RESPONSES TO PHOSPHATE DEPRIVATION IN WHITE CLOVER (*TRIFOLIUM REPENS* L.)

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Abstract

Four breeding lines of white clover (*Trifolium repens* L.) were obtained from AgResearch Grasslands, Palmerston North, New Zealand, that had been shown previously to differ in terms of specific growth responses to added phosphate (P) in the field. These were designated Breeding Line (BL) 43 (low performer on low P; low performer on high P), BL 45 (low performer on low P; high performer on high P), BL 47 (high performer on low P; high performer on high P), and BL 49 (high performer on low P; low performer on high P). These breeding lines and five selected genotypes that were propagated from each line (designated 43-7, 43-8, 45-14, 45-4 and 47-9) were rooted in half-strength Hoagland solution in vermiculite for two weeks and then transferred to half-strength Hoagland liquid media for five weeks prior to the initiation of the experiments. For the breeding line screening, plants were acclimatized in a constant temperature environment for one week prior to treatments, while for the genotypic screening, plants were maintained in a temperature-controlled glasshouse. These lines and genotypes were characterized in relation to P uptake and utilization efficiency by growing in P-sufficient media (+P; 0.5 mM KH_2PO_4) and P-deficient media (-P; 0 mM KH_2PO_4) for 3, 5, 7 and 14 days (for the breeding line screening) and 7, 14 and 21 days (for the genotype screening). Over the time course, inorganic phosphate (Pi) content in leaves, non-specific acid phosphatase (APase) activity in intact roots (both as a total soluble activity and a cell-wall-associated activity), isoenzyme analyses, shoot dry weight (DW) and fresh weight (FW), leaf area, weight of an individual leaf (designated as the weight of the first fully expanded leaf), root FW, and the root:shoot (R:S) ratio were determined.

Pi deprivation enhanced the induction of one major low mobility cell wall acidic isoform, two minor high mobility cell wall acidic isoforms and one major low mobility cell wall basic isoform in all genotypes. Furthermore, the activity of one major low mobility cell wall basic isoform was more higher in genotype 45-14 and one minor high mobility cell wall basic isoform was induced only in genotype 45-14 in response to Pi deprivation.

In terms of individual BLs and genotypes, the screening results showed that BL 49 and genotype 45-14 displayed a constant Pi content and a slow induction of APase activity in the –P media, and had the highest total biomass FW in both +P and –P media.

Overall (in both treatments) BL 49 and genotype 45-14 are the most efficient at utilizing available P as they produced the largest biomass FW, produced more roots in P-deprived media when compared with the other BLs and genotypes, and were more efficient in utilizing the P for the synthesis of biomass. BLs 43 and 45 and genotypes 43-7 and 43-8 are less efficient at utilizing available P, while under P deprivation, BL 45 and genotype 45-14 are the most efficient at utilizing P compared to the other BLs and genotypes. The study also showed that the Pi content in leaves and APase activity in roots was found to be the plant parameter most sensitive to Pi deprivation, and the results suggest that the selection of white clover germplasm for satisfactory performance under low P availability can be carried out using these two parameters as criteria.

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Table of Contents

List of Figures

List of Tables

List of Appendices

Abbreviations

$A_{405\ nm}$	absorbance [log(*Io/I*)] in a 1 cm light path at 405 nm
3-PGA	3-phosphoglyceric acid
APase	acid phosphatase
APS	ammonium persulphate
AtACP5	*Arabidopsis thaliana* acid phosphatase type 5
AtPAP12	*Arabidopsis thaliana* purple acid phosphatase type 12
AVG	aminoetoxyvinylglycine
BL	breeding line
BM	biomass
cDNA	complementary deoxyribonucleic acid
cv	cultivar
d	day
DNA	deoxyribonucleic acid
DOT	days of treatment
DTT	dithiothreitol
DW	dry weight
FW	fresh weight
G	genotype
g	gram
g	acceleration due to gravity (9.81 m s^{-2})
h	hour
kD	kiloDalton (unit of molecular mass)
kPa	kiloPascal
L	litre
LePS2	*Lycopersicon esculentum* phosphate starvation-induced gene type 2
M	molar, moles per litre
mg	milligram
min	minute
MilliQ water	water that has been purified by passing through a MilliQ ion exchange column

µg	microgram
µM	micromolar
miR	microRNA
mL	milliliter
mm	millimeter
mM	millimolar
NIL	near isogenic line
nm	nanometer
NS	not significant
°C	degree Celsius
OD	optical density at x nm in a 1 cm light path
PAE	phosphorus acquisition efficiency
PAGE	polyacrylamide gel electrophoresis
PEP	phospho(enol) pyruvate
PGA	phosphoglycerate
Pht	phosphate transporter
Pi	inorganic phosphate
psr1	phosphate starvation response type 1
PUE	phosphorus utilization efficiency
pup1	phosphate under-producer mutant type 1
R:S	root per shoot
RNA	ribonucleic acid
RNase	ribonuclease
RO	reverse osmosis
s	second
S-APase	secreted acid phosphatase
SPT2	*Saccharomyces cerevisiae* phosphate transporter type 2
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	tris (hydroxymethyl) methylamine
V	Volt (kg m^2 s^{-3} A^{-1})
v/v	volume per volume
W	Watt (kg m^2 s^{-3})
w/v	weight per volume
ρNPP	ρ-nitrophenyl phosphate

Chapter One: Introduction

Overview

Phosphorus (P) is the second most limiting plant mineral after nitrogen. As world phosphorus reserves are becoming depleted, it is becoming increasingly important to understand the molecular mechanisms involved in the P-deficiency response in plants. Although plant roots readily absorb inorganic phosphate, most soil phosphate is inaccessible to plants as it is bound to many soil constituents such as iron, aluminium and calcium phosphate, forming complexes of limited availability to plants. Plants have evolved different strategies to liberate P including the secretion of organic acids, piscidic acids, and secretory acid phosphatases (S-APases) from roots. It has been suggested that S-APases secreted from roots can hydrolyze organic phosphates from the soil so liberating inorganic phosphate that can subsequently be absorbed and utilized by the plants. Acid phosphatases (APases) have also been found to play a major role in salvaging phosphate from the soil.

White clover is one of the most important forage legumes in the temperate regions of the world, and is of significance to the beef cattle and dairy industries. This project examines the responses of four breeding lines of white clover plants to P-deprivation. These breeding lines have been selected from the field, based on their growth responses to added phosphate fertilizer. The responses are characterised in terms of changes in the activity of acid phosphatase in roots, levels of inorganic phosphate (Pi) in leaves and growth responses during Pi deprivation.

1.1. Mineral nutrition in plants

Mineral nutrient deficiencies constitute the major prevalent limitation for crop productivity worldwide. Phosphorus (P), potassium (K) and nitrogen (N) (macro nutrients) are classified into essential mineral nutrients that require the greatest agricultural investment. For micronutrients, Fe is the most limiting micronutrient to agricultural yields (Kochian, 2000). High agricultural yields depend strongly on fertilizer application, with the use of the three main mineral elements - nitrogen, phosphorus, and potassium - rising steadily from 112 million metric tonnes in 1989 to 143 million metric tonnes in 1990 (Lauriente, 1995). However, most crop plants, only

use less than half of the fertilizer applied (Loomis and Connor, 1992). The remaining minerals may leach into groundwater, become fixed in the soil, or contribute to air pollution.

Over the past decade, there has been considerable research investigating the molecular and physiological mechanisms of P, K and Fe acquisition and to understand more about mineral-related genes and the proteins they encode. Research has shown that mineral nutrient acquisition and homeostasis is a highly regulated and complex set of processes. The studies have demonstrated that changes in plant mineral status results in signals that ultimately are transduced and result in the alteration in expression of mineral nutrition-related genes and proteins, causing changes in mineral uptake and utilization which is beneficial to the plant (Wang et al. 2002).

1.2. Phosphorus and plant growth

Phosphorus (P) is one of the most important elements for plant growth and development (Abel et al. 2002). Phosphorus availability is seldom adequate for optimal plant growth as it is commonly bound to many soil constituents, forming complexes of limited availability to plants (Sample, et al. 1980). Unlike the nitrogen cycle, the phosphorus cycle is open and tends toward depletion (Stevenson, 1986). In weathered soils, Fe and Al oxides (and in some cases recalcitrant organic matter) bind native and applied phosphorus into forms with limited availability to plants (Anderson, 1980; Tarafdar and Claassen, 1988). Therefore, low phosphorus availability is a primary limitation to terrestrial plant productivity, and is often acutely limiting in the tropics and subtropics.

The Pi concentration in the soil solution is less than 10 μM (Marschner 1995) and so this makes the uptake of Pi into living cells a problem since the Pi concentration within most plant cells is 10,000-fold higher (1-10 mM, Bieleski 1973). As a result, under normal physiological conditions, plants have to acquire Pi against this huge concentration gradient (Bieleski and Ferguson, 1983). Application of phosphorus fertilizers is not an entirely satisfactory solution to this problem, because of the limited availability, high cost, and marginal effectiveness of phosphorus fertilizers for low input farmers. Further, environmental pollution results from excessive use of phosphorus fertilizers in developed countries (Ryden et al. 1973; Sanchez and Buol, 1975; Sanchez, 1976). Globally, phosphate-rich ore deposits are a non-renewable mineral resource that

may be depleted within the next century (Cathcart, 1980). An alternative or complementary approach is the development of crops with higher adaptation to low phosphorus availability ('phosphorus efficiency'). Such crops would yield better in low-input agroecosystems, and would require reduced fertilizer application (and reduced environmental pollution) in higher input systems (Lynch, 1998a).

Phosphorus efficiency is defined as the ability of a plant to acquire P from the soil and to incorporate or utilize it in yield production (Blair, 1993). To distinguish efficient genotypes from their genetic yield potential, Gerloff and Gabelmen (1983) proposed that germplasm differing in yields under nutrient stress could only be designated as efficient or inefficient if they have similar yields when an optimal nutrient is applied. Thus it can be hypothesized that the efficiency of genotypes is in fact their response to P-deficiency stress, and genotypes having the same yield potential may behave differently with respect to their P-deficiency stress tolerance. Genotypes can also be categorized as P responsive if they have the capacity to increase uptake or yield as the supply of the nutrient to roots is increased. For these reasons, it is important to categorize the available germplasm for their P-efficiency, as well as P-responsiveness (Gerloff, 1976).

Many studies have been documented intra-specific variations for P efficiency in various plant species and these have been proposed as a possible tool to overcome the problem of P-deficiency stress in soils (Fageria and Baligar, 1993) with the application of P fertilizers. Salinas and Sanchez (1976) divided intra-specific variations for P-efficiency in plants in two classes: the differences in relation to external critical levels of P (in the soil) and internal critical levels (in the plant). Genotypic difference in response to P-deprivation has been reported in many crops. These include wheat (Batten et al. 1986a; 1986b; Jones et al. 1989; Fohse et al. 1991; Graham et al. 1992; Batten et al. 1993; Horst et al. 1993; Gill et al. 1994; Gahoonia and Nielsen, 1996; Gahoonia et al. 1997; Schulthess et al. 1997; Yeseen et al. 1998; Egle et al. 1999; Fageria and Baligar, 1999; Osborne and Rengel, 2002; Hayes et al. 2004), barley (Clarkson and Scattergood, 1982; Baon et al. 1993; Gahoonia and Nielsen, 1996; Gahoonia et al. 1997; Römer and Schenk, 1998), maize (Elliott and Läunchli, 1985; Bottacin et al. 1990; Jungk et al. 1990; Sachay et al. 1991; da Silva and Gabelman, 1992; da Silva et al. 1993; Hajabbasi and Schumacher, 1994; Fageria and Baligar, 1997; Krisztina et al. 1997; Ciarelli et al. 1998; Gaume et al. 2001; Yun and Kaeppler, 2001; Machado and Furlani, 2004; Zhu and Lynch, 2004), rice (Ponnamperuma and Castro, 1971; Fageria et al. 1976; Fageria

et al. 1988; Majumder et al. 1990; Hedley et al. 1994; Ni et al. 1996; Fageria and Baligar, 1997; Ming et al. 2002; Lim et al. 2003), sorghum and millets (Wieneke et al. 1990; Payne et al. 1995; 1996; Brown and Jones, 1977), forage and tree legumes (Jones, 1974; Sanginga et al. 1989; Davis, 1991; Mugwira and Haque, 1991; Mugwira and Haque, 1993; Sanginga et al. 1995), cotton (Ashraf et al. 1996; Ahmad et al. 1998), and in other crops including bean (Whiteaker et al.1976), tomato (Coltman et al. 1987), *Plantago major* (Baas and van Bausichem, 1990), Arabidopsis (Krannitz et al. 1991), *Eucalyptus grandis* (Kirschbaum et al. 1992), pigeon pea (Ae at al. 1993), chickpea (Hamud-ur-Rahman et al. 1998), common bean (Yan et al. 1995a; 1995b; Nielsen et al. 2001; Yan et al. 2001), and in soybean (Raposo et al. 2004).

The response of plants to low P is complex, involving P sensing, increased uptake and metabolic shifts promoting P recycling (Abel et al. 2002; Franco-Zorrilla et al. 2004). There are two groups of genes identified in response to Pi deprivation. The early genes that respond rapidly and often non-specifically to P deficiency and the 'late' genes that alter the morphology, physiology or metabolism of plants upon prolonged P deficiency. These late genes generally improve the acquisition of P or promote the efficient use of P within the plant. In *Arabidopsis* short-term responses including induction of phosphatases (Duff et al. 1994; Trull and Deikman, 1998; del Pozo et al. 1999; Haran et al. 2000; Coello 2002), Pi transporters (Smith et al. 1997), RNases (Bariola et al. 1994; Green, 1994; Chen et al. 2000; Müller et al. 2004) and response regulators (Coello and Polacco, 1999).

1.2.1. Effect of Pi deprivation on root growth

Many workers have shown that root growth and development are dependent on the P status of the plants since P is relatively unavailable and immobile in many soils (Silberbush and Barber, 1983; Barber, 1994). The effect of P deficiency on root growth is still unclear. Several authors have observed an enhanced root growth on P deficient plants (Narayanan and Reddy, 1982; Anuradha and Narayanan, 1991; Rychter and Randall, 1994), whilst other authors have reported a reduction of root growth under P deficiency (Radin and Eidenbock, 1984; Hajabbasi and Schumacher, 1994; Kondracka and Rychter, 1997; Mollier and Pellerin, 1999). For example, studies by Narayanan and Reddy (1982) on several plant species and Anuradha and Narayanan (1991) on horsegram reported increased primary and secondary root elongation in P-deprived

plants. Similar results are also reported by Rychter and Randal (1994) on root biomass of bean. Other studies by Anghonini and Barber (1980) observed an increase in root length and dry weight on 12-d-old maize plants when the duration of the P starvation increased between 1 and 6 d. Thus effects of P deficiency on root biomass and root length are still controversial.

In contrast, some workers observed a reduction in root length and biomass for a wide range of species and experimental conditions in P-deficient plants (Atkinson, 1973; Tennant, 1976; Amijee et al. 1989; Bruce et al. 1994; Hajabbasi and Schumacher, 1994; Rosolem et al. 1994). Other studies by Mollier and Pellerin (1999) showed that root growth was slightly enhanced a few days after P starvation, but strongly reduced thereafter. Other researchers reported that root growth is independent of Pi deprivation (Radin and Eidenbock, 1984). For example, Hayes et al. (2004) using two cultivars of wheat (cvs Brookton and Krichauff) which differ in P-uptake efficiency in the field, observed no significant different in root weight when grown in solution culture in P-sufficient or P-deficient media. They also showed that there were also no significant effects of cultivar or P treatment on measured root surface areas. Also, Khamis et al. (1990) using maize and Sicher and Kremer (1988) using barley reported no effect of P deprivation on root biomass. Other studies in soybean have shown that root growth was much less affected by low-P, and no significant reduction in root growth was observed until day 17 (Freeden et al. 1989). At day 21, low-P plants had a 24% reduction in root DW. Studies by Nielsen et al. (2001), using common bean, showed that although genotypes have no significant difference in carbon assimilation, low phosphorus plants utilized a 40% fraction of their daytime net carbon assimilation to root respiration while medium and high phosphorus plants allocated only about 20% of their daytime net carbon assimilation to root respiration. They also found no significant difference in P absorption per unit root weight and plant growth per unit P absorbed. Furthermore, relative to P-inefficient genotypes, P-efficient genotypes allocated a larger fraction of their biomass to root growth under low P conditions. They also showed that a lower root respiration rate in efficient genotypes enables them to maintain a greater root biomass allocation without increasing root carbon costs.

The mechanism by which P deficiency affects root growth still remains unanswered. Anuradha and Narayanan (1991) proposed that P deficiency affects root elongation through its effect on H^+ ion excretion by roots and subsequent effects on cell wall loosening. Other workers, for example Amijee et al. (1989), suggest a direct effect

of the P inflow in roots on their density of branching. Mollier and Pellerin (1999) suggested that P deficiency mainly affects the carbon budget of the plant and has no direct effect of P deficiency on root morphogenesis. These results are in accordance with the results of many workers who observed a higher root:shoot ratio in P-deficient plants which associated with a higher proportion of carbohydrates being partitioned to the roots and a higher sugar concentration in the roots (Freeden et al. 1989; Khamis et al. 1990; Paul and Stitt, 1993; Cakmak et al. 1994; Rychter and Randal, 1994; Ciereszko et al. 1996).

1.2.2. Effect of Pi deprivation on shoot growth

Shoot weight and Pi uptake were shown to be the most sensitive plant parameters to P deficiency (Fageria and Baligar, 1997; 1999) and reduced shoot growth is generally observed for plants during P-deprivation (Whiteaker et al. 1976). A study by Hammond et al. (2003), using Arabidopsis, showed no significant difference in shoot fresh weight of plants grown in +P or –P for at least 72 h after P was withdrawn. However, 216 h after P was withdrawn, shoot FW of plants grown without P was significantly lower relative to plants grown in P-sufficient solutions. In soybean, low-P treatment reduced shoot growth significantly 7 days after treatment began (Freeden et al. 1989). After 21 days, plants grown in low-P had a shoot DW of less than 17% of that of high P-plants. In another study, Hayes et al (2004) used two cultivars of wheat that differ in P-uptake efficiency in the field and compared their performances in solution culture. In soil, with similar biomass accumulation, the cv. Brookton accumulated 32% more P than the cv. Krichauff. In solution culture at 1 μM Pi and 10 μM Pi, cv. Krichauff (the less efficient cultivar) grew better compared to cv. Brookton (Hayes et al. 2004). Other workers suggested that the increase in shoot growth in P-deprived plants is a direct consequence of a reduction of leaf expansion and reduced leaf initiation (Lynch et al. 1991) possibly by decreasing root hydraulic conductance (Radin and Eidenbrock, 1984) and by reducing cytokinin transport from root to shoot (Salama and Wareing, 1979; Horgan and Wareing, 1980).

1.2.3. Effect of Pi deprivation on Root:Shoot Ratio

A general response to low P availability is to increase the relative biomass allocation to roots. An increase in the root-to-shoot ratio is often observed in plants in

response to P derivation (Atkinson, 1973; Anghinoni and Barber, 1980; Sicher and Kremer, 1988; Fredeen et al. 1989; Smith et al.1990; Lynch et al. 1991; Paul and Stitt, 1993; Cakmak et al. 1994; Rosolem et al. 1994; Rychter and Randall, 1994; Ciereszko et al. 1996; Horst et al. 1996; Zhu and Lynch, 2004). This increase in root:shoot ratio may enhance phosphorus acquisition as well as reduce growth rates by diverting carbon production to the roots (Fredeen et al. 1989; Khamis et al. 1990; Paul and Stitt, 1993; Cakmak et al. 1994; Rychter and Randal, 1994; Ciereszko et al. 1996; Trull et al. 1997). Zhu and Lynch (2004) reported an increased root:shoot ratio of maize plants by approximately 39% in plants grown in low P media. In wheat, the R:S ratio is reportedly highly dependent on the wheat genotype (Sadhu and Bhaduri, 1984). Furthermore, under P deficiency, P-efficient wheat varieties tend to enhance their root growth (Römer et al. 1988). With more root length per unit above ground biomass, the wheat plants are able to access more P resources in the soil and improve P uptake.

Studies by Nielsen et al. (2001) using four common bean genotypes with different adaptations to low P availability in the field showed that although these common bean genotypes had similar rates of P absorption per unit root weight and plant growth per unit P absorbed, P-efficient genotypes allocated a 20% fraction of their biomass to root growth especially under low P conditions. It was also shown that efficient genotypes had lower rates of root respiration when compared with inefficient genotypes. This suggested that efficient genotypes are able to maintain greater root biomass allocation without increasing overall root carbon costs (Nielsen et al. 2001).

1.2.4. Effect of Pi deprivation on leaf area and weight of individual leaf

Reduction in leaf area is commonly observed during Pi deprivation. Phosphorus deficiency was shown to severely reduce the leaf area of several species. These include cassava (Pellet and El-Sharkawy, 1993), sunflower (Colomb et al. 1995; Rodriguez et al. 1998c), and wheat (Rodriguez et al. 1998a; 1998b). This is consistent with the results of other authors who have reported a rapid and severe effect of Pi deprivation on leaf growth (Atkinson, 1973; Radin and Eidenbock, 1984; Sicher and Kremer, 1988; Freeden et al. 1989; Rao and Terry, 1989; Lynch et al. 1991; Qui and Israel, 1992; Halsted and Lynch, 1996; Mollier and Pellerin, 1999). For example, Radin and Eidenbock (1984) showed that Pi deprivation in cotton caused reduction in leaf

expansion through interactions with water transport, while Lynch et al. (1991) showed that reduced leaf area development was associated mainly with reduced leaf appearance and its morphological determinants, rather than reduced elongation of individual leaves and final leaf size. Further, in soybean, Freeden et al. (1989) showed that total leaf area was slightly affected by low-P treatment with a decrease to 15% in plants grown in low-P treatment. This is followed by reductions of 67% in mean leaf area, and 43% in leaf emergence. In addition, foliar application did increase the final leaf area at all P levels. Other work by Mollier and Pellerin (1999) reported that Pi deprivation caused reduction in leaf area by about 20% relative to the control. By day 16, total leaf area of P deprived plants was reduced to about 80% of that of the control. These workers thus proposed that this was the consequence of a slower rate of leaf appearance and a reduced final size of individual leaves.

1.2.5. Effect of Pi deprivation on biomass accumulation

Phosphorus deficiency substantially reduced total biomass accumulation. This reduction in total biomass accumulation is determined by many physiological and biochemical changes in plants under P deficiency. Plants grown in high phosphorus soil produced significantly more total dry matter than those grown in the low phosphorus soil from day 24 onwards. Further Gaume et al. (2001) showed that P deficiency in hydroponic culture resulted in decreased dry matter production of the four maize genotypes. The decrease was especially evident in the low-P tolerant *NTS* and acid-tolerant *Sikuani*. A significant difference in biomass accumulation was observed by Hayes et al. (2004) using two cultivars of wheat in solution culture that differ in P-uptake efficiency in the field. In soil, with similar biomass accumulation, cv. Brookton (the P efficient cultivar) accumulated 32% more P than cv. Krichauff (the less efficient cultivar). However in solution culture, at 1 μM Pi and 10 μM Pi, cv. Krichauff grew better. Other workers have shown that the final reduction of biomass production during Pi deprivation is as a result of reduction in leaf area and reduction of net photosynthesis per unit leaf area (Rao and Terry, 1989; Jacob and Lawlor, 1991; Qui and Israel, 1994; Rodriguez et al. 1998a). However, several authors have shown that plant growth under P deficiency is usually reduced before the photosynthesis rate per unit leaf area was observed (Jacob and Lawlor, 1991; Qui and Israel, 1994; Rao and Terry, 1989).

1.3. Strategies adopted by plants to withstand Pi-deprivation

Plants have different metabolic, biochemical and developmental strategies for adapting to limited Pi supply. These comprise (1) those aimed at a conservation of P, and (2) those directed toward enhanced acquisition or uptake (Lajtha and Harrison, 1995; Horst et al. 2001; Vance 2001, Playsted et al. 2006). The former processes include decreased growth rate, increased growth per unit of P uptake, remobilization of internal Pi, modifications in carbon metabolism that bypass P-requiring steps, and alternative respiratory pathways (Schachtman et al. 1998; Plaxton and Carswell, 1999; Uhde-Stone et al. 2003a; 2003b).

