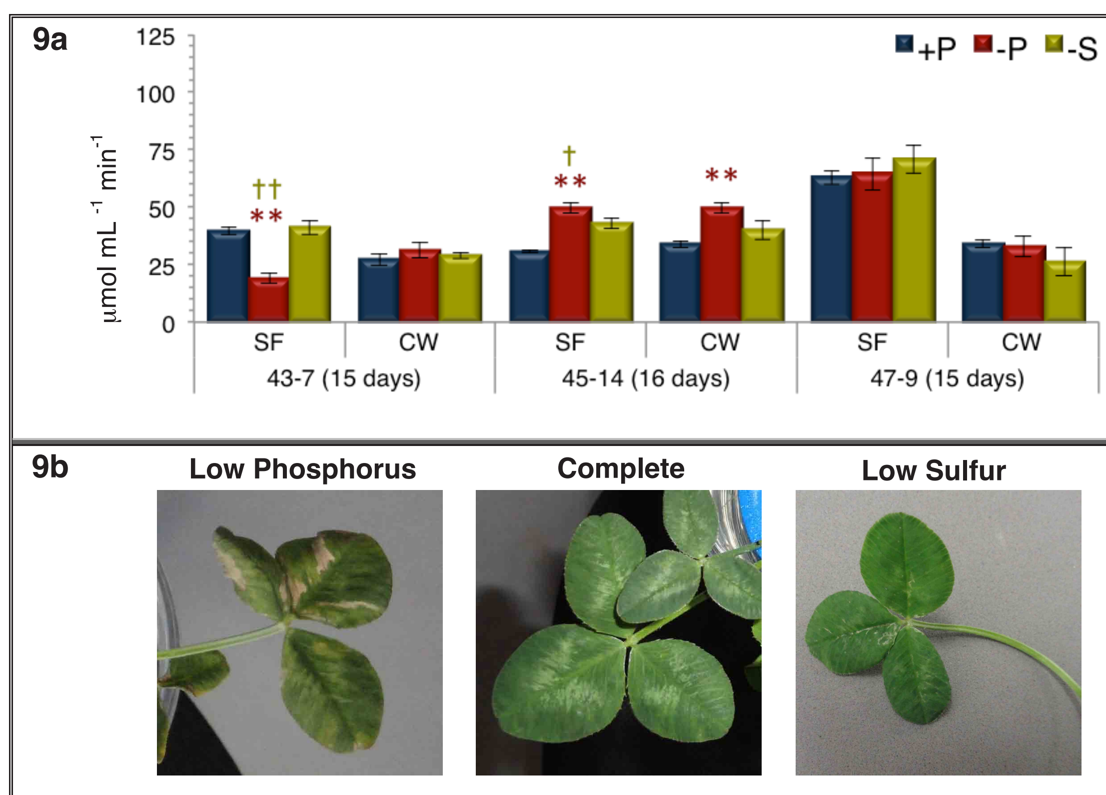


Figure 3.9. Acid phosphatase activity and leaf necrosis in 45-14

a. Acid phosphatase activity in the soluble fraction (SF) and cell wall fraction (CW) in the harvested roots. The calculations were based on a minimum of five plants. Significant differences were calculated using the student's T-test. Differences between +P and –P are indicated in red (*P<0.05, **P<0.01) and differences between –P and –S are indicated in olive green (†P<0.05, ††P<0.01). **b.** Necrosis in the second leaf from the apex in 45-14 was visible on the day of harvesting, which was after 26 days post excision and 16 days of treatment, and differed between the low P (left image) and low S (right image) treatment groups. No necrosis was observed in the plants grown in complete media (middle image), or in either of the other genotypes.

Leaf P content and fresh weight

There were no significant differences in the fresh weight any of the harvested leaves in 43-7 and 47-9 (Figure 3.10a, c). The observation of visual stress in the leaves under limited P-supply (at harvest) is consistent with the higher fresh weight of 45-14 (between 100 mg and 200 mg) when compared with the other two genotypes that had all of the leaves under 100 mg (Figure 3.10). Another difference between 45-14 and the other two genotypes is that the fresh weight increased with the age of the stolon post excision compared with the 43-7 and 47-9 that had a relatively consistent distribution of fresh weight in all of the leaves (Figure 3.10).

In 43-7, the P-content of the leaves of the plants grown in complete media was higher in the alternate leaves (Apex, L1, and L3), compared to the P-stressed leaves that contained less than 0.05 μg/gFW, in all four of the harvested leaves (Figure 3.11a). The fresh weight of the S-stressed leaves was higher than the P-stressed leaves, and slightly lower than the sufficient leaves except L1. In the S-stressed plants, the P content followed a similar trend as the plants grown in complete media, except the P content was lower in L2 and L4 (Figure 3.11b).

The higher distribution of P content in alternate leaves did not occur in 45-14 and 47-9 (Figure 3.11c, d, e and f). In 45-14, the P-stressed plants had a significantly lower fresh weight at harvest when compared with the P-sufficient and S-stressed plants.
The P content was slightly higher in L2 and L3 of the P and S-sufficient leaves, which was reflected in the fresh weight (Figure 3.10b and Figure 3.11d). The P-content in the S-stressed plants of 45-14 and 47-9 was slightly higher compared to the P-sufficient plants, and followed the same trend (Figure 3.10c and Figure 3.11d). In 47-9, the leaf fresh weight and P-content was evenly distributed in the first four leaves with no significant differences between treatments (Figure 3.10c and Figure 3.11e, f). The low fresh weight and similar P-content between treatment groups observed in the first four leaves of 47-9 is consistent with the slower growth in the first 27 days post excision observed in the previous experiments.

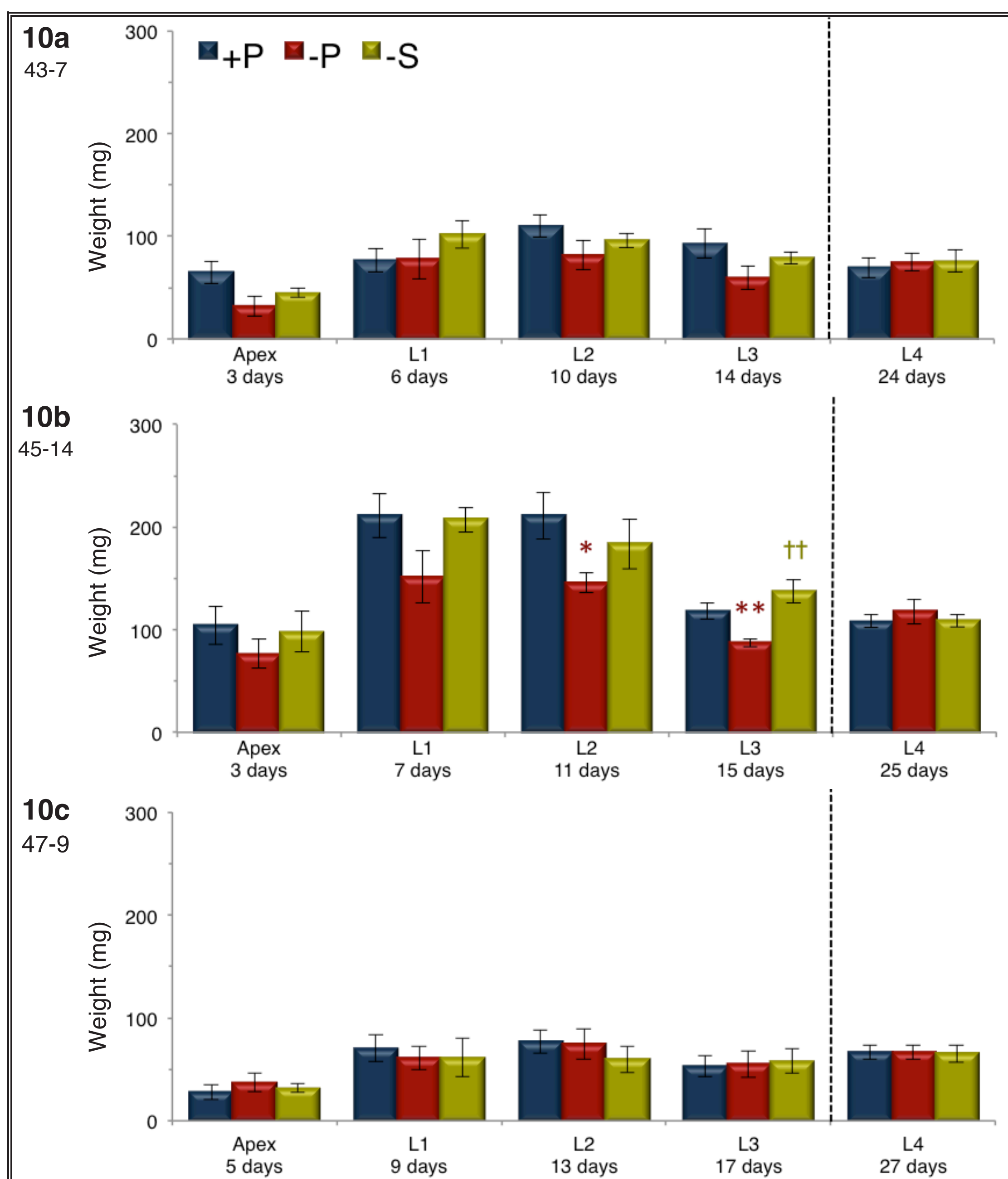


Figure 3.10. Weight and age profile of apex and four leaves

Harvested fresh weight of the apex and four emerged leaves of stolons grown in media containing P and S sufficient (1 mM KH_2PO_4 and 1.125 mM $MgSO_4$) media compared with limited P-supply (10 μM KH_2PO_4) and limited S supply (28 μM $MgSO_4$). The leaves to the left of the dotted line were initiated in the respective treatment media. The age of the leaf at harvest is on the x-axis. **a.** 43-7, **b.** 45-14, **c.** 47-9. The standard error and significant differences were calculated from a minimum of five plants using the student's T-test. Differences between +P and -P (red), +PS and -S (olive green) $P< 0.05$ (*), $P<0.01$ (**). Differences between -P and -S ($P<0.01$ ††).

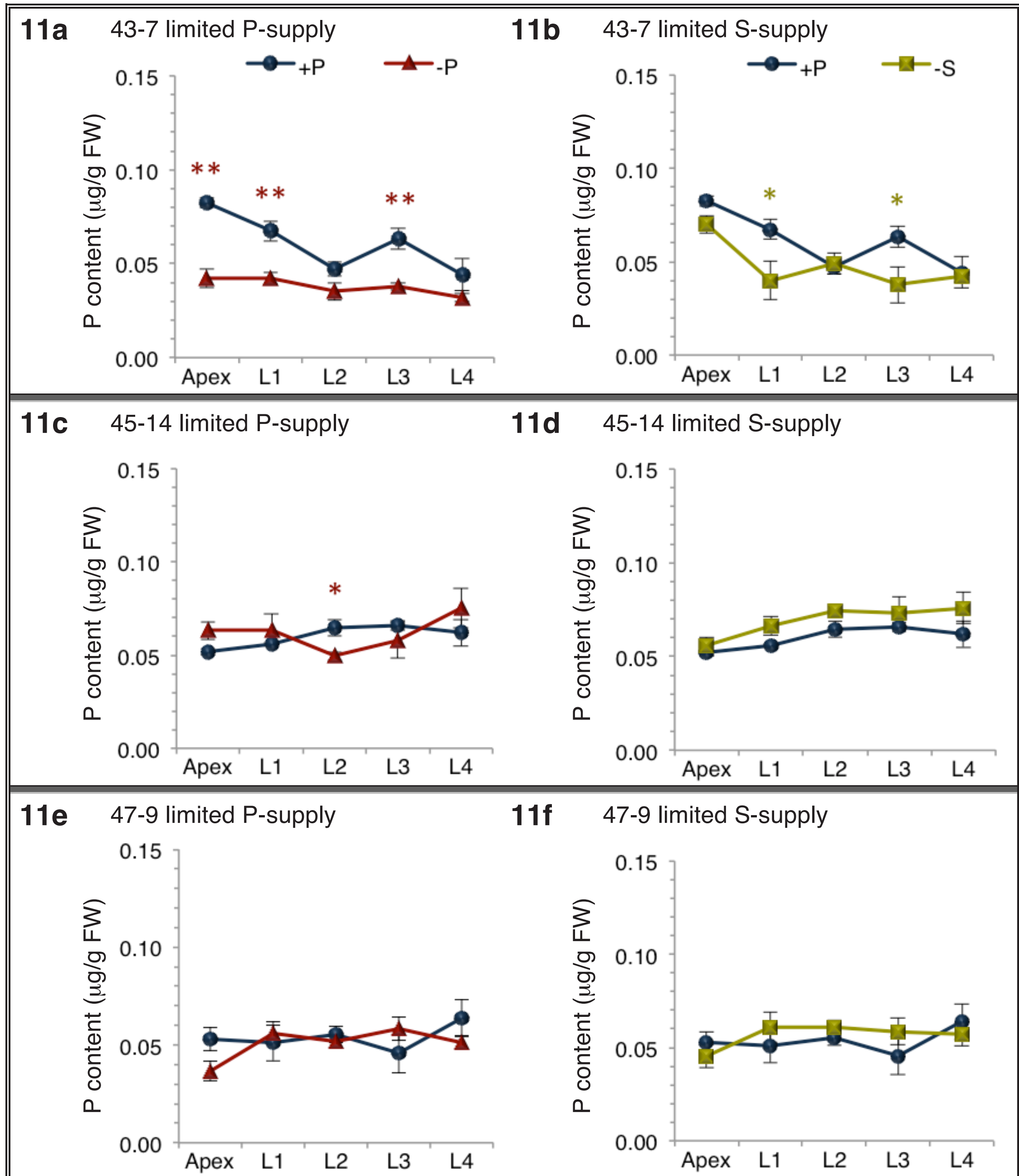


Figure 3.11. Leaf phosphate content in P or S-stressed plants

Phosphate content in the apex and four emerged leaves of stolons grown in media containing P and S sufficient (1 mM KH_2PO_4 and 1.125 mM $MgSO_4$) media compared with limited P-supply (left 10 µM KH_2PO_4) and limited S-supply (right 28µM $MgSO_4$). **a, b.** 43-7, **c, d.** 45-14, **e, f.** 47-9. The standard error and significant differences were calculated from a minimum of five plants, using the student's T-test (*P> 0.05 and **P>0.01).

Overview of root system architecture at harvest

Each genotype has distinct root morphology in P-sufficient media (Figure 3.12 left). The mean dominant primary root length in 45-14 was 1.8 fold longer than 43-7 and 2.5 fold longer than 47-9 (Figure 3.12, left). There were no significant differences in primary root length in the P-stressed (Figure 3.12, middle) or S-stressed plants at harvest in any of the treatment groups at harvest (Figure 3.12, right).

The lateral root density was calculated by dividing primary root length by the number of visible lateral roots, and expressed as lateral roots per mm of primary root length (LR/mm). The lateral root density was the highest in 47-9 by 1.7 fold when compared with 43-7 and 1.3 fold greater than 45-14. The lateral root density was similar between treatment groups in 43-7 that contained the least number of lateral roots on the dominant primary root with respect to length (Figure 3.12a). The lateral root density in 45-14 was similar in the P-sufficient plants and P-stressed plants and slightly decreased in the S-stressed plants at harvest. In 47-9, the P-stressed plants had significantly more lateral roots on the primary root (higher lateral root density, 1.53 LR/mm) compared with the P-sufficient (1.99 LR/mm) and S-stressed roots (1.85 LR/mm) (Figure 3.12c).

Overview of responses in root system architecture between genotypes

A detailed description of the differences in root system architecture in limited P or S-supply in experiment II is described in sections 3.3.1 (43-7), 3.3.2 (45-14) and 3.3.3 (47-9), and is characteristic for each genotype. The response to P-stress that is observed within seven days from the reduction in P-supply and is consistent with experiment I except for 43-7.

The characteristic response to P-stress in 43-7 is decreased elongation of lateral roots (section 3.1) and primary roots (in experiment II only). In 45-14 the lateral root emergence is decreased (section 3.3.2), and in 47-9 the primary roots and lateral roots are stimulated (section 3.3.3). In experiment II (only), the branching zone is increased in 47-9 in the P-stressed plants with respect to time when compared with the P-sufficient plants (Figure 3.15f). There were no changes to the branching zones in 43-7 and 45-14 because the number of lateral roots was proportional to the primary root length (Figure 3.15d and e).