Developmental responses mainly involve changes in root architecture that enhance the root surface/soil volume ratio and, as a result, the ability of the plant to access soil phosphate. These changes include, for example, increases in the root-to-shoot ratio, the number of lateral roots and the number and length of root hairs (Bates and Lynch, 1996; 2000, Ma et al. 2001; Williamson et al. 2001; López-Bucio et al. 2002; Wang et al. 2004; Zhu and Lynch, 2004). Furthermore, some plants have the ability to form cluster roots or to establish a symbiotic association with mycorrhizal fungi (Harrison, 1999; Watt and Evans, 1999; Burleigh et al. 2002; Lambers et al. 2003; Vance et al. 2003; Karandashov et al. 2004; Glassop et al. 2005). Moreover, at extremely low P supply, nonmycorrhizal Cyperaceae species can form dauciform roots (rootlets densely covered with long hairs) that comprise up to a quarter of a root biomass (Shane et al. 2005). It has also been shown that lateral roots prefer to proliferate in areas of high Pi content and are retarded in the low Pi areas (Drew, 1975; Robinson, 1994; Shane et al. 2005).

1.4. Pi deprivation induced changes in gene expression

In an effort to understand the molecular mechanisms underlying P stress, attempts have turned toward the isolation of genes regulated by P supply. This also permits insight into their functions and the pathways that lead to their expression. Although several responses of plants to P deprivation, including short term metabolic and physiological changes, may not require changes in gene expression, the majority are predicted to rely on alterations in gene expression. The most important questions to be asked with respect to P deprivation are: (1) what genes are induced or repressed during

Pi deprivation? (2) what is the function of the encoded gene products?, and (3) how are these genes regulated? It is clear now that many of the biochemical, physical and morphological changes which occur in response to Pi starvation are associated with altered gene expression (Plaxton and Carswell, 1999; Raghothama, 1999).

Altered gene expression in response to phosphorus deprivation has been demonstrated in the roots of *Arabidopsis*, tomato, rice, white lupin, and white clover. These genes encode proteins involved in P metabolism, carbon metabolism, glycolysis, and lipid metabolism, as well as coding for a high affinity Pi transporter, lignin synthesis-related genes and secondary metabolites. Many of these genes have been cloned and their responsiveness has demonstrated the importance of transcriptional control in the regulation of these responses in plants. For example, a *LePS2* (a gene involved in internal remobilization of P from tomato) transcript was detected within 24 h after Pi-starvation in roots and shoots and it continued to increase with an extended duration of the Pi starvation period reaching a maximum at day 5. In cell culture, rapid induction of *LePS2* was observed within 3 h after transferring tomato cell cultures to a Pi-deficient media. This indicates a rapid response to Pi deficiency in the culture media. This result is in agreement with the data showing that APase activity was induced within 24 h of transferring tomato cells to Pi-deficient medium (Goldstein et al. 1988b).

1.5. Pi homeostasis and signal transduction during Pi deprivation

In order to overcome problems with P availability, plants have evolved a series of adaptive responses to maintain Pi homeostasis. These responses include conservation and remobilization of internal P and enhanced acquisition of internal P (Raghothama, 1999; Poirier and Butcher, 2002). It has been shown that Pi homeostasis plays an important role within plant cells. This requires monitoring of the Pi concentration in cellular compartments/organelles such as the cytoplasm, vacuole, plastids and mitochondria. When a plant is Pi sufficient, about 85-95% of the total Pi is stored in the vacuole (Bieleski and Ferguson, 1983). However, in Pi-deprived plants, almost all Pi is found in the cytoplasm and chloroplasts. This represents parts of the 'metabolic pool' of Pi in the plant (Bieleski and Ferguson 1983; Marschner, 1995). Studies in maize roots showed that under Pi deprivation, the vacuole acts as a Pi reservoir to maintain the

cytoplasmic Pi pool constant and the latter does not decrease until Pi stress becomes severe (Lee et al. 1990).

Studies using the *Arabidopsis* mutants *pho1* and *pho2* which are defective in Pi homeostasis, showed that Pi concentration in the leaves of *pho1* was strongly reduced while Pi concentration in the roots was similar to that of wild type plants (Poirier et al. 1991). This suggested that the *pho1* mutant was impaired in a protein involved in loading of Pi into the xylem in the root. In Arabidopsis, Chiou et al. (2006) showed that the mechanism of Pi homeostasis involves the suppression of an ubiquitin-conjugating E2 enzyme by a specific mircoRNA, namely miR399. Under Pi deprivation, the miR399 is upregulated and its target gene, E2, is downregulated. Using transgenic Arabidopsis overexpressing miR399, they showed that the accumulation of the E2 transcript is suppressed. Further they observed that transgenic plants accumulated five- to six-fold higher Pi levels in the shoots and showed Pi toxicity symptoms similar to E2 mutant. It is further shown that Pi toxicity is caused by an increase in Pi uptake, translocation and retention in the shoots. Unlike wild-type plants, remobilization of Pi in miR399 transgenic plants was impaired (Chiou, et al. 2006). These results prove that miRNA controls Pi homeostasis by regulating the components of the proteolysis machinery in plants.

1.6. Pi starvation and secondary metabolism

A change from primary to secondary metabolism is commonly found in plants under nutrient limitation. Phosphorus starvation often results in accumulation of secondary metabolites such as flavonoids and indole alkaloids (Plaxton and Carswell, 1999). Shih and Kao (1996) reported that the accumulation of the polyamine putrescine is involved in mediation of growth inhibition in response to P starvation. Furthermore, studies by Yamakawa et al. (1983), using *Vitis* cell cultures, reported that P deprivation induced the accumulation of anthocyanin, while application of P induced primary metabolism and inhibited anthocyanin synthesis. The increase in anthocyanin production in P deficient plants is thought to protect plants from photoinhibitory damage to chloroplasts (Takahashi et al. 1991; Trull et al. 1997). Exudation of phenolic compounds into the rhizosphere in response to P deficiency has been observed in several plant species for example, alfafuran from alfalfa roots (Masaoka et al. 1993),

piscidic acid from pigeon pea roots (Ae et al. 1990) and isoflavonoids from cluster roots of white lupin (Neumann et al. 2000).

1.7. Pi deprivation and root architecture

Root architecture, defined as the spatial configuration of a root system (Fitter, 1991; Lynch, 1995), may be especially important for phosphorus acquisition by plants, since the relative immobility of phosphorus in soils makes phosphorus acquisition very dependent on soil exploration in time and space (Barber, 1995). One of the principal components of root architecture is root gravitropism, or the tendency of roots to grow with a certain orientation with respect to gravity (Evans, 1991). Since root gravitropism determines how much shallower a root system remains in the soil (Chu and Chang, 1966; Pothuluri et al. 1986), a shallower root system may be advantageous for phosphorus acquisition in many soils (Lynch, 1998b; Playsted et al. 2006; Walk et al. 2006).

Comparative analysis of genotypes of common bean (*Phaseolus vulgaris* L.) contrasting in adaptation to low phosphorus availability suggests that several root architectural parameters are related to phosphorus efficiency. These include lateral root branching, density and length of root hairs, adventitious rooting, and root growth plasticity (Lynch and Beebe, 1995; Snapp et al. 1995; Lynch, 1998b; Miller et al. 1998; Yan and Lynch, 1999). Furthermore, phosphorus availability regulates root gravitropism in bean and other legumes, and phosphorus efficient genotypes have shallower root systems (Bonser et al. 1996). Geometric modelling has shown that shallower root systems are inherently more efficient in acquiring phosphorus because of colocalization of soil nutrient availability and root activity, and less inter-root competition (Ge et al. 2000).

1.8. Pi deprivation and Pi transporters

Enhanced Pi uptake during Pi deficiency has been correlated with an increased number of high-affinity Pi transporters assembled in the plasma membrane (Drew and Saker, 1984; Shimogawara and Usuda, 1995; Muchhal and Raghothama, 1999). Genes encoding high-affinity phosphate (Pi) transporters have been identified in several species, including *Arabidopsis* (Muchhal et al. 1996; Okumura et al. 1998, Smith et al.

1997; Rausch et al. 2001; Mudge et al. 2002; Misson et al. 2004; Shin et al. 2004;), rose (Kai et al. 1997), *Medicago truncatula* (Liu et al. 1998b), tomato (Daram et al. 1998), potato (Leggewie et al. 1997, Rausch et al. 2001, Gordon-Weeks et al. 2003), and barley (Schünmann et al. 2004). Studies showed that many of the genes of phosphate transporters are expressed preferentially in roots in response to Pi deprivation (Muchhal et al. 1996; Leggewie et al. 1997; Smith et al. 1997; Daram et al. 1998; Liu et al. 1998a; Raghothama, 1999; 2000; Liu et al. 2001; Gordon-Weeks et al. 2003) which suggests a role in Pi capture and uptake (Daram et al. 1998; Liu et al. 1998a; Chiou et al. 2001; Karthikeyan et al. 2002; Mudge et al. 2002). It has been shown that the transcript levels of these transporters increase both with duration and severity of Pi deficiency. Liu et al. (1998a) observed the transcript accumulation of high affinity transporters within 3 to 6 h after cell cultures were subjected to Pi deprivation. These results showed that plants activate Pi-deprivation response mechanisms before depletion of cellular Pi. In contrast, *pht1;*4 was induced during short-, medium-, and long-term treatments, indicating rapid, sustained induction of this gene in response to Pi deprivation, which is consistent with its proposed role in both acquisition and mobilization of Pi (Karthikeyan et al. 2002; Misson et al. 2004; 2005). In potato, a high-affinity transporter, SPT2, is expressed mainly in the elongation zone at the root tip (Gordon-Weeks et al. 2003). This suggests an adaptation strategy of plants to grow under Pi deprivation. Furthermore, Pi replenishment studies using two genes encoding phosphate transporters, *Pht1;7* (a high-affinity transporter) and *Pht2;*1 (a low-affinity transporter) showed that these Pi transporters were highly expressed in both roots and shoots of P-starved plants and the transporters' transcripts could not be detected after transferring to the Pi-rich medium.

Unlike high-affinity Pi transporters, the low-affinity Pi transporter, *Pht2;1* is predominantly expressed in green tissue and transcript levels were independent of P status (Daram et al. 1999; Versaw and Harrison 2002). It has been shown by Daram et al. (1999) that a very low transcript level was evident in roots, which increased steadily during Pi deprivation, while Rausch et al. (2004) found that the expression of *Pht2;*1 is strongly upregulated by light in potato and *Arabidopsis* leaf tissues. They suggested that the Pht2;1 protein is involved in cell wall metabolism of young, rapidly growing tissue. Studies by Versaw and Harrison (2002) detected no *Pht2;1* transcripts in roots of either P-deprived or in P-sufficient plants. Versaw and Harrison (2002) further localized Pht2;1 fused to green fluorescent protein to the chloroplast envelope. These data

suggested that Pht2;1 is important for P transport in leaves. Using a null mutant *pht2;1*-1, these authors further demonstrated that Pht2:1 activity affects the level of Pi and re-distribution among leaves during P starvation (Versaw and Harrison, 2002). In another study using a low-affinity Pi transporter *pht2;1* Müller et al. (2004) demonstrated that this transporter appeared to be constitutively expressed in shoots, whereas in roots the transcript level clearly decreased in response to Pi resupply.

1.9. Pi deprivation and the role of ethylene

It has been suggested that plant hormones are involved in the transduction of environmental signals into growth and developmental responses (Voesenek and Blom, 1996). For that reason, it is logical that hormones would mediate signals generated by low phosphorus availability to promote the development of adaptive responses. Studies have shown that ethylene may mediate the adaptive changes in roots during low P supply. Both phosphorus deficiency and ethylene cause similar changes in root systems, such as aerenchyma formation, altered root growth angle, and stimulated root hair development (He et al. 1992; Lynch and Brown, 1997; Borch et al. 1999; Lynch and Brown 2001; Ma et al. 2003). Borch et al. (1999) found that phosphorus-deficient bean roots produced twice as much ethylene per unit dry weight as roots supplied with adequate phosphorus. In contrast, Schmidt and Schikora (2001) found that phosphorus deficiency increased root hair density of Arabidopsis even in the presence of the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG).

How the ethylene signalling pathway interacts with the Pi signalling pathway at the genetic and molecular level is still unclear. Using ethylene insensitive mutants, Zhang et al. (2003) showed that these mutants reduce responses to low phosphorus. Growth in low phosphorus resulted in smaller, more numerous cortical cells. When using the ethylene precursor, ACC, they found that the cortical cell number was not affected, indicating that ethylene does not participate in this response. In similar experiments using exogenous ethylene (ethephon), Hou et al. (2005) showed that exogenous ethylene did not affect the expression of the *OsIPS1* and *OsIPS2* genes (members of the TPSII/*Mt4* family genes with unknown function cloned from a Pi-starvation-induced cDNA library from rice roots).

1.10. The role played by APase in P-deprived roots

1.10.1. Acid phosphatase biochemistry and terminology

Phosphatases (E.C.3.1.3) are a class of enzymes that have been used as vacuolar markers in cellular compartmentalization studies, protein "fingerprints" in isozyme analysis (Paul and Willamson, 1987), and markers of the Pi status of plants (Ascencio, 1994). Phosphatases are non-specific orthophosphoricmonoester phosphohydrolases, cleaving Pi from larger molecules at their ester linkage sites. Phosphatases are divided into two broad groups based on their pH optima; alkaline phosphatases (E.C.3.1.3.1) and acid phosphatases (E.C.3.1.3.2) (Vincent et al. 1992). Unlike alkaline phosphatases, acid phosphatases are enzymes with non-specific substrate specificity. However, there are two distinct categories of plant APases that can be distinguished based on their relative substrate specificity. The first type of plant APases are those truly 'non-specific' enzymes that do show little or no substrate specificity. The second category of plant APases are specialized enzymes. The non-specific enzymes occur in a wide variety of species and tissue and show considerable diversity regarding their physical properties. The specific enzymes, for example, 3-P-glycerate (3-PGA) phosphatase from maize leaves, have a clear but non-absolute substrate specificity (Randall et al. 1971). Another example is phosphoenolpyruvate (PEP) phosphatase that accumulates in *Brassica nigra* (black mustard) suspension cell cultures (Duff et al. 1989a). Microbes produce both acid and alkaline phosphatases, while higher plants and animals exclusively produce acid phosphatases (Dick and Tabatabai, 1984).

The non-specific phosphatases are active in soil. Their broad substrate specificity makes these enzymes good candidates for action on a variety of organic phosphate (P_o) substrates found in soils. Although soil P_o is dynamic and difficult to characterize, it is primarily made up of inositol hexaphosphate, glucose-6-phosphate, glycerol phosphate, nucleoside monophosphates and polynucleotides (Dalal, 1977; Pant et al. 1999). These compounds are all phosphate esters on which phosphatases can act to liberate P_i. While most enzymes are generally short-lived in soil, phosphatases can be immobilized on or within soil clays and humates that preserve their activity (Burns, 1986). Further, phosphatases that persist in soils have pH optima in the same range as the surrounding soil pH (Dick and Tabatabai, 1984; Rojo et al. 1990).

Tarafdar and Jungk (1987) showed that phosphatase activity increased within 3.1 mm of *Trifolium aestivum*, *Trifolium alexandrinum* and wheat roots, and that this increase significantly correlates (r=0.97 and r=0.99) with a zone of P_o depletion around these roots. Haussling and Marschner (1989) also showed that soil acid phosphatase activity increases in the rhizosphere of a mature Norway spruce stand when compared to bulk soil, and this increase correlates with a decrease in P_o. Plants can alter their rhizospheric phosphatase activity levels by secreting phosphatases from their roots. Plant-derived acid phosphatases are hardy enzymes that are able to maintain activity in soils. Secreted plant phosphatases maintain >50% activity over broad pH range (4.0-7.6), maintain >80% activity over a broad temperature range (22-48°C), and maintain stability at temperature as high as 60°C (LeBansky et al. 1991; Li and Tadano 1996). Plants grown in nutrient solutions can mobilize organic forms of P to fulfil their complete P nutritional requirements (Furlani et al. 1987; Tarafdar and Claasen, 1988; Yan et al. 1996), so at least under these controlled conditions the phosphatases that plants secrete are sufficient.

In the soil, plant phosphatases are supplemented with microbial phosphatases. Free-living soil microbes are concentrated around root systems because sloughed off plant cells and root secretions are a source of carbon and nutrients (Tate et al. 1991; Marschner, 1995). Phosphatases from soil microbes contribute significantly to the phosphatase activity of soils.

1.10.2. Distribution, localization and function of plant APases

Acid phosphatases are widely distributed in plants and have been found in seeds (Lorenc-Kubis and Morawiecka, 1980; Basha, 1984; Park and van Etten, 1986; Ullah and Gibson, 1988; Biswas and Cundiff, 1991; Biswas et al. 1996; Kawarasaki et al. 1996; Pasqualini et al. 1996; Granjeiro et al. 1999), in roots (Ridge and Rovira, 1971; McLachlan, 1980a, 1980b; Panara et al. 1990; Ozawa et al. 1995; Li and Tadano, 1996; Ascencio, 1997; Penheiter et al. 1997; Hayes et al. 1999; Hunter and Leung, 2001; Gaume et al. 2001; Lim et al. 2003; Machado and Furlani, 2004), in leaves (De Leo and Sacher, 1970; Besford 1979a; Jonsson 1981; Barrett-Lennard et al. 1982; McLachlan, 1984; McLachlan et al. 1987; Tanaka et al. 1990; Staswick et al. 1994; Shih and Kao, 1998; Yan et al. 2001; Raposo et al. 2004), in fruit (Turner and Plaxton, 2001), in cotyledons (Kaneko et al. 1990), in seedlings (Bhargava and Sachar, 1987), in the cell

wall (Hunter et al. 1999; Zhang and McManus, 2000), in storage tubers (Sugawara et al. 1981; Kruzel and Morawiecka, 1982; Gellathly et al. 1994), in bulb tissue (Guo and Pesacreta, 1997), and in cells derived from various tissue cultures (Ueki and Sato, 1971; Suzuki and Sato, 1976; Kaneko et al. 1990; Duff et al. 1991; LeBansky et al. 1992; Theodorou and Plaxton, 1996; Kaneko et al. 1998.

The induction of acid phosphatases (APases) is a common response of higher plants to P deprivation (Duff et al. 1994). Acid phosphatases are thought to break down both intracellular and extracellular organic molecules and liberate phosphate, making it available to the plant. Many plants including rice, wheat, and tomato secrete increased amounts of acid phosphatase from the roots in response to phosphorus stress (Tadano et al. 1993). The acid phosphatases secreted by lupin and tomato roots have been isolated and are both homodimers (Ozawa et al. 1995; Li and Tadano, 1996). The activity of acid phosphatase was also shown to increase in the shoots of wheat plants, and specific isozymes were detected under phosphorus-starvation conditions (McLachlan et al. 1987).

Changes in specific isoforms of phosphatases under Pi deprivation are generally observed (Goldstein et al. 1988a; Tadano et al. 1993; Trull and Deikman 1998; Yun and Kaeppler, 2001). Trull and Deikman (1998) have isolated the *pup1* mutant to dissect the Pi-starvation response in *Arabidopsis*. This mutant shows reduced histochemical staining for APase activity in Pi-starved roots. Further characterization showed that the *pup1* mutants are able to respond normally to phosphorus-deficient conditions, except for the expression of one specific acid phosphatase isoform (Trull and Deikman, 1998). Similar results identified by Yun and Kaeppler (2001) using two maize genotypes suggested that one of the minor isozymes identified increased specifically in response to P starvation. Other studies by Tomscha et al. (2004) using pup3 exudates, demonstrated that one of the two exudate isoforms is recognized by a polyclonal antibody raised to an Arabidopsis purple APase recombinant protein (AtPAP12). Using conditional genetic screening in Arabidopsis, Chen et al. (2000) has identified a Pi-starvation-inducible mutant, namely *psr1* (*phosphate starvation response*). This mutant had significantly reduced activities of phosphatase-starvation-inducible isoforms of ribunuclease and acid phosphatase under phosphate-limiting conditions.

1.10.2.1. Intracellular APases

Intracellular plant APases appear to be ubiquitous since they have been found in

dormant seeds, developing seeds, germinating seeds, leaves, stems, roots, storage tubers, flowers and cultured cells. Intracellular forms are mainly found in the vacuole (Nishimura and Beevers, 1978;) or in the cytoplasm. Histochemical and subcellular fractionations studies have shown the presence of APase in the cell vacuole of possibly all plant cells (Nishimura and Beevers 1978 Boller and Kende 1979, Vogeli-Lange et al. 1989, Duff et al. 1991). Unlike extracellular APases, intracellular APase are usually more stable and remain stable for hours to days (Goldstein et al. 1988a; Duff et al. 1989a; 1991; Miller et al. 2001). However, it is not clear if vacuolar APases exist since nutrient sufficient plants store excess phosphorus in their vacuole in the form of Pi (Theodorou and Plaxton, 1993). Intracellular APases are induced during P deficiency and function to release P from senescent tissue for remobilizing and in bypassing the P-requiring steps in C metabolism (Duff et al. 1989b; Plaxton and Carswell, 1999).

1.10.2.2. Extracellular APases

Extracellular APases occur in the root apoplast and are frequently released from cell suspension cultures. Extracellular types are usually localized in the cell wall, outer surface of root epidermal cells, and in the root apical meristem. Root extracellular APases are more likely to be involved in acquisition from soil (Marschner et al. 1986; Tarafdar and Claasen, 1988). Although the acquisition of P from soil organic matter is equivocal, Miller et al. (2001) postulated that secretion of even low-affinity P-cleaving enzymes into soils with high organic P is an effective mechanism to provide additional sources of P for plant growth.

1.10.3. Acid phosphatases and changes in gene expression during Pi deprivation

The literature abounds with reports of APases secreted from roots of P-deficient plants (Goldstein et al. 1988a; Li et al. 1997a, 1997b; Gilbert et al. 1999; Hayes et al. 1999; Hunter and McManus, 1999; Hunter and Leung, 2001). However, only recently have APase genes and proteins from Arabidopsis, tomato and lupin cluster roots been fully characterized (del Pozo et al. 1999; Wasaki et al. 1999; Haran et al. 2000; Baldwin et al. 2001; Miller et al. 2001; Wasaki et al. 2003). Hewitt and Tatham (1960), demonstrated an 18-fold increase in APase activity in tomato leaves during Pi starvation.

del Pozo et al. (1999) isolated and characterized a type 5 purple acid phosphatase, *AtACP5*, gene from *Arabidopsis*. The gene contained a 1014 –bp open reading frame (ORF) encoding 338 amino acid residues. The protein had a 31 amino acid *N*-terminal extension with characteristics of a membrane targeting signal peptide. Both roots and shoots showed highly induced expression of *AtACPA5* under P deficiency and the addition of P could reverse the induction. Induction of *AtACP5* also occurred in senescence tissues and in response to salt stress. Although AtACP5 was highly expressed in roots under P-stress, the authors could find no evidence for secretion of the AtACP5 protein. They concluded that the protein was tightly anchored to the cell wall or plasmalemma. The *AtACP5* promoter, when fused to GUS and used as a reporter, showed high activity in response to P-deficiency, senescence, and salt stress. They concluded that *AtACP5* was involved more in internal P remobilization than P acquisition. Another APase (*LePS2*) implicated in internal remobilization of P has been characterized from tomato (Baldwin et al. 2001). *LePS2* contains an 810-bpORF encoding a 269 amino acid protein. The protein contains no 5'-signal peptide and is postulated to be in the cytosol. *LePS2* was highly induced in all tissues in P-deficient plants, but expression was not induced by other nutrient stresses. Induction of *LePS2* transcripts could also be reversed by addition of P to stressed plants. Both *AtAPC5* and *LePS2* were rapidly induced under P-deficient conditions, suggesting that plant molecular responses to P are tightly controlled.

1.10.4. Correlation between APase activity and Pi content during Pi deprivation

Acid phosphatases are suggested to be important in regulating P nutrition in plants, particularly with respect to the role of cell wall/secreted enzymes in accessing P from the soil (Duff et al. 1994). There are now many studies which have shown a correlation of APase activity in response to P-concentrations in tissues of several plant species. These include, *Cucumis sativus* L. (Besford, 1978), *Lycopersicon esculentum* Mill (Besford, 1979a; 1979b), *Triticum aestivum* L. (McLachlan, 1982), *Oryza sativa* L .(Zaini and Mercado, 1985), *Phaseolus vulgaris* L. (Breseghelo et al. 1992), *Saccharum officinarum* L. (Silva and Basso, 1993), *Phaseolus vulgaris* L. and *Vigna unguiculata* L. Walp (Fernandez and Ascencio, 1994), *Cajanus cajan* L. and *Gossypium hirsutum* L.

(Ascencio, 1994), *Bactris gasipaes* Kunth (Bovi et al, 1998), *Zea mays* (Machado and Furlani, 2004), and *Glycine max* (Raposo et al. 2004).