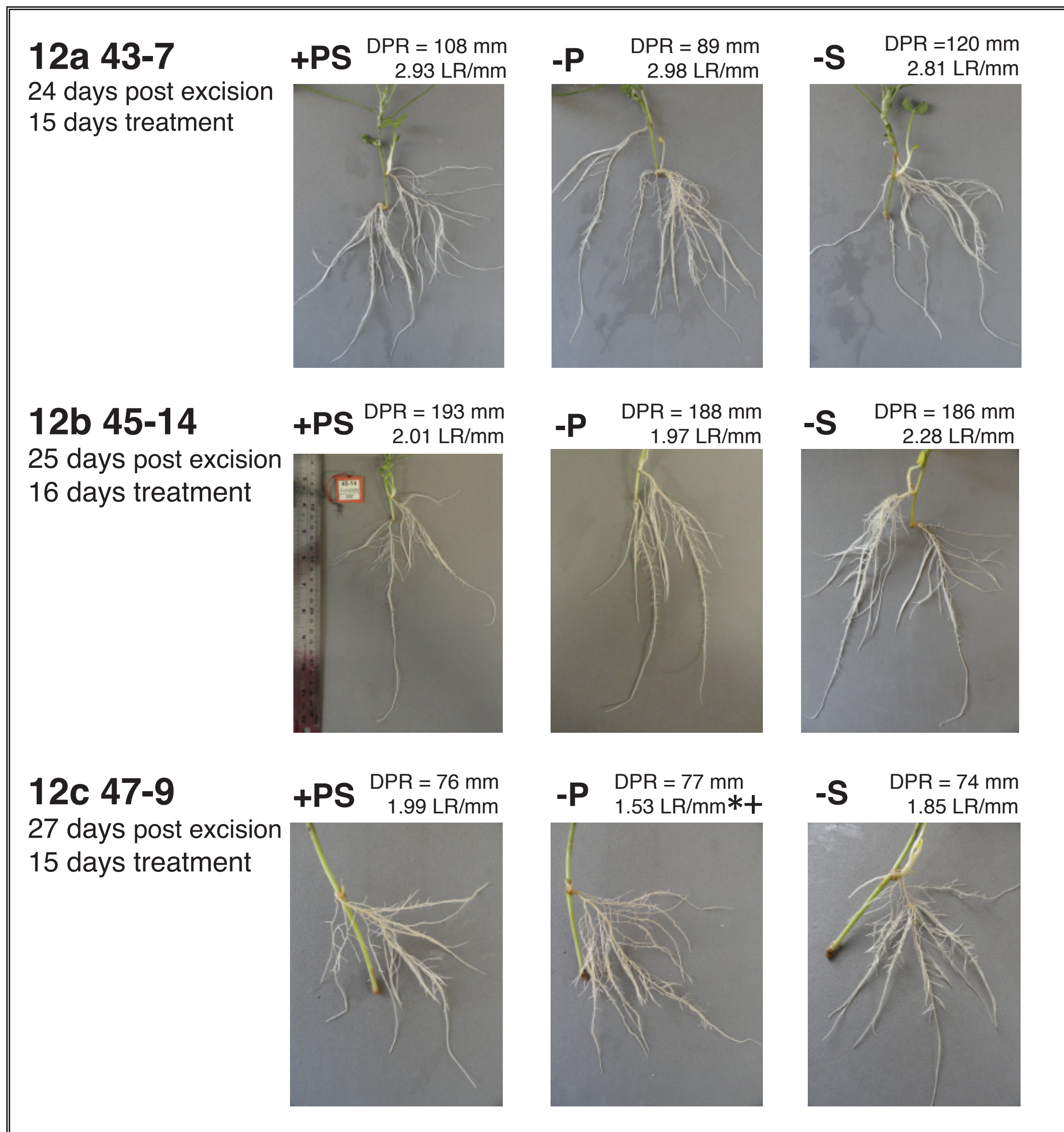


Figure 3.12. Root system architecture in 43-7, 45-14 and 47-9

a. 43-7, **b.** 45-14, **c.** 47-9. Contrasting root system architecture of the three genotypes from experiment II. The plants were grown for a total of 24 (43-7), 25 (45-14) and 27 (47-9) days in Hoagland's media containing 1 mM KH_2PO_4 and 1.25 mM $MgSO_4$ (+PS) or 1 μM KH_2PO_4 (-P) or 28 μM $MgSO_4$ for 15 (43-7 and 47-9) or 16 days (45-14). The camera settings for each photo are detailed in Appendix VI, and the mean dominant primary root (DPR) length and lateral root density (LR/mm) is at the top of each picture for perspective. A low value for lateral root density indicates a higher number of lateral roots on the primary root (more dense) than a lower number (less dense). The density of the P-stressed roots of 47-9 was significantly higher than the +P and –S roots, ($*^{\dagger}$ $P<0.05$) using the student's t-test.

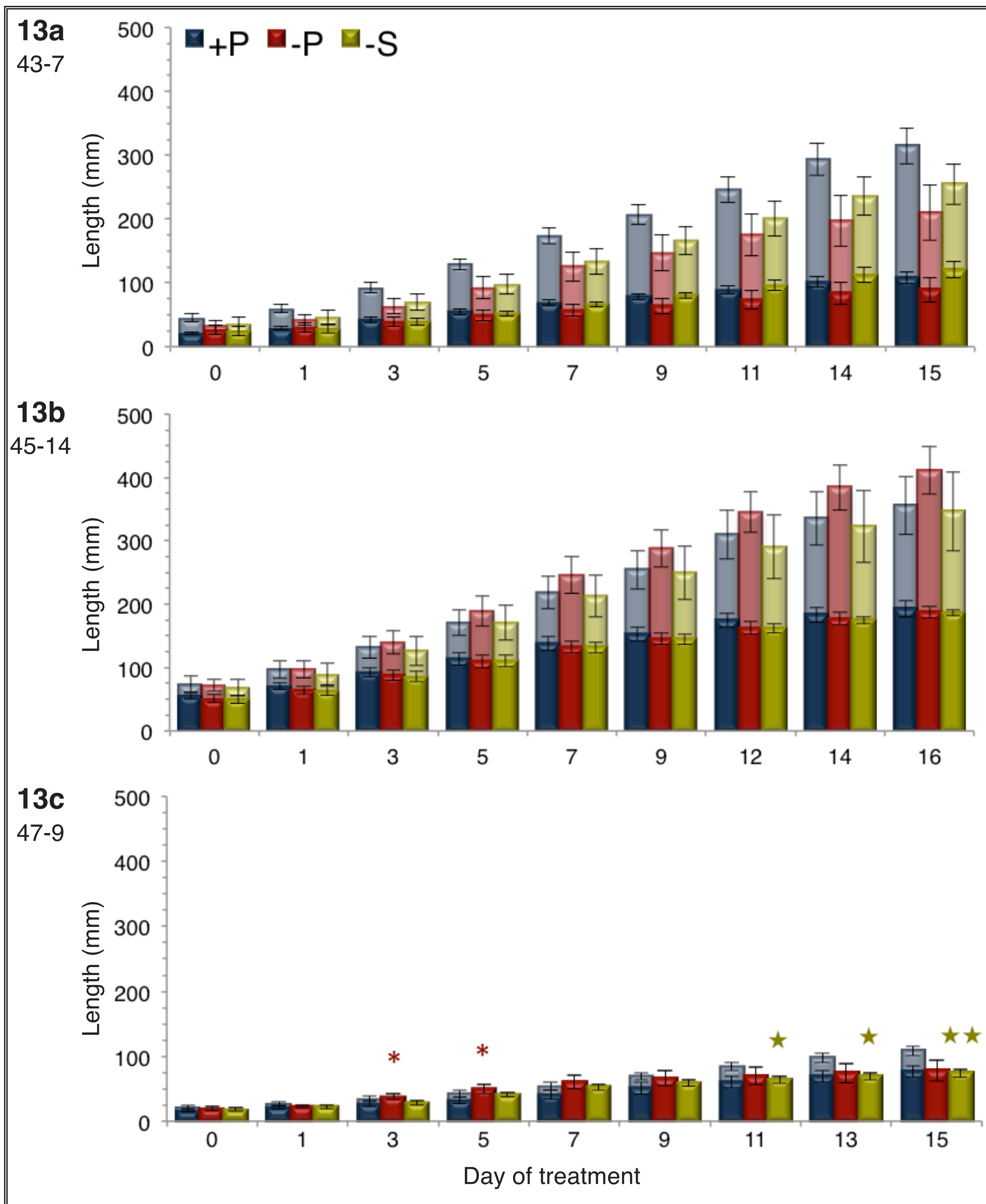


Figure 3.13. Total primary root length

a. 43-7, **b.** 45-14, **c.** 47-9. Mean dominant primary root length (solid) as a proportion of the total primary root length (transparent). The values are calculated from a minimum of five plants in each treatment group. The significant differences in the dominant primary root between +P and –P (*P<0.05) and total length between +P and –S (⋆ P<0.05, ⋆⋆ P<0.01) were calculated using the student's t-test.

3.3.1. Decreased primary and lateral root elongation in 43-7

At the start of treatment, the dominant primary root contributed to 65% of the total primary root length and the contribution decreased as the supporting primary roots elongated (Figure 3.13a). At harvest, the dominant primary root contributed 35% (complete media), 43% (-P) and 48% (-S) to the total primary root length (Figure 3.13a). The limited P-supply decreased the initiation and elongation of supporting primary roots in 43-7 from day three of treatment, and from day five of treatment for the S-stressed plants (Figure 3.13a). The differences between the P-sufficient and P-stressed plants were significant between the $P < 0.05$ and $P < 0.10$ level from day three of treatment until harvest, and were not significant for the S-stressed plants (Figure 3.13a). At harvest, the total primary root length of the plants grown in complete media was 315 mm, compared with 210 mm (-P) and 255 mm in the S-stressed plants (Figure 3.13a). The mean final dominant primary root length for the P-sufficient plants was 108mm, and was higher the P-stressed dominant primary root (89 mm), and lower than the S-stressed dominant primary root (120 mm) (Figure 3.14a).

There was no difference in the branching zone between treatment groups because the total number of lateral roots was proportional to the primary root length (Figure 3.15a, d). On day seven of treatment (16 days post excision) the branching zone represented between 65% and 67% of the dominant primary root length, and continued to increase gradually until harvest (25 days post excision). At harvest, the branching zone was between 80% and 84% of the primary root length in all three of the treatment groups (Figure 3.15d).

In the P-stressed plants, the dominant primary root was shorter from day seven of treatment, which reduced the total number of lateral roots compared to the other two treatment groups (Figure 3.14a and Figure 3.15a). The branching zone was proportional to the primary root length in the three treatment groups (Figure 3.15d). The elongation of the longest lateral roots on the dominant primary root was reduced in the P-stressed plants from day 17 days post excision (eight days treatment) (Figure 3.17a).
The reduced elongation of the lateral roots impacted the length of the longest lateral root in the P-stressed plants, but not the S-stressed plants, and delayed the initiation of its tertiary roots by three (–P) and two (-S) days (Figure 3.17a).

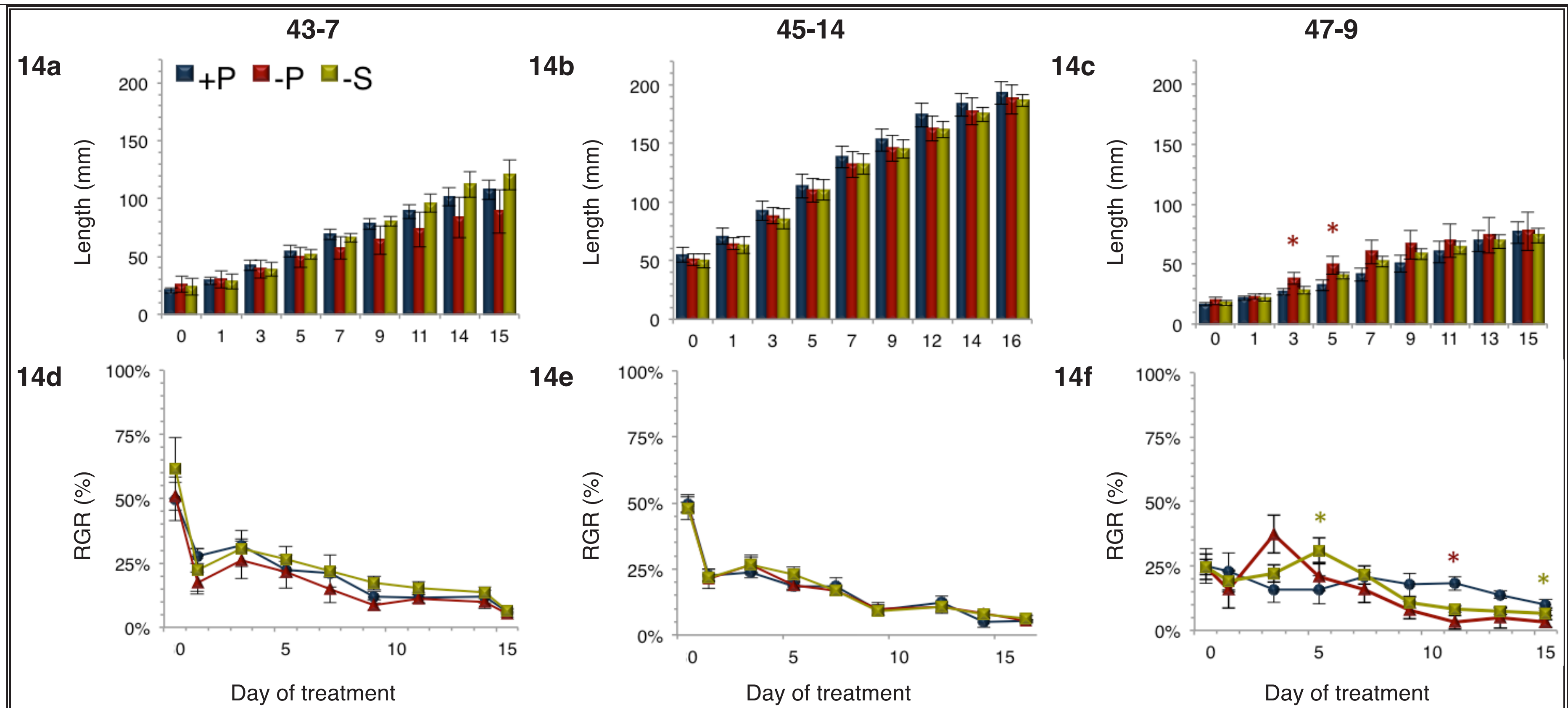


Figure 3.14. Dominant primary root length and relative growth rate
a. 43-7, **b.** 45-14, **c.** 47-9. Length of dominant primary root **d.** 43-7, **e.** 45-14, **f.** 47-9. Relative growth rate of the dominant primary root length. The significant differences and standard errors P< 0.05 (*) between +P and –P (red), or +P and –S (olive) were analysed using the student's T-test from a minimum of five plants.

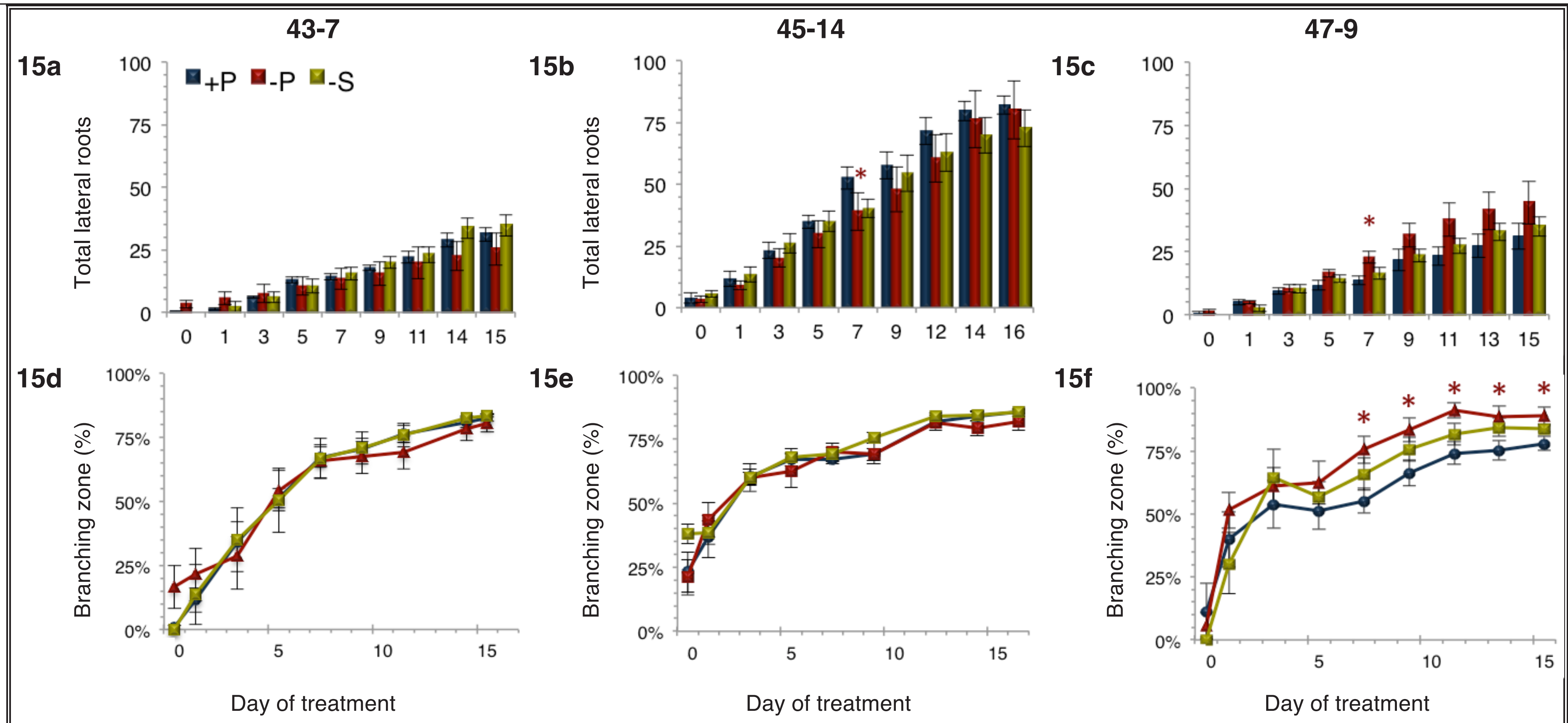


Figure 3.15. Branching zone on the dominant primary root

a. 43-7, **b.** 45-14. **c.** 47-9. Number of visible lateral roots. **a.** 43-7, **b.** 45-14. **c.** 47-9. Branching zone as the proportion of dominant primary root length.

The significant differences and standard errors P< 0.05 (*) were analysed using the student's T-test from a minimum of five plants.

3.3.2. Decreased lateral root emergence in 45-14

At the start of treatment, the mean dominant primary root length contributed to 78%, of the total primary root length. The proportion decreased to 60% (+P) at harvest and 63% in the S-stressed plants due to the contribution of the supporting primary roots (Figure 3.13b). In the P-stressed plants, the dominant primary root length contributed 47% of the total primary root length due to the extended growth of the supporting primary roots (Figure 3.13b) that contributed a higher total number of lateral roots in the P-stressed plants (data not shown). There were no differences in the relative growth rate of the primary root length between treatments in the dominant primary root (Figure 3.14e), or the plant total (data not shown). This is the opposite effect on the elongation of supporting primary roots that was observed in 43-7 (refer section 3.3.1).

On day five of treatment (15 days post excision), the dominant primary root in 45-14 was 110 mm (-P, -S), and 114 mm in the plants grown in complete media with a slightly lower branching zone in the low P plants (62%) compared with 68% in the other two treatment groups (Figure 3.15e). The number of newly emerged lateral roots increased rapidly in the first five days of treatment (14 days post excision) (Figure 3.16b). On day five, the P-stressed roots had a significantly lower number of newly emerged lateral roots that affected the number of lateral roots that were greater 10 mm from day seven (Figure 3.16e). The number of newly emerged lateral roots fluctuated in the S-stressed plants, with equivalent numbers of elongated lateral roots to the P-stressed plants (Figure 3.16b, e).

By day seven of treatment (16 days post excision), the P-sufficient plants had a total of 53 visible lateral roots, and was significantly higher than the P-stressed plants with 39 lateral roots, but not significantly higher than the S-stressed plants with 40 lateral roots (Figure 3.16b). At harvest (25 days post excision), the plants grown in complete media had 82 visible lateral roots compared with 80 in the P-stressed plants, and the S-stressed plants had slightly less with 73 lateral roots, which was not significant (Figure 3.16b). The length of the longest lateral root was not affected in the P or S-stressed plants (Figure 3.17b). The emergence of tertiary roots was delayed by three days in the low P media when compared with the plants grown in complete media and two days when compared with the plants grown in low S media (Figure 3.17b).

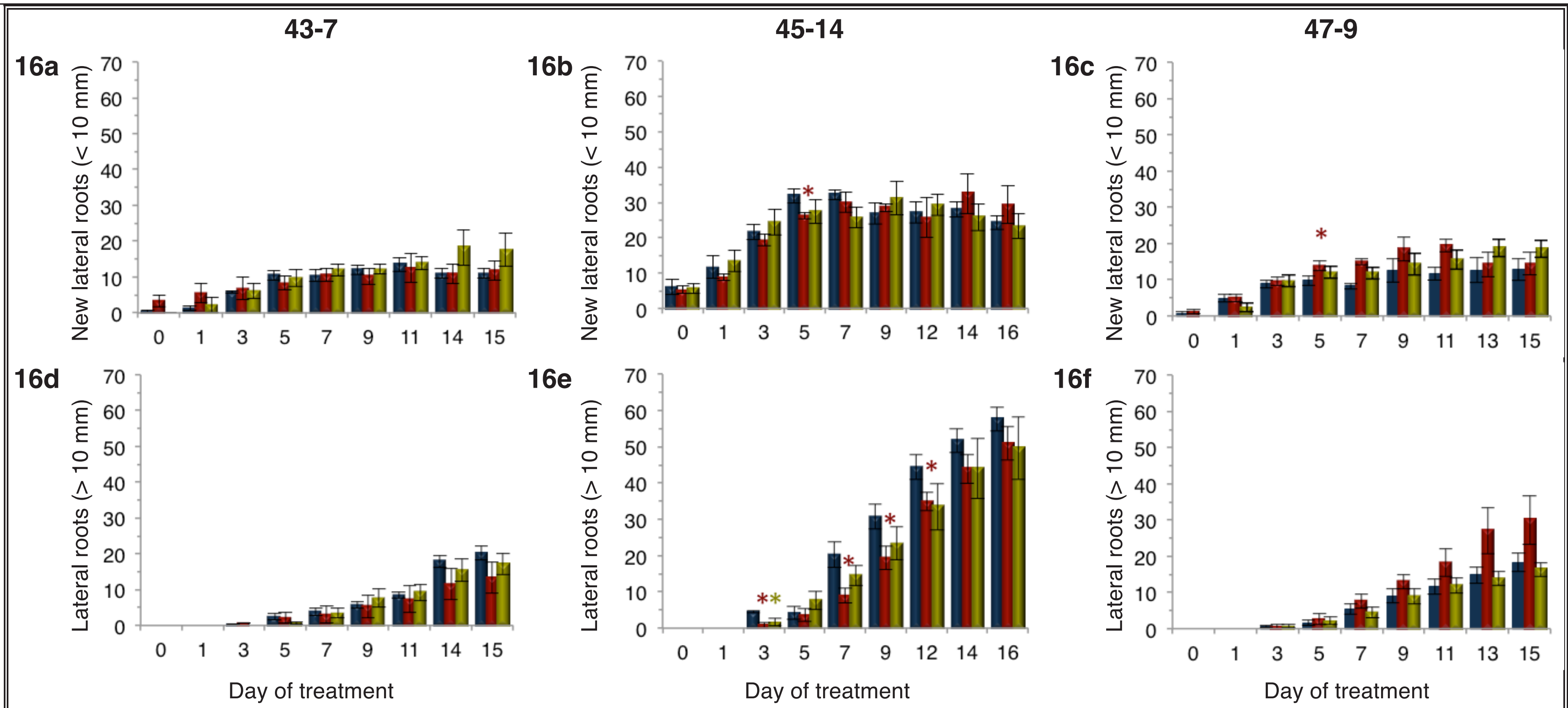


Figure 3.16. Emergence and elongation of visible lateral roots
a. 43-7, **b.** 45-14. **c.** 47-9. Lateral roots less than 10 mm. **a.** 43-7, **b.** 45-14. **c.** 47-9. Lateral roots greater than 10 mm. The significant differences and standard errors P< 0.05 (*) between +P and –P (red), or +P and –S (olive) were analysed using the student's T-test from a minimum of five plants.

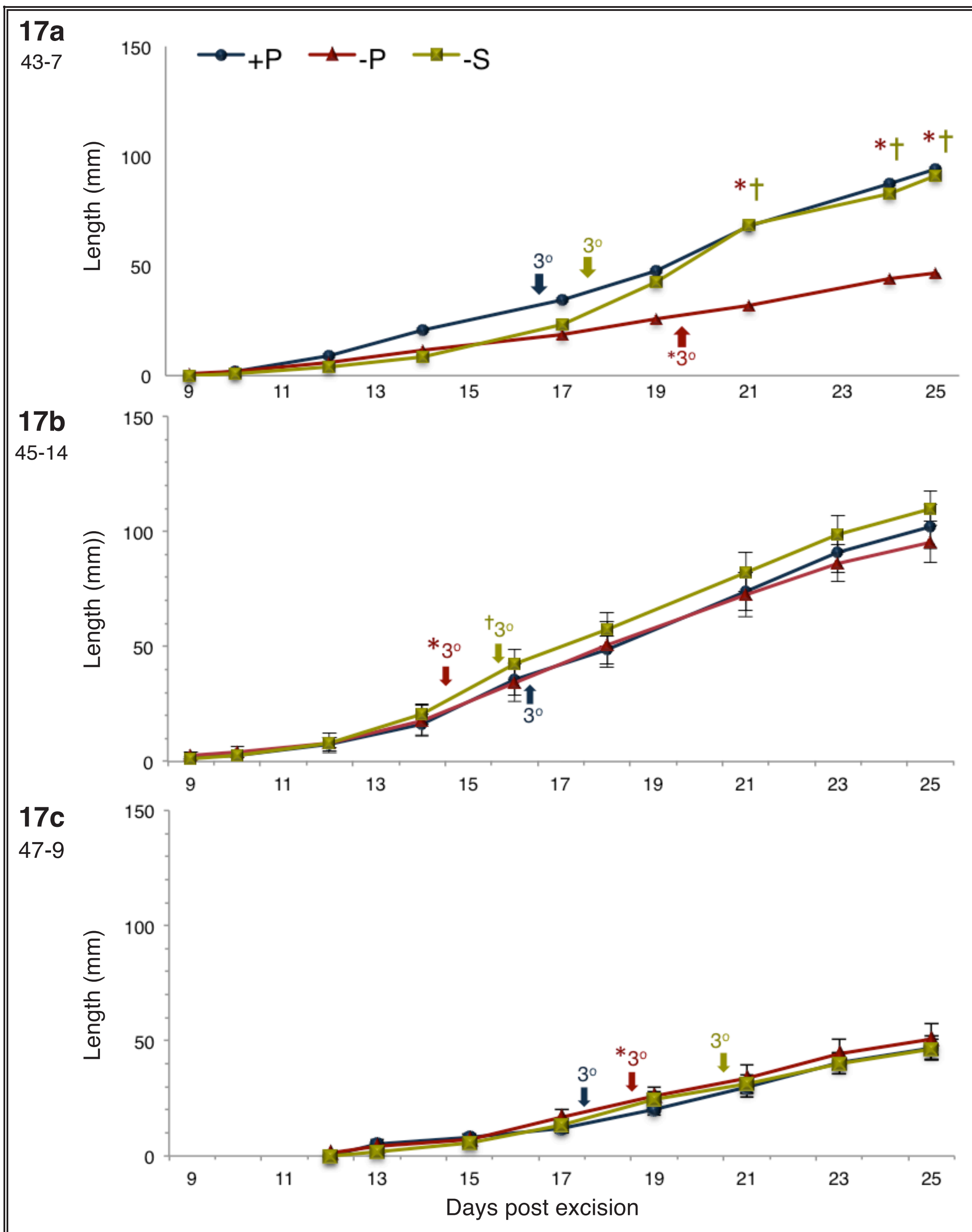


Figure 3.17. Initiation of tertiary roots on the longest lateral root

a. 43-7, **b.** 45-14, **c.** 47-9. The length of the longest lateral root measured on the dominant primary root. The day of tertiary root emergence that was first observed is marked by an arrow and indicated by 3°. Differences (student's T test) between C and P (red), C and S in olive green (*P< 0.05, **P<0.01). Differences between P and S in olive green (†P< 0.05).

3.3.3. Increased lateral root density in 47-9

In 47-9 the dominant primary root contributed 100% total primary root length for the P-stressed and S-stressed plants because there were no supporting roots (Figure 3.13c). In the P-sufficient plants, the dominant primary root contributed to 82% of the total primary root length at the start of treatment, with only two of the five plants in the group having one supporting root (data not shown). The single root in 16 of the 18 plants selected for the experiment in 47-9 was a random event that occurred in the stolons that were cut from the stock plants and the treatment groups were selected on the total primary root length and fresh weight before treatment started. The limited P and limited S media had no effect on the initiation of supporting primary roots in 47-9, but because there were no supporting primary roots initiated in the P-sufficient plants during the treatment period (Figure 3.13c).

The length of the dominant primary root in the P-stressed plants was significantly longer than P-sufficient plants on days three and five of treatment, and the trend continued until day 11, with no difference in length with 15 days in limited P-supply (Figure 3.14c). The S-stressed plants were also slightly longer than the plants grown in complete media from days five to 11 (Figure 3.14c). The relative growth rate of the primary root length was higher (although not significantly) in the P-stressed plants on day three of treatment, and declined to below 10% by day 11 (Figure 3.14f). A similar trend occurred in the S-stressed plants, with a peak after five days, and was lower than the plants grown in complete media from day nine (Figure 3.14f).

The emergence and elongation of lateral roots increased the total number of lateral roots in the P-stressed plants, which increased the total lateral roots in the branching zone from day five of treatment (Figure 3.15c, f and Figure 3.16c, f). On day seven, the branching zone was rapidly increased to 75% of the dominant primary root length in the P-stressed plants, and was maintained at 89% from day 11 (23 days post excision) (Figure 3.14f). In contrast, the branching zone was 55% of the dominant primary root length on day seven P-sufficient plants, and 66% of the dominant primary root length in S-stressed plants (Figure 3.15f). On day 15 of treatment (27 days post excision), the branching zone was 78% (+P) and 84% of the dominant primary root length in the S-stressed plants (Figure 3.15f).

The difference between the branching zones between –P and –S ranged between 10% on day seven and 5% at harvest (Figure 3.15f). The difference was doubled in the P-sufficient plants with 20% on day seven, and 11% on day 15 (Figure 3.14f).
The increased lateral root density of the P-stressed roots in 47-9 at harvest is the result of the shorter elongation zone and longer branching zone compared to the P-sufficient and S-stressed plants (Figure 3.12c). There were no differences observed in the elongation of the longest lateral root in any of the treatment groups (Figure 3.17c). However, the initiation of the tertiary roots was delayed by two days (-P), and four days (-S) (Figure 3.17c). The lateral emergence of the tertiary roots in 47-9 possibly reduced the fresh weight biomass that was observed in the lower root-to-shoot ratio (Figure 3.8h).

3.4 Comparisons between experiments I and II

Two major objectives of this thesis are to identify differences in the primary and lateral roots between the three genotypes in response to a limited P-supply and confirm that the changes are specific to P-stress. The specific changes to the primary and lateral roots are described in sections 3.3.1 (43-7), 3.3.2 (45-14) and 3.3.3 (47-9), and are mostly consistent with experiment I (section 3.2.3). These results are interesting because the reduction in P supply differed by the number of days post excision in 47-9 for experiment I and II compared with the other two genotypes (refer Table 2).

Two general observations (discussed in sections 3.4.1 and 3.4.2) were made during experiments I and II that form the basis of a short-term P-stress response time course (experiment III) between 45-14 and 47-9 (refer Table 2 and section 3.4.3). The first is a comparison of signature responses to P-stress in experiments I and II of this study (Figure 3.18 and Table 5), and the genotype screen performed by Effendy (2007).
The second observation is the consistency of the branching zone on the primary root in P-sufficient media (Table 7 in section 3.4.2), and differences in the responses to the change in P-supply with respect to time post excision (Figure 3.6 and Figure 3.15).

3.4.1. Comparison of signature responses to P-stress

One indication that the low P environment affected the roots was a change in root colour that occurred when the P-supply was reduced, which was irrespective of the stage of root development (Figure 3.18). The caramel/brown colour was observed in the P-stressed roots of all three of the genotypes after three days from the change in P concentration, and was absent in the P-sufficient and low S-stressed roots (Figure 3.18c). The intensity of the colour increased with the duration of time spent in limited P-supply and age of the roots (Figure 3.18). The images in Figure 3.18 show roots after 12, 25 and 40 days post excision with three, 15 and 17 days in limited P-supply respectively. The root tip and elongation zone remained white and the older parts of the roots turned from caramel to light brown (Figure 3.18c, d). There was also an increased number of root hairs that were visible in the emerging lateral roots at the junction between the elongation zone and branching zone of the younger P-stressed roots in the microscopic images, which exclude the root tip because it did not fit into the field of view (Figure 3.18a, b). The specific cause for the colour change in the P-stressed roots is an area for future investigation.

Comparisons between the length of treatment and signature responses to P-stress in this study and the genotype screen (Effendy, 2007)

In the genotype screen performed by Effendy (2007), the signature responses to long term P-stress included the final harvested fresh weight and the root-to-shoot ratio, the decline in P-content, and the induction of acid phosphatase in the soluble and cell wall fractions of the root (summarised in Table 5). The specific root length was estimated for each genotype to compare the root system architecture at the different stages of development at harvest from experiments I and II (Figure 3.18e). The specific root length is an estimate because the length of the lateral roots was not taken into account in the calculation (refer Table 3). The specific root length was higher in the older roots from experiment I (left) compared with experiment II (right) due to the increased root development (Figure 3.18e). There were no significant differences between treatment groups in either of the experiments. Interestingly, 47-9 had a higher specific root length in experiment I compared with 45-14 that had longer primary roots (Figure 3.18e).

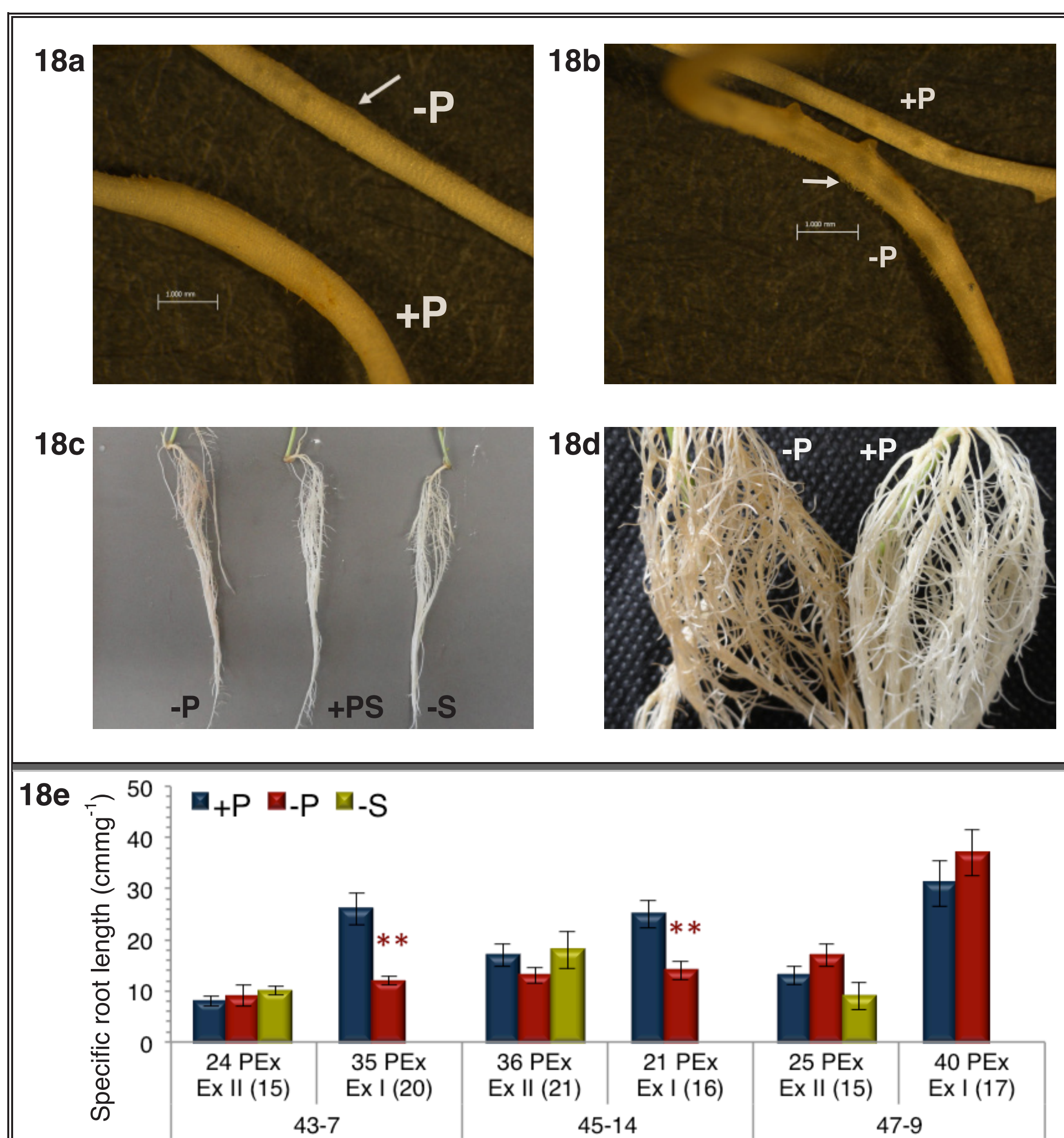


Figure 3.18. Colour of P-stressed roots and specific root length

a. b. Images of roots 12 days post excision taken with a dissecting microscope with assistance from Jianyu Chen at the Manawatu Microscopic Imaging Center (MMIC) at Massey University. The scale represents 1 mm and the first visible lateral root is located 36 mm from the root tip (excluded from the field of view). **a.** Onset of caramel colour around the elongation zone in P-stressed roots (arrow) after three days treatment. **b.** Root hairs visible at elongation zone of P-stressed roots (-P). **c.** Harvested roots from experiment II after 25 days (+P) or 16 days in –P or –S treatment. **d.** Harvested roots from experiment I after 40 days (+P, right) or 17 days in limited P-supply (left). **e.** Estimated specific root length (SRL) in harvested roots. SRL = total primary root length (cm) divided by the harvested root fresh weight (mg) (Crush *et al.*, 2008).
The number of days post excision (PEx) and treatment are indicated for each experiment.