Some reports propose a direct link between APase activity and specific aspects of P nutrition. McLachlan (1976), working with four plant species (buckwheat, rye, crimson clover and subterranean clover), established a negative relationship between root APase activity and P uptake and efficiency of use. Other studies by Elliot and Läuchli (1986) using a leaf disc APase assay for diagnosis of P starvation found that APase activity per unit area increased 2–3 times in leaves of P-deficient maize plants compared with P-sufficient leaves. Similarly, intracellular APase activity increased in wheat shoots under P-deficient conditions (McLachlan et al. 1987). Remarkable difference in levels of APase secretion from roots under P deficient conditions has been observed for many plant species (Tadano and Sakai, 1991). Secretion of APases increases in response to P starvation in cell suspension cultures of maize and other plant species (Goldstein et al.1988b; Lefebvre et al.1990; Miernyk, 1992). In cell suspension systems, both the synthesis and secretion of APase are altered under P deficiency (Ueki and Sato, 1977). Other studies have also observed the negative correlation between APase activity and Pi content (Hunter and Leung, 2001; Yun and Kaeppler, 2001; Ming et al. 2002; Lim et al. 2003; Raposo et al. 2004; Sharma et al. 2005; Playsted et al. 2006).

Conversely, P deficiency did not result in a consistent response by root acid phosphatase activity. Although there are many reports of increased phosphatase with P deficiency, there are also instances where phosphatase activity does not respond to P nutrition (Ascencio 1996). When plant-derived APase secretion was compared between efficient and inefficient P genotypes in maize, the results correlated positively (Gaume et al. 2001). Other studies have also shown a positive relationship between root APase and phosphorus uptake from inositol hexaphosphate in bean (Helal, 1990), barley (Lee, 1988; Asmar et al. 1995; Manske et al. 2000). P efficiency is conferred by multiple traits even between recombinant inbred lines of the same species (Yan et al. 2001), which may explain why another study found no significant effect of root-associated APase activity between efficient and inefficient white clover genotypes (Hunter and McManus, 1999). Other workers also demonstrated that APase activity is independent

of P level (Barrett-Lennard et al 1982; Szabo-Nagy et al. 1992; Fernandez and Ascencio 1994; Shih and Kao, 1998).

2.0. White clover

White clover (*Trifolium repens* L.) is the plant used in this thesis for studying the responses to phosphate deprivation. White clover belongs to the genus *Trifolium* which consists of 250 to 300 species. This genus is classified taxonomically in the family of Leguminosae (Heywood, 1971). White clover is a stoloniferous plant. The stolon consists of a series of internodes separating the nodes which form as a result of growth at the apical bud. White clover leaves are trifoliate except the first unifoliate leaves. The main stem is short, with only two or three terminal nodes without stolons. Each node consists of a single trifoliate leaf, two root primordial and an axillary bud which either remains dormant, produce an inflorescence flowers, or produce a lateral stolon. Production of lateral stolons leads to the vegetative spread of a plant. When the root primordium at a node is in contact with a moist substratum, it grows out into nodal roots, providing a degree of nutritional independence of each lateral stolon (Thomas, 1987). The growth of a stolon is indeterminate with plants in summer often possessing up to 6 branching (Brock et al. 1988). A lateral stolon with roots can be cut out from the parental plant after which it develops into an independent plant. Therefore, it is advisable to use stolon cuttings for the production of clonal material for the experiments.

2.1. White clover in New Zealand

White clover is one of the most important forage legumes in the temperate regions of the world. Grown alone or as a companion species with grasses, white clover produces high yields and good quality forage in a wide range of soil types, pH, and environmental conditions. In New Zealand pasture, it has significant benefits to pastoral agriculture including: (1) the conversion of atmospheric nitrogen into a plant usable form, (2) the reduction of N fertiliser use, (3) the improvement of soil structure, and (4) the flexibility of use; high protein content, digestibility, mineral content and high intake (Abberton and Marshall, 2005). As one of the important forage legumes in New

Zealand, Caradus et al. (1996) estimated that the total financial contribution, comprised of indirect (N fixation, increased forage yield) and direct (seed, honey), of white clover to the New Zealand economy to be $3 billion in the mid 1990s. A large part of this is derived from marketing of white clover cultivars globally (Mather et al. 1996)

2.2. White clover and phosphate uptake and utilization

In New Zealand, white clover is an important pasture legume. Because New Zealand soils are phosphorus deficient, often the growth and productivity of these pastures are limited by the availability of soil phosphorus to the clover. The continuing rise in the cost of phosphatic fertilizers has led to the search for pasture species that are more efficient at utilizing phosphorus (Caradus et al. 1992). Selection of increased tolerance on low-P soils and selection for increased efficiency of utilization of P on medium- to high-P soils could lead to identifying a cultivar of white clover which requires less P to sustain the same production as that of present cultivars. Further, producing more dry matter with the same amount of P would also be of important value in maintaining the profitability of pastoral farming.

Early reviews (da Silva and Gabelman, 1992; Manske et al. 2000; Yun and Kaeppler 2001; Ming et al. 2002; Lim et al. 2003; Machado and Furlani, 2004; Raposo et al. 2004) have indicated that within many species there is substantial intraspecific variation in response to P and that scope exists for selection for accession with improved P efficiency. Wedderburn et al. (2005) examined the wide edaphic tolerance within *T. repens*, and concluded that this 'is due, at least in part, to ecotypic differentiation'. They examined several natural populations of white clover in field-based reciprocal transplant experiments, and two-controlled-environment experiments were conducted using plants removed from the 8-year field experiments. They found that accessions from regions with soils high in available phosphorus showed larger reductions in growth at low phosphate levels than did those from regions with soils with low available phosphate. The drift by both 'Tahora' and 'Huia' away from the potential of the original seed attributes towards the resident type demonstrates the adaptive nature of the traits found in the resident population that favour a conservative growth strategy. Caradus and Dunn (2000), using white clover breeding lines developed as high and low P-responsive, however, found the difference between P-response groups were transitory and biologically insignificant and concluded that selection for differences in response to

P in a controlled environment was not successful in identifying white clover germplasm adapted to low P hill-country soils.

Screening germplasm for shoot dry mass or harvestable product in low P conditions may provide the best estimate of productivity in low P soils. Gourley et al. (1993) studied the response to P supply to differentiate two white clover cultivars (Gandalf, P efficient and Huia, moderately efficient) and lucerne (EG2, low P tolerant and IG2, P intolerant cultivars). These studies showed that Gandal and EG2 would be the preferred germplasm over Huia and IG2. Whether the superior performance in low P conditions is truly related to a specific mechanism enhancing P uptake or utilization needs to be taken into consideration before a germplasm can be categorized as "P efficient" or "P inefficient". Metabolic activities such as phytohormone production, photosynthesis rate, photoperiodism, and production of ATP, can increase nutrient uptake and utilization by influencing root morphology and function (Wilkins, 1984). An efficient genotype, regardless of the mechanism, is likely to result in higher yields independent of P availability and could require less nutrient for normal metabolic processes than an inefficient germplasm. Differences among germplasm in nutrient uptake per unit root dry mass or length, or differences in root morphological characteristics such as shoot:root ratio or root fitness, may also indicate mechanisms for increased nutrient acquisition at low nutrient availability (Caradus, 1990). Formation of greater amount of vesicular-arbuscular mycorrhizal association increased the efficiency of P uptake and yields at low levels of P while similar yields are obtained when effectively and ineffectively inoculated plants when adequate P is available (Smith et al. 1992).

Studies using white clover showed an induction of phosphatase activity in both leaf and root tissues following the onset of phosphate deprivation (Caradus and Snaydon 1987; Hunter et al. 1999). Hunter et al. (1999) investigated soluble and cell wall acid phosphatase activity in leaf and root tissues of white clover grown in P-deprived media. The results showed that no significant enhancement of activity was observed in isoenzymes from soluble extracts of leaf tissue. In contrast, they found an increase in cell wall isoforms from leaf tissue. In the soluble root extract, they found an induction of one major staining lower mobility isozyme and one minor staining higher mobility isozyme (Hunter et al. 1999). Previous studies using white clover genotypes PgH_2 and 10 F (Hunter et al. 1999; Zhang and McManus, 2000) showed an induction in Pi and APase activity in response to Pi deprivation.

In present study, for the first time, the responses to Pi deprivation are investigated using four selected breeding lines. These have been selected at two fertility levels at the Ballantrae Hill Country Research Farm, AgResearch and bred in isolation, and then selected based on their responses to added P. An interesting question is to see if the intraspecific variation in response to P at two different soil fertility levels in the field would continue to do so when plants are grown in liquid media in a climatic-controlled room or in a glasshouse? A further question is whether the superior performance of C23147 (BL 47) or C23149 (BL 49) in low P conditions is truly related to a specific mechanism enhancing P uptake or utilization, such that these lines will show the same performance when grown in –P in a liquid media? To examine these lines, a glass house experiment and a climatic-controlled room experiment were conducted to test the hypothesis that:

1. Genetic variation occurred within the four breeding lines and selected genotypes of white clover in response to +P or –P treatment;
2. Breeding lines that performed better in the field under high-P condition would continue to do this when grown in the liquid media;
3. These four breeding lines and selected genotypes will show different responses in term of their Pi content in leaves, acid phosphatases in roots and growth responses during Pi deprivation; and
4. There is a correlation between Pi content and the responses to added P across the breeding lines and selected genotypes.

3.0 Thesis Aims

1. To screen four breeding lines which have been bred based on their responses to added P fertilizer in the field and compare these breeding lines with regard to their response to non-limiting and limiting P supply and their P acquisition efficiency (PAE) and P utilization efficiency (PUE).
2. To identify the response of four breeding lines and selected genotypes of white clover to phosphate deprivation by measuring levels of inorganic phosphate (Pi) in leaves, acid phosphatase activity in roots and several growth parameters.
3. To determine if there is differential induction of acid phosphatases in four breeding lines and selected genotypes previously selected and bred based on their responses to added P fertilizer

4. To identify acid phosphatase isoforms in the roots of these four breeding lines and selected genotypes of white clover grown under P-deprived conditions and to compare this with those grown in P-sufficient condition.

Chapter Two: Materials and Methods

2.1. Plant Material

Seeds of four breeding lines of white clover (*Trifolium repens* L.) were obtained from the New Zealand Pastoral Agricultural Research Institute Inc. (AgResearch Grasslands) at Palmerston North, New Zealand. The four phosphate selections were made from empirical field screening for clover yield at two fertility levels at the AgResearch Ballantrae Hill Country Research Station near Woodville. The trial was planted with cloned genotypes of Tahora and Prestige white clover in June 1997 and the performance of individual genotypes was measured over the next three years. Genotypes were selected and crossed in four groups to provide four breeding lines (BLs):

1. LLHH C23143 (**BL 43**) = Poor performance in low fertility and high performance in high fertility
2. LHHH C23147 (**BL 47**) = High performance in low fertility and high performance in high fertility
3. LHHL C23149 (**BL 49**) = Low performance in high fertility and high performance in low fertility
4. LLHC C23145 (**BL 45**) = Low performance in low fertility and low performance in high fertility.

For genotype screening, five genotypes were chosen. These genotypes were chosen from a preliminary experiment (designated Screen 1) based on differences in the time of induction APase activity and Pi content during Pi deprivation (Table 2.1). The genotypes selected were designated as follow:

A photographic record of each of these five selected genotypes can be seen in Figures 2.1a and b

Table 2.1. Five selected genotypes used in genotypic screening. Genotypes selected over replicates of all genotypes using Screening 1 (Breeding line screening).

Genotype	Origin	Pi content (measured in –P media)	Cell wall APase activity (measured in –P media)
43-7	BL 43	Low Pi content	Low APase activity APase peak at d 3
43-8	BL 43	Medium Pi content	High APase activity APase peak at d 5
45-14	BL 45	High Pi content	Medium APase activity. APase peak at d 5
45-4	BL 45	Low Pi content	Medium APase activity APase peak at d 7
47-9	BL 47	Medium Pi content	Medium APase activity APase activity peak at d 5

2.2. Growth of white clover plants

2.2.1. Stock Plants

Stock plants of white clover were grown in pots and watered daily and supplemented with half-strength Hoagland's solution [68.04 g/L KH_2PO_4, 50.55g/L KNO_3, 118.1 g/L $Ca(NO_3)2$, 123,24 g/L $Mg(SO_4)_2$, 5 g/L FeEDTA ($[CH_2N(CHCOO)_2]2FeNa$), (2.86 g/LH_3BO_3, 1.81 g/L $MnCl_2.4H_2O$, 0.22 g/L $ZnSO_4.7H_2O$, 0.08 g/L $CuSO_4.5H_2O$, 0.02 g/L $(NH_4)6MO_7(O_2)4.4H_2O$] (Hoagland and Arnon, 1950) twice a week. Plants were maintained in a heated glasshouse (15°C minimum, vented at 25°C, without supplementary illumination) at Massey University Palmerston North for the preliminary breeding line-based screenings (Screenings 1 and 2) and the genotype-based screening (Screenings 3 and 4). A final breeding line-based screening (designated as Screening 5), was also carried out using a controlled environment chamber in the National Climate Laboratory, Horticulture and Food Research Institute of New Zealand, Palmerston North.

The summary of BL-based screening (Screenings 1 and 2) and genotype-based screening (Screening 3) can be seen in Table 2.2. In this thesis, only Screening 4 and Screening 5 will be discussed.

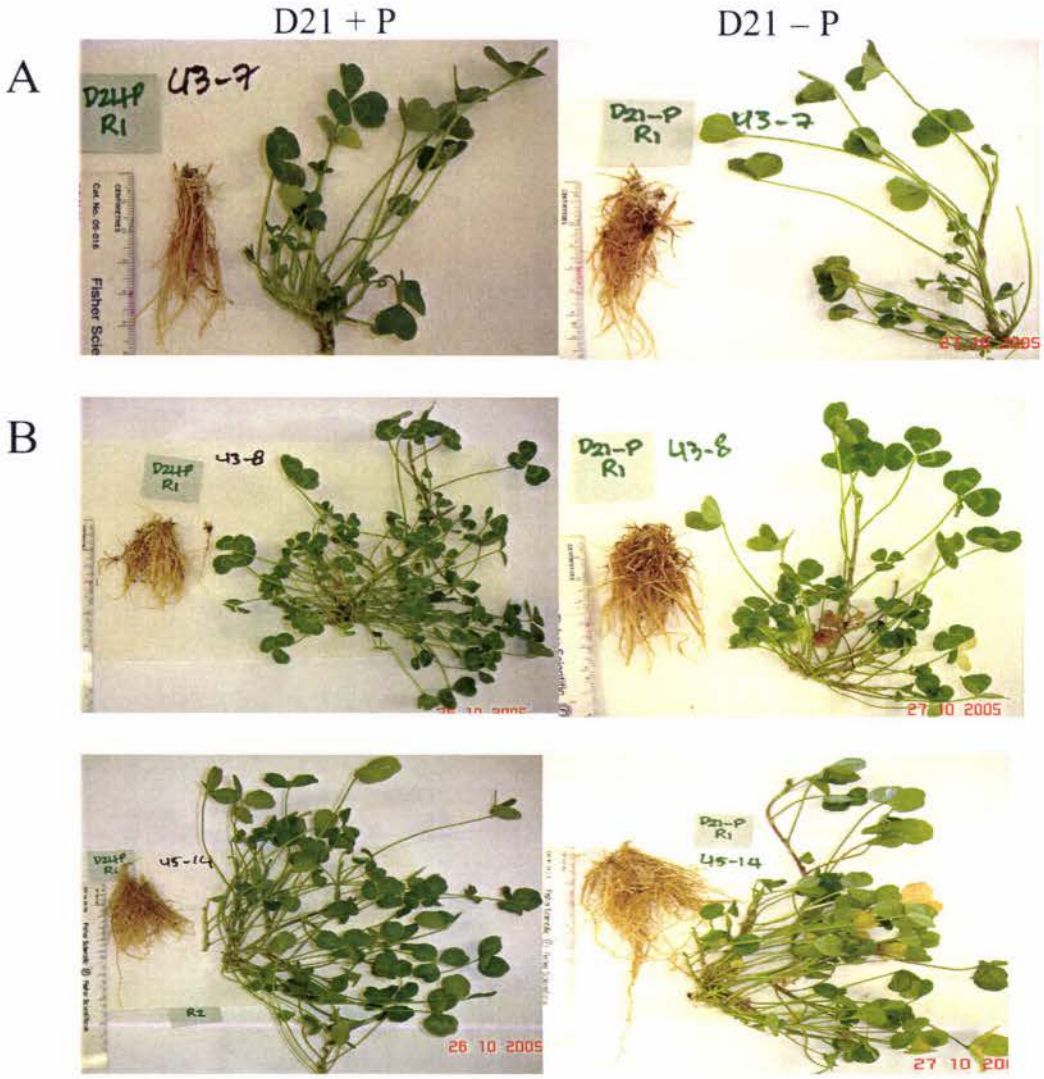


Figure 2.1a. The growth of five selected white clover genotypes grown in P-containing and P-deprived media at day 21:

A. Genotype 43-7 (left hand side: +P media, right hand side: –P media)
B. Genotype 43-8 (left hand side: +P media, right hand side: –P media)
C. Genotype 45-14 (left hand side: +P media, right hand side: –P media)

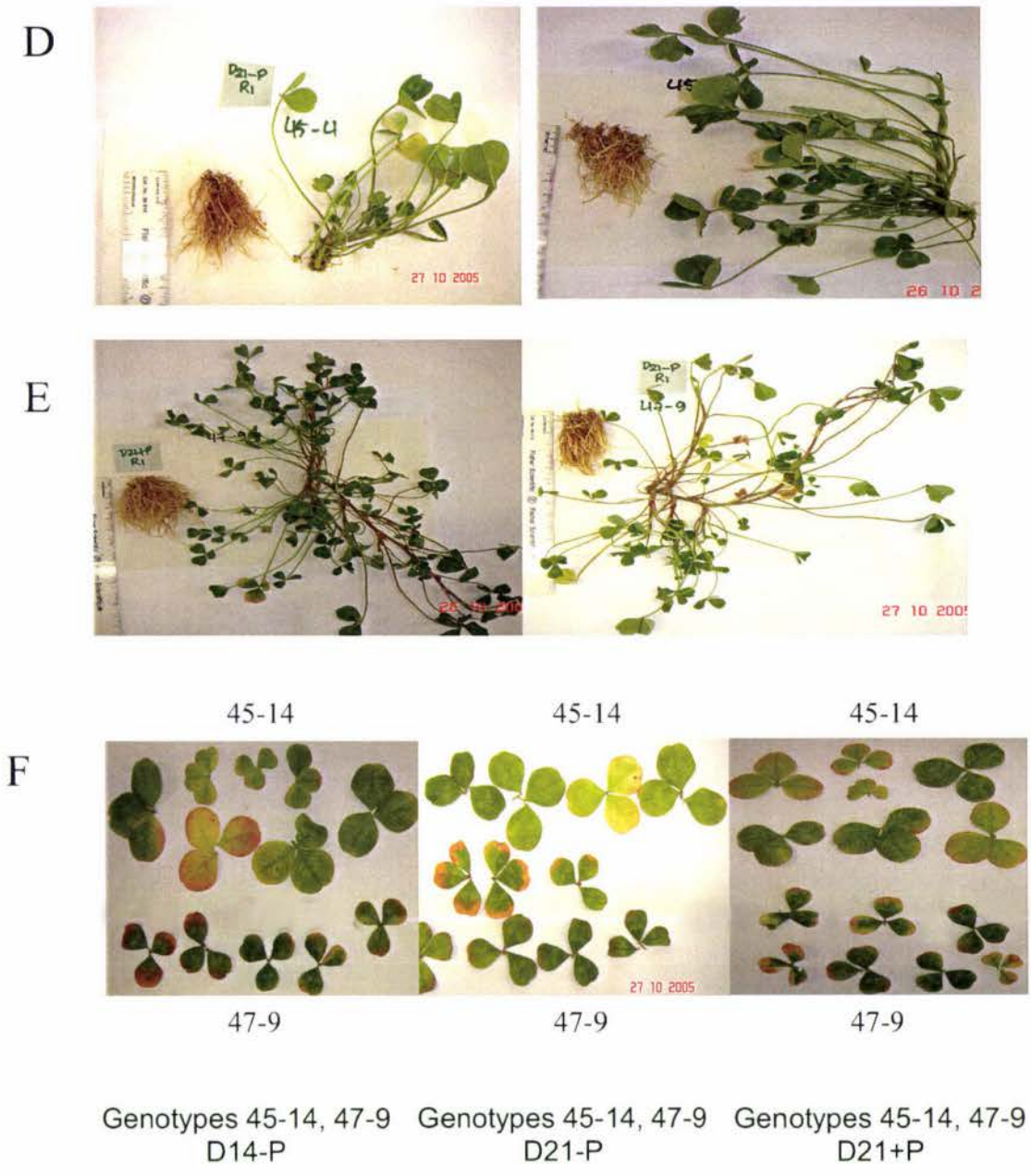


Figure 2.1a. The growth of five selected white clover genotypes grown in P-containing and P-deprived media at day 21:

D. Genotype 45-4 (left hand side: +P media, right hand side: –P media)
E. Genotype 47-9 (left hand side: +P media, right hand side: –P media)
F. Leaf coloration observed in genotypes 45-14 and 47-9 grown at d 14 in –P, d 21 in –P, and d 21 in +P media.

Table 2.2. Summary of preliminary BL (Screenings 1 and 2) and genotype (Screening 3) screenings based on –P/+P of Pi content and cell wall APase activity at the conclusion of the screens (data not shown in this thesis).

Screening Number	Origin	Outcome (At the conclusion of screens)	
		Pi Content	Cell wall APase activity
Screening 1 (BL Screening)			
BL 43	BL 43	High Pi content	Low cell wall APase
BL 45	BL 45	Low Pi content	High cell wall APase
BL 47	BL 47	High Pi content	Medium cell wall APase
BL 49	BL 49	Low Pi content	Medium cell wall APase
Screening 2 (BL Screening)			
BL 43	BL 43	Low Pi content	High cell wall APase
BL 45	BL 45	High Pi content	Low cell wall APase
BL 47	BL 47	Medium Pi content	Low cell wall APase
BL 49	BL 49	Medium Pi content	High cell wall APase
Screening 3 (Genotypic Screening)			
43-7	BL 43	High Pi content	Medium cell wall APase
43-8	BL 43	Medium Pi content	Low cell wall APase
45-14	BL 45	Medium Pi content	High cell wall APase
45-4	BL 45	Low Pi content	Lowest cell wall APase
47-9	BL 47	Low Pi content	Low cell wall APase
49-6	BL 49	Medium Pi content	Medium cell wall APase

2.2.2. Growth of white clover in liquid media

Half-strength Hoagland's solution was used in liquid medium for all experiments. White clover apical cuttings with 3-4 leaf nodes were excised from stock plants to grow as experimental plants. These cuttings were planted in trays containing vermiculite for 2 weeks in half-strength Hoagland's liquid medium. After two weeks in vermiculite, cuttings were transferred to 2 L dark colour plastic trays covered with polythene to exclude light. The roots were placed through the hole and submerged in liquid media. After growth in liquid medium for eight weeks, the white clover plants were then used for either genotype or breeding line experiments. For screens 1-4 plants were maintained subsequently in the glasshouse as described previously. For screen 5, eight-week old white clover plants were acclimatized in the controlled environment room for 1 week. The room supplied plants with a photosynthetic photon flux density of 650 μmol m^{-2} s^{-1} and a 12-h photoperiod with the temperature 22°C, relative humidity 70% and vapour pressure deficit 0.8 kPa.

To regulate P supply, plants were transferred to liquid media consisting of half-strength Hoagland's solution with the phosphate component (KH_2PO_4) removed (P-deprived) while the remaining plants were maintained in half-strength Hoagland's solution (P-containing) with 0.5 mM KH_2PO_4. The media was changed twice weekly. After 3, 5, 7 and 14 days (for breeding line screening) and 7, 14 and 21 days (for genotype screening), plants were harvested. To harvest root tissue, whole plants were removed from the media, the roots were then cut *ca.* 1 cm below the crown, washed in RO water, blotted dry between tissue papers, weighed, wrapped in aluminium foil, frozen in liquid nitrogen and stored at -80°C until further use. To harvest leaf tissue for leaf Pi content measurement, the first fully expanded leaf (usually subtending from node number 3 above the crown) was collected, weighed and wrapped in aluminium foil, frozen in liquid nitrogen and stored at -80°C until required. The responses are characterized in terms of changes in the activity of APase in roots and levels of inorganic phosphate in leaves. Breeding lines and genotypes were also evaluated in terms of their growth parameters such as leaf area, leaf and stolon fresh weight, leaf and stolon dry weight, Root:Shoot ratio and individual leaf weight. For measuring shoot dry matter at the specific times indicated, shoots were harvested above the crown and then were dried in an oven at 80° C for 24 h and weighed. Shoot dry matter of each genoype and breeding line was harvested and determined at 3, 5 7, 14 days (for BLs) and 7,

14, randomly at fully developed leaf (mean of 6 leaflets for BLs and mean of 3 leaflets for genotypes collected from leaf position number 4 from the top).