Table 5. Differences in long-term signature responses to P-stress

Summary of the response observed in P-stressed plants with respect to the P-sufficient plants in experiments I and II from Figures 3.2, 3.3, 3.8 and 3.9 and the genotype screen performed by Effendy, (2007). The significant differences with respect to the low P treatment group are indicated with arrows (↓, ↑) and a cross (x) indicates no significant difference.
Abbreviations include: Fresh weight (FW), Root acid phosphatase (APase), soluble fraction (SF), cell wall fraction (CW).

Genotype	Measurement	**Treatment prior to LR emergence** (Experiment II)	**Treatment after LR emergence** (Experiment I)	**Established root Effendy, (2007)** (Genotype screen)
43-7	Days post excision – start	9	15	56
	Days treated	15	20	21
	Days post excision – harvest	24	35	77
	Difference in harvested FW	x	↓ 58%	x
	Root-to-shoot-ratio	x	↑	x
	APase activity in SF	↓	↑	x
	APase activity in CW	x	↑	x
	Leaf 1 P content	↓	↓	↓ Slow (14 days)
	Leaf 2 P content	x	Not tested	Not tested
	Leaf 3 P content	↓	↓	Not tested
45-14	Days post excision – start	9	15	56
	Days treated	16	21	21
	Days post excision – harvest	25	36	77
	Difference in harvested FW	x	↓ 59%	x
	Root-to-shoot-ratio	x	↑	↑
	APase activity in SF	↑	↑	x
	APase activity in CW	↑	↑	↓ Fast (7 days)
	Leaf 1 P content	x	↓	↓ Fast (7 days)
	Leaf 2 P content	↓	Not tested	Not tested
	Leaf 3 P content	x	X	Not tested
47-9	Days post excision – start	12	23	56
	Days treated	15	17	21
	Days post excision – harvest	27	40	77
	Difference in harvested FW	x	X	x
	Root-to-shoot-ratio	x	↑	x
	APase activity in SF	x	↑	x (↑ 7 days)#
	APase activity in CW	x	X	x
	Leaf 1 P content	x	↓	↓ Fast (7 days)
	Leaf 2 P content	x	Not tested	Not tested
	Leaf 3 P content	x	↓	Not tested

#No reduction in SF after 21 days, with a higher APase in SF after seven days

Signature responses to P-stress in 43-7

The estimated specific root length in 43-7 was the lowest of the three genotypes in experiment II in all three of the treatment groups (Figure 3.18e). In experiment II, the acid phosphatase activity was reduced in the soluble fraction of the P-stressed roots and reduced P-content in leaf 1 and leaf 3 after 15 days treatment (Table 5). In experiment I, the fresh weight was reduced by 58% in the P-stressed plants, the root-to-shoot ratio, root acid phosphatase activity in both fractions were elevated and there was a decline in leaf P content after 20 days treatment (Table 5). There was no induction of acid phosphatase in the genotype screen performed by Effendy (2007) after 21 days treatment, and the decline in P content was observed after 14 days, indicating that P was stored in the leaves. The differences in the signature responses to P-stress between this study and the genotype screen performed by Effendy (2007) suggest that P utilisation differed (post excision) in response to P-sufficient or low P media (Table 5).

Signature responses to P-stress in 45-14

The estimated specific root length was lower in the P-stressed roots in experiments I and II when compared with the P-sufficient and S-stressed plants (Figure 3.18e).
In experiment I, there was a 59% difference in harvested fresh weight between treatment groups that was not observed in experiment II or the genotype screen performed by Effendy, (2007). In experiment II, there was no significant difference in the root-to-shoot ratios between treatment groups, and the P-content was only reduced in leaf 2 (Table 5). The older plants in experiment I and the genotype screen (Effendy, 2007) had a higher root-to-shoot ratio after 21 days that correlated with a reduction in P-content in leaf 1 (Table 5). The acid phosphatase activity in the cell wall fraction of the P-stressed plants was consistently induced in the three treatment regimes listed in Table 5. The acid phosphatase activity in the soluble fraction of the P-starved roots was not induced in the genotype screen performed by Effendy (2007) however, the levels were elevated in the P-stressed plants in experiments I and II of this study (Table 5).
It should be noted that the term 'P-starved' in the genotype screen (performed by Effendy, 2007) refers to the complete withdrawal of P-supply (0 μM KH_2PO_4), and the term 'P-stressed' in this study refers to 10 μM KH_2PO_4.

Signature responses to P-stress in 47-9

The only genotype to have a higher specific root length in the P-stressed roots in both experiments is 47-9, which also had the overall highest estimated specific root length experiment I (Figure 3.18e). However, the estimated specific root length in the S-stressed plants was decreased in experiment II, and reflects the decreased root-to shoot ratio (Figure 3.8h and Figure 3.18e). The high estimated specific root length is consistent with the increased primary root length and increased number of lateral roots (and higher lateral root density Figure 3.12a and c) in the P-stressed plants that contributed to the root fresh weight in experiment II (Figure 3.14c and Figure 3.16c). For example, the estimated specific root length was lower in 43-7, compared to 47-9 in the P-sufficient plants in experiment II. There were 31 lateral roots in 43-7 and 47-9 at 25 days post excision in the P-sufficient plants in experiment II, but the dominant primary root was 108 mm in 43-7 and 76 mm in 47-9 (Table 6a and c). The specific root length was calculated using the fresh weight of the harvested roots. The dominant primary root contributed 35% of the total root length in 43-7 compared with 82% contribution of the total primary root length in 47-9 due to the lower number of supporting primary roots (Figure 3.13a and c).

There were no differences in fresh weight, root-to-shoot ratios, acid phosphatase activity and P-content after 15 days treatment in experiment II (Table 5). The fresh weight of the P-stressed (experiments I and II) and P-starved plants (Effendy, 2007) was not reduced, and the root-to-shoot ratios were increased in experiment I after 17 days treatment, but not after 21 day treatment in the genotype screen (Effendy, 2007). This suggests that the timing of the reduced (or withdrawn) P-supply influences the acquisition and/or utilisation of P more than the length of time treated. In experiment I, the decreased P content in leaf 1 and leaf 3 suggested that 47-9 was able to maintain the plant and leaf fresh weight with the P reserves in the leaves (Table 5, Figure 3.2g and Figure 3.3c). In the genotype screen (Effendy, 2007), the acid phosphatase activity was reduced after seven days, and there was not significant difference after 21 days P-withdrawal. This suggests the P-supply in the vacuole was depleted compared with experiment I in this study that showed increased acid phosphatase activity after 17 days P-stress (Table 5).

3.4.2. Establishment of the branching zone on the primary root

A snapshot of the root morphology at 15 and 25 days post excision shows similar branching zones as a proportion of the primary root length in all three of the genotypes is consistent for experiments I and II (Table 6). This is important because the experimental regime was slightly modified for 47-9 in experiment I (refer section 3.2). Generally, the branching zone is approximately 50% of the primary root length for all three genotypes after 15 days post excision and approximately 80% after 25 days post excision when the plants are grown in P-sufficient media (Table 6). In experiment I, the branching zone was maintained between 85% and 90% from 30 days post excision in all of the genotypes (Figure 3.6d, e, and f). Interestingly, the supporting primary roots also have branching zones that are similar to the dominant primary root of each genotype (data not shown). The individual responses to P-supply differ between the genotypes however; there is a trend that suggests the elongation of the primary root and initiation of lateral roots changed in response to P-supply to achieve a branching zone between approximately 80% and 90% of the primary root length from 24 to 30 days post excision (Table 6).

Branching zone in 43-7

In 43-7, the dominant primary root length was 55 mm and 59 mm for experiments I and II respectively for the plants grown in P-sufficient media at 15 days post excision with similar dominant primary root lengths in the other two treatment groups after three days in low P or low S media (Table 6a). The branching zone was 55% and 51% respectively for experiment I and II. At 24 days post excision, the branching zone was between 78% and 84% because the number of lateral roots in the branching zone was proportional to the length (Table 6). At 35 days post excision (experiment I), the dominant primary root was significantly longer in the P-stressed plants, but the branching zone was maintained between treatment groups.

In summary, the branching zone is not affected by the nutrient supply in 43-7 because the number of lateral roots is proportional to the primary root length, and suggest that 43-7 equally sensitive to P-sufficient and low P media.

Table 6. Summary of dominant primary root development

Data from experiments (Expt) I and II in the dominant primary root at 15 and 25 days post excision for the different treatment groups. **A.** 43-7 **B.** 45-14, **C.** 47-9. The number of days pre-treatment is shown for each treatment group, and is broken down into the number of days in vermiculite and liquid media (+P) indicated respectively in superscript. *Significant differences between +P and –P were calculated using the student's T-test ($P < 0.05$) and analysed using a minimum of five plants. There were no significant between –S and the other treatment groups. The term "growth" refers to the growth of the root post excision.

A. 43-7	**Growth**	**Expt**	**Days pre treatment**	**Days treated**	**Media**	**Length (mm)**	**Branching zone**	**Total lateral roots**
	15	I	15 $^{(9+6)}$	15	+P	59	55%	12
		II	9 $^{(6+3)}$	6	+P	55	51%	13
		II	9 $^{(6+3)}$	6	-P	49	54%	11
		II	9 $^{(6+3)}$	6	-S	52	50%	10
	25	I	15 $^{(9+6)}$	10	+P	121	79%	36
		I	15 $^{(9+6)}$	10	-P	121	78%	33
		II	9 $^{(6+3)}$	15	+P	108	83%	31
		II	9 $^{(6+3)}$	15	-P	89	81%	25
		II	9 $^{(6+3)}$	15	-S	120	84%	35
	35	I	15 $^{(9+6)}$	20	+P	157	85%	
		I	15 $^{(9+6)}$	20	-P	192*	83%	

B. 45-14	**Growth**	**Expt**	**Days pre treatment**	**Days treated**	**Media**	**Length (mm)**	**Branching zone**	**Total lateral roots**
	15	I	15 $^{(9+6)}$	15	+P	105	44%	20
		II	9 $^{(6+3)}$	6	+P	114	67%	35
		II	9 $^{(6+3)}$	6	-P	110	63%	30
		II	9 $^{(6+3)}$	6	-S	110	68%	35
	25	I	15$^{(9+6)}$	10	+P	196	83%	65
		I	15 $^{(9+6)}$	10	-P	182	80%	58
		II	9 $^{(6+3)}$	16	+P	193	86%	82
		II	9 $^{(6+3)}$	16	-P	188	82%	80
		II	9 $^{(6+3)}$	16	-S	186	86%	73
	36	I	15 $^{(9+6)}$	21	+P	225	91%	
		I	15 $^{(9+6)}$	21	-P	249	87%*	

C. 47-9	**Growth**	**Expt**	**Days pre treatment**	**Days treated**	**Media**	**Length (mm)**	**Branching zone**	**Total lateral roots**
	15	I	15 $^{(9+6)}$	15	+P	52	54%	14
		II	12 $^{(7+5)}$	3	+P	27	54%	9
		II	12 $^{(7+5)}$	3	-P	38	61%	10
		II	12 $^{(7+5)}$	3	-S	28	64%	10
	25	I	23 $^{(9+14)}$	2	+P	88	85%	37
		I	23 $^{(9+14)}$	2	-P	101	82%	39
		II	12 $^{(7+5)}$	13	+P	76	78%	31
		II	12 $^{(7+5)}$	13	-P	77	89%*	44
		II	12 $^{(7+5)}$	13	-S	74	84%	35
	40	I	23 $^{(9+14)}$	17	+P	138	87%	
		I	23 $^{(9+14)}$	17	-P	158*	88%	

Branching zone in 45-14

In 45-14, the dominant primary root length of the plants in P-sufficient media was similar after 15 days (post excision) in experiments I and II however, the branching zones were 44% and 67% for experiment I and II respectively (Table 6B). The higher branching zones were also observed in the low P and low S plants with three days treatment and is the result of the earlier transfer from vermiculite to P-sufficient media (as described in section 3.3). At 25 days post excision, the primary root length ranged between 182 mm and 196 mm and the branching zone ranged from 80% to 86% with 10 and 16 days treatment for experiments I and II respectively. A difference was observed in the number of lateral roots between the two experiments, at 15 and 25 days post excision. This is possibly due to the number of days in vermiculite (pre-treatment), because the numbers of lateral roots also differ between experiments I and II for the P-sufficient plants. In summary, the results suggest that 45-14 is sensitive to P-sufficient media because the branching zone increases in P-sufficient media (experiment II) and decreases in a low P supply (experiment I) (Figure 3.6e, Figure 3.7b, right and Table 6B).

Branching zone in 47-9

In 47-9, the length of the dominant primary root was 52 mm in experiment I after nine days in vermiculite and six days in P-sufficient media at 15 days post excision.
In experiment II (15 days post excision), the dominant primary root length was 27 mm after seven days in vermiculite and eight days in P-sufficient media but the branching zone was 54% for both experiments, which means the lateral root density was higher in experiment II (Table 6C). In experiment II, the P-stressed plants had three days treatment at 15 days post excision, and although not significant, the stimulatory response is evident in the P and S treatment groups. At 25 days post excision, the primary root length was similar, and the branching zone was significantly higher in experiment II with 13 days treatment. In experiment I, the P-supply was reduced after 23 days post excision when the branching zone approached 80% of the primary root length, and the stimulatory response occurred in the P-stressed roots.

However, the branching zone was above 80% of the primary root length and similar between treatment groups. At 40 days post excision, the P-stressed roots were longer than the P-sufficient roots in experiment I, but there was no difference in the branching zone that increased by 2% (+P) and 6% (-P) from 25 days post excision.

In summary, the results suggest that 47-9 is sensitive to low P media, and low S media because the branching zone increases by elongating the primary root and increasing the number of lateral roots.

3.4.3. Experiment III – Seven day time course with 45-14 and 47-9

The signature responses to P-stress differed between the genotypes in experiment I, compared with the younger plants (post excision) in experiment II (Table 5).
The signature responses to P-stress were similar between 43-7 and 45-14 in experiment I, and the harvested fresh weights were similar between 43-7 and 47-9 in experiment II. It is possible that the timing of treatment in experiment I may have contributed to the similar fresh weights between treatment groups in 47-9 (Figure 3.2g, Figure 3.8g and Table 5). In experiment I, the branching zone was 55% of the dominant primary root length in 45-14 when the P-supply was changed (15 days post excision) compared with 47-9 that had a branching zone of 74% when the P-supply was changed (23 days post excision).

Experiment III was designed to observe the changes in fresh weight and primary root length as the branching zone approached 80% of the primary root length, and is a refined version of the treatment regime that was used for 47-9 in experiment I.
The seven-day time course was timed to reduce the P-supply as the branching zone approached 80% of the primary root length in 45-14 and 47-9 because the two genotypes had different responses in the branching zone in experiments I and II after the change in P-supply. The plants were harvested after one, two, three, five and seven days and received 1 mM KH_2PO_4 (P-sufficient) or 10 µM KH_2PO_4 (P-stressed) during the seven-day treatment period (Table 7).

Table 7. Analysis of harvested plants from seven day time course

The mean measurements of all of the plants was recorded as day zero, and then recorded again on the day they were harvested (1, 2, 3, 5 or 7 days) with three plants in each treatment group (+P, 1 mM KH_2PO_4 or –P 10 μM KH_2PO_4). The age of the stolon post excision ("growth") is indicated on the top of each table and the number of days treated is recorded in brackets below. The number of leaves was determined by scoring the number of fully emerged leaves as 1 and the unemerged leaf was scored as 0.5. There were no significant differences between treatment groups in any of the measurements between treatment groups using the student's T-test. The specific root length is an estimate because lateral root length was not measured.

45-14		Growth (top) and treatment (below)					
	+P or -P	21 (0)	22 (1)	23 (2)	24 (3)	26 (5)	28 (7)
Weight (grams)	+	0.987	1.073	1.167	1.167	1.900	2.310
	-	0.987	1.047	1.300	1.438	1.810	2.170
Number of leaves	+	3.33	3.50	3.50	4.17	4.50	4.83
	-	3.33	3.17	3.50	4.17	4.50	4.83
Specific root length (cm/mg^{-1})	+	10	8	10	17	17	13
	-	10	7	14	11	16	20
Total primary root length (mm)	+	247	345	374	354	450	557
	-	247	318	349	420	410	455
Dominant primary root length (mm)	+	132	156	177	177	185	199
	-	132	179	165	166	189	206
Branching zone proportion (%)	+	71%	73%	76%	84%	83%	80%
	-	71%	79%	79%	79%	85%	87%

47-9		Growth (top) and treatment (below)					
	+P or -P	22 (0)	23 (1)	24 (2)	25 (3)	27 (5)	29 (7)
Weight (grams)	+	0.647	0.693	0.830	0.753	0.897	1.310
	-	0.647	0.637	0.677	0.883	1.000	1.410
Number of leaves	+	2.67	3.33	3.50	3.50	4.50	5.17
	-	2.67	2.50	3.50	3.67	4.17	4.67
Specific root length (cm/mg^{-1})	+	7	8	11	14	11	15
	-	7	7	9	16	11	14
Total primary root length (mm)	+	133	156	146	122	148	172
	-	133	153	143	117	151	198
Dominant primary root length (mm)	+	66	84	95	83	101	114
	-	66	76	87	111	83	119
Branching zone proportion (%)	+	68%	80%	78%	82%	81%	80%
	-	68%	87%	75%	83%	76%	78%

Treatment regime and harvested fresh weight for experiment III

The stolons were grown in P-sufficient media for 21 days (47-9) or 22 days (45-14), with the extra day to allow 47-9 to initiate primary roots in vermiculite. Three plants were harvested on day zero as a baseline for both genotypes, and the remaining stolons were split into groups of three plants with the mean fresh weight and primary root length consistent with the baseline. The baseline fresh weight and total primary root length was 0.987 g and 247 mm respectively for 45-14, and 0.647 g and 133 mm for 47-9 (Table 7). The plant fresh weight doubled in both genotypes and treatment groups over the seven-day period with no significant differences in either of the genotypes (Table 7).