2.3. Chemicals

Unless specified otherwise, all the chemical reagent used were analytical grade, obtained from BDH Laboratory Supplies (Poole, BH15 1 TD, England), Sigma Chemical Company (St. Louis, Mo., USA), Bio-Rad Laboratories (Richmond, Ca, USA), and Pharmacia Biotechnology (Uppsala, Sweden). The laboratory supply of purified water used for making solutions was produced by reverse-osmosis (RO), followed by passing through a microfiltration system containing ion exchange, solvent exchange, organic and inorganic removal catridges (MilliQ, Millipore Corp, Bedford, Massachusetts, USA).

2.4. Measurement of leaf phosphate

At specific time intervals, the first fully expanded leaf from a single stolon, excised from node number 3 above the crown of each phosphate sufficient and phosphate-deprived plants were weighed, wrapped singly in aluminium foil, frozen in liquid nitrogen, ground to a fine powder and transferred to falcon tubes. To each tube, three volumes of 5.0 M H_2SO_4 was added and after vortexing at room temperature for 1 min, 30 volumes of water were added. The tubes were mixed and then centrifuged at 13 000 x *g* for 10 min. Quadruple 50 µl aliquots of the supernatant were removed, diluted 5-fold with water and 50 µl of each pippetted into microtitre plates (A/S Nunc. Rothkilde, Denmark). A series (50 µl) of phosphate standards, containing 0, 40, 80, 120, 160 and 200 µM KH_2PO_4 were also pippetted, in quadruple onto the plate. To each sample or standard, 200 µl of phosphate assay reagent [1.75% (w/v) l-ascorbic acid in 13.5% (v/v) concentrated assay reagent (16 mM ammonium molybdate, 2.25 mM H_2SO_4, 0.15 mM antimony potassium oxide (+) tartrate)] were added, mixed and the absorbance of each well measured at 595 nm using an Anthos HT plate reader (Anthos Labtec Instruments, Salzburg, Austria). The percentage of phosphate per gram fresh weight was calculated using the phosphate standard curve (Figure 2.2). Leaf Pi content was measured in quadruple samples.

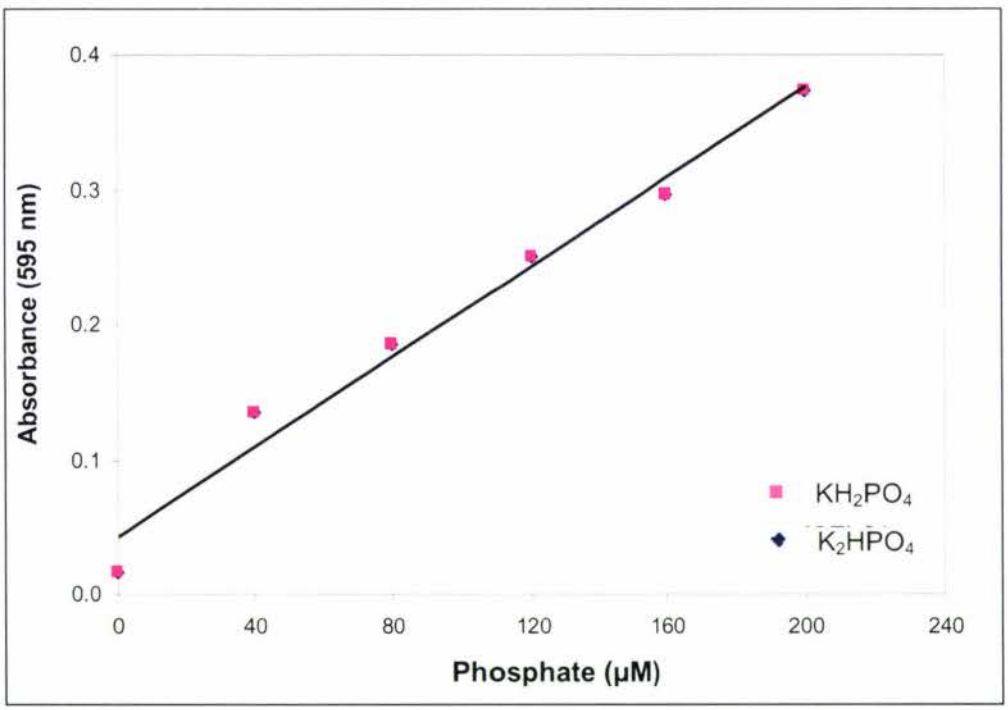


Figure 2.2. Phosphate standard curve for leaf phosphate content determination.

2.5. *Extraction of soluble and cell wall proteins*

At specific time intervals, root tissue was excised from plants maintained in P-deprived or P-containing media, washed with water, blot dried, weighed and then frozen in liquid nitrogen. The frozen tissue was powdered and extracted with 1 mM DTT for 30 min on ice, at a 3:1 extractant:tissue fresh weight ratio. The slurry was centrifuged at 12000 x *g* for 10 min at 4°C, the supernatant removed and designated as the total soluble fraction in the acid phosphatase assay. The pellet then resuspended in three volumes of 1 mM DTT. This extraction procedure was repeated five times with 1 mM DTT with the supernatant being discarded after each wash. The final DTT wash extraction was removed from the pellet by centrifuging for 10 min and the pellet extracted with one volume of 1.0 M NaCl at 37°C for 1 h. The slurry was centrifuged as before, the supernatant decanted and the pellet resuspended and extracted with one volume of 1.0 M NaCl on ice for 30 min and then one volume of 1.0 M NaCl at 4°C for 18 h. After centrifugation, the (three)1.0 M NaCl extracts were pooled and designated the cell wall extract, and also used in the acid phosphatase assay.

2.6. *Acid phosphatase assays*

To measure activity in the ionically-bound (1 M salt-extractable) cell wall and total soluble extracts, typically 5 to 20 µl of extract was made up to 50 µl with 50 mM sodium citrate buffer (pH 5.8) and then pipetted into a microtitre plates (A/S Nunc. Rothkilde, Denmark). To each well, 200 µl substrate (0.5 mg/mL ρ-nitrophenyl phosphate) (Sigma Chemicals, St Louis, Mo, USA) in 50 mM sodium citrate sodium citrate buffer (pH 5.8) was added and the reaction was incubated at 37°C for 12 min. This time interval was determined in preliminary assays to give sufficient colour development. The reaction was terminated with the addition of 50 µl of 1.0 M NaOH, and the absorbance read at 405 nm using the Anthos HTII plate reader (Anthos Labtec Instruments, Salzburg, Austria). The amount (in moles) of product formed was determined from a standard curve of ρ-nitrophenyl constructed for each experiment (Figure 2.3).

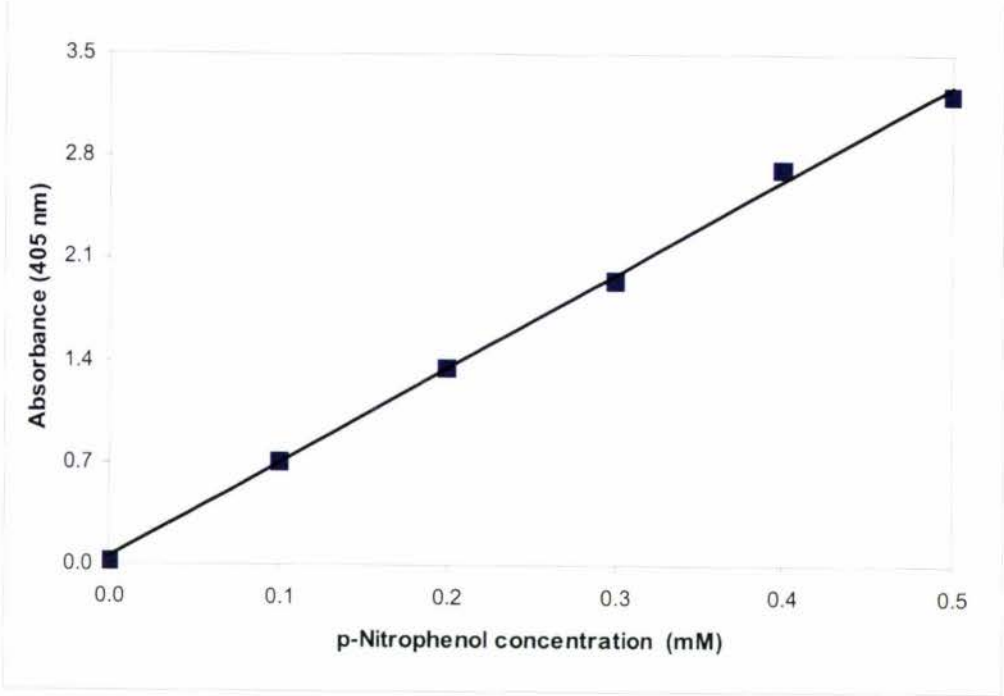


Figure 2.3. Nitrophenol (ρNP) standard curve for the determination of the amount of ρNP liberated by hydrolysis of acid phosphatase.

2.7. Acid phosphatase isoenzyme analyses

Cell wall and total soluble APases extracts were separated through 10% polyacrylamide gels using the method of Davis (1964) to resolve acidic (low pI) proteins or Reisfeld et al. (1962) to resolve basic (high pI) proteins.

2.7.1. High pH non-dissociating discontinuous buffer system (Davis Method)

Reagents:

- 40% (w/v) acrylamide stock solution (Bio-Rad)
- 8 x resolving gel buffer: 3M Tris-HCl, pH 8.8
- 4 x stacking gel buffer: 0.5 M Tris-HCl pH 6.8
- APS (ammonium persulfate) 1.5% (w/v)
- TEMED (N,N,N',N'-Tetramethylethylenediamine)
- Running buffer: 3.0 g Tris, 14.4 glycine, water to 1 L (pH 8.3)
- 2 x Loading buffer: 36 mM Tris-HCl pH 6.8, 25% glycerol, 0.1% bromophenol blue.

A 10% acrylamide resolving gel solution was prepared by mixing the components in the order outlined in Table 2.3 for high pH discontinuous native gel and Table 2.4 for low pH discontinuous native gel. The resolving gel solution was the poured into a slot sandwiched between two glass plates until the gel level was 1 cm below the top of the shorter glass plate. Water was then layered onto the glass surface to protect from atmospheric oxidation and the gel allowed to polymerise for about 1 hr or until separate layers had formed. The layer of water was then discarded. The gel then pre-run with resolving gel buffer to remove any excess of salt for about 2 hr at 50 V, the resolving gel buffer then discarded, the well-forming comb inserted, and gel stacking solution added. After polymerisation for about 30 min, the sandwich apparatus was placed in the electrophoresis chamber. Running buffer was added to both inner and outer chambers and the comb removed. In preparation for loading buffer, all samples were prepared for loading by adding one fifth volume of 2 x gel loading buffer, vortexed and then centrifuged for 1 min. For the high pH discontinuous native gel, electrophoresis was conducted at 150 V for 2 hr or until the bromophenol blue had migrated at the end of the

Table 2.3. Composition of resolving and stacking gels used for high pH discontinuous native gel.

Order of Addition	Components	Resolving gel solution (mL)	Stacking gel solution (mL)
1.	water	2.95	2.2
2.	8 x resolving gel buffer	0.75	
3.	4 x stacking gel buffer		1.0
4.	Acrylamide stock solution	2.0	0.5
5.	APS	0.3	0.3
6.	TEMED	0.003	0.003

glass. For the low pH discontinuous native gel, electrophoresis was conducted at 50 V for at least 2 hr or until the methyl green dye had migrated about 75% into the separating gel.

2.7.2. Low pH non-dissociating discontinuous buffer systems (Reisfeld Method)

Reagents:

- 40% (w/v) acrylamide stock solution (Bio-Rad)
- 8 x resolving gel buffer: 3M acetic acid-KOH pH 4.3
- 4 x stacking gel buffer: 0.5 M acetic acid-KOH pH 6.8
- APS (ammonium persulfate) 1.5% (w/v)
- TEMED (N,N,N',N'-Tetramethylethylenediamine)
- Running buffer: Acetic acid-β-alanine (pH 4.5): 31.2 g β-alanine and 8.0 ml glacial acetic acid; water to 1 L.
- 2 x Loading buffer: 25% (v/v) glycerol, 0.1% (w/v) methyl green.

2.7.3. Staining high and low non-dissociating discontinuous native gels

Reagent:

- Activity staining solution: 0.02% (w/v) β-napthyl acid phosphate in 100 mM citrate buffer (pH 5.8) containing 0.01% (w/v) Fast Garnet and 0.4% (w/v) $MgCl_2$.

To visualize separated acid phosphatase isoenzymes, the gel after conclusion of electrophoresis was immersed in 50 mM sodium citrate buffer pH 5.8 for 10 min, rinsed with water and then incubated in staining solution until activity bands reached the

appropriate intensity (usually overnight). The reaction was terminated by washing the gel with MilliQ water several times.

Table 2.4. Composition of resolving and stacking gels used for low pH discontinuous native gel.

Order of Addition	Components	Resolving gel solution (mL)	Stacking gel solution (mL)
1.	water	2.62	2.2
2.	8 x resolving gel buffer	0.75	
3.	4 x stacking gel buffer		1.0
4.	Acrylamide stock solution	2.0	0.5
5.	APS	0.6	0.3
6.	TEMED	0.03	0.003

2.8. Statistical Analyses

Results were submitted to statistical analyses using the SAS – System for windows 9.1 (SAS Institute Inc., Cary, NC, USA. 2003) program, through the GLM procedure. Analysis of variance was performed. When the F test was significant, the Duncan's test ($\alpha = 0.05$) for multiple mean comparisons was applied to identify differences among breeding lines and genotypes.

Chapter Three: Results

3.1. White clover growth in phosphate deprived conditions

3.1.1. Visual changes in white clover plants maintained in –P conditions

White clover apical cuttings, with all but the first two emerged leaves removed, were rooted on vermiculite in half-strength Hoagland's solution for 2 weeks. Plants were then divided into two groups. One group of plants was transferred to half-strength Hoagland's medium without phosphate (-P medium) while the second group remained in complete half-strength Hoagland solution (+P). Phosphorus availability affected the growth of white clover, and each breeding line responds differently to P stress. For example, BL 43 showed no visual changes during the first two weeks, while BLs 45 and 49 started to show symptoms as early as two weeks in the P-deprived media. Leaves in these BLs were observed to develop reddish coloured spots starting at the terminus of each trifoliate leaf, after which the leaves turned yellow. These colour spot changes and yellowing were observed on the older leaves only (data not shown).

3.1.2. Selection of root material for the extraction and characterization of acid phosphatase

Preliminary genotypic screening studies showed that after three weeks in P-deprived media, a decrease in APase activity and Pi content was observed in root and leaf tissues. Furthermore, growth indicators such as root FW, shoot FW, leaf area and total fresh biomass all showed reductions in all genotypes tested. In addition, severe phosphate deficiency symptoms were observed after three week in –P media which included thinner stolons and petioles, and the leaves developed the purplish spots and then turned yellow. By comparison, plants after two weeks in –P media demonstrated a significant induction of APase activity but did not show the leaf yellowing (necrosis) which was commonly observed at the basal end of the stolon. Consequently, for screening, root tissue was harvested from plants no later than two weeks in P-deprived and P-sufficient media and used in further experiments.

In this chapter, plant responses to Pi deprivation in liquid media are divided into two parts: a selected breeding line screening and a genotypic screening.

3.2. Selected breeding line screening

3.2.1. Onset of P-deficiency in selected breeding lines of white clover leaf tissue

For the breeding line-based screening, four breeding lines were selected. These breeding lines were BL 43, BL 45, BL 47 and BL 49 and have been selected based on their growth responses to added phosphate fertilizer (see Section 2.1). In this study, responses are further characterized in terms of changes in the activity of acid phosphatase in roots, levels of organic phosphate (Pi) in leaves, and several growth parameters. Phosphate levels were measured in the first fully expanded mature leaf excised from plants grown in phosphate-containing media and phosphate-deprived media for 3, 5, 7 and 14 days (d). Analysis of variance showed that BLs, days of treatment and P treatment all have a significant effect on Pi content (Table 3.1). Further statistical analyses were performed on each source of variance to see the difference in each BL, days of treatment and P treatment. The result showed that the highest Pi content was observed in BL 47, BL 43 had a medium Pi content, while BLs 45 and 49 have the lowest Pi content (Table 3.2). It was also observed that Pi content in leaves decreased after d 7 (Table 3.2). Furthermore, it is shown that a high level of Pi content in leaves depends on P application (Table 3.2). To examine the performance of each BL at specific time intervals grown in +P and –P, one factorial statistical analyses were performed. In these, relative to +P treatment, BLs 45 and 49 showed a significant decrease in Pi content as early as d 3, BL 47 at d 5 and BL 43 at d 7 (Table 3.3).

When compared the interaction between BL, D and P, the results in Table 3.1 show strong interactions between BL x D, BL x P, D x P, and BL x D x P on Pi content in leaves. The complete analyses of the effect of those interactions on Pi content in leaves can be seen in Appendices 1, 2, 3, and 4, respectively.

The effect of breeding line on the level of Pi content is shown in Figure 3.1A. Relative to the +P treatment, a decrease in Pi content can be seen at d 7 after P withdrawal in BL 43, and at d 14 in BLs 45 and 49 (Figure 3.1A). A rapid reduction in Pi content can be seen in BL 47, while BLs 45 and 49 showed a slighter slower rate of Pi reduction (Figure 3.1A). The results show a trend in the decrease in Pi content upon prolonged Pi withdrawal (Figure 3.1A). The results also show no difference in Pi

Table 3.1. Probability of F for Pi content and APase activity of selected breeding lines of white clover.

Source of Variation	df	Pi content (%P.g^{-1}FW)	Acid phosphatase activity	
			Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
Breeding Line (BL)	3	*	***	NS
Days of Treatment (D)	3	***	***	***
Phosphorus (P)	1	***	***	**
BL x D	15	***	***	***
BL x P	7	***	***	NS
D x P	7	***	***	***
BL x D x P	31	***	***	***

*, **, *** significance at the 0.05, 0.01 and 0,001 probability level, respectively.
NS = not significant at $p = 0.05$

Table 3.2. The effect of breeding lines (BL), days of treatment (D) and phosphorus (P) application on Pi content and APase activity of selected breeding lines. Plants were maintained in either P-containing (0.5 mM P) or P-deprived (0 mM P) media. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at 0.05 level according to Duncan's multiple range test.

Source of Variance	Pi content (%P.g^{-1}FW)	Acid phosphatase activity	
		Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
Breeding Line (BL)			
BL43	0.01047^{ab}	1.998^{a}	11.2700^{a}
BL45	0.00977^{b}	1.735^{bc}	10.5400^{a}
BL47	0.01086^{a}	1.845^{ab}	10.3500^{a}
BL49	0.00981^{b}	1.621^{c}	11.0900^{a}
Days of Treatment (D)			
D3	0.01084^{a}	1.3400^{c}	8.2900^{b}
D5	0.01093^{a}	1.7040^{b}	9.6200^{b}
D7	0.00996^{b}	1.8010^{b}	12.2700^{a}
D14	0.00919^{b}	2.3540^{a}	13.0800^{a}
Phosphorus (P)			
0 mM P	0.00877^{b}	2.2450^{a}	10.0100^{b}
0.5 mM P	0.01169^{a}	1.3540^{b}	11.6200^{a}

Table 3.3. Comparison of Pi content and APase activity from each breeding line grown in +P and –P at the specific time indicated (DOT). Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi content and APase activity were determined. Values are the means from six independent plants. The different letters above in the same row indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Breeding Line	DOT	Pi content (%P.g^{-1}FW)		Acid phosphatase activity			
				Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)		Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)	
		+P	–P	+P	–P	+P	–P
43	3	0.012^{a}	0.009^{a}	1.446^{a}	1.595^{a}	9.040^{a}	7.630^{a}
	5	0.012^{a}	0.011^{a}	1.704^{a}	2.115^{a}	8.590^{a}	10.220^{a}
	7	0.012^{a}	0.008^{b}	1.381^{a}	2.764^{a}	11.73^{a}	15.330^{a}
	14	0.011^{a}	0.007^{b}	1.681^{b}	3.298^{a}	11.89^{a}	15.730^{a}
45	3	0.013^{a}	0.009^{b}	1.043^{a}	1.586^{a}	8.910^{a}	7.540^{a}
	5	0.010^{a}	0.010^{a}	1.253^{b}	1.997^{a}	9.020^{a}	11.300^{a}
	7	0.011^{a}	0.009^{a}	1.216^{b}	2.261^{a}	12.840^{a}	12.460^{a}
	14	0.009^{a}	0.007^{b}	1.806^{b}	2.717^{a}	9.780^{b}	12.500^{a}
47	3	0.012^{a}	0.010^{a}	0.969^{a}	1.555^{a}	8.200^{a}	7.580^{a}
	5	0.014^{a}	0.010^{b}	1.303^{a}	2.081^{a}	8.290^{a}	9.390^{a}
	7	0.011^{a}	0.009^{b}	1.135^{b}	2.005^{a}	9.010^{a}	11.880^{a}
	14	0.015^{a}	0.007^{b}	1.796^{b}	3.916^{a}	11.620^{a}	16.870^{a}
49	3	0.012^{a}	0.009^{b}	0.980^{a}	1.551^{a}	8.960^{a}	8.510^{a}
	5	0.011^{a}	0.009^{b}	1.356^{b}	1.823^{a}	10.440^{a}	9.680^{a}
	7	0.011^{a}	0.009^{b}	1.041^{b}	2.602^{a}	10.730^{a}	14.190^{a}
	14	0.010^{a}	0.008^{b}	1.557^{a}	2.057^{a}	11.150^{a}	15.080^{a}

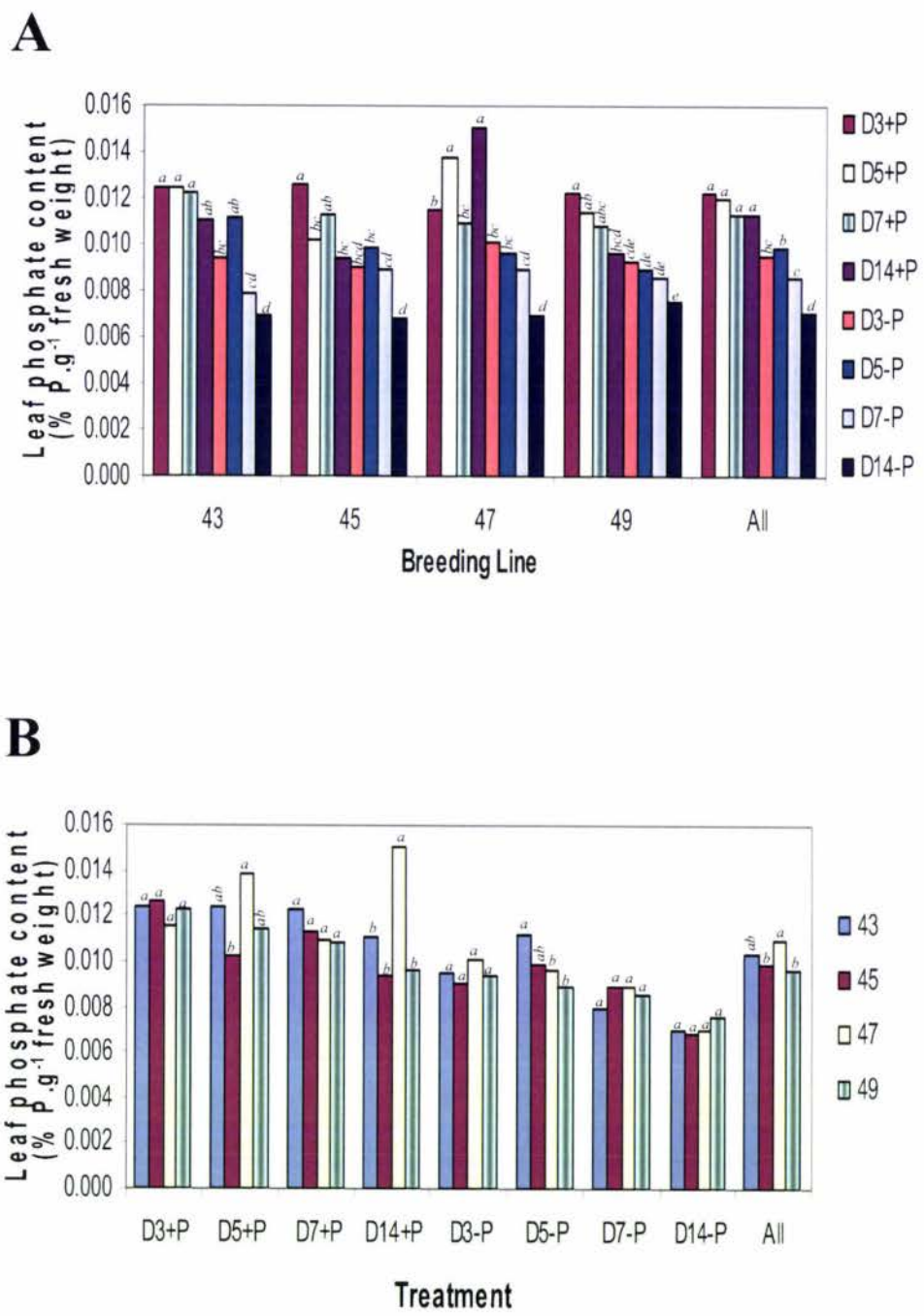


Figure 3.1. Phosphate contents in the first mature leaf from four breeding lines grown in P+ or P– media and sampled at day 3, 5, 7, and 14. Values are the means from individual leaf extracts excised from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test

content in BLs grown in P-sufficient media (Figure 3.1A). For each treatment given, all breeding lines showed no significant difference in Pi content at d 3 and d 7 for plants maintained in P-containing or P-deprived media (Figure 3.1B). The effect of treatment on the Pi content of each breeding line showed that at d 5 in P-deprived media, only breeding line 43 was significantly different from BLs 47 and 49 (Figure 3.1B). At d 7 onwards after P withdrawal, no significant difference was observed between each breeding line. Overall, the results showed that these four breeding lines have different responses to Pi deprivation. BLs 43 and 47 showed a high response to P application and performed relative well in +P media (a higher Pi content), but performed poorly in –P media (a rapid reduction in leaf Pi content), while BLs 45 and 49 showed a lower Pi content in leaves in +P media, and shows medium performance in –P media (a slow reduction in leaf Pi content).