Fresh weight and P content of leaf one and leaf two in 45-14

The fresh weight and P content were measured in leaf 1 (L1) and leaf 2 (L2) that were numbered from the apex, because they were common to the different time points in the three genotypes (Figure 3.19). At the start of treatment, 45-14 had three fully emerged leaves and one unfolded leaf (Table 7). The newest leaf (L1) was 70 mg on day zero of treatment (21 days post excision), and the fresh weight of L2 was 93 mg (Figure 3.19a and b). On day three of treatment the fourth fully emerged leaf represented L1 and was mostly developed in the low P media, together with L1 on days five and seven of treatment. In contrast, L2 was developed in P-sufficient media on day zero of treatment for all of the time points (Figure 3.19b). On day seven of treatment (28 days post excision), the fresh weight of L1 was 157 mg in the P-sufficient plants, and the P-stressed plants were 137 mg, but were not significantly different (Figure 3.19a).
The P-content in the L1 was significantly different between treatment groups on days one, three, five and seven (Figure 3.19c). This could be due to the distribution of P in two leaves on day 0 compared with four leaves on day seven. The fresh weight of L2 increased over the seven-day period, and the P-content declined over the seven day period with a significant difference between P treatments observed on day seven (Figure 3.19b, d). The P content in L2 was generally higher compared with L1, in both treatment groups, and the decline in P content did not reduce the fresh weight on day seven, which were 277 mg (+P) and 267 mg (-P) (Figure 3.19b).

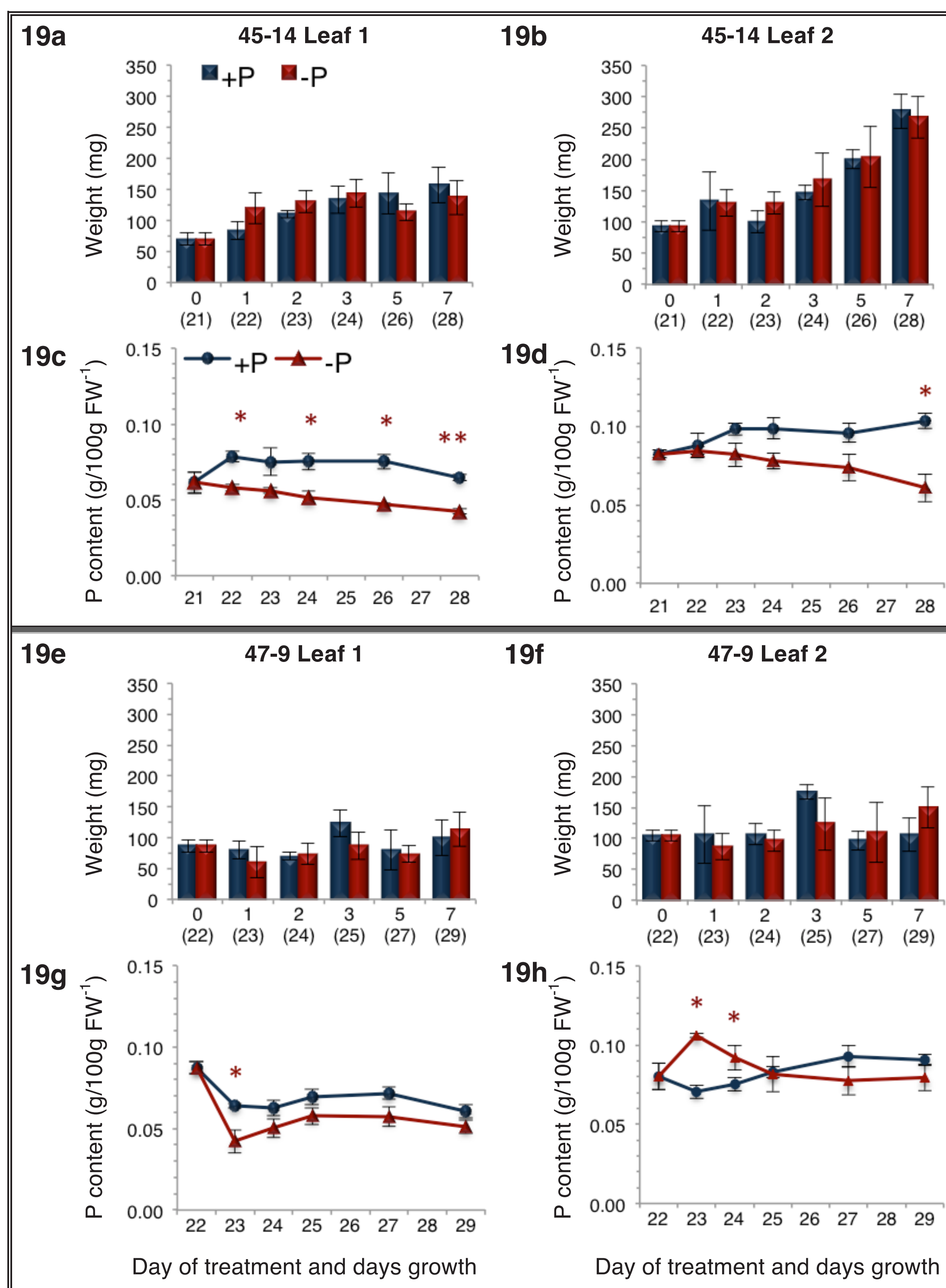


Figure 3.19. Changes in leaf fresh weight and phosphate content

Leaf freah weight for 45-14 (**a.** L1 **b.** L2.) P content for 45-14 **(c.** L1, **d.** L2**)**. Leaf fresh weight for 47-9 (**e.** L1, **f.** L2**)**. P content for 47-9 (**g.** L1, **h.** L2). The significant differences were calculated using the student's T-test ($P < 0.05$ * and $P < 0.01$), from three individual leaves at each time point. The term "growth" refers to the number of days post excision.

Fresh weight and P content of leaf one and leaf two in 47-9

At the start of treatment, 47-9 had two fully emerged leaves and one emerging leaf (Table 7). On days two and three of treatment (24 and 25 days post excision), the third emerged leaf (designated L1 at harvest) and most of the devopment occured in low P media. From day five of treatment, L1 was fully developed in low P media together with L1 on day seven of treatment (29 days post excision). L2 was developed in P-sufficient media throughout the treatment period, which is consistent with 45-14 (Table 7). In 47-9, the fresh weight of L1 was similar over the seven-day time course, and ranged between 87 mg and 123 mg, with a generally lower P content that was significantly different on day one of treatment in the P-stressed plants (Figure 3.19 g). The fresh weight of L2 ranged from 105 mg to 175 mg from day zero (22 days post excision) to day seven (29 days post excision) of treatment (Figure 3.19h). The P content of L2 was also generally higher in L2 compared with L1 in 47-9, and on days one and two, the P-content of the P-stressed plants was significantly higher than the P-sufficient plants (Figure 3.19h).

Root system architecture

The estimated specific root length increased over the seven days in both genotypes and treatment groups (Table 7). The increase over the seven-day period highlights the importance of the root development as the branching zone approaches 80% of the primary root length. The estimated specific root length was similar to the values observed in experiment II for both of the genotypes, which suggests that the number of days post excision reflects root development with respect to time. In experiment III, the estimated specific root length was higher in 45-14 compared with 47-9 because in 45-14, the emergence of lateral roots was not inhibited in the P-sufficient media during the pre-treatment phase that was up to 21 days post excision (compared with experiment II). The root-to shoot ratios were generally higher in the P-stressed plants in 45-14 from day two of treatment, and higher on day one, three and seven of 47-9 (data not shown). In 45-14, the total primary root length was higher in the P-sufficient plants after five days, and it was higher in the P-stressed plants for 47-9 after seven days, which is consistent with the previous experiments.

There were no differences in the dominant primary root length between treatments for both genotypes, and the similar dominant primary root length on days two and three was due to a mean difference of 10 mm on day zero (Table 7). The development of the branching zone with respect to the number of days post excision occurred as expected in both genotypes (Table 6). The branching zone was 68% of the dominant primary root length in 45-14 at the start of treatment and 67% for 47-9, which is consistent with other experiments for 21 or 22 days post excision. The branching zone on the primary root increased to 80% for the P-sufficient plants of 45-14, and was 87% in the P-stressed plants after seven days. In 47-9, the final branching zones on the primary root were similar at 80% (+P) and 78% (-P). The differences in the branching zones were not significant between treatment groups as expected (Table 7).

Acid phosphatase activity and relative expression of *TrPAP26*

The acid phosphatase activity was significantly higher in the soluble fraction of the P-stressed roots in both genotypes on day seven (Figure 3.20a, c). There was also a significant difference in 47-9 after two days (24 days post excision) in the low P media (Figure 3.20c). The P-stressed plants had a similar acid phosphatase activity in the cell wall fraction in both treatment groups and genotypes up to five days in low P (Figure 3.19b, d). On day seven, the acid phosphatase activity in the P-stressed plants of 45-14 was significantly higher than the P-sufficient plants (Figure 3.20b) with no difference in 47-9 (Figure 3.20d).

The relative expression of *TrPAP26* was six times higher in the P-stressed plants of 45-14 on day three of treatment (24 days post excision) and correlates with the emergence of the fourth leaf (Table 7 and Figure 3.20e). However, the qPCR analysis would need to be repeated to confirm the significance of the result because the qPCR was carried out on the pooled sample of three plants at each time point, and does not correlate with the acid phosphatase activity observed from the analysis of the individual roots on day three of treatment (Figure 3.20a, b). This result was not observed in 47-9 that had very low expression of *TrPAP26* (Figure 3.20e).

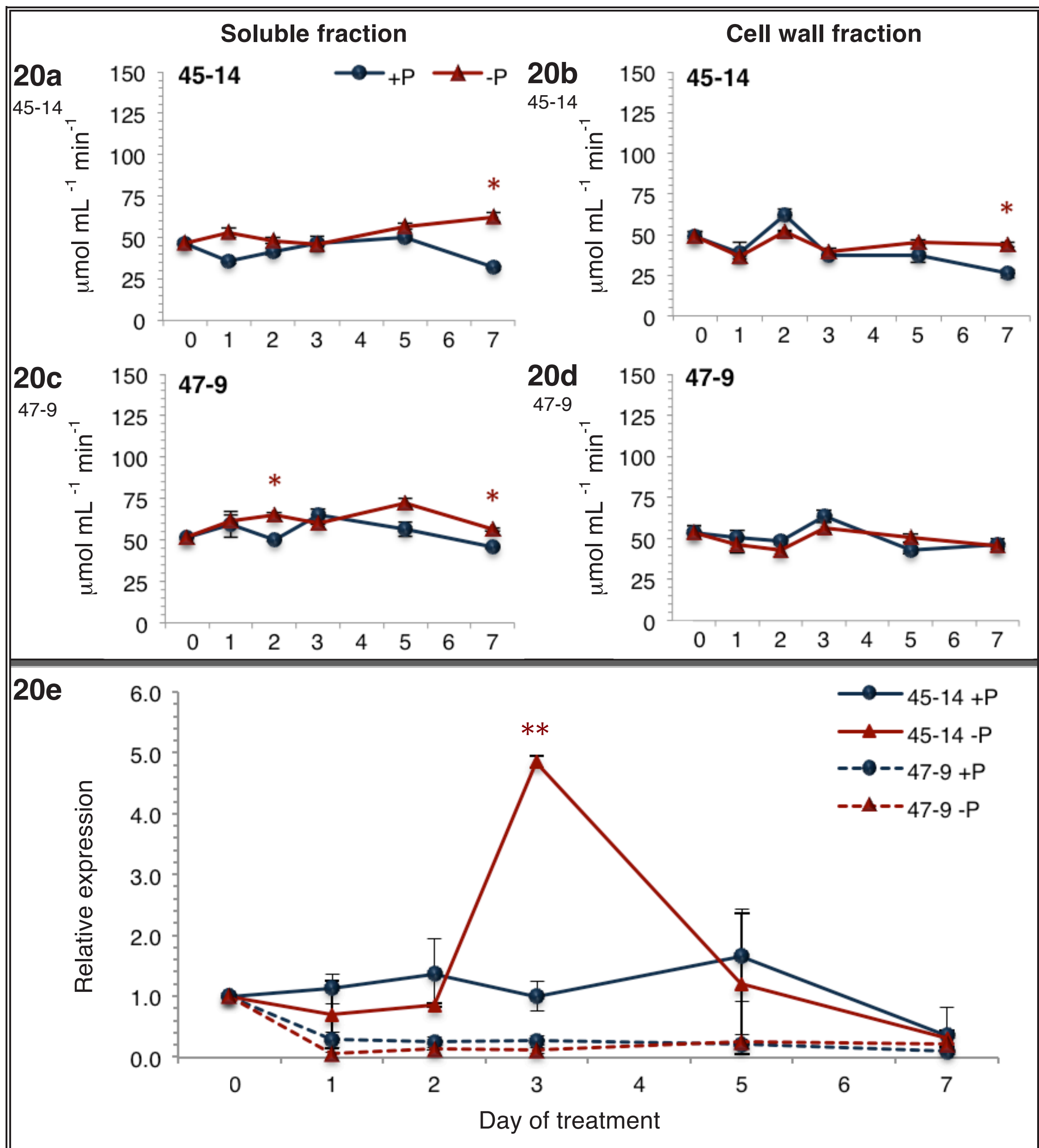


Figure 3.20. Acid phosphatase activity and relative expression of *TrPAP26*

Acid phosphatase activity in soluble and cell wall fractions from harvested roots **a. b**. 45-14 and **c. d**. 47-9. The significant differences were calculated using the student's T-test from three individual plants at each time point (*P<0.05, **P<0.01). **e.** Relative expression of *TrPAP26* in 45-14 and 47-9. Susanna Leung performed the RNA extraction and qPCR analysis with pooled root material from three individual plants at each time point. The standard error and significant differences were calculated using the geomean of three technical replicates (**P<0.01).

Preliminary results from the relative expression of two root P-transporters

Since the conclusion of this study, Susanna Leung and Afsana Islam carried out a preliminary qPCR analysis of two high affinity transporters. The first high affinity transporter was *PHT1*, which transports P from the vacuole in *Arabidopsis*; the second was *PHT1;5*, which mobilises P from the shoot to the root during P-stress, and is upregulated by ethylene (Mudge *et al*., 2002; Raghothama *et al*., 2002; Bayle *et al*., 2011; Nagarajan *et al*., 2011). The expression of *PHT1* increased in the plants grown in P-sufficient media in both genotypes and correlates with the increase in the number of leaves from three to five and the development of the branching zone to 80% of the primary root length during the treatment period (Table 7 and Appendix Va, c).

On day three of treatment (24 and 25 days post excision), the relative expression of *PHT1* increased in the P-stressed plants of both genotypes, and correlates with the emergence of the fourth leaf (Appendix Va, c). By day seven of treatment, the expression of *PHT1* increased in both treatment groups and genotypes, with further increases in the P-stressed plants observed in both of the genotypes (Appendix Va, c). The expression of *PHT5* was low in both genotypes and treatment groups and indicates that there was a low demand to mobilise P from the shoots to the roots (Appendix Vb, d). It would be interesting to test the expression of these P-transpoters in the leaves to correlate the decreasing P content with increased fresh weight in 45-14 (Figure 3.19a, b, c and d) compared with 47-9 that has similar fresh weights slightly lower leaf P content (Figure 3.19 e, f, g and h).

CHAPTER 4. DISCUSSION

The macronutrient phosphorus (P) is essential for plant growth. For example, it forms a component of adenosine triphosphate (ATP) and also phospholipids, which are important for cell structure (discussed in sections 1.2 and 1.4.3). The concept of Phosphorus Use Efficiency (PUE) in plants has been defined as the acquisition and utilisation of P, and has been developed through a world-wide interest in sustainability of crop production for food because there is decline in world phosphate rock reserves (Raghothama, 1999; Wang *et al*., 2010b; White and Brown, 2010; Veneklaas *et al*., 2012). The world interests in establishing biological targets for P-efficiency in plants are discussed in sections 1.2 and 1.4. Generally, when the roots sense a local change in external P, long distance signaling between the roots and shoots maintain the root meristem and growth of younger leaves, and divert non essential resources to the roots for exploration of a new supply (Doerner, 2008; Hammond and White, 2011; Peret *et al*., 2011; Jain *et al*., 2012). The responses to low P-supply occur sequentially and change P metabolism by recycling it from stores in the vacuole and/or remobilising P-esters through hydrolysation by acid phosphatases (Misson *et al*., 2005; Thibaud *et al*., 2010; Tran *et al*., 2010a).

For New Zealand, white clover is an important pasture legume that compliments the growth of perennial rye grass in pastoral farming by fixing nitrogen, and increasing the nutritional content of pastures (discussed in section 1.5.1). The objectives of the white clover breeding programme described by Dunlop *et al.,* (1988) were to identify all of the traits in the legume that assist with tolerance to a low P environment. The proposed desirable root morphology characteristics for optimising growth and uptake of P in white clover are long roots (similar to 43-7 and 45-14) with frequent branching (similar to 47-9) (Crush *et al*., 2005; Crush *et al*., 2008). In the field, the stolons extend horizontally along the ground so it is beneficial for a root to be sensitive to a P-sufficient environment and adapt root growth by remobilising P (as required) to further extend (Chapman and Hay, 1993; Brock *et al*., 2000). In addition, the roots should be sensitive and responsive to low P environments in order to explore new sources or exploit the existing environment through horizontal and vertical growth (Crush *et al*., 2005; Crush *et al*., 2008; Peret *et al*., 2011).

The genotypes (43-7, 45-14 and 47-9) selected for this study were selected based on the utilisation of P after growth in P-sufficient media (1 mM KH_2PO_4) for eight weeks which was then withdrawn (0 mM KH_2PO_4) for 21 days (Effendy, 2007). At the conclusion of the genotype screen, Effendy (2007) suggested that 45-14 was the most efficient genotype, 43-7 was the least efficient genotype, and 47-9 showed potential through the performance of the breeding line in soil and field trials (refer sections 1.5.3 and 1.6). Although the root system architecture was not examined in detail, Effendy (2007) observed a longer primary root compared with the other genotypes, which was of interest for further investigation in this study (M.T. McManus *pers comm*).
In addition, 45-14 was the only genotype to show a response in terms of the induction of acid phosphatase in the cell wall fraction of the roots, which correlated with a higher fresh weight (Effendy, 2007).

This study extends the analysis of 43-7, 45-14 and 47-9 that was first described by Effendy (2007) in terms of root system architecture (discussed in section 4.1) and the timing of the change in P-supply in terms of P utilisation (discussed in section 4.2).
The roots are a major interest in the research field of P-efficiency because of the direct interactions with the environment (discussed in section 1.3), and the hydroponic growth system was a non-destructive method for observing root development.