3.2.2. Comparison of acid phosphatase (APase) activity of selected breeding lines of white clover grown in P-containing and P-deprived media.

At specific time intervals, plants were harvested and the roots extracted to yield two fractions, the water-soluble whole tissue fraction and the cell wall fraction that contains the ionically bound (1M salt-extractable) cell wall proteins. The assay of acid phosphatase activity in four breeding lines reveals differential responses of these BLs to P-deprivation (Table 3.1). BLs, days of treatment and P application have a significant effect on both APase activity except that BL has no effect on total soluble APase activity (Table 3.1). For each BL tested, BL 43 had the highest cell wall APase activity and was significantly different from BL 45 and 49, but not different from BL 47 (Table 3.2). The longer the days of treatment, the more APase activity is induced up to d 14 in the cell wall fraction and d 7 in the total soluble fraction (Table 3.2). In both fractions, a higher activity is observed in extracts from P-deprived plants (Table 3.2). At the specific times assayed, significant differences in cell wall APase activity in plant grown in +P and –P media were observed (Table 3.3). Relative to the +P treatment, in BLs 45 and 49, the difference was observed at d 5, for BL 47 at d 7, and for BL 43 at d 14 (Table 3.3).

Very significant interactions were observed between BL x D, BL x P, D x P, and BL x D x P in both APase activity, but no significant difference in the interaction

between BL x P for the total soluble fraction (Table 3.1). The complete analyses of the effect of those interactions on both APase activity can be seen in Appendices 5, 6, 7, and 8, respectively.

The effect of breeding line on cell wall APase activity is shown in Figure 3.2A. The result showed that there is a significant difference in APase accumulation in each treatment given, and a rapid induction of APase activity in BL 47 at d 14 in P-deprived media was observed and a similar trend can be observed in BLs 43 and 45, but not in BL 49 (Figure 3.2A). Total soluble APase activity in the different BLs is shown in Figure 3.2B. The results show a rapid increase in total soluble APase activity at d 14 in P-deficient media for BL 47, while BL 45 showed no difference in APase activity in +P or –P media at the same day Figure 3.2B). No significant difference was observed in total soluble APase activity for BL 49 (Figure 3.2B). The figure showed a positive trend in the induction of APase activity upon prolonged P-deficiency. The effect of treatment on cell wall APase activity of all breeding lines is shown in Figure 3.3A. In this analysis, there was no significant difference in cell wall APase activity for plants grown in P-contained and P-deprived media up to d 7 (Figure 3.3A). The rapid induction of APase activity can only be observed at d 14 in P-deprived media, at which time BL 47 was significantly different from BL 49 but not from BL 43 and BL 45 (Figure 3.3A). Overall, the result showed that BL 43 has the highest phosphatase activity and was significantly different with BL 49 and not statistically different from BL 45 and 47 (Figure 3.3A). The effect of treatment on total soluble APase activity of all BLs is shown as Figure 3.3B. The figure showed that there was no significant difference in total soluble APase activity for plants grown in P-contained or P-deprived media for all treatments given (Figure 3.3B).

3.2.3. Relative plant growth in response to Pi deprivation

For breeding line-based screening, 7 parameters of plant growth in response to Pi deprivation were measured at d 3, 5, 7, and 14. The result showed that these BLs varied greatly for all parameters measured (Table 3.4). No significant effect of P treatment on leaf area, weight of an individual leaf and shoot DW was observed (Table 3.4). It was observed that BLs 45 and 49 have the largest leaf area and a more heavy leaf and produce more shoot DW compared to the other two BLs (Table 3.5). BL 47 has the

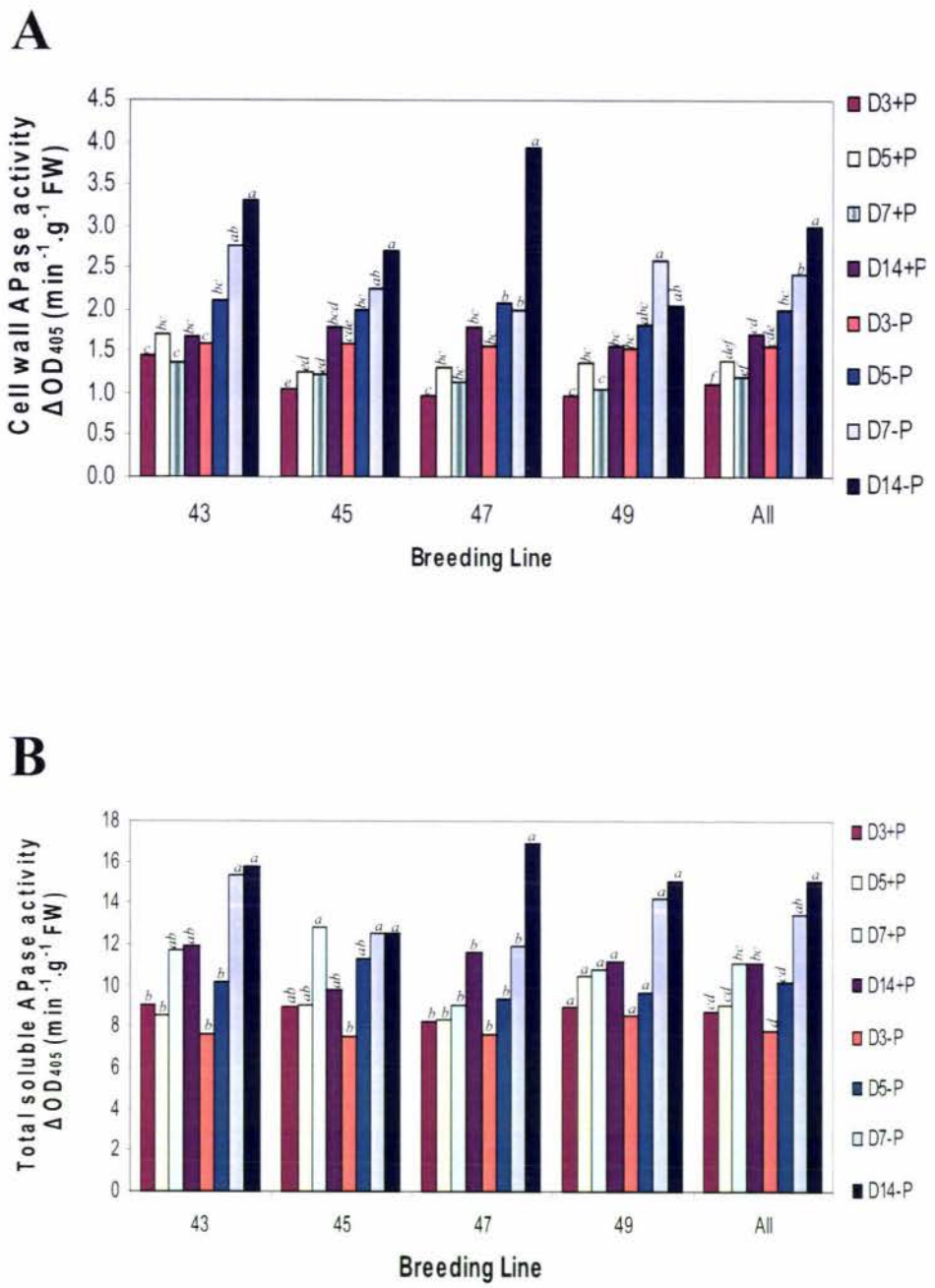


Figure 3.2. The effect of BLs on cell wall and total soluble APase activity in the roots from four breeding lines grown in P+ or P– media and sampled at day 3, 5, 7, and 14. Values are the means from individual root extracts excised from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test

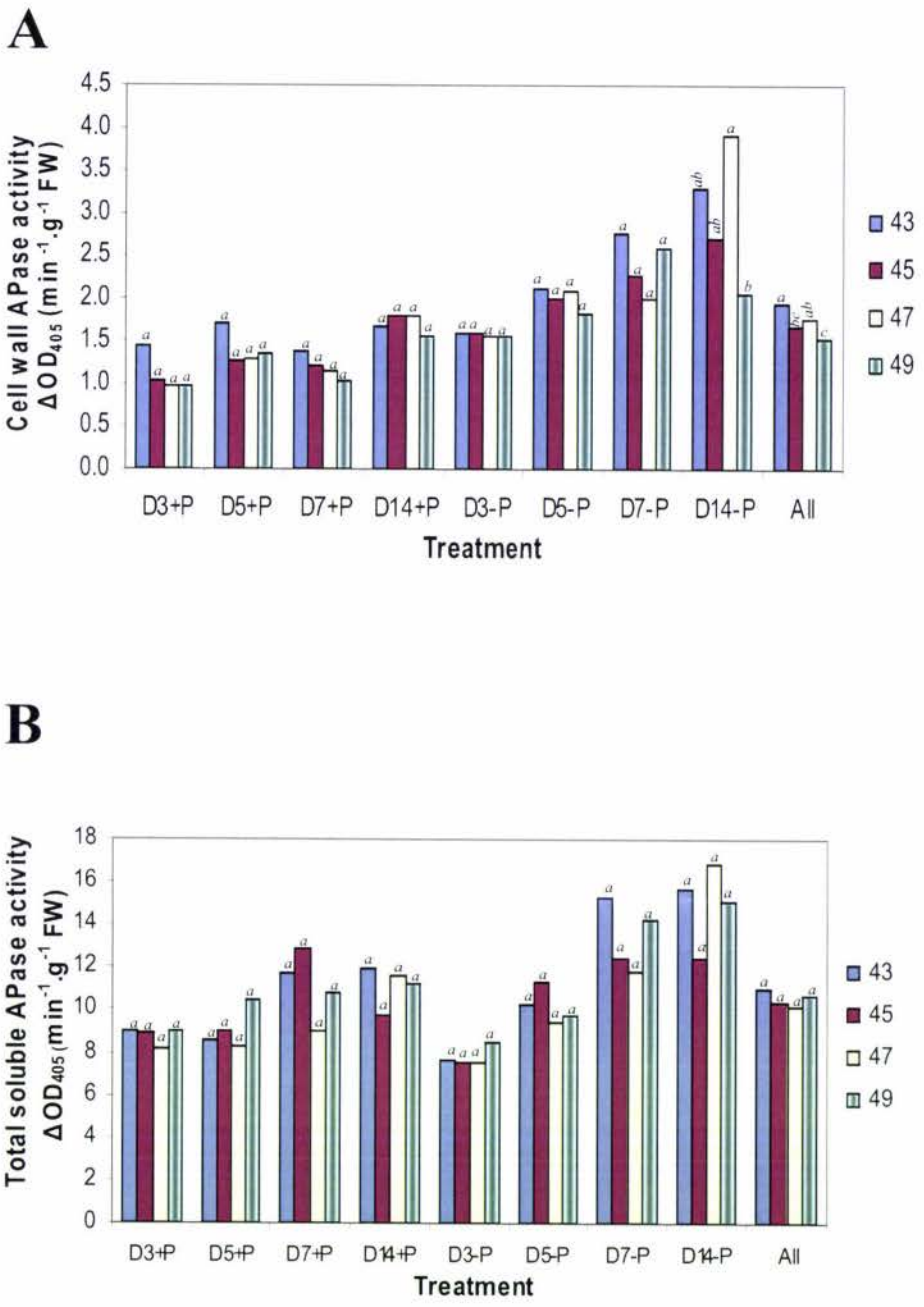


Figure 3.3. The effect of treatments on cell wall and total soluble APase activity in the roots from four breeding lines grown in P+ or P– media and sampled at day 3, 5, 7, and 14. Values are the means from individual root extracts excised from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Table 3.4. Probability of F for leaf area, weight of an individual leaf, shoot DW, fresh biomass yield and R:S FW ratio.

Source of Variation	df	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)	Fresh biomass yield (g/plant)			R:S FW ratio
					Root	Shoot	Biomass	
Breeding Line (BL)	3	***	**	***	***	**	**	*
Days of Treatment (D)	3	*	***	***	*	***	***	NS
Phosphorus (P)	1	NS	NS	NS	NS	NS	NS	NS
BL x D	15	***	***	***	***	***	***	*
BL x P	7	***	*	**	***	*	*	*
D x P	7	*	***	***	**	***	***	***
BL x D x P	31	***	**	***	***	***	***	*

*, **, *** significance at the 0.05, 0.01 and 0,001 probability level, respectively.
NS = not significant at $p = 0.05$

Table 3.5. The effect of breeding lines, days of treatment and phosphorus application to leaf area, weight of an individual leaf and shoot DW of selected breeding lines. Plants maintained in either P-containing (+P) or P-deprived (-P) media. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at 0.05 level according to Duncan's multiple range test.

Source of Variance	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)
Breeding Line (BL)			
BL43	356.6^{b}	0.1050^{b}	1.1350^{b}
BL45	452.8^{a}	0.1366^{a}	1.9220^{a}
BL47	291.6^{c}	0.0923^{b}	1.7560^{a}
BL49	451.6^{a}	0.1388^{a}	1.9560^{a}
Days of Treatment (D)			
D3	447.4^{a}	0.1069^{b}	1.3610^{b}
D5	355.4^{b}	0.1219^{ab}	1.2580^{b}
D7	396.4^{ab}	0.1306^{a}	1.6070^{b}
D14	353.4^{b}	0.1133^{ab}	2.5420^{a}
Phosphorus (P)			
0 mM P	395.8^{a}	0.1136^{a}	1.7050^{a}
0.5 mM P	380.4^{a}	0.1227^{a}	1.6790^{a}

smallest leaf area, no difference in weight of an individual leaf with BL 43, but produced more shoot DW relative to BL 43 (Table 3.5). BL 43 has the lower shoot DW relative to the other 3 BLs (Table 3.5). The largest leaf area is at d 3, while the heaviest weight of an individual leaf is at d 7 (Table 3.5). When the effect of P treatment on the weight of an individual leaf at the specific times indicated for each BL is compared, no significant difference in leaf area, weight of an individual leaf and shoot DW is observed in plants grown in both media, except for weight of an individual leaf for BL 45 at d 14 and BL 49 at d 7 (Table 3.6).

When the interaction between BL, D and P is compared, the results in Table 3.4 show strong interactions between BL x D, BL x D x P for leaf area, weight of an individual leaf and shoot DW. Further, Table 3.4 show weak interactions are observed in D x P (leaf area), BL x P (weight of an individual leaf) and medium interactions for BL x P (shoot DW), BL x D x P (weight of and individual leaf). The complete statistical analyses of these interactions can be seen in Appendices 5, 6, 7, and 8.

Although not significant, BL 45 showed an increase in leaf area after 14 days in P-deprived media (Figure 3.4A). A similar result can be observed when the weight of an individual leaf is compared among BLs. Relative to other BLs, only BL 45 showed a trend of an increase in the weight of an individual leaf upon prolonged P deprivation (Figure 3.5B). No significant difference in leaf area and weight of an individual leaf for BLs 43 and 47 grown in +P or –P media was observed (Figures 3.4A and 3.5A). For the effect of treatment on leaf area and weight of an individual leaf, BLs 45 and 49 show a higher leaf area and weight on an individual leaf for plants grown in +P or –P media when compared with BLs 43 and 47 (Figures 3.4B and 3.5B). However, for BL 45, there were significant differences in shoot dry matter from plants grown in +P or –P (Figure 3.6A). For BL 45, after 2 weeks in P-deprived media an increase in the production of shoot dry matter and a 75% increase in the production of shoot dry weight compared to +P plants was observed (Figure 3.6A). Although not statistically significant, BL 45 produced more shoot dry matter than other BLs at d 14 after P-withdrawal (Figure 3.6B). For BLs 43 and 47, although both have similar leaf area and weight of an individual leaf, BL 47 produced more shoot DW relative to BL 43 (compare Figures 3.4A, 3.5A, and 3.6A). At 14 d in P-deprived media, although not statistically significant, only BL 49 showed a reduction in shoot dry weight compared to the control plants at the same period (Figure 3.6A). At d 14 in +P media, BL 49 is

Table 3.6. Comparison of leaf area, weight of an individual leaf and shoot DW from each breeding line grown in +P and –P at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf and shoot DW were determined. Values are the means from six independent plants. The different letters above in the same row indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Breeding Line	DOT	Leaf area (mm^2)		Weight of an individual leaf (g)		Shoot DW (g)	
		+P	–P	+P	–P	+P	–P
43	3	426.00^a	398.83^a	0.106^a	0.094^a	1.208^a	1.092^a
	5	317.33^a	332.00^a	0.128^a	0.089^a	0.912^a	0.991^a
	7	356.00^a	376.50^a	0.115^a	0.111^a	1.125^a	0.777^a
	14	320.67^a	325.17^a	0.093^a	0.104^a	1.077^a	1.898^a
45	3	559.33^a	554.67^a	0.123^a	0.097^a	1.604^a	1.679^a
	5	356.83^a	415.17^a	0.151^a	0.122^a	1.506^a	1.351^a
	7	513.00^a	427.67^a	0.172^a	0.152^a	1.882^a	1.865^a
	14	335.00^a	460.50^a	0.113^b	0.163^a	2.005^a	3.487^a
47	3	331.00^a	309.83^a	0.096^a	0.083^a	1.428^a	1.330^a
	5	253.50^a	284.00^a	0.095^a	0.099^a	1.401^a	1.115^a
	7	281.67^a	292.17^a	0.086^a	0.098^a	1.986^a	1.675^a
	14	259.17^a	321.83^a	0.080^a	0.101^a	2.377^a	2.736^a
49	3	520.33^a	479.17^a	0.145^a	0.111^a	1.291^a	1.259^a
	5	404.67^a	479.50^a	0.151^a	0.141^a	1.218^a	1.573^a
	7	459.67^a	464.67^a	0.171^a	0.141^b	1.868^a	1.681^a
	14	393.00^a	411.50^a	0.141^a	0.111^a	3.978^a	2.778^a

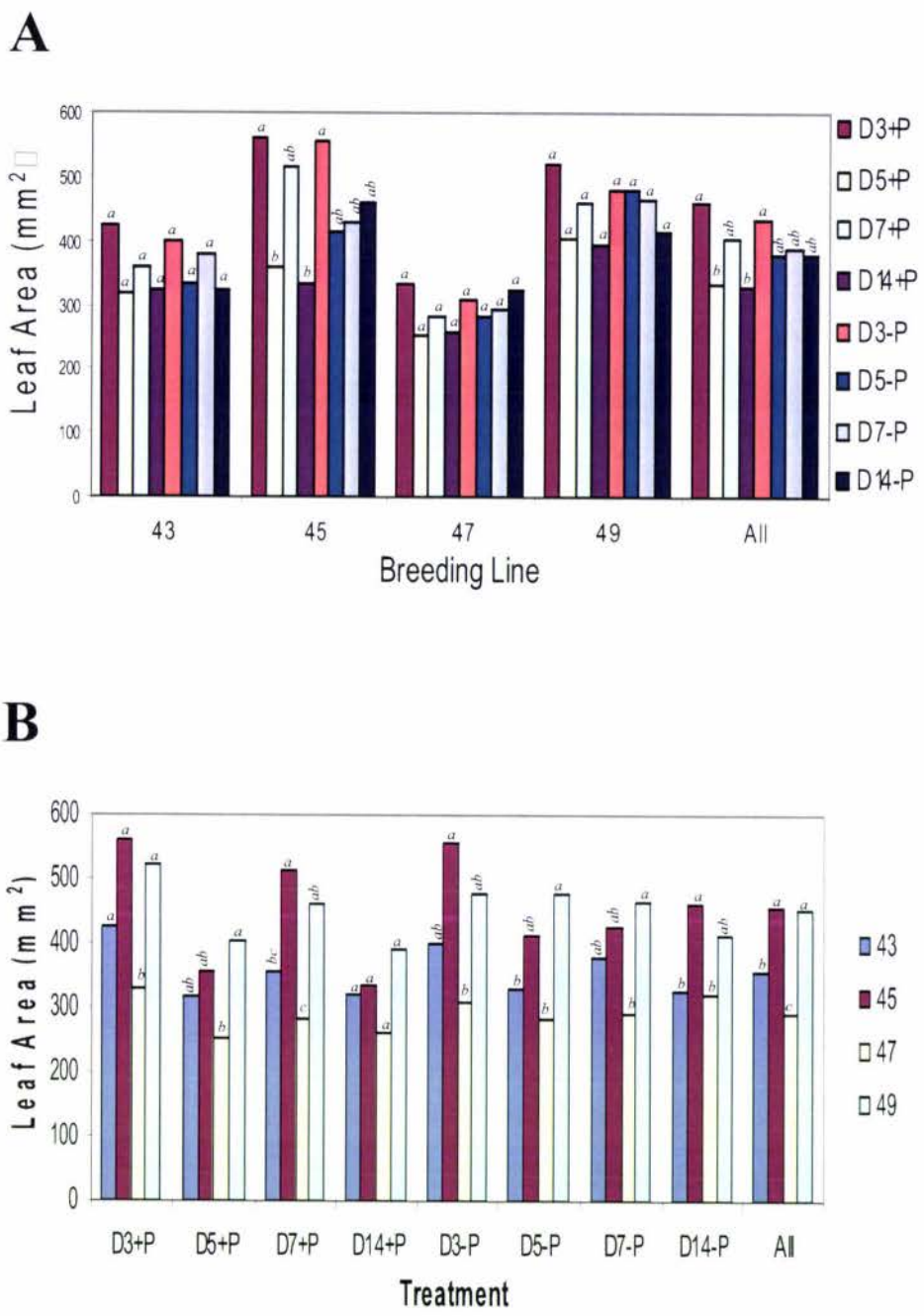


Figure 3.4. Leaf area determination of four breeding lines of white clover grown in P-containing (P+) or P-deprived (P–) media and sampled at day 3, 5, 7, and 14. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