The main objective of this study was to identify phenotypes in response to the change in P-supply in terms of primary and lateral roots (refer section 1.6). In this study, root development was observed in P-sufficient media in two preliminary experiments (Table 4A). The preliminary experiments formed the basis for two further experiments that were designed to characterise the response to the change in P-supply in terms of changes to the primary and lateral roots (experiment I, section 3.2 and experiment II, section 3.3). However, the final treatment regime for 47-9 differed in experiment I and II because the primary roots were slower to elongate after initiation post excision when compared with 43-7 and 45-14. The treatment regime for 47-9 in experiment I formed the basis of experiment III (section 3.4.3) to test how the timing of the change in P-supply influences the responses as the branching zone approaches between 80% and 90% of the primary root length (discussed in section 4.2).

In this study, root development was directed towards achieving a branching zone that is between 80% and 90% of primary root length between 24 and 30 days post excision (Table 6). The branching zone is established through the elongation of the primary root, and emergence of visible lateral roots in a first stage of growth (refer Figure 2.2). A second stage of growth occurs when the branching zone is maintained above approximately 85% of the primary root length and secondary stolon branches emerge as the lateral and tertiary roots extend (Figure 3.6 and Table 7).

Although the development of the stolons in hydroponic media was similar between the three genotypes, the strategies for growth differ in terms of P acquisition and utilisation by the leaves and roots. This is important to understand persistence of the genotypes in low and high P-supply in the field.

The results from the different treatment regimes used in this study suggest

1. The branching zone in 43-7 is equally sensitive to +P and -P nutrient supply because there are a high number of primary roots with a relatively low lateral root density. The elongation of supporting primary roots and lateral roots is reduced in a limited P-supply. P is stored in the leaves and utilised for the branching of secondary stolons that extend horizontally from the main stolon.

2. The metabolism in 45-14 rapidly converts the nutrient supply to plant biomass by increasing the acid phosphatase activity. The roots are sensitive to P-sufficient media, and the priority is to elongate the primary roots. The root biomass is higher as a total proportion of total fresh weight before the branching zone is between 80% and 90% of the primary root length, and reduced after the branching zone is established.

3. The branching zone in 47-9 is sensitive to a limited P-supply and the roots respond to low P by increasing the lateral root density in the branching zone. The acquisition and utilisation of P is good in +P and –P, but compromised in low sulfur as the branching zone is established to between 80% and 90% of the primary root length (experiment II, section 3.3).

4.1 Analysis of the roots in this study

In this study, the term root system architecture refers to the roots as a whole and includes all of the primary and lateral roots. Each of the cut stolons had four root primordia (two at each node) that were able to initiate primary roots at node three and node four. The final effects of P-stress on the roots were assessed at harvest (summarised in Table 5). The mechanisms involved in changing the root system architecture (as a whole), or local changes to the primary and/or lateral roots in response to P-stress originate from a combination of local signals that alter the cell cycle and maintenance of the root apical meristem and change the sensitivity of hormones and systemic signals that change the root-to shoot ratio (Ticconi *et al*., 2004; Sanchez-Calderon *et al*., 2005; Devaiah *et al*., 2007a; Devaiah *et al*., 2007b; Perez-Torres *et al*., 2008). For example, systemic signals that modify the root-to-shoot ratio are regulated by a combination of sugar, auxin and cytokinin signaling (Jain *et al*., 2007; Hammond and White, 2011; Shin, 2011). The local changes to the primary and lateral roots originate from the perception of external P in the environment at root tip however; the exact signal that is involved in the perception of local low external P is unknown (Svistoonoff *et al*., 2007; Perez-Torres *et al*., 2008; Peret *et al*., 2011; Dinh *et al*., 2012; Li *et al*., 2012b).

The sensitivity of hormones is increase during the early stages of P starvation (P-stress) and is integrated with development (Misson *et al*., 2005; Nibau *et al*., 2008; Jain *et al*., 2012; Li *et al*., 2012b). Therefore, it was important (in this study) to compare the final effects of a limited P-supply between two developmental stages (Table 5), and establish if the changes to the primary and lateral roots were specific to the reduction in P-supply (discussed in sections 4.1.3 and 4.2.1). In the study by Dinh *et al.,* (2012), the primary root length was measured and the number of lateral roots were counted, but this did not demonstrate if the increase in the number of lateral roots was proportional to the primary root length in the limited P-supply, auxin and ethylene treatments. This is important to understand if lateral root density influences P acquisition, although the study did not answer the question directly.

The method of analysis used in this study has not been previously carried out in white clover and suggests there is a common programmed root development pattern shared between the genotypes (Table 6). The main method for root system architecture analysis in white clover uses scanners and image analysis that measure the root surface area, number of root folks, tips and diameter (Crush *et al*., 2005; Crush *et al*., 2008; Jahufer *et al*., 2008).

In this study, the length of the primary roots differed between the genotypes but the proportion of the primary root length containing lateral roots (branching zone) was very similar between genotypes and experiments with respect to time (Table 6). (discussed in section 4.1.2).

The method of root analysis in this study showed that

- The root system architecture (as a whole) differs between genotypes in terms of the number of primary roots, estimated specific root length and proportion of the plant fresh weight at harvest (discussed in section 4.2.1).
- The dominant primary root is representative of all of the primary roots in terms of the development of the branching zone (discussed in section 4.1.1).
- The branching zone is a simple and sensitive method for analysing primary root elongation and lateral root emergence with respect to time (discussed in sections 4.1.1).
- The timing of the change in P-supply influences the response observed in the branching zone however; the changes to the primary and lateral roots are a specific response to P-stress and characteristic to each genotype (discussed in sections 4.1.2 and 4.1.3).

4.1.1. The branching zone as a method for root analysis in this study

In this study, the branching zone contained all of the visible lateral roots compared with the elongation zone that contained the root tip and the root apical meristem (refer Figure 2.2).

The contribution of the dominant primary root to the total primary root length differed between genotypes and the course of the experiments, with more supporting primary roots in 43-7 compared with 45-14 and 47-9 (Figure 3.5 and Figure 3.13).

The selection a single dominant primary root standardised the lateral root analysis to accommodate the variation between stolons (refer Figure 2.2b). The changes in the branching zone measured primary root elongation and emergence of visible lateral roots with respect to time (Williamson *et al*., 2001; Dubrovsky and Forde, 2012).
This method extended the analysis beyond 24 days post excision, which was when they could be physically counted (refer Figure 2.2a and c). Although the data from the dominant primary root was only presented in this study (Table 6), all of the other primary roots in each of the three genotypes and treatment groups also had a branching zone that was approximately 80% of the primary root length at about 25 days post excision (data not shown).

4.1.2. Developing the branching zone for optimum P acquisition

The reduction in P-supply induced specific changes to the primary and lateral roots and these differed between the genotypes (Figure 3.12). In the P-stressed plants, the elongation of lateral roots decreased in 43-7, the emergence of lateral roots was decreased in 45-14, and primary root elongation and lateral root emergence increased in 47-9 within seven days from when the P-supply was reduced from 1 mM KH_2PO_4 to 10 μM KH_2PO_4 in experiments I and II. Therefore the hypothesis that the changes to the primary and lateral roots would occur in response to the change in P-supply is supported. This is important because the timing of the change in P-supply was not consistent between the genotypes (Table 2, discussed in section 3.4). The difference in the timing of the change in P-supply is also interesting from a practical point of view, and raises a question about the how to 'decide' (fairly) when to reduce the P-supply in an experimental situation because natural variation is a factor. For example, in experiment II, the mean branching zone was below 10% in all of the genotypes when the P-supply was reduced after the pre-treatment phase. This was nine days post excision in 43-7 and 45-14, and 12 days post excision for 47-9 because the elongation of the primary roots was slower in 47-9 (refer Table 2 and Figure 3.8a, b and c).

Although the number of days post excision were the same in experiment I for 43-7 and 45-14, the branching zones were 55% and 44% of the primary root length respectively 15 days post excision (Table 6). The branching zones were similar 15 days post excision between experiments in 43-7 and 47-9 suggesting that the pre-treatment phase had no effect on the development of the branching zone (Table 6A and C). In 45-14, the branching zone differed between experiments 15 days post excision because the roots responded to three additional days in P-sufficient media in the pre-treatment phase in experiment II compared with experiment I (Table 6B).

In this study, the timing of the change in P-supply did not affect to observed response to the primary and lateral roots (within seven days) even though the branching zone differed between genotypes and experiments. The responses to P-supply observed in the branching zone could be a mechanism to optimise P acquisition, and if the treatment regime for 47-9 was not delayed in experiment I, the comparison with the other two genotypes could not have been made. Although there was no difference in fresh weight between the treatment groups in 47-9, the 58% and 59% reduction in fresh weight observed in 43-7 and 45-14 respectively suggests that the timing of the reduction in P-supply played a role in P acquisition (Figure 3.2g, and Table 5). The treatment time in experiment III did not extend far enough to evaluate the long-term reduction in fresh weight in 45-14. However, it is possible that the difference in fresh weight would have been less than 59%.

In a large-scale analysis of *Arabidopsis* ecotypes, the rate of the development of the branching zone varied, and was thought to be an indication of the sensitivity to P-sufficient and low P media (Chevalier *et al*., 2003). The length of the primary root and the length from the root tip to the first lateral root were measured after 14 days growth in four ecotypes (Chevalier *et al*., 2003). Two ecotypes (Cvi-0, and Ws-1) were slow at developing lateral roots on the primary root, and the other two (Col-4 and Ler-1) were considered to be fast in P-sufficient media. If the data from the *Arabidopsis* study is converted to the branching zones as a proportion of the primary root length, the two slow accessions had about 47% (Ws-1) and 49% (Civ-0) of the primary root length as the branching zone, and the fast accessions had about 60% and 65% for Col-4 and Ler-1 respectively (Chevalier *et al*., 2003).

The branching zone was reduced by 20-35% under P-starvation because there was a reduction in the primary root length, which decreased the number of lateral roots that could emerge (Chevalier *et al*., 2003).

In an earlier study, the expression of P transporters was compared in the *Arabidopsis* ecotype Ws and Col in P-sufficient and low P media, with differences observed between the two ecotypes (Misson *et al*., 2004). In Ws (slow developer), the expression of *PHT1;5* was expressed in the roots of P-sufficient and low P plants, and was not expressed in Col (fast developer) that expressed *PHT1;7* in P-sufficient media instead (Misson *et al*., 2004). Both of the P transporters were expressed in low P media, with a higher amplification of the PCR product of both transporters observed in Ws-1 compared with Col (Misson *et al*., 2004). The expression of all of the P transporters was not tested in experiments I and II. However, it is possible that there is a different expression pattern between the three genotypes based on the leaf P content in experiments I and II (Figure 3.3b and c and Figure 3.11).

4.1.3. Characteristic responses to P-supply for each genotype

The reduction in P-supply induced changes to the primary and lateral roots that were characteristic for each genotype within seven days. If the response in the branching zone is an indication of the sensitivity to nutrient supply, then 43-7 and 47-9 are slower at developing the branching zone compared with 45-14 in P-sufficient media (Table 6). In this context, the term ‘sensitive’ refers to the responsiveness of the branching zone to maintain the proportion of lateral roots with respect to time by elongating the primary root or the emergence of lateral roots (Table 6).

The contrasting responses to the change in P-supply observed in the branching zones of 45-14 and 47-9 suggest that the root growth is redirected towards exploitation (horizontal) or exploration (vertical) of the environment (Crush *et al*., 2005). In 45-14 the emergence of lateral roots was slower at the ‘expense’ of elongating the primary root in limited P-supply (Figure 3.6b, Figure 3.7b, Figure 3.14b). However, tertiary roots were initiated two days earlier than the in the P-stressed roots indicating that growth is directed horizontally to ‘explore’ the environment compared with explorative (vertical) growth in P-sufficient media (Crush *et al*., 2005).

The phenotype in response to the change in P-supply in 45-14 is similar to the observations by Li *et al.,* (2012) in maize, so it is possible that cytokinin is involved in the observed changes.

In contrast, 47-9 is sensitive to low P-supply and root growth is exploitive (horizontal) in P-sufficient media and explorative (vertical) in limited P-supply because the primary and lateral root growth is increased in response to a change in P-supply (Figure 3.6c Figure 3.15f). The response is increased when the P-supply is changed before the emergence of visible lateral roots and lateral root density increases together with the development of the branching zone (experiment II, Figure 3.15f).

An increase in the primary root length and number of lateral roots was observed in the white clover genotype Challenge 10F in response to low P (Dinh *et al*., 2012). The data in the Dinh (2012) study suggests that number of lateral roots is proportional to the primary root length, which is consistent with, experiment I (47-9) in this study (Figure 3.6c and g). The lateral root density and branching zone increased in 47-9 in experiment II, and is consistent with the external application of 5 nM NAA in P-sufficient media, suggesting the perception through auxin signaling results in changes to the primary and lateral roots (Dinh *et al*., 2012). A comparison of the response to low P-supply between Challenge 10F and 47-9 is an area required for further investigation because the branching zone was not measured in the Dinh (2012) study.

The branching zone is proportional to the primary root length in 43-7 and is maintained by minor adjustments to the primary roots or lateral roots in response to nutrient supply (Figure 3.6d and Figure 3.15d). The growth of 43-7 is exploitative (vertical) through the length of primary roots, and exploitive through the higher number of primary roots compared with the other two genotypes (discussed in section 4.2). However, the elongation of the lateral roots and delayed emergence of tertiary roots reduces the explorative (horizontal) growth of 43-7 in limited P-supply (Figure 3.17).

4.2 Lessons from the treatment regimes

In this study a branching zone comprising of 85% of the dominant primary root length was established in a first stage in growth that includes the initiation of the first four leaves (experiment II, section 3.3). It is possible that this first stage is important in establishing the roots to opitimise P-acquisition (discussed in section 4.1.2).

The role of sulfur (S) during this first stage of growth suggests that the genotypes also differ in S metabolism (discussed in section 4.2.2). The later treatment start time for 47-9 in experiment I and experiment III suggest that a second stage of growth occurs when the branching zone is maintained above 85% of the primary root length and secondary stolon branches emerge as the lateral and tertiary roots extend. The strategies for growth during these two stages differ between the three genotypes in terms of P acquisition and utilisation by the leaves and roots (discussed in section 4.2.1).

4.2.1. Acquisition and utilisation of phosphorus

One of the suggestions for improving the P nutrition in plants in a recent review on P-efficiency was to understand the utilisation of the pool of P within plants, which includes the signals that contribute to the P-stress response (Jain *et al*., 2012; Veneklaas *et al*., 2012). The P pool includes the P acquired and stored in plentiful supply and the rapid utilisation to maintain growth (Wang *et al*., 2010b), which was the basis for the selection of the genetic screen performed by Effendy (2007).

The strategies for growth that were observed in this study included differences in;

- root biomass as a proportion of plant fresh weight at harvest;
- and long term P-stress before and after the branching zone is 80% of the primary root length in experiments II and I respectively;
- acid phosphatase activity in the roots during short term P-stress and preliminary results for P transport by in the vacuole by *TrPHT1* and shoot to root by *TrPHT5* and *TrPAP26* expression in 45-14 and 47-9 (experiment III, section 3.4.3 and Appendix V).

Root biomass as a proportion of plant fresh weight

Some economical benefits of high dry matter and high leaf P content in white clover include increased live weight in sheep, increased milk solids (per cow and per hectare), and increased nitrogen fixation that result in more days in milk from pasture and reduced fertiliser costs (Mackle *et al*., 1996; Chapman and Caradus, 1997; Harris *et al*., 1998; Crush *et al*., 2006). The growth in P-sufficient media is important to compare the responses to limited P-supply, and the efficiency of 45-14 to convert nutrients to fresh weight also extends to its growth in a limited P-supply when compared with the other two genotypes in a similar time frame (Figure 3.8g). In this study, the proportion of root biomass decreased in 45-14 after the branching zone was established to 80% of the dominant primary root length (Appendix IIIA). In contrast, the root biomass (as a proportion of the plant total at harvest) increased with the number of days post excision in 43-7 and 47-9 in P-sufficient media (Appendix IIIA and Appendix IV).

An increasing fresh weight biomass during the seven-day time course that is observed between 21 and 28 days post excision (in 45-14 and 47-9) suggests that an increase in shoot fresh weight and a reduction in root fresh weight occurs between 29 days and 36 days post excision (Table 7). It is possible that the allocation of resources to promote root growth as the branching zone approaches 80% of the primary root length (experiment II) and shoot growth after 29 days post excision in 45-14 contributes to the efficiency in P-sufficient media, which differs in 47-9 (Appendix IV).

A similar study to this one by Chapman and Hay (1993) compared the growth of two white clover genotypes with contrasting leaf sizes in P-sufficient media, and were consistent with the root fresh weight biomass observed in 43-7 (Appendix IIIa).
The genotype with small leaves (Grasslands Tahora) had a higher allocation of root biomass than the larger leaved genotype (Grasslands Kopu), and a higher root-to shoot ratio (Chapman and Hay, 1993). The larger leaved genotypes in this study (45-14 and 47-9) both had a lower root fresh weight ratio than 43-7 (33%) at the end of experiment I, which were 23% and 25% respectively, after 36 and 40 days growth in P-sufficient media with further increases in the P-stressed plants (Appendix IIIA).

Grasslands Tahora was one of the original parental cultivars used for the breeding line screen (Chapman and Caradus, 1997), and it is possible that the high root fresh weight in 43-7 is inherited.

The fresh weight of 43-7 and 47-9 was similar in the P-sufficient plants at the conclusion of the preliminary experiments (Table 4B), and P-stress experiments (Figure 3.2g and Figure 3.8g). However, their root and leaf morphology differed. The higher number of primary roots and lower lateral root density in 43-7 could explain the consistency in sensitivity to high and low concentrations of P. The contribution of the dominant primary root length to the plant total was the lowest in 43-7, and the initiation and elongation of supporting primary roots was inhibited in low P media (Figure 3.5 and Figure 3.13). It is possible that 43-7 requires several primary roots because it prostrate growth of its small leaves and branches requires the diversion of nutrients in a different way to 47-9 (Chapman and Hay, 1993).

The efficiency of the single root in 47-9 was supported by the higher estimated specific root length in the P-stressed treatment group (experiment II) that was higher in experiment I compared with 43-7 and 45-14 with several long roots (Figure 3.18e). However, the efficiency of 47-9 is only evident after the roots are established with a branching zone above 80% of the primary root length (Figure 3.2) because the harvested fresh weight in experiment II was the lowest of the three genotypes (Figure 3.8g).

Relationships between acid phosphatase activity and plant growth

The relationship between high acid phosphatase activity and increased fresh weight (or dry matter) is another biological target to improve P-efficiency in plants (Tran et al., 2010a; Wang et al., 2010b; Zamani et al., 2012). In low P-supply, protein synthesis decreases as resources are diverted to maintain the growth of the roots and new leaves, so the hydrolysis of esterified P through the induction of acid phosphatase is thought to assist with the tolerance to low P-supply and maintain biomass (Morcuende et al., 2007; Tran et al., 2010a; Zamani et al., 2012). The regulation of P-stress induced acid phosphatase in white clover is not fully understood (discussed in section 1.5.3).

This could be because the timing of the change in P-supply varies between studies (Caradus and Snaydon, 1987b; Hunter and McManus, 1999; Hunter *et al*., 1999; Zhang and McManus, 2000; Effendy, 2007). If acid phosphatase is regulated by mechanisms that regulate P homeostasis, instead of through the PHR1 stress response pathways (discussed in section 1.4.4), then the P acquired by the genotypes in the pre-treatment phases of the experiments could influence the levels activity that is observed.
For example, the activity of acid phosphatase on the root surface was induced within an hour of being placed on P-deprived agar, and was localised to the older parts of the roots in after 19 days post excision (Hunter *et al*., 1999). The acid phosphatase activity was visible from staining with XP (5-bromo-4-choro-3-indolyl phosphate *p*-toluidine) in the older parts of the P-stressed white clover roots, which require P for the elongation of the secondary and tertiary roots (Hunter *et al*., 1999).

In this study, the P-stressed plants in 45-14 consistently responded by increasing the acid phosphatase activity in the soluble and cell wall fractions of the harvested roots (Figure 3.3a, Figure 3.9a, Figure 3.20a, b and Table 5). This indicates that P-metabolism is altered by the hydrolysation of P-esters or other substrates in 45-14 (Hunter and McManus, 1999; Hunter *et al*., 1999; Zhang and McManus, 2000; Tran *et al*., 2010a). The acid phosphatase activity in the vacuole also indicates that P is stored and hydrolysed during P-stress in 45-14 in this study, and the genotype screen performed by Effendy (2007). An acid phosphatase isoform was identified in 45-14 by a discontinuous native gel at pH 8.8 that was induced after seven days of P-starvation in a separate experiment from the genotype screen (Effendy, 2007).

As Effendy (2007) did not include 43-7 and 47-9 in the discontinuous native gel, there is no previous knowledge that P-stress induced isoforms of acid phosphatase exist in these two genotypes. The acid phosphatase activity in the cell wall fraction of the roots in experiment I suggest that 43-7 also has an acid phosphatase isoform that is induced under P-stress (Figure 3.3a) that was not observed in the genotype screen performed by Effendy, (2007), (Table 5). In the genotype screen performed by Effendy (2007), 43-7 had a slow response for the decline in leaf P content after 14 days and no response to the induction of cell wall acid phosphatase activity in the roots after 21 days (Table 5).

In experiment I, the acid phosphatase in the cell wall and soluble fraction in 43-7 were higher in the P-stressed roots after 20 days treatment together with a decline in leaf P content and fresh weight (Figure 3.3). This suggests that there was sufficient P stored in the plant to withstand the withdrawal of P in the genotype screen (Effendy, 2007) compared with experiment I in this study (Figure 3.2g). This also supports the proposal that acid phosphatase is induced through systemic signals involved in P homeostasis such as ethylene rather than the phosphate stress response pathway induced by PHR1 (Nagarajan and Smith, 2012). In addition, the different acid phosphatase activity in 43-7 observed in the genotype screen by Effendy (2007) and in experiment I explains the conclusion reached by Caradus and Snaydon (1987b) that acid phosphatase is not a good indicator of P tolerance in white clover (discussed in section 1.5.3).

The different levels of acid phosphatase activity between genotypes and studies also provide some insight about P utilisation during growth. For example, at 19 days post excision, the branching zone was 66% of the dominant primary root length in experiment I in 45-14 (Figure 3.6e). The results from 43-7 and 47-9 in this study (Table 6) suggest that in the Hunter *et al.,* (1999) study, the branching zone was of similar proportions. In this study, 19 days post excision correlates with the growth of the secondary stolons that extend from the main stolon in 43-7 (in P-sufficient media, data not shown). In 45-14 the branches of secondary stolons occurs 24 days post excision and 26 days post excision in 47-9 (data not shown, discussed in section 3.2.2).
The growth of the leaves was not discussed in the Hunter *et al.,* (1999) study, however it is possible that the induction of acid phosphatase within an hour of the change in P-supply 19 days post excision could be due to the demand of P at the time of growth that correlated with a change in P-supply. In this study, the secondary stolons in the P-stressed plants were delayed in 43-7 and 45-14 by eight and five days respectively in experiment I, but not in 47-9 (discussed in section 3.2.2). This suggests that acid phosphatase plays a role in the requirement of P during stolon branching that differs between genotypes.

Preliminary results for P-transport in experiment III

The aim of experiment III was to investigate if the change in P-supply influenced P acquisition as the branching zone approached 80% of the primary root length (sections 3.4.3 and 4.2.3). The increased expression of *PHT1* in experiment III (Appendix V) offers an explanation about the relationship between P-acquisition in P-sufficient media and the difference in harvested fresh weight between treatment groups in 47-9 in experiment I (Figure 3.2g and Table 5 discussed in section 3.4). The preliminary qPCR analysis of *PHT1* in experiment III in 45-14 and 47-9 suggest that P in the vacuole is increased as the branching zone approaches 80% of the primary root length (Appendix V).

The preliminary qPCR analysis in 45-14 suggest that *TrPAP26* is induced in the P-stressed roots after three days treatment (24 day post excision), which further suggests that P is hydrolysed and transported in the vacuole when taken together with qPCR analysis of *TrPHT1* in both treatment groups (Figure 3.20e and Appendix Va). However, the qPCR analysis was only carried out once with pooled biological replicates from three plants at each time point compared with experiments I and II that had a minimum of five plants and a maximum of eight plants (refer Table 1). The qPCR analysis would need to be repeated to establish if the expression of *TrPAP26* was induced by three days of P-stress or if TrPAP26 is required to recycle P for the growth of the fourth leaf or secondary stolons (as previously discussed).

In *Arabidopsis*, *PHT1;1* is regulated by the transcription factor WKRY75, which is also thought to be responsible for regulating lateral root growth (discussed in section 1.4.1) through the homeostatic regulation pathways (Devaiah *et al*., 2007a). The qPCR analysis also needs to be carried out on the harvested root material from experiments I and II in all three of the genotypes, to investigate how the age of the root influences the expression of *PAP26* and the P transporters and compared with the number of days treated.

4.2.2. Acquisition and utilisation of sulfur

The expression of transporters change with varying degrees of available macronutrients to maintain the levels required for optimal growth (Schachtman and Shin, 2007; Nibau *et al.*, 2008; Bao *et al.*, 2011; Shin, 2011). A holistic view of P-efficiency traits in plants could also include the up-regulation of the other mineral transporters in times of P-stress to maintain growth (Misson *et al.*, 2005; Lai *et al.*, 2007; Rouached *et al.*, 2011a). For example, studies with the *Arabidopsis* ecotype Colombia observed that the levels of other minerals such as iron, potassium, sulfur and zinc accumulate in the roots and/or leaves when the P-supply is low, and can alter the balance of endogenous hormones and mineral homeostasis (Misson *et al.*, 2005; Ward *et al.*, 2008; Shin, 2011; Giehl *et al.*, 2012; Jain *et al.*, 2012). Potassium is required for cell division and expansion and is necessary for root growth (Misson *et al.*, 2005; Lai *et al.*, 2007; Peret *et al.*, 2011), and is consistent with the elongation of the primary roots in 43-7 and 45-14 in experiment I (Figure 3.6a and b).

The higher acquisition of other minerals in 45-14 cannot be ruled out. For example, in 45-14, the influx of zinc, iron and sulfur correlates with the structural requirement of these minerals in acid phosphatase (Misson *et al.*, 2005; Anand and Srivastava, 2012). In experiment II, the P-stressed plants in all of the genotypes had 64% of their time post excision growing in 10 μM KH_2PO_4 (-P) or 28 μM $MgSO_4$ (-S) and 45-14 still achieved a greater leaf, root and total fresh weight compared with 43-7 and 47-9 (Figure 3.8g and Figure 3.10b). It is possible that P and S were stored in the stolon (from the stock plants) and utilised during the experiment. However, this could not be tested because it requires a destructive harvest.

The three genotypes were only tested for S-starvation up to 28 days post excision in experiment II of this study (section 3.3). The role of S in the development of the first four leaves and branching zone to 80% of the primary root length was investigated in experiment II of this study, with the reduction in S-supply occurring before the emergence of visible lateral roots (Figure 3.8d, e, f). Sulfur plays a large role in metabolism because it is required for the synthesis of methionine and cysteine (Kopriva, 2006).

The higher acquisition of S is thought to be required in the replacement of phospholipids for sulfolipids during P-stress (Essigmann *et al*., 1998; Misson *et al*., 2005). Two enzymes in the S assimilation pathway in P-stressed *Arabidopsis* plants are upregulated together with the influx of Fe and S (Morcuende *et al*., 2007). When S is taken up in the cell, it is converted to adenosine 5′-phosphosulfate (APS) that can either be activated by adenosine 5′-phosphosulfate (APS) kinase to form 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to form glucosinolates or reduced by APS reductase (APR) in the cysteine and methionine biosynthetic pathways (Takahashi *et al*., 2011).

The levels of APS reductase (APR) and APS kinase were weakly induced in the P starved plants and were unaffected when P was resupplied (Morcuende *et al*., 2007). It was also suggested that P-stress reduces S-assimilation in *Arabidopsis* because the methionine levels were lower in the P-stressed seedlings after 24 hours (Morcuende *et al*., 2007). In 45-14, necrosis was observed both in the P-stressed and S-stressed leaves at harvest, and may be an indication of the interactions between P and S metabolism in 45-14, together with the elevated levels of acid phosphatase activity in the soluble fraction of the roots (Figure 3.9).

The interactions between the different classes of transporters, and their regulation during depletion of other macronutrients are not well characterised. The expression of three transporters for P (*AtPHT1;2*), N (*AtNRT2.1*) and S (*SULTR1;1*) in *Arabidopsis* were compared in a recent study (Bao *et al*., 2011). Six-week-old plants were starved of N, P or S for two and a half days, and then resupplied with the respective mineral for three hours. The expression of *AtPHT2;1* and *AtNRT2* were up regulated by fluctuations in starvation of the either of the other two minerals. The expression of *SULTR1;1* increased by 526% after S starvation and re-supply compared with continuous full nutrient supply, and was not significantly regulated by fluctuations in N or P-supply (Bao *et al*., 2011). A common regulatory pathway between P, S and N stress has been identified in *Arabidopsis* through two MYB transcription factors (MYB75 and MYB90), and are the first step in anthocyanin biosynthesis (Shin, 2011).

In 45-14 and 47-9, a reddish colour was observed in the older leaves of the P-sufficient and P-stressed plants in experiment I, which could be due to the accumulation of anthocyanin that are known to play a role protecting plants from UV damage (Vance *et al*., 2003; Plaxton and Tran, 2011; Shin, 2011). The reddish colour in the leaves was also reported by Effendy (2007) and would require further investigation about when the alternate metabolic pathways are being utilised by identifying the colour of the leaves as a symptom (Effendy, 2007; Plaxton and Tran, 2011).

Although the P and S and N transporters were not investigated in experiment II, the results suggest that the three genotypes utilise S in the leaves with different requirements for the mineral. In 43-7, the P content was significantly reduced in L1 and L3 of the P-stressed and S-stressed plants when compared with the plants grown in complete media (Figure 3.11a and b). In contrast, the P content in the leaves of the S-stressed plants was slightly higher than the plants grown in complete media in 45-14 and 47-9 (Figure 3.11d and f). The lower acid phosphatase activity in the soluble fraction of the P-stressed plants on 43-7 suggests that P could not be recycled from the vacuole in the roots (Figure 3.9a), because P is transported to the leaves together with S in the first 25 days post excision. The exact reason for this observation would require further investigation, and could be related to the recent studies in white clover that proposed that the N metabolism is reduced in white clover under S-stress (Varin et al., 2010).

In 45-14, the fresh weight and root-to-shoot ratios of the S-stressed plants were comparable with the plants grown in complete media (Figure 3.8g, h). The acid phosphatase activity of the cell wall fraction was significantly higher than the plants grown in complete media, but lower than the P-starved plants (Figure 3.9a).
The induction of acid phosphaste in the roots possibly occurred later in the treatment period because necrosis was observed in L2 of the P-stressed and S-stressed plants on the day of harvesting (Figure 3.9b).

The changes to the primary and lateral roots in response to P and S in 47-9 are consistent with changes in auxin levels in *Arabidopsis*, although the mechanisms differ (Kutz *et al*., 2002; Nacry *et al*., 2005; Nibau *et al*., 2008).

In *Arabidopsis* S-stress results in a stimulation of the primary root and increase in the number of lateral roots and is thought to be the result of the synthesis of the auxin precursor indole-3-acetonitrile, which increases the levels of auxin and increases the lateral root formation (Kutz *et al*., 2002; Nibau *et al*., 2008).

This differs from P-stress in *Arabidopsis* and the white clover genotype Challenge 10F where auxin accumulates at the root tip and increases the sensitivity, which also increases the number of lateral roots (Nacry *et al*., 2005; Perez-Torres *et al*., 2008; Dinh *et al*., 2012). The transcription factors that regulate these changes are *PHR1* and *SIZ1* for P-stress and *SLIM1* for S-stress (Miura *et al*., 2005; Miura *et al*., 2011; Shin, 2011). The interactions of hormones (discussed in section 1.3.4) with P and S are an area that requires further investigation in 47-9 together with N metabolism, because the reduction in root biomass in white clover has previously been reported under S-stress (Varin *et al*., 2010).

CHAPTER 5. SUMMARY AND FUTURE WORK

5.1 Summary

The two aspects involved in P-efficiency in plants are phosphorus (P) acquisition by the roots and utilisation of internal P for growth and development (Raghothama, 1999; Vance *et al*., 2003; Wang *et al*., 2010b; Plaxton and Tran, 2011; Jain *et al*., 2012; Veneklaas *et al*., 2012). There are several biological targets that exist for optimising the processes involved in P-efficiency in plants and include the mechanisms that regulate P homeostasis and local signals in the roots that sense P in the environment (discussed in section 1.4). The local and systemic signals induced by the P-stress response result in physiological and morphological changes to plant growth to optimise P acquisition and utilisation (Raghothama, 1999; Vance *et al*., 2003; Bari *et al*., 2006; Ramaekers *et al*., 2010; Thibaud *et al*., 2010; Peret *et al*., 2011; Jain *et al*., 2012). The changes that occur to optimise P-acquisition differ with and between plant species, and the field of phosphorus efficiency has two major branches of research with respect to root system architecture (discussed in sections 1.3 and 1.4).

1. The plant selection approach to identify cultivars and genotypes that adapt root system architecture in low P environments such as bean, maize, white lupin and white clover (Bonser *et al*., 1996; Vance *et al*., 2003; Crush *et al*., 2008; Lynch and Postma, 2011).
2. The molecular approach to characterise the P-stress response signaling that result in changes to root morphology, such as the studies in *Arabidopsis* that does not have specialised root structures or form any associations with mycorhizal fungi that occur in maize, lupin, bean and white clover (discussed in sections 1.3 and 1.4).

The relationships between adaptive root system architecture, P acquisition and P utilisation are not fully understood (Wang *et al*., 2010b; Peret *et al*., 2011; Veneklaas *et al*., 2012). Higher (natural or transgenic) levels of acid phosphatase in roots has been associated with a high fresh weight because P is recycled for efficient metabolism (Plaxton and Tran, 2011; Wang *et al*., 2011; Zamani *et al*., 2012).

In the genotype screen performed by Effendy (2007), 43-7, 45-14 and 47-9 were grown in media containing 1 mM KH_2PO_4 for eight weeks. Then, response to the withdrawal of P (0 mM KH_2PO_4) was measured in terms of the decline in leaf P content and induction of root acid phosphatase over a three-week period together with a series of agronomic parameters such as leaf area, fresh weight and root-to-shoot ratios (Effendy, 2007). The results of this study suggest that there is a correlation between high fresh weight in 45-14 and acid phosphatase activity that was first established by Effendy, (2007). The results suggest that the induction of acid phosphatase is regulated by P-homeostasis because an elevated level of acid phosphatase was observed in the cell wall fraction of the P-stressed roots in 43-7 (Figure 3.3a) that was not observed in the genotype screen (discussed in section 4.2.1).

In 45-14, the nutrients are rapidly converted to fresh weight, and the P-stressed plants consistently had higher acid phosphatase activity (Table 5 and Figure 3.20). The roots are sensitive to P-sufficient media, and the elongation of the primary root and development of the branching zone is rapid. The root biomass as a percentage of the total fresh weight decreases with the number of days post excision in 45-14, compared with an increase in 43-7 and 47-9 (Appendix III), suggesting this strategy of P utilisation assists with P-efficiency.

The main objective of this study was to characterise the changes in the primary and lateral roots of the white clover genotypes grown in P-sufficient media (1 mM KH_2PO_4) and any differences that occur in reduced P-supply (10 μM KH_2PO_4). The specific changes in root phenotype in response to the perception of low external P-supply observed in white clover and *Arabidopsis* formed the basis for the hypothesis in this study (Williamson *et al.*, 2001; Svistoonoff *et al.*, 2007; Perez-Torres *et al.*, 2008; Dinh *et al.*, 2012). In this study, the elongation of the primary roots and the emergence of lateral roots was measured with respect to time and expressed as a proportion of the primary root that contains lateral roots, designated the branching zone (refer Figure 2.2). A common developmental programme was observed in all of the genotypes as the roots grew post excision (Table 6). The branching zone is approximately 50% of the primary root length 15 days post excision (Table 6).

By 25 days post excision, the branching zone is approximately 80% to 85% of the primary root length and maintained between 85% and 90% after 30 days post excision (Figure 3.6 and Table 6). It is possible that establishing the branching zone has a biological function for optimising P acquisition (discussed in section 4.1.2). The method of analysis supports the hypothesis (refer section 1.6) and distinguishes the response to the change in P-supply (within seven days) from root development in P-sufficient media (Table 6 and section 4.1.3) irrespective of the timing of treatment between genotypes (Table 2). In addition, the responses to S-stress (28 μM $MgSO_4$) were not significantly different to the roots grown in P-sufficient media with respect to primary root length and number of lateral roots, indicating a specific response to the limited P-supply (experiment II, section 3.3).

In summary, the genotypes have similarities and differences with respect to root system architecture (Figure 3.12) and the treatment regimes provide some insight about how P is utilised for growth (discussed in section 4.2).