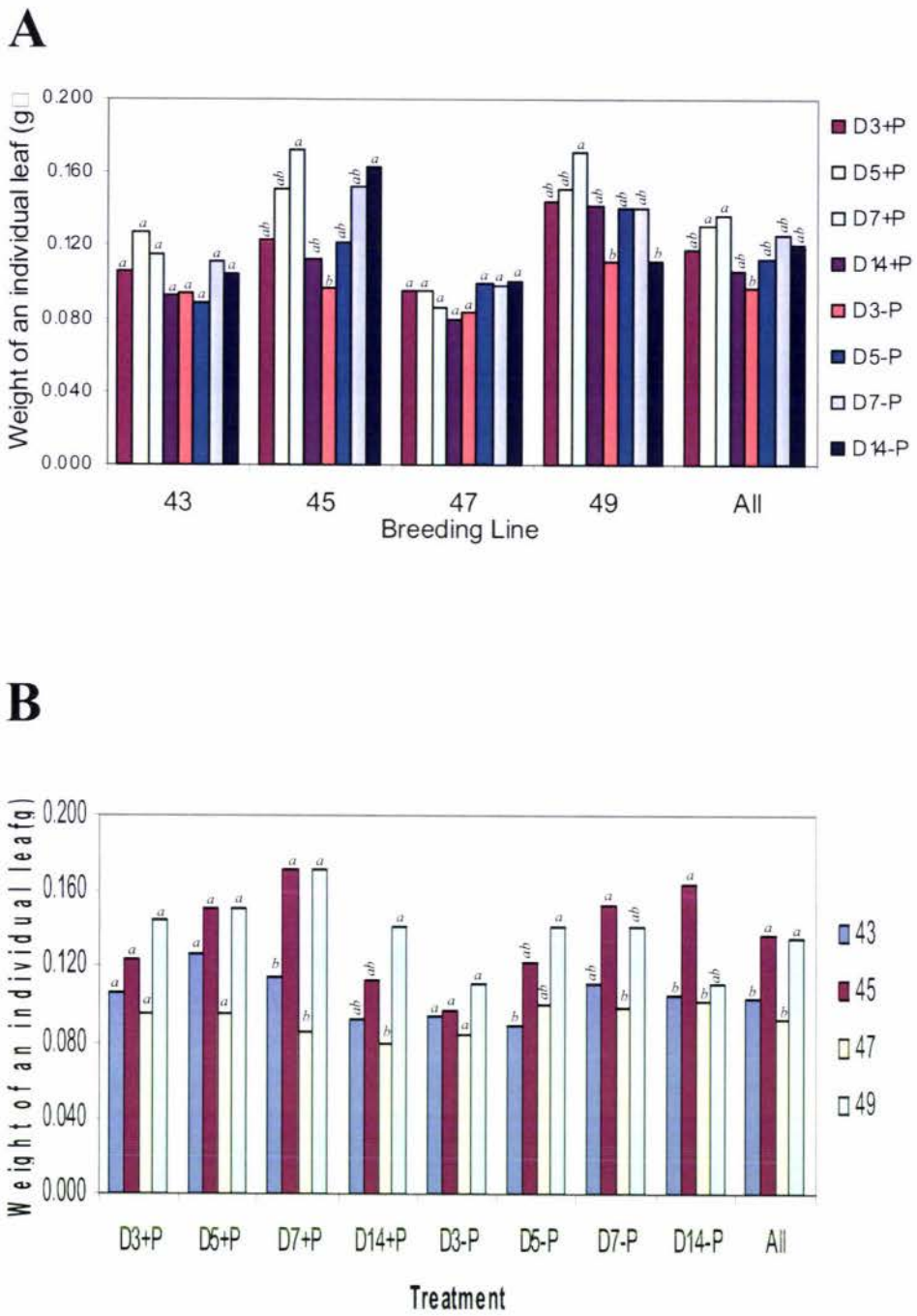


Figure 3.5. Weight of an individual leaf of four breeding lines of white clover grown in either P-containing (P+) or P-deprived (P–) media and sampled at day 3, 5, 7, and 14. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test

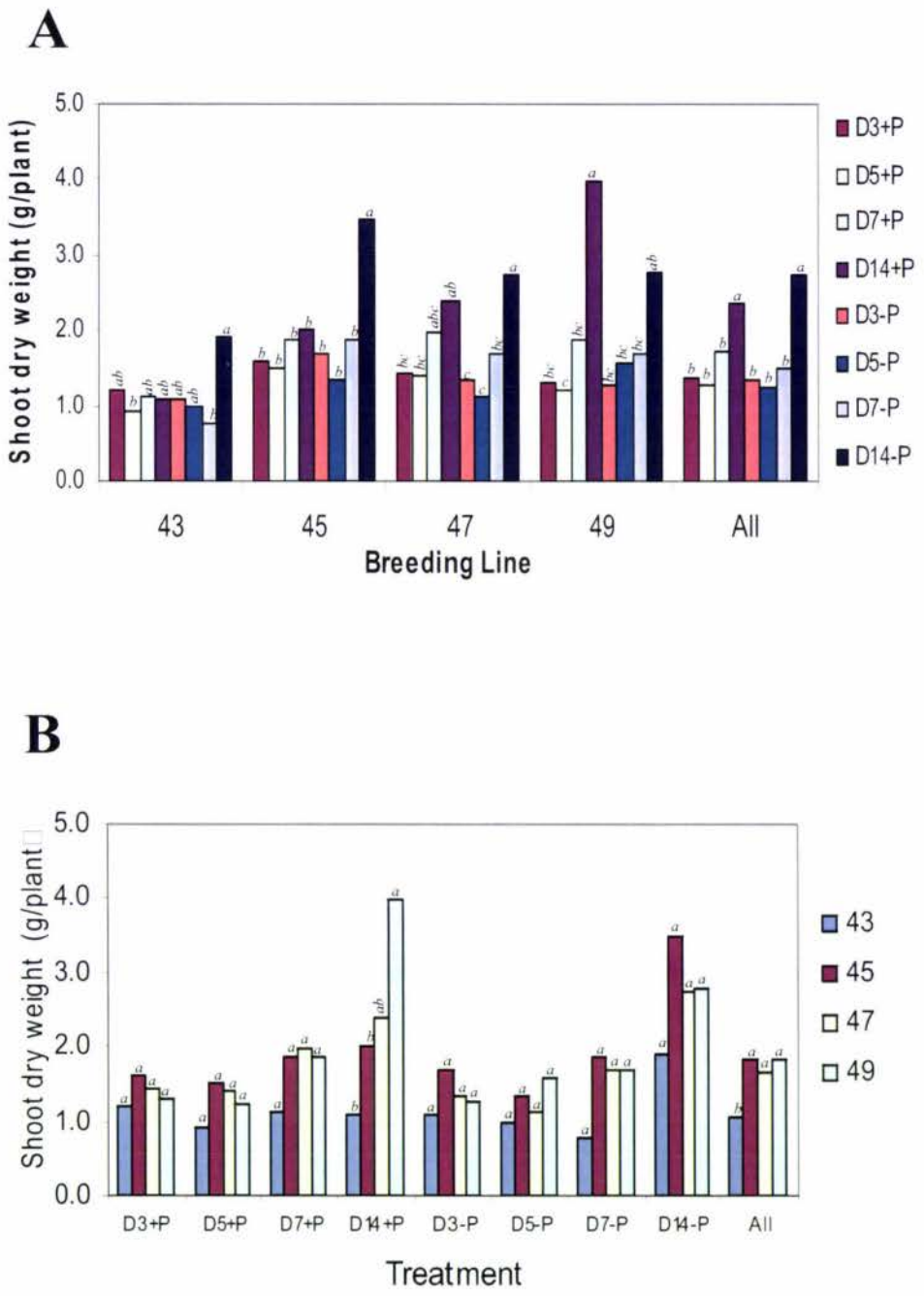


Figure 3.6. Shoot dry weight determinations of four breeding lines of white clover grown in either P-containing (P+) or P-deprived (P–) media and sampled at day 3, 5, 7, and 14. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test

significantly different with BL 43 and 45, but not with BL 47 (Figure 3.6B). Further, the result showed that BL 45 had a higher shoot DW in –P media (Figure 3.6B).

BLs and days of treatment have a significant effect on the biomass fresh weight (Table 3.4). However, days of treatment had no effect on root:shoot ratio (Table 3.4). It is also observed that P treatment alone had no significant effect on all of the growth parameter measured (Table 3.4). BL 43 had the lowest root FW, shoot FW, and total biomass FW, but it had the highest R:S ratio (Table 3.7). On the other hand, BL 49 had the highest root FW, shoot FW, total biomass FW and it had also the highest R:S ratio (Table 3.7). Although BL 45 had a higher fresh biomass yield, it did have a lower root FW, and so a lower R:S ratio compared to BL 49, and while BL 47 had a higher fresh biomass yield, it did have a lower R:S ratio compared to BL 49 (Table 3.7). The longer the days of treatment, the higher weight of fresh biomass observed, except for the R:S ratio (Table 3.7). When the effect of P treatment on each BL at a specific time indicated is compared, no significant difference was found for each BL grown in +P and –P media (Table 3.8).

When comparing the interaction between BL, D and P, the results in Table 3.4 show strong interactions between BL x D, BL x P, D x P, and BL x D x P, while weak interactions are observed in BL x D (R:S ratio), BL X P (shoot FW, biomass FW and R:S ratio) and BL x D x P (R:S ratio). The complete statistical analyses of these interactions can be seen in Appendices 9, 10, 11, and 12.

Total shoot fresh weight at d 14 in P-containing and P-deprived media was not reduced significantly within the duration of the experiment, and the white clover lines appeared able to cope with P deprivation (at least BLs 43, 47 and 49). Relative to the –P media, BL 49 produced a higher root FW, shoot FW, biomass FW in +P media, but no difference in R:S ratio in both media (Figures 3.7A, 3.8A, 3.9A, and 3.10A). In contrast, BL 45 produced more root FW, shoot FW, biomass FW in –P media (Figures 3.7A, 3.8A, 3.9A, and 3.10A). BL 43 had the lowest root FW, shoot FW, biomass FW, but had a higher R:S ratio, while BL 47 produced medium root FW, shoot FW, biomass FW and R:S ratio. Although not statistically significant, BL 45 did produce a greater weight of roots in P-deprived media than the other BLs (Figure 3.7B). If a level of 0 mM P is used to indicate plant performance under a severe degree of P stress, marked yield differences between breeding lines were recorded. At this P level, BL 49 was in the highest group in terms of root (Figure 3.7B) and shoot fresh weight yield (Figure

Table 3.7. The effect of breeding lines, days of treatment and phosphorus application on fresh biomass yield and root:shoot FW ratio of selected breeding lines of white clover. Plants were maintained in either P-containing (+P) or P-deprived (-P) media and harvested at the time indicated. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at 0.05 level according to Duncan's multiple range test.

Trait	Fresh biomass yield (g/plant)			Root:Shoot FW ratio
	Root	Shoot	Total	
Breeding Line (BL)				
BL43	1.8660^{b}	6.9500^{b}	8.8200^{b}	0.2808^{a}
BL45	2.4110^{ab}	10.4900^{a}	12.9000^{a}	0.2444^{b}
BL47	2.4810^{a}	10.8200^{a}	13.3000^{a}	0.2466^{b}
BL49	2.8160^{a}	10.6400^{a}	13.4600^{a}	0.2768^{a}
Days of Treatment (D)				
D3	2.1960^{b}	8.7740^{bc}	10.9700^{bc}	0.2664^{a}
D5	1.8800^{b}	7.1530^{c}	9.0300^{c}	0.2760^{a}
D7	2.3000^{b}	9.7160^{b}	12.0200^{b}	0.2531^{a}
D14	3.1980^{a}	13.2550^{a}	16.4500^{a}	0.2532^{a}
Phosphorus (P)				
0 mM P	2.4320^{a}	9.5290^{a}	11.9600^{a}	0.2713^{a}
0.5 mM P	2.3550^{a}	9.9200^{a}	12.2700^{a}	0.2530^{a}

Table 3.8. Comparison of fresh biomass yield and R:S ratio FW from each genotype grown in +P and –P at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and R:S ratio FW were determined. Values are the means from individual leaf extracts excised from six independent plants. The different letters above in the same row indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

BL	Day	Fresh biomass yield (g/plant)						Root:Shoot FW ratio	
		Shoot		Root		Total			
		+P	–P	+P	–P	+P	–P	+P	–P
43	3	8.555^a	7.383^a	2.0919^a	2.0781^a	10.647^a	9.461^a	0.2367^a	0.2913^a
	5	5.504^a	5.703^a	1.3815^a	1.7896^a	6.886^a	7.493^a	0.2763^a	0.3220^a
	7	7.170^a	4.798^a	1.7333^a	1.2495^a	8.903^a	6.048^a	0.2480^a	0.2744^a
	14	6.398^a	10.100^a	2.1147^a	2.4911^a	8.513^a	12.591^a	0.3493^a	0.2487^a
45	3	10.269^a	9.690^a	1.994^a	2.6956^a	12.263^a	12.386^a	0.2073^a	0.2946^a
	5	8.324^a	7.105^a	1.9005^a	1.7166^a	10.225^a	8.822^a	0.2467^a	0.2504^a
	7	11.279^a	9.935^a	2.4968^a	2.1844^a	13.776^a	12.119^a	0.2422^a	0.2429^a
	14	9.912^a	17.379^a	2.4755^a	3.8254^a	12.388^a	21.204^a	0.2555^a	0.2159^a
47	3	9.552^a	8.934^a	2.1381^a	2.782^a	11.690^a	11.716^a	0.2247^a	0.3255^a
	5	8.211^a	6.783^a	2.1174^a	1.7161^a	10.328^a	8.499^a	0.2779^a	0.2631^a
	7	13.418^a	11.070^a	2.6805^a	2.5634^a	16.099^a	13.633^a	0.2013^a	0.2411^a
	14	13.969^a	14.589^a	3.0311^a	3.1507^a	17.000^a	17.740^a	0.2281^a	0.2110^a
49	3	8.072^a	7.738^a	1.9018^a	2.2226^a	9.974^a	9.961^a	0.2440^a	0.3075^a
	5	7.095^a	8.494^a	1.7228^a	2.6934^a	8.818^a	11.187^a	0.2669^a	0.3047^a
	7	11.478^a	8.576^a	2.9996^a	2.4909^a	14.478^a	11.067^a	0.2800^a	0.2947^a
	14	19.507^a	14.183^a	4.9032^a	3.5947^a	24.410^a	17.778^a	0.2637^a	0.2533^a

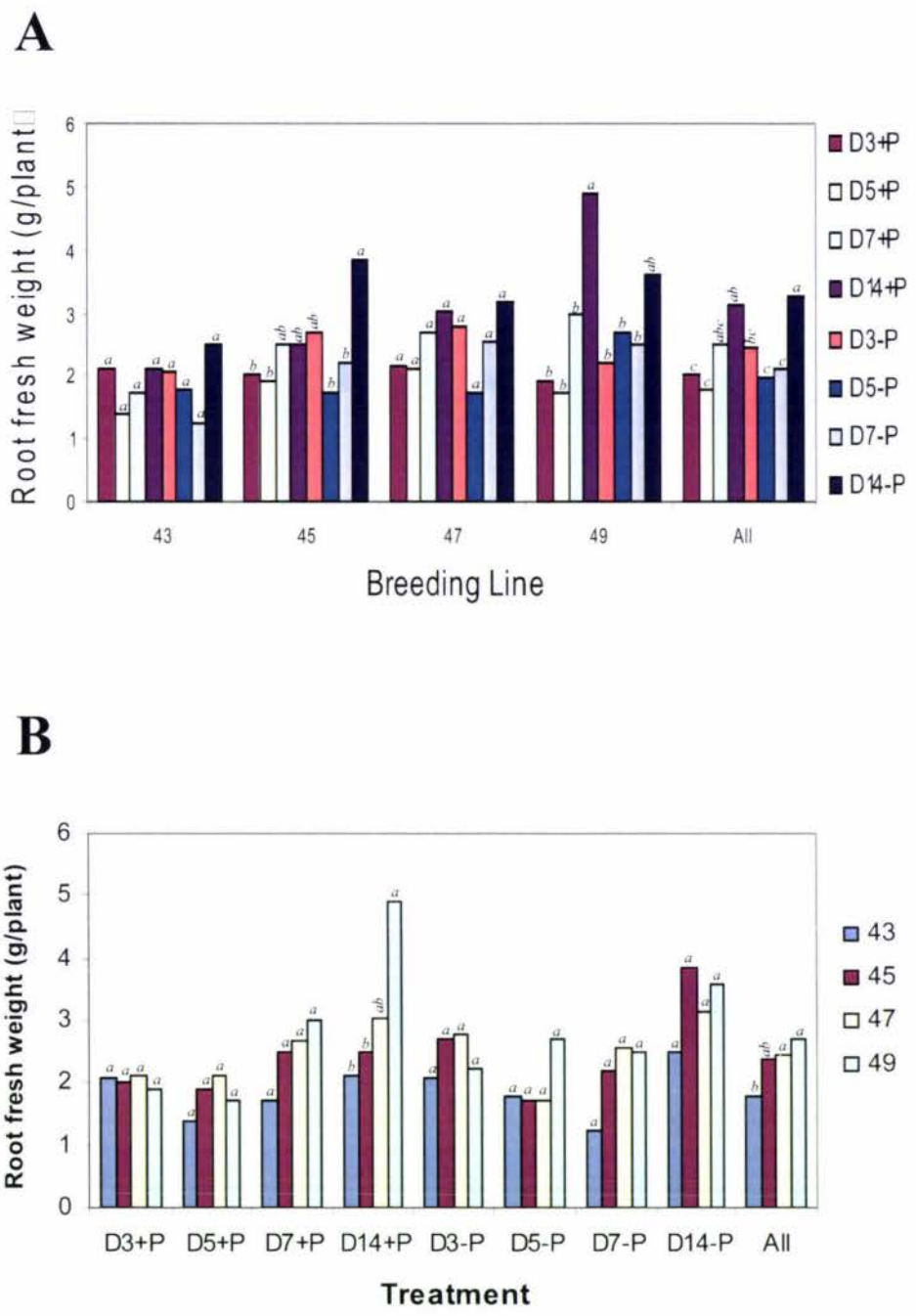


Figure 3.7. Root fresh weight from four breeding lines grown in P+ or P– media and sampled at day 3, 5, 7, and 14. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

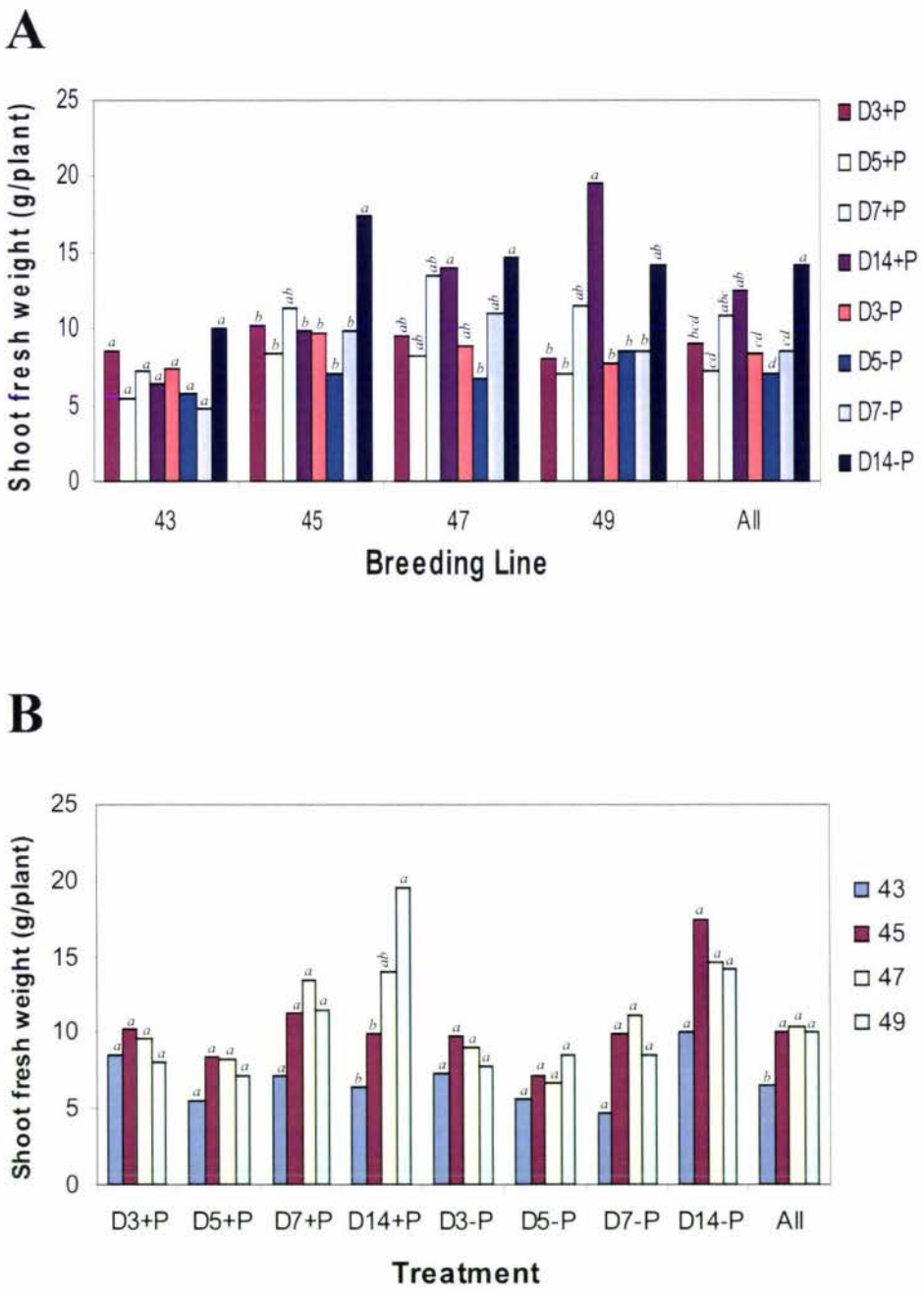


Figure 3.8. Shoot fresh weight from four breeding lines grown in P+ or P– media and sampled at day 3, 5, 7, and 14. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

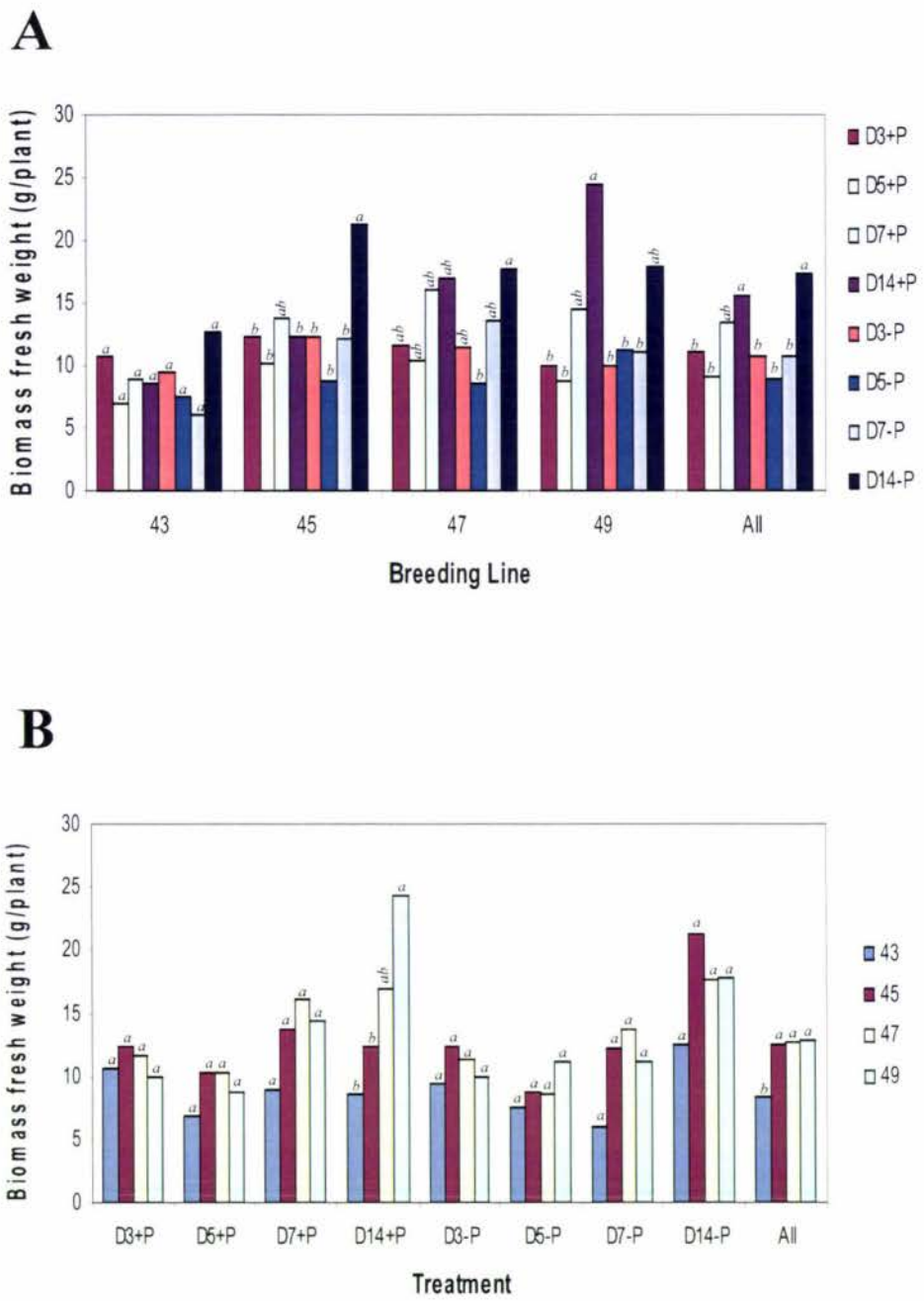


Figure 3.9. Biomass fresh weight determinations of four breeding lines of white clover grown in either P-containing (P+) or P-deprived (P–) media and sampled at day 3, 5, 7, and 14. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

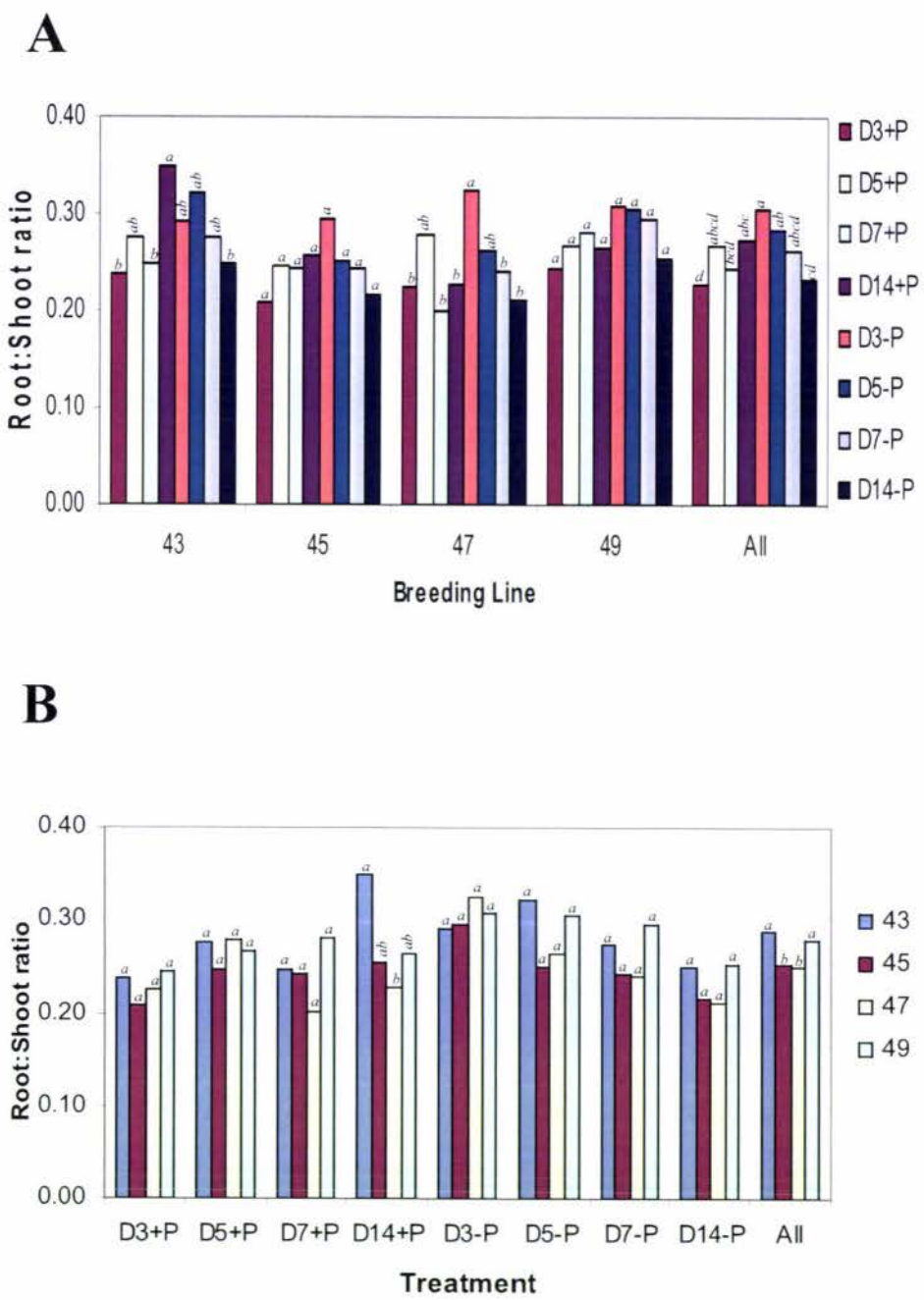


Figure 3.10. Root:Shoot fresh weight ratio from four breeding lines grown in P+ or P– media and sampled at day 3, 5, 7, and 14. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

3.8B), had the highest fresh biomass yield (Figure 3.9B) and had the same R:S ratio in both media (Figure 3.10B). By contrast root, shoot, and biomass yields were lowest in BL 43, but it had a higher R:S ratio (Figures 3.7B, 3.8B, 3.9B, and 3.10B).

3.3. Selected Genotypic Screening

3.3.1. Onset of P-deficiency in selected genotypes of white clover leaf tissue

For the genotype-based screening, five genotypes were selected. These genotypes were designated 43-7, 47-8, 45-14, 45-4 and 47-9, and were selected based on their performances in preliminary screenings (see Chapter 2 for a description). Phosphate levels were measured in the first fully expanded mature leaf excised from plants grown in phosphate-containing and phosphate deprived media at 7, 14, and 21 d. Genotypes, days of treatment and P application all had a significant effect on Pi content (Table 3.9). The results also showed that the average Pi content was high in genotype 43-7, followed by genotypes 43-8 and 47-9, and a low Pi content can be observed in genotype 45-14 and 45-4 (Table 3.10). It was also shown that Pi content in leaves decreased after d 21 (Table 3.10). Further, the results show an increase in leaf Pi content observed in all genotypes grown in +P media (Table 3.10). For genotypes 43-7 and 43-8, the decrease in leaf Pi content can be observed at d 14 onward in plants grown in –P media, while for genotypes 45-14, 45-4 and 47-9, these decreases in leaf Pi content can be observed earlier at d 7 in P-deprived media (Table 3.11).

Statistical analyses using Duncan Least Significant Differences showed that all genotypes showed a similar trend of a decrease in Pi content upon prolonged deficiency (Figure 3.11A). A rapid decrease in Pi content can be seen in genotype 43-7, a medium decrease in genotype 47-9, while genotypes 45-14 and 45-4 showed a slighter slower rate decrease (Figure 3.11A). For plants grown in P-sufficient media, genotype 43-7 had the highest Pi content, while genotypes 43-8 and 47-9 had a medium Pi content and genotypes 45-14 and 45-4 had the lowest Pi content (Figure 3.11A). For each treatment given, there was a significant difference in the Pi content of all genotypes grown in P-sufficient media (Figure 3.11). A decrease in Pi content upon prolonged Pi withdrawal was observed as early as d 7, with genotype 43-7 significantly different from the other

Table 3.9. Probability of F for Pi content and APase activity of selected genotypes of white clover.

Source of Variation	df	Pi content ($\%P.g^{-1}FW$)	Acid phosphatase activity	
			Cell wall fraction	Total soluble fraction
			OD_{405} ($min^{-1}g^{-1}FW$)	OD_{405} ($min^{-1}g^{-1}FW$)
Genotype (G)	4	***	*	***
Days of Treatment (D)	2	***	NS	*
Phosphorus (P)	1	***	***	***
G x D	14	***	*	***
G x P	9	***	***	***
D x P	5	***	***	***
G x D x P	29	***	***	***

*, **, *** significance at the 0.05, 0.01 and 0.001 probability level, respectively.
NS = not significant at $p = 0.05$

Table 3.10. The effect of genotypes, days of treatment and phosphorus application to Pi content and APase activity in selected genotypes of white clover. Plants were maintained in either P-containing (+P) or P-deprived (-P) media. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at 0.05 level according to Duncan's multiple range test.

Source of variation	Pi content ($\%P.g^{-1}FW$)	Acid phosphatase activity	
		Cell wall fraction OD_{405} ($min^{-1}g^{-1}FW$)	Total soluble fraction OD_{405} ($min^{-1}g^{-1}FW$)
Genotype (G)			
43-7	0.017979^{a}	1.5578^{b}	9.1539^{ab}
43-8	0.015466^{b}	2.0225^{a}	8.1009^{b}
45-14	0.012364^{c}	1.6686^{ab}	8.0830^{b}
45-4	0.012833^{c}	1.4839^{b}	9.5564^{ab}
47-9	0.015018^{b}	1.5311^{b}	10.9341^{a}
Days of Treatment (D)			
D7	0.016383^{a}	1.6087^{a}	10.8026^{a}
D14	0.014892^{a}	1.8369^{a}	10.2199^{a}
D21	0.012921^{b}	1.5129^{a}	6.4745^{b}
Phosphorus (P)			
0 mM P	0.011973^{b}	2.0467^{a}	10.4333^{a}
0.5 mM P	0.017492^{a}	1.2588^{b}	7.8980^{b}

Table 3.11. Comparison of Pi content and acid phosphatase activity from each genotype grown in +P and –P at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi content and acid phosphatase activity were determined. Values are the means from individual leaf extracts excised from three independent plants. The different letters above in the same row indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Genotype	DOT	Pi content (%P.g^{-1}FW)		Acid phosphatase activity			
				Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)		Total soluble OD_{405} (min^{-1}g^{-1}FW)	
		+P	–P	+P	–P	+P	–P
43-7	7	0.022775^{a}	0.020392^{a}	1.0542^{a}	1.9565^{a}	12.1437^{a}	12.4085^{a}
	14	0.021716^{a}	0.012284^{b}	1.2396^{a}	2.1079^{a}	8.1209^{a}	8.4046^{a}
	21	0.020206^{a}	0.010500^{b}	1.1198^{a}	1.8689^{a}	6.1031^{a}	7.7425^{a}
43-8	7	0.014941^{a}	0.014392^{a}	1.4124^{a}	2.4011^{a}	8.4235^{a}	10.5611^{a}
	14	0.024275^{a}	0.012000^{b}	1.5372^{b}	3.0108^{a}	8.6316^{a}	10.8196^{a}
	21	0.016549^{a}	0.010637^{b}	1.2718^{a}	2.5020^{a}	4.7726^{a}	5.3969^{a}
45-14	7	0.015088^{a}	0.012157^{b}	1.7686^{a}	2.0713^{a}	8.3541^{a}	10.4791^{a}
	14	0.013765^{a}	0.010922^{b}	0.9905^{b}	2.5997^{a}	5.7122^{a}	11.0718^{a}
	21	0.013510^{a}	0.008745^{b}	1.1507^{a}	1.4307^{a}	4.9681^{a}	7.9128^{a}
45-4	7	0.015824^{a}	0.012627^{b}	0.9792^{b}	1.6413^{a}	8.1460^{a}	11.5384^{b}
	14	0.013892^{a}	0.011431^{b}	1.2024^{b}	2.0896^{a}	9.8864^{a}	12.7806^{a}
	21	0.013980^{a}	0.009245^{b}	1.2806^{a}	1.7106^{a}	6.7525^{a}	8.2343^{a}
47-9	7	0.021608^{a}	0.014029^{b}	1.2087^{a}	1.5933^{a}	9.8675^{a}	16.1036^{b}
	14	0.017961^{a}	0.010676^{b}	1.4868^{a}	2.1041^{a}	10.5169^{a}	16.2549^{a}
	21	0.016284^{a}	0.009549^{b}	1.1803^{a}	1.6135^{a}	6.0716^{a}	6.7904^{a}

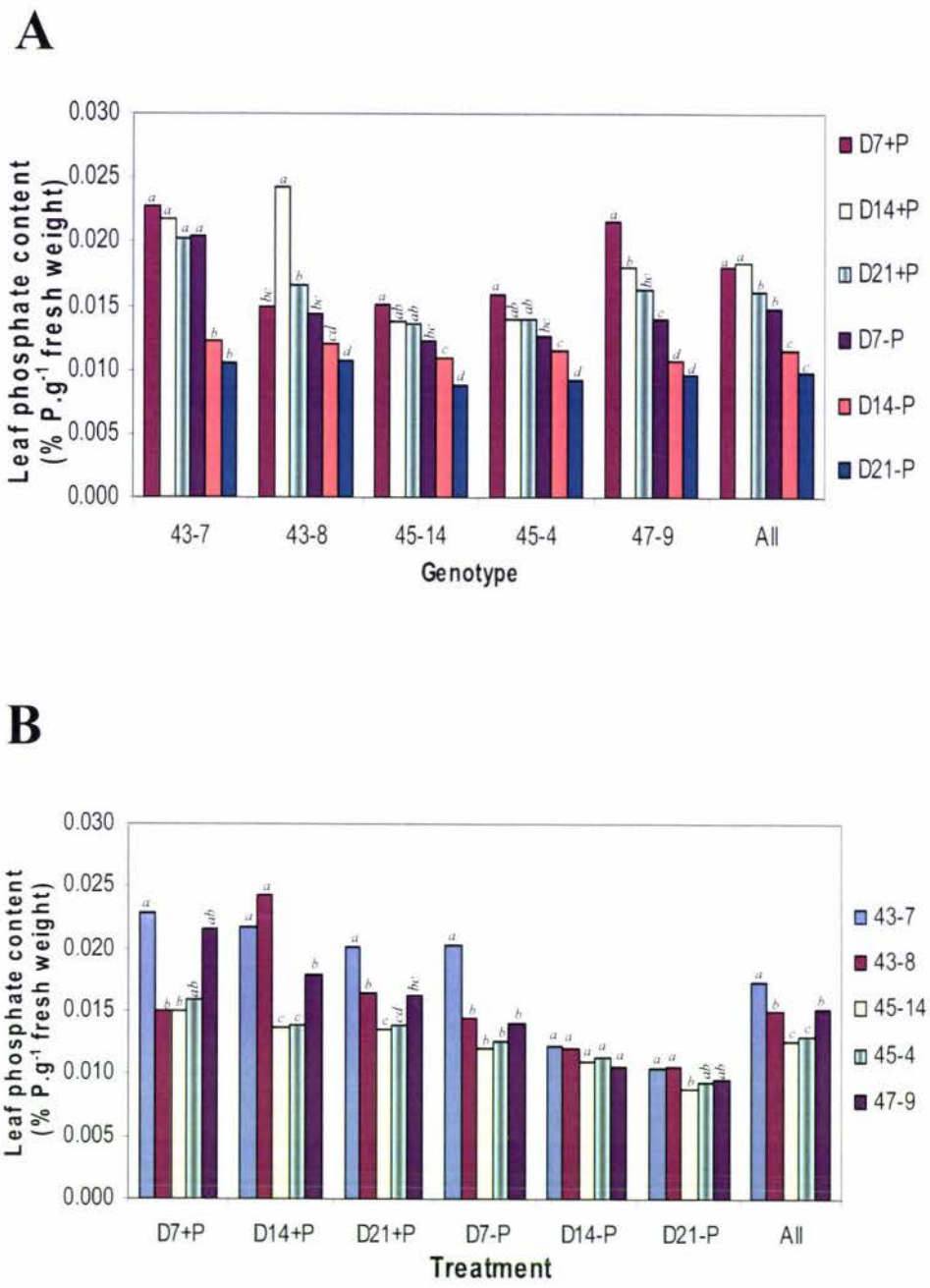


Figure 3.11. Phosphate contents in the first mature leaf from selected genotypes grown in P+ or P– media and sampled at day 7, 14, and 21. Values are the means from individual leaf extracts excised from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

genotypes tested (Figure 3.11B). The level of Pi was not significantly different at d 14 in the –P media, then at d 21 in P-deprived media, genotypes 43-7 and 43-8 were significantly different from genotype 45-14, but not from the other two genotypes (Figure 3.11B).

Very significant interactions were observed between G x D, G x P, D x P, G x D x P on Pi content in leaves (Table 3.9). The complete analyses of these effects of those interactions on Pi content in leaves can be seen in Appendices 13, 14, 15, and 16, respectively. The results show that these five genotypes have different responses to Pi deprivation. Genotype 43-7 showed a high response to P application and performed relatively well in +P media but performed poorly in –P media (a rapid reduction in leaf Pi content). Genotypes 43-8 and 47-9 had a medium performance in +P media (medium leaf Pi content), and a gradual decrease in leaf Pi content in –P media. Genotypes 45-14 and 45-4 had the lowest leaf Pi content in +P media (a poor performance in +P) but show a slow reduction in Pi content in –P media.

3.3.2. Comparison of acid phosphatase (APase) activity in white clover grown in P-containing and P-deprived media

At specific time intervals, plants were harvested and the roots extracted to yield two fractions, the water-soluble whole tissue fraction and the cell wall fraction that contains the ionically bound (1M salt-extractable) cell wall proteins. Assay of acid phosphatase activity in each fraction of five genotypes of white clover reveals a differential response of these genotypes to P-deprivation. Traits such as genotype, days of treatment and P level have significant effects on both APase activities, except that days of treatment has no effect on cell wall APase activity (Table 3.9). The highest cell wall APase activity can be observed in genotype 43-8, with medium activity for genotype 45-14, while genotypes 43-7, 45-4 and 47-9 have the lowest APase activity(Table 3.10). When the total soluble APase activity is compared, only genotype 47-9 is significantly different from genotypes 43-8 and 45-14, but is not different from genotypes 43-7 and 45-4 (Table 3.10). It is also shown that the total soluble APase activity decreased at d 21, and no significant difference was observed in the cell wall fraction for days of treatment (Table 3.10). In both the cell wall and soluble extracts from roots, a higher activity is recorded in extracts from P-deprived plant (Table 3.10). No significant difference was observed in cell wall APase activity at the specific

times assayed for genotypes 43-7 and 47-9 grown in +P or –P media (Table 3.11). However, a lower enzyme activity can be observed in genotypes 43-8 and 45-14 at d 14 and d 21 in P-sufficient plants (Table 3.11). For genotype 45-4, the difference in cell wall APase activity can be seen from d 7 to d 14 when plants grown in +P or –P media are compared (Table 3.11). No significant difference was observed at the specific times assayed in APase activity for each genotype when total soluble APase activity is compared for plants grown in +P or –P media (Table 3.11). However, the only difference in total soluble APase activity was observed in genotypes 45-4 and 47-9 at d 7 in –P media (Table 3.11).

When interactions between G, D and P are compared, the results in Table 3.9 show strong interactions between G x P, D x P, and G x D x P and weak interactions between G x D for cell wall APase activity. For total soluble APase activity, strong interactions were observed between G x D, G x P, D x P, and G x D x P (Table 3.9). The complete analyses of the effects of those interactions on both APase activities can be seen in Appendices 13, 14, 15, and 16, respectively.

The analysis of genotypes and the cell wall APase activity is shown in Figure 3.12A. All genotypes show an increase in cell wall APase activity in the –P media (Figure 3.12A). For genotype 43-8, a significantly higher accumulation of APase activity is observed in d 14 relative to the +P values (Figure 3.12A). The same trend can also be seen for the other genotypes. The figure also shows a rapid decrease in cell wall APase activity at d 21 in –P media for genotype 45-14. Overall, Figure 3.12A shows a positive trend in the induction of APase activity until d 14 in P-deprived media and by d 21, all genotypes showed a decrease in APase activity. The figure also shows a higher phosphatase activity in P-deprived media when compared with P-sufficient media for all treatments given. Relative to the +P treatments, cell wall APase activity in genotypes grown in –P media were significantly different at d 7, reach a peak at d 14 and then decrease to the same level to d 7 at d 21 (Figure 3.12A). For the total soluble APase activity, the highest activity can be observed in genotype 47-9 at d 7 and d 14 and this is significantly different from plants grown in +P media (Figure 3.12B). For all genotypes, the difference in the total soluble APase activity was significant at d 14, and then the activity rapidly decreases at d 21 (Figure 3.12B).

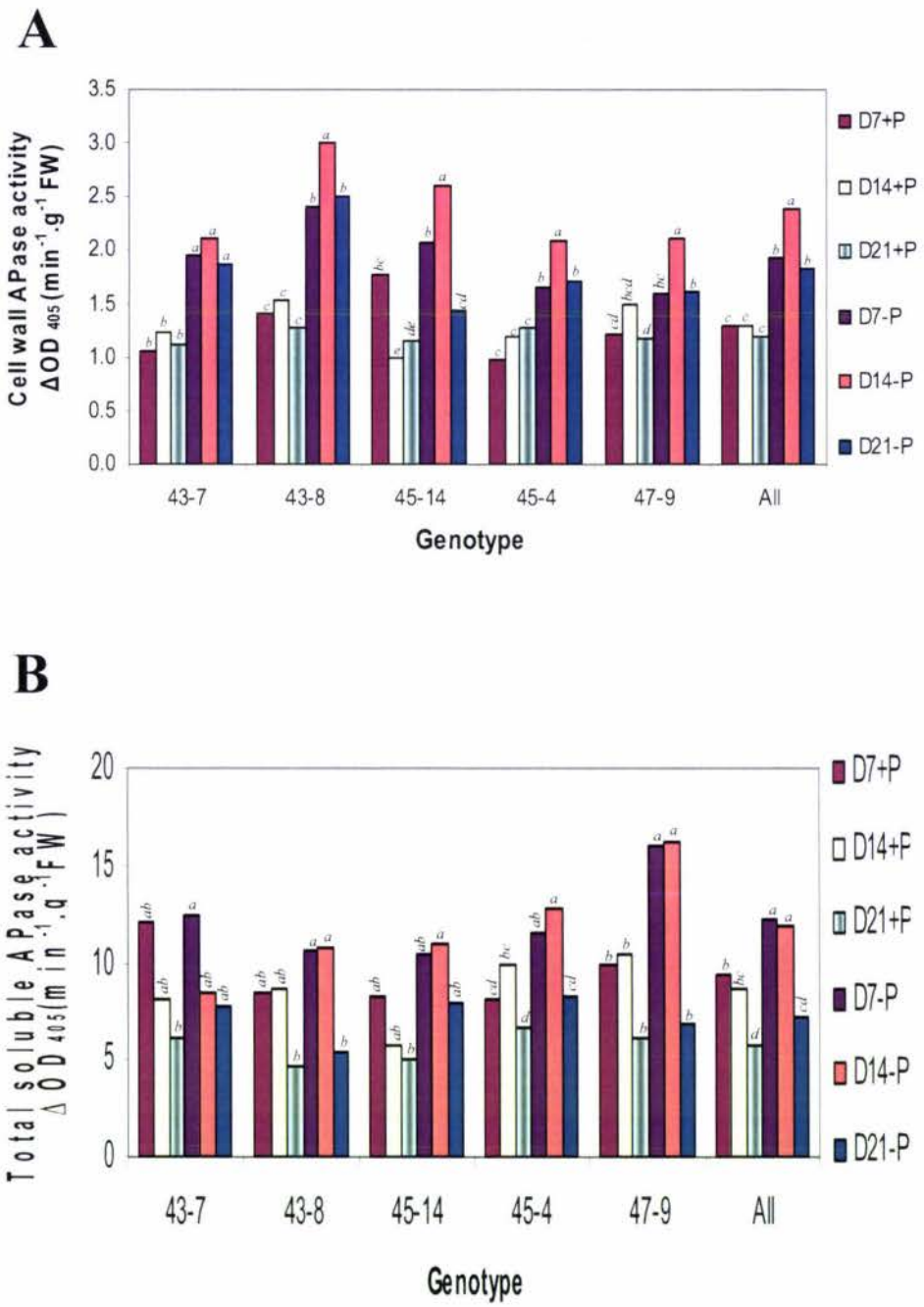


Figure 3.12. The effect of genotypes on cell wall and total soluble APase activity in the roots from selected genotypes grown in P+ or P– media and sampled at day 7, 14, and 21. Values are the means from individual root extracts excised from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

The effect of treatment on the cell wall APase activity is shown in Figure 3.13A. In this experiment, there was no significant difference in APase activity in plants grown in P-contained and P-deprived media, except at d 7 in P-sufficient media, where genotype 45-14 has a significantly higher activity than genotypes 43-7 and 45-4 (Figure 3.13A). Overall for all treatments given, genotype 43-8 had a higher APase activity and was significantly different from genotypes 43-7, 45-4 and 47-9, but not different from genotype 45-14 (Figure 3.13A; Table 3.10). There was no significant difference in the cell wall APase activity of plants grown in P-deprived media for all genotypes tested (Figure 3.13A). Although not statistically different with other genotypes, genotype 43-8 had a higher cell wall APase activity that started at d 7 in P-deprived media, had a peak at d 14 and then decreased again at d 21 (Figure 3.13A). Compared to other genotypes, genotypes 45-4 and 47-9 showed the lowest APase activity in P-deprived media, and a decrease in APase activity was observed in all genotypes at d 21 in P-deprived media (Figure 3.13A). A significant difference in total soluble APase activity can be observed at d 14 in –P media with genotype 47-9 having the highest total APase activity and was significantly different from genotype 43-7, but is not different from genotypes 43-8, 45-14, and 45-4 (Figure 3.13B).