5.2 Future work

There are three major influences that contribute to the change in root morphology in response to P-supply. The first is the sensing of the external low P environment that induces the Phosphate Stress Induced (PSI) genes to make changes in the short, medium and long term (Raghothama, 1999; Hammond *et al*., 2003; Misson *et al*., 2005; Peret *et al*., 2011; Plaxton and Tran, 2011).

This study identified specific responses to the primary and lateral roots of three white clover (*Trifolium repens* L.) genotypes by regulating the P-supply prior to the emergence of visible lateral roots in experiment II, and after the emergence of lateral roots in experiment I (discussed in section 4.1.3). The genotypes differ with respect to elongation of the primary root and emergence of lateral roots in P-sufficient and low P media, therefore the molecular signals that regulate the changes to root system architecture (as a whole) and primary and lateral roots is one area for future investigations.

It is reasonable to conclude that the fate of the meristematic cells is not changed in low P media (as observed in *Arabidopsis*), however detailed microscopy studies of the developing primary root would confirm the differences between the genotypes at a cellular level. The recently developed lateral root promordia index could be used to standardise the analysis (Dubrovsky *et al*., 2009).

The roots of the white clover genotypes makes it suitable for split root studies because the primary roots all follow the same developmental pattern with respect to developing the branching zone on the primary root (Table 6). The development of the individual primary roots in liquid media is staggered, the branching zone is consistently between 80% and 90% from 25 to 30 days post excision. Therefore, the primary roots on the same plant could be exposed to different external P-supplies and transcriptional analysis could be carried out on each of the genotypes to observe the local and systemic signaling that has previously been observed in *Arabidopsis* (Thibaud *et al*., 2010). Bioinformatics studies could also be carried out to identify the PHR1 targets to determine if the regulation of stress response differs between the genotypes (Rouached *et al*., 2011a).

The results of this study suggest that the development of stolons post excision influences the phosphate stress induced responses, and could be directly tested by a series of 14-day time courses. The stolons are cut on Day 0 and grown in vermiculite for seven days with P-sufficient media, then transferred to P-sufficient liquid media with treatment starting after 10 days post excision and at five-day intervals from ten to 35 days post excision (six experiments). The plants are grown over a 14 day time period in treatment media and harvested after 2, 5, 7, 10 and 14 days treatment. The expression of phosphate stress induced genes such as *PAP10* or *PAP12, SQD1*, *PHT1;1, PHT1,4, PHT1;5, PHT1;8* and *PHT1;9* sulfur transporters *SULTR1;1* and *SULTR1;2* (and perhaps *PHO1* and *PHR1*) would serve as indicators of the level of P-stress over time. In addition a full mineral profile of the leaves and analysis of lipid composition and metabolisable energy (an indicator for cattle nutrition) could be carried out on the harvested plant material. In addition, the plants could be resupplied with P to study P homeostasis (Liu *et al*., 2012). A similar set of experiments could be used to test the interactions of hormones during development in response to low P.

One observation that was not pursued in this study was the difference in pH in the low P media (data not shown). A reduction in P in the media from plant uptake results in the media becoming alkaline due to the influx of H^+ ions from active transport (Raghothama, 1999).

In experiment I of this study control pipes containing only media were used to assess fluctuations and identify when the plants excreted acids after the reduction of P in the media. The absence of KH_2PO_4 increased the pH of the low P media compared to the media containing P (data not shown). After seven days, the pH of the low P media remained high, and the pH was reduced in the low P media that contained plants. In comparison, the pH of the P-sufficient media with and without plants remained between 6.0 and 6.1 and was consistent for the three genotypes (data not shown). The alkaline low P media also occurred in experiment II and the pH of the low S media was similar to the P and S sufficient media. Samples of the media were collected in 15mL falcon tubes and frozen, so it is possible to test the changes during the course of the experiments together with mineral analysis. A detailed analysis of media was carried out as part of the investigations contribute to the relationships between P and Fe in *Arabidopsis* (Ward *et al*., 2008), and it would be interesting to observe if Fe also plays a role in changing the primary and lateral roots in white clover. This would be of interest to coastal regions of NZ that have high levels of iron sand in the soil.

Finally, in experiment I, the PVC pipes were weighed with the intention of measuring water (media) uptake in P-sufficient and low P plants compared with PVC pipes without plants. Differences were observed between treatment groups, but the data appeared to be more accurate after 30 days post excision because the roots were taking up more water (media). For example, the difference in 45-14 was approximately 20 mL per day between treatment groups 32 days post excision (data not shown). If these observations were investigated further, it would add to the knowledge of P-efficiency traits in the three white clover genotypes.

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APPENDICIES

Appendix I. Modified Hoagland's media

Stock solutions of the modified Hoagland's media containing half strength macronutrients containing 1 mM KH_2PO_4, and full strength micronutrients. The ingredients were mixed in the order listed to maximise solubility. A 10 times stock solution of the macronutrients and 100 times strength stock solution for the micronutrients was made with 2 L milli-Q water, then diluted with RO water to fill 5 L containers The treatment solutions contained adjusted and replaced amounts of either low P or low S, and the pH was adjusted 6.0 with 5 M KOH. The recipe for liquid media was modified from (Gibeaut *et al.*, 1997).

Macronutrient	MW (g.mol.L)	Complete (mM)	Low P (mM)	Low S (mM)
KH_2PO_4	136.09	1.0	0.01	1.0
$Ca(NO_3)_2$	236.15	2.25	2.25	2.25
KNO_3	101.11	1.8	3.36	1.8
$MgSO_4$	246.48	1.125	1.125	0.028
$Mg(NO_3)_2$	256.41	-	-	1.097

Micronutrient	Concentration (μM)	Weight (mg/L) of 100 times stock solution
KCl	50.0	372.8
H_3BO_3	50.0	309.2
$MnSO_4$	10.0	151.0
$ZnSO_4$	2.0	57.5
$CuSO_4$	1.5	37.5
$(NH_4)_6Mo_7O_{24}$	0.075	9.3
NaO_3Si	0.10	2.10
Fe NaEDTA	72.0	2642.8

Appendix II. RNA extraction buffer for hot borate method

Chemical	Mr (g/mol)	Wt (g) chemical for volume desired		
		250mL	500mL	1L
0.2 M Sodium borate decahydarate	381.4	19.07	38.14	76.28
0.03 M EGTA	380.4	2.853	5.706	11.412
1% SDS		2.5	5.0	10.0
1% Sodium deoxycholate salt		2.5	5.0	10.0
Other components added immediately before use				
10mM DTT				
1% Nonidet P-40, also called IGEPAL				
2% PVP (PVP-40)				

Extraction Buffer (A+B), prepared fresh, pre-warmed at 80°C,

Tissue fresh weight (g)	Buffer XT (mL)	1M DTT (μL)	IGEPAL (μL)	PVP (g)
1	5	50	50	0.10
4	20	200	200	0.40
6	30	300	300	0.60
8	40	400	400	0.80
10	50	500	500	1.00

PVP = Polyvinylpyrrolide mean MW 40,000 (Sigma Cat # PVP40T)

Stock Solutions			Wt (g) of chemical per certain volume of solution				
Conc	Chemical	Mr (g/mol)	1mL	50mL	100mL	200mL	250mL
1 M	DTT	154.3	0.1543				
2M	KCl	74.55		7.46	14.91	29.82	37.28
3 M	NaOAc**	136.08		20.41	40.82	81.64	102.06
4 M	LiCl	42.39		8.48	16.96	33.91	42.39

** 3 M NaOAc = 3 M sodium acetate. Adjust to pH 5.2 with glacial acetic acid.

Other Reagents/Enzymes

Proteinase K - 20mg/mL in DEPC-treated water. Store at -20°C.

24:1 (v/v) chloroform:isoamyl alcohol

Isopropanol

80% ethanol

Sterile DEPC-treated water

DNase

Appendix III. Root biomass as a percentage of total plant fresh weight

Root fresh weight biomass as a percentage of the total harvested fresh weight. **A.** Experiments (Expt) I and II. *The term "growth" refers to the number of days post excision at harvest. The term 'treatment refers to the total number of days in –P or –S at harvest. In experiment II, the P-supply was changed after nine days post excision for 43-7 and 45-14, and 12 days post excision in 47-9. In experiment I, the P-supply as changed after 15 days post excision for 43-7 and 45-14, and after 23 days post excision in 47-9. **B.** Experiment III seven-day time course. The P-supply was changed 21 days post excision for 45-14 and 22 days post excision for 47-9 for a total of seven days.

A. Genotype	Expt	Treatment (Days)	Growth * (Days)	+PS	-P	-S
43-7	II	15	24	27%	22%	26%
	I	20	35	33%	43%	
45-14	II	16	25	33%	35%	32%
	I	21	36	23%	32%	
47-9	II	16	27	17%	22%	12%
	I	17	40	25%	29%	

B. Genotype	Treatment (Days)	Growth * (Days)	+P	-P
45-14	0	21	24%	-
	1	22	20%	22%
	2	23	33%	35%
	3	24	32%	30%
	5	26	32%	36%
	7	28	32%	32%
47-9	0	22	19%	-
	1	23	19%	17%
	2	24	24%	21%
	3	25	24%	26%
	5	27	26%	30%
	7	29	28%	28%

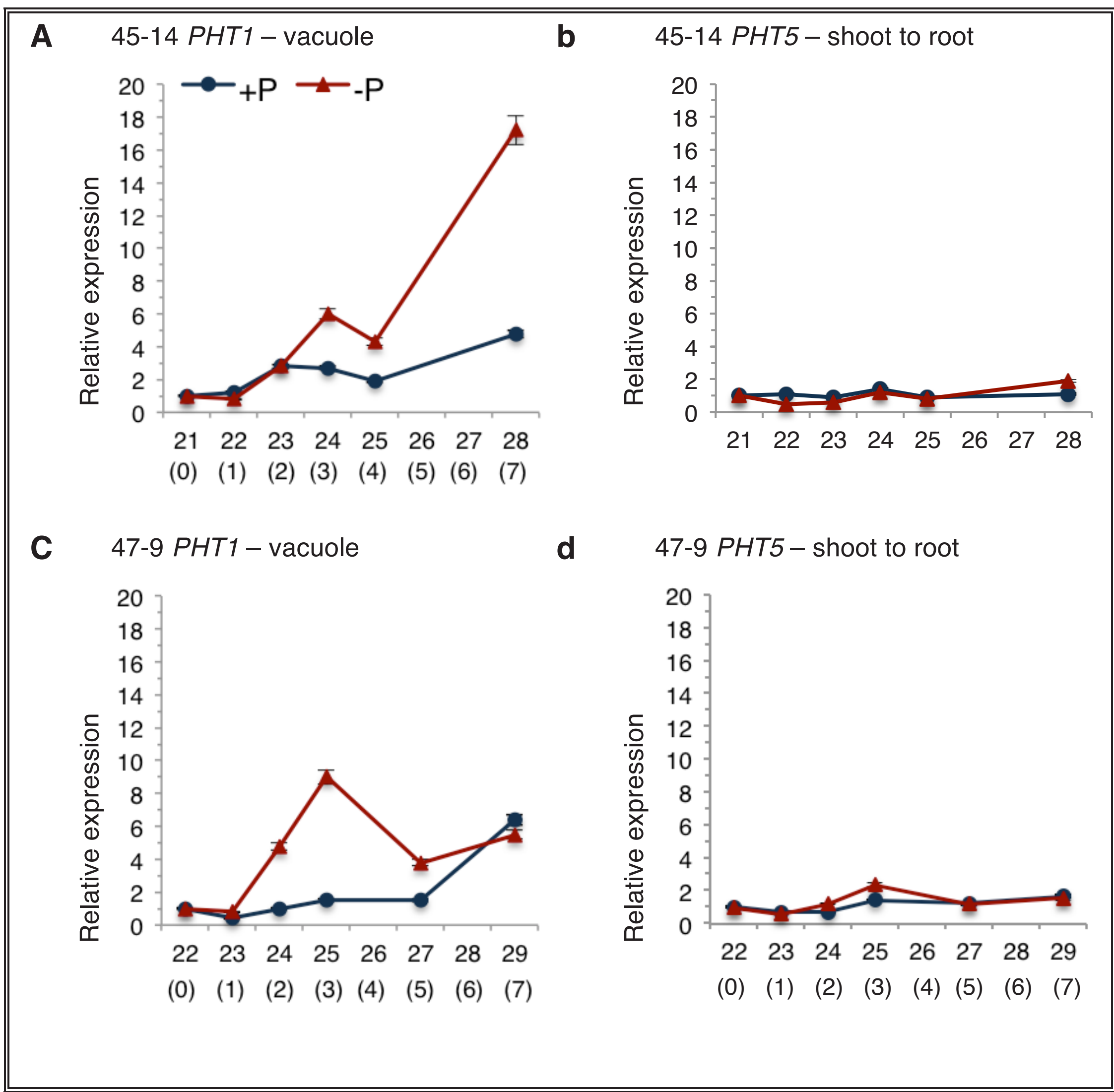
Appendix IV. Increasing fresh weight in 47-9 between 23 and 30 days post excision

Combined results of fresh weight and branching zone as a proportion of the primary root length in 47-9 from experiments I, II and III between 23 and 30 days post excision. Significant differences for experiment II were calculated from a mean of at least five plants using the student's T-test (P<0.05).

Experiment	Days post excision	Days treated	Treatment	Fresh weight	Branching zone as proportion of PR length
I	23	-	+P	86 mg	77%
II	23	11	+P	Not recorded	74%
II	23	11	- P		*91%
II	23	11	-S		82%
III	23	-	+P	69 mg	80%
III	23	1	- P	64 mg	87%
I	27	4	+P	Not recorded	84%
I	27	4	- P		78%
II	27	-	+P	80 mg	78%
II	27	15	- P	69 mg	*89%
II	27	15	-S	63 mg	84%
III	27	-	+P	90 mg	81%
III	27	5	- P	100 mg	76%
III	29	-	+P	1310 mg	80%
III	29	7	- P	1410 mg	78%
I	30	-	+P	1550 mg	85%
I	30	7	- P	1780 mg	84%

Appendix V. Preliminary results of the relative expression of two root phosphate transporters *TrPHT1* and *TrPHT5* from experiment III

Preliminary results from qPCR analysis of two white clover phosphate transporters using the pooled harvested roots from experiment III. In *Arabidopsis* PT1 transports P from the vacuole and PT5 transports P from the shoots to the roots. **a.** PT1 from 45-14, **b.** PT5 from 45-14. **c.** PT1 from 47-9, **b.** PT5 from 47-9. The graphs represent the geomean, with 5% error represented by the error bars. Susanna Leung and Afsana Islam did not carry the statistical analysis when they performed this analysis.



Appendix VI. Camera settings for photographs

All of the photographs were taken from a Sony DSC-H55 digital camera, and the ISO, aperture, shutter speed were set and recorded automatically by the camera.

Figure	Photo description	ISO	Aperture	Shutter speed	Zoom
Figure 2.1a	Stolons in vermiculite	80	F3.5	1/50	4.2mm
Figure 2.1b	Plants with lids	400	F5	1/30	28.4mm
Figure 2.1c	PVC pipes	80	F3.5	1/400	4.2mm
Figure 2.2	Stolon measurements	400	F3.5	1/13	4.2mm
Figure 2.2b	Node 3 DPR (left)	320	F3.5	1/30	4.2mm
Figure 2.2b	Node 4 DPR (right)	400	F3.5	1/10	4.2mm
Figure 3.1a	43-7 – 28 days	200	F4	1/40	7.1mm
	43-7 – 42 days leaves	80	F3.5	1/30	4.2mm
	43-7 – 42 days plant	200	F3.5	1/30	4.2mm
Figure 3.1b	45-14 – 28 days	200	F4	1/40	6.1mm
	45-14 – 42 days leaves	200	F3.5	1/30	4.2mm
	45-14 – 42 days plant	80	F3.5	1/40	4.2mm
Figure 3.1c	47-9 – 28 days	200	F4	1/40	6.5mm
	47-9 – 42 days leaves	250	F3.5	1/30	4.2mm
	47-9 – 42 days plant	80	F3.5	1/30	4.2mm
Figure 3.2a	43-7 Expt 1 Day 0	400	F3.5	1/8	4.2mm
Figure 3.2b	45-14 Expt 1 Day 0	400	F3.5	1/13	4.2mm
Figure 3.2c	47-9 Expt 1 Day 0	250	F3.5	1/30	4.2mm
Figure 3.2d	43-7 Expt 1 Harvest	250	F3.5	1/30	4.2mm
Figure 3.2e	45-14 Expt 1 Harvest	400	F3.5	1/20	4.2mm
Figure 3.2f	47-9 Expt 1 Harvest	200	F4.5	1/50	7.9mm
Figure 3.8a	43-7 Expt 2 Day 0	400	F3.5	1/20	4.2mm
Figure 3.8b	45-14 Expt 2 Day 0	200	F3.5	1/30	4.2mm
Figure 3.8c	47-9 Expt 2 Day 0	80	F3.5	1/100	4.2mm
Figure 3.8d	43-7 Expt 2 Harvest	160	F3.5	1/30	4.2mm
Figure 3.8e	45-14 Expt 2 Harvest	250	F3.5	1/30	4.2mm
Figure 3.8e	47-9 Expt 2 Harvest	320	F3.5	1/30	4.2mm
Figure 3.9	45-14 P necrosis	200	F3.5	1/30	4.2mm
Figure 3.9	45-14 No necrosis	160	3.5	1/30	4.22m
Figure 3.9	45-14 Sulfur necrosis	400	F3.5	1/25	4.2mm
Figure 3.12a	RSA 43-7 +PS	160	F3.5	1/30	4.2mm

Figure	Photo description	ISO	Aperture	Shutter speed	Zoom
Figure 3.12a	RSA 43-7 –P	250	F3.5	1/30	4.2mm
Figure 3.12a	RSA 43-7 –S	200	F3.5	1/30	4.2mm
Figure 3.12b	RSA 45-14 +PS	320	F3.5	1/30	4.2mm
Figure 3.12b	RSA 45-14 – P	400	F3.5	1/10	4.2mm
Figure 3.12b	RSA 45-14 –S	400	F3.5	1/30	4.2mm
Figure 3.12c	RSA 47-9 +PS	200	F3.5	1/30	4.2mm
Figure 3.12c	RSA 47-9 –P	160	F3.5	1/30	4.2mm
Figure 3.12c	RSA 47-9 –S	125	F3.5	1/30	4.2mm
Figure 3.18c	Root colour (+PS, -P, -S)	250	F3.5	1/30	4.2mm
Figure 3.18d	Root colour (-P, +P)	160	F3.5	1/30	4.2mm