3.3.3. Relative plant growth in response to Pi deprivation

Seven parameters of plant growth in response to Pi deprivation were measured at 7 d, 14 d. and then 21 d for five selected genotypes grown in P-containing and P-deprived media. The results showed that these genotypes varied in leaf area, weight of an individual leaf and shoot DW (Table 3.12). It was observed that genotype 45-14 has the largest leaf area, the heaviest weight of an individual leaf and the highest shoot DW and is significantly different with other genotypes (Table 3.13). Genotypes 43-7 and 43-8 had the smallest leaf area, the lowest weight of an individual leaf and the lowest shoot DW (Table 3.13). Genotypes 43-7 and 43-8 had the lowest leaf area, weight of an individual leaf and shoot DW, while BL 45-14 had the highest leaf area, weight of an individual leaf and shoot DW (Table 3.13). Relative to genotype 47-9, genotype 45-4 had a higher leaf area, a higher weight of an individual leaf, but was not different in shoot DW when compared with genotype 47-9 (Table 3.13). The longer the days of

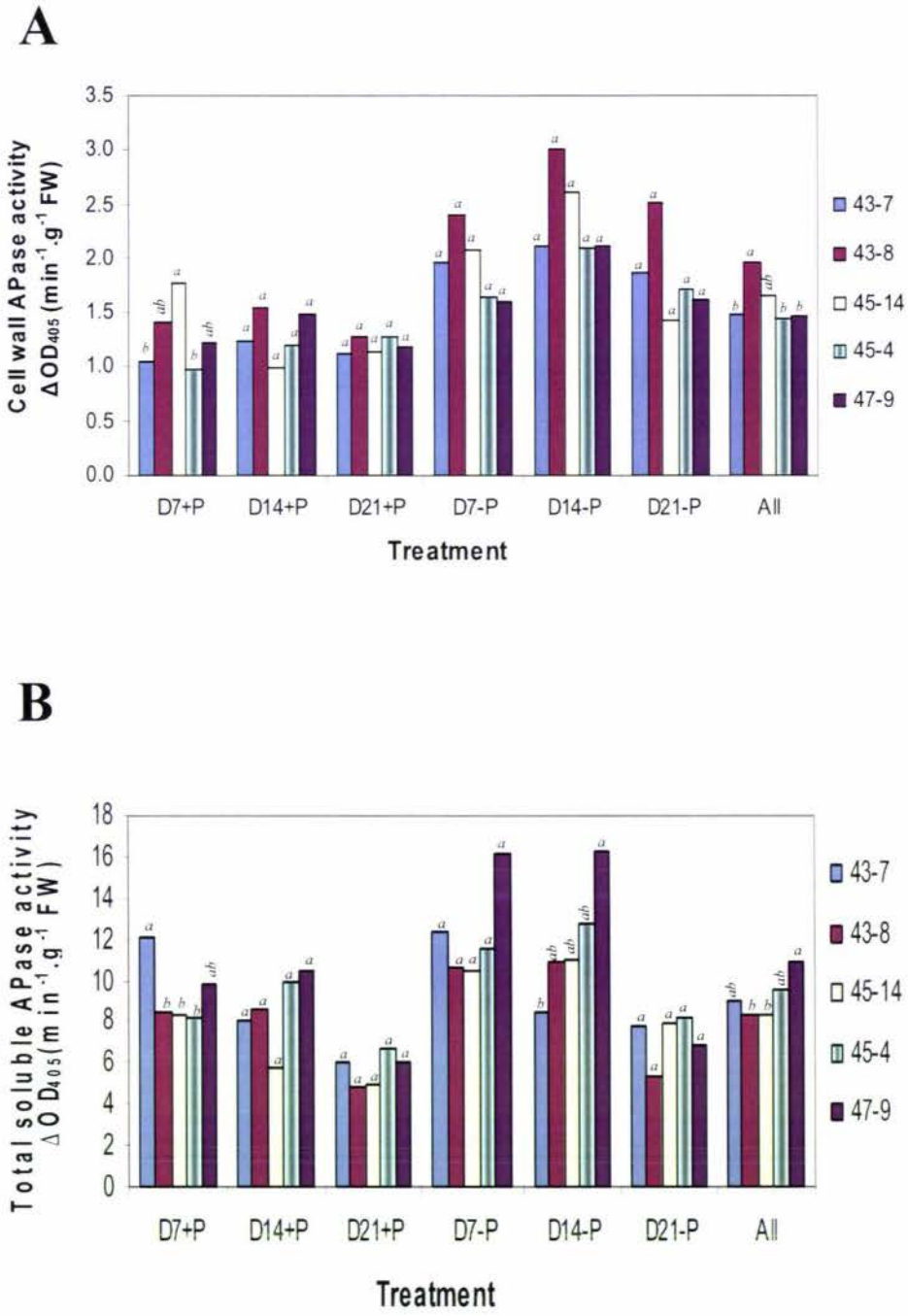


Figure 3.13. The effect of treatments on cell wall and total soluble APase activity in the roots from selected genotypes grown in P+ or P– media and sampled at day 7, 14, and 21. Values are the means from individual root extracts excised from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Table 3.12. Probability of F for several growth parameters of selected genotypes of white clover.

Source of Variation	df	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)	Fresh biomass yield (g/plant)			R:S FW ratio
					Root	Shoot	Biomass	
Genotype (G)	4	***	*	***	***	***	***	***
Days of Treatment (D)	2	NS	NS	*	*	*	*	NS
Phosphorus (P)	1	NS	NS	NS	NS	NS	NS	NS
G x D	14	***	***	***	***	***	***	***
G x P	9	***	***	***	***	***	***	***
D x P	5	NS	NS	**	*	*	*	*
G x D x P	29	***	***	***	***	***	***	***

*, **, *** significance at the 0.05, 0.01 and 0,001 probability level, respectively.
NS = not significant at $p = 0.05$

Table 3.13. The effect of genotypes, days of treatment and phosphorus application to leaf area, weight of an individual leaf and shoot DW in selected genotypes of white clover. Plants were maintained in either P-containing (+P) or P-deprived (-P). Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at 0.05 level according to Duncan's multiple range test.

Source of variation	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)
Genotype (G)			
43-7	138.33^{d}	0.05256^{d}	0.1974^{c}
43-8	160.50^{cd}	0.06050^{d}	0.5222^{bc}
45-14	461.33^{a}	0.19289^{a}	1.8656^{a}
45-4	409.61^{b}	0.13011^{b}	0.8037^{b}
47-9	199.94^{c}	0.08506^{c}	0.8856^{b}
Days of Treatment (D)			
D7	265.97^{a}	0.09207^{a}	0.5296^{b}
D14	269.80^{a}	0.10060^{a}	0.7759^{b}
D21	286.07^{a}	0.12000^{a}	1.2591^{a}
Phosphorus (P)			
0 mM P	277.60^{a}	0.10700^{a}	0.9666^{a}
0.5 mM P	270.29^{a}	0.10144^{a}	0.7432^{a}

treatment, the higher shoot DW (Table 3.13). When the effect of P treatment on leaf area, weight of an individual leaf and shoot DW at the specific times indicated are compared, no significant difference on leaf area and the weight of an individual leaf observed in plants grown in +P or –P media are observed except for genotype 43-7 at d 14 for leaf area and genotype 47-9 at d 14 for shoot DW (Table 3.14).

When the interaction between G, D and P is compared, the results in Table 3.12 show strong interactions between G x D, G x P, G x D x P on leaf area, weight of an individual leaf and shoot DW (Table 3.12). No effect of interaction between D x P on leaf area and weight of an individual leaf (Table 3.12). The complete statistical analyses of these interactions can be seen in Appendices 17, 18, 19, and 20.

No significant difference in leaf area for all genotypes grown in +P or –P media, except for genotype 45-14 (Figure 3.14A). Figure 3.15A showed that genotypes 43-7, 43-8, and 45-14 have no significant different in weight of an individual leaf for plants maintained in +P or –P media (Figure 3.15A). Further, in Figure 3.15B, relative to other genotypes, genotype 45-14 had no difference in weight of an individual leaf for plants grown in +P or –P media. Although not significantly different with plants grown in P-sufficient media, genotype 45-14 had the higher leaf area in P-deprived media (Figure 3.14A). Genotypes 45-14 and 45-4 have a large leaf area when compared with other genotypes (Figure 3.14B). Overall there was no effect of P treatment on the weight of an individual leaf for each genotype tested (Figure 3.15A). For the weight of an individual leaf, genotype 45-14 had the heaviest leaves when compared with the other genotypes (Figure 3.15B). The results also shown that although genotypes 43-7 and 43-8 had the same leaf area and weight of an individual leaf, genotypes 43-8 produced more shoot DW relative to genotype 43-7 (Figures 3.14A, 3.15A, and 3.16A). Relative to genotype 47-9, genotype 45-4 had a higher leaf area, a higher weight of an individual leaf but was not different in shoot DW (Figures 3.14B, 3.15B, and 3.16B). From this table, based on leaf area and the leaf weight, genotype 45-14 can be categorized as a line with a large size leaf, genotype 45-4 as medium size leaf, genotype 47-9 as small medium size leaf, while genotypes 43-7 and 43-8 are of smaller leaf size.

Genotypes and days of treatment have a significant effect on root, shoot, biomass and R:S ratio, but days of treatment has no effect on R:S ratio (Table 3.12). It is also observed that P treatment alone had no significant effect on all of the growth parameters measured (Table 3.12). Genotypes 43-7 had the lowest root FW, shoot FW, total FW and had a higher R:S FW ratio, while genotype 45-14 had the highest root FW,

Table 3.14. Comparison of leaf area, weight of an individual leaf and shoot DW from each genotype grown in +P and –P at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf and shoot DW were determined. Values are the means from individual leaf extracts excised from three independent plants. The different letters above in the same row indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Geno-type	DOT	Leaf area (mm^2)		Weight of an individual leaf (g)		Shoot DW (g)	
		+P	–P	+P	–P	+P	–P
43-7	7	118.67^a	110.67^a	0.043667^a	0.040667^a	0.1050^a	0.1690^a
	14	120.33^b	150.33^a	0.044333^a	0.066667^a	0.2127^a	0.2180^a
	21	156.67^a	173.33^a	0.060667^a	0.059333^a	0.1497^b	0.3303^a
43-8	7	151.33^a	171.33^a	0.058667^a	0.051000^a	0.2890^a	0.4183^a
	14	157.67^a	164.67^a	0.053333^a	0.067333^a	0.5813^a	0.3237^a
	21	181.33^a	136.67^a	0.065333^a	0.067333^a	0.8943^a	0.6263^a
45-14	7	463.00^a	492.33^a	0.188333^a	0.166667^a	0.9277^a	1.3080^a
	14	438.00^a	548.33^a	0.186000^a	0.214333^a	1.5417^a	2.6437^a
	21	463.33^a	363.00^a	0.233667^a	0.168333^a	2.2520^a	2.5207^a
45-4	7	393.33^a	381.00^a	0.080333^a	0.127333^a	0.3957^a	0.5523^a
	14	317.33^a	427.33^a	0.092333^a	0.137667^a	0.5790^a	0.8870^a
	21	484.33^a	454.33^a	0.186000^a	0.157000^a	1.2800^a	1.1280^a
47-9	7	179.00^a	199.00^a	0.073667^a	0.090333^a	0.2863^a	0.8450^a
	14	179.00^a	195.00^a	0.063333^a	0.080667^a	0.2367^b	0.5357^a
	21	251.00^a	196.67^a	0.092000^a	0.110333^a	1.4167^a	1.9933^a

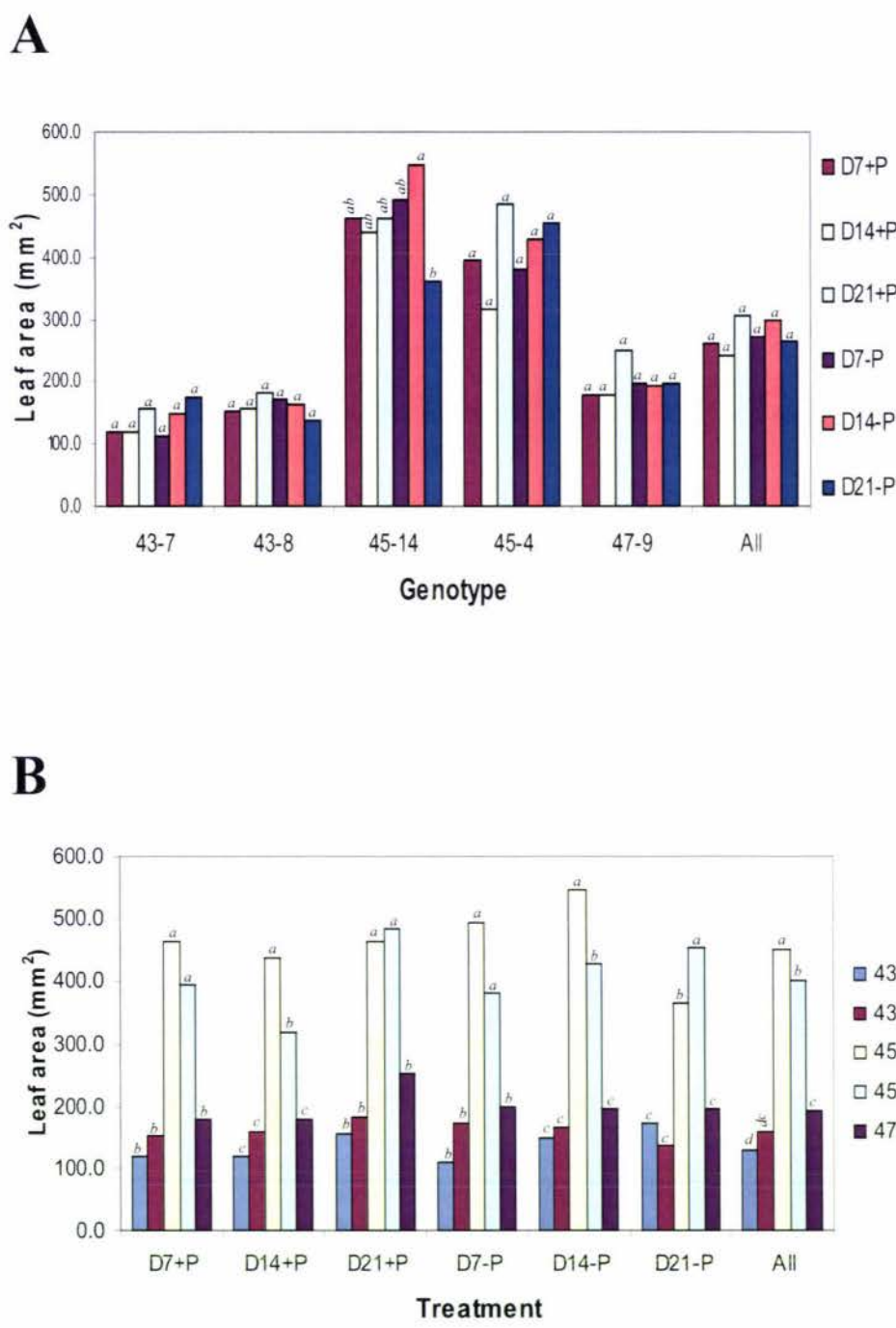


Figure 3.14. Leaf area of selected genotypes grown in P+ or P– media and sampled at day 7, 14, and 21. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

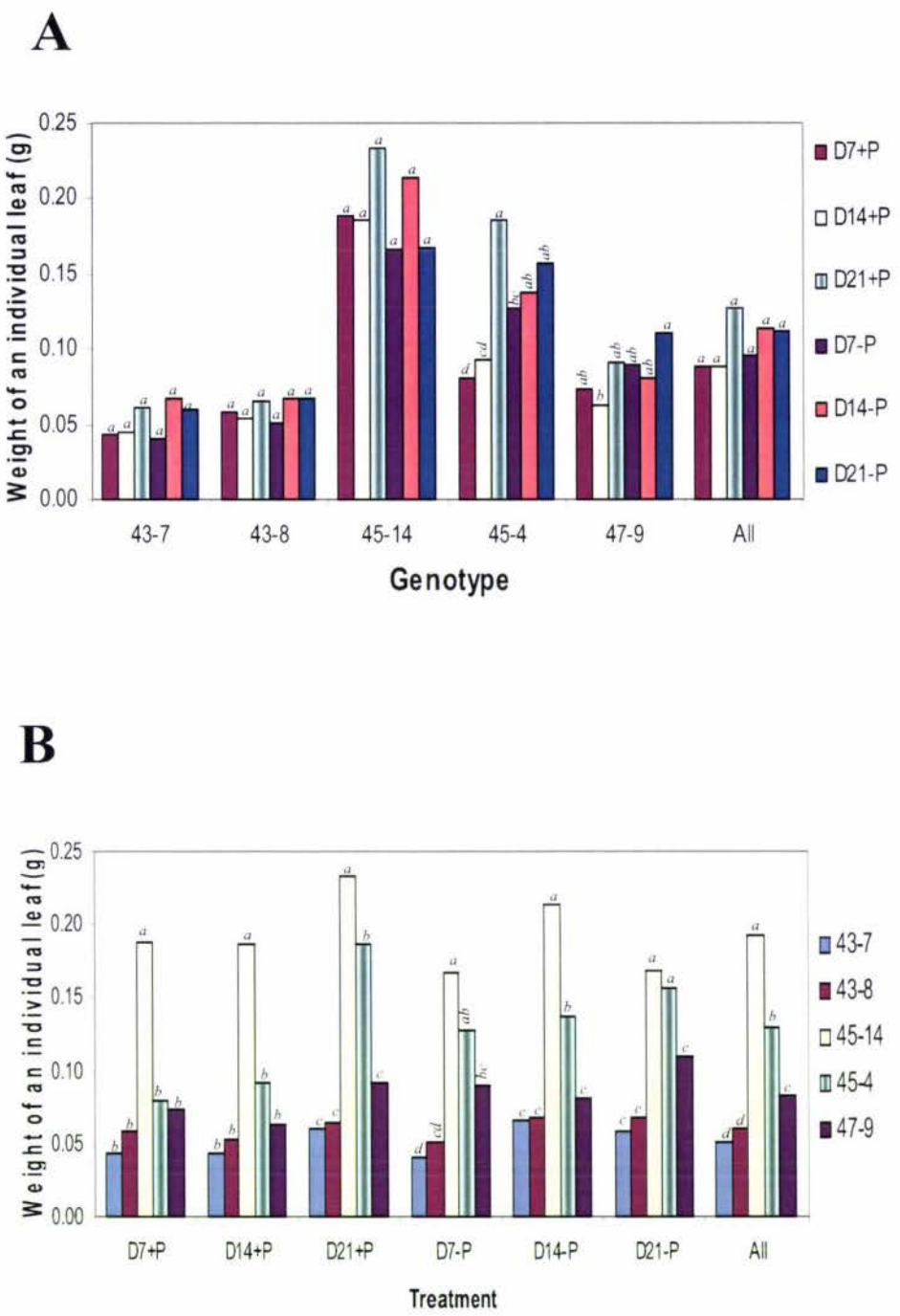


Figure 3.15. Weight of an individual leaf of selected genotypes of white clover grown in either P-containing (P+) or P-deprived (P–) media and sampled at day 7, 14, and 21. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

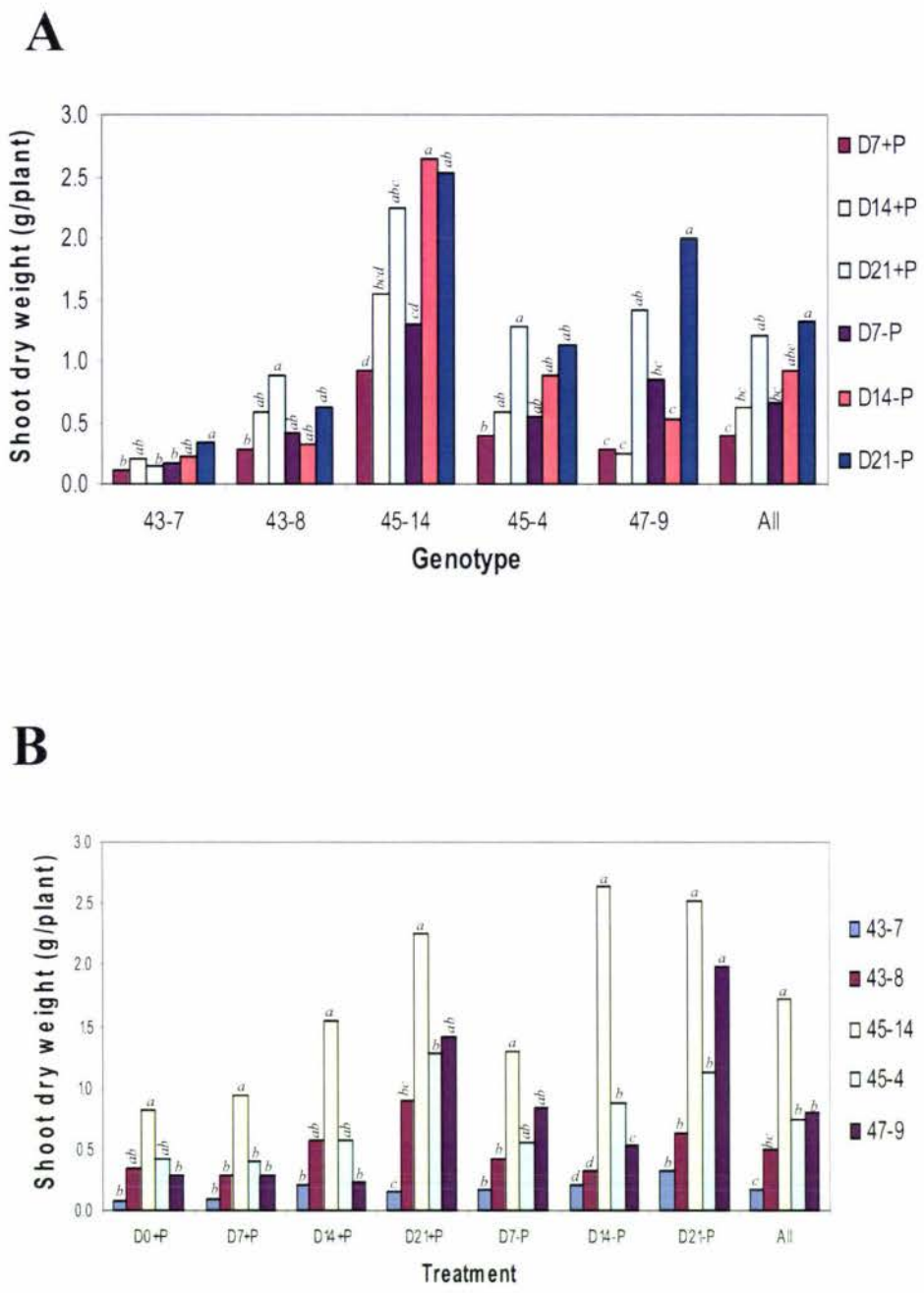


Figure 3.16. Shoot dry weight determinations of selected genotypes of white clover grown in either P-containing (P+) or P-deprived (P–) media and sampled at day 7, 14, and 21. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

shoot FW, total biomass FW, but had a lower R:S FW ratio (Table 3.15). Genotypes 43-8, 45-4 and 47-9 had a medium root FW, shoot FW, total biomass FW but only genotype 47-9 had a lower R:S FW ratio (Table 3.15). When the effect of P treatment on each genotype at the specific time indicated is compared, no significant difference was found for each genotype grown in +P and –P except for root FW in genotype 43-7 at d 21, R:S FW ratio for genotype 47-9 at d 14, and genotypes 45-14 and 45-4 at d 21 (Table 3.16).

When the interactions between genotype, D and P are compared, the results in Table 3.12 show strong interactions between G x D, G x P, G x D x P and weak interactions between D x P for root FW, shoot FW, biomass FW and R:S FW ratio (Table 3.12). The complete statistical analyses of these interactions can be seen in Appendices 21, 22, 23, and 24.

The highest accumulation of root fresh weight in genotype 45-14 was observed at d 14 in P-deprived media, and is significantly different from plants grown in P-sufficient media at d 7 and d 14 (Figure 3.17A). In genotypes 45-4 and 47-9, there was no significant difference in root mass accumulation for plants maintained in +P or –P media (Figure 3.17A). The total root fresh weight was also compared in five genotypes (Figure 3.17B). At d 7, 14 and 21 in either P-containing or P-deprived media, genotype 45-14 has the highest accumulation of root fresh weight, while genotype 43-7 has the lowest accumulation of root fresh weight (Figure 3.17B). The effect of treatments on shoot fresh weight for all genotypes showed that the highest production of shoot fresh weight grown in +P and –P media was observed at d 21, and the lowest at d 7 in P-containing media (Figure 3.18A). No difference in total biomass accumulation at d 21 in P-sufficient or P-deficient media was observed for genotype 45-14 (Figure 3.19A). Genotype 45-14 produced the highest total biomass compared to other genotypes (Figure 3.19B). Compared to genotype 43-7, genotype 45-14 produced 10 x more total biomass, while genotypes 43-8, 45-4 and 47-9 produced only a moderate increase in total biomass (Figure 3.19B). Genotypes 45-14 and 47-9 have a lower root:shoot ratio compared to the other three genotypes (Figure 3.20A). At 21 days in P-deprived media, all genotypes have a higher root:shoot ratio compared to plants grown in P-containing media at the same period (Figure 3.20B). Overall, genotypes 43-8, 45-4, and 47-9 had no difference in root FW, shoot FW, biomass FW, but only genotype 47-9 had a lower R:S FW ratio (Figures 3.17B, 3.18B, 3.19B, and 3.20B). Further, genotype 45-14 had the highest root FW, shoot FW, biomass FW but had a lower R:S ratio, while

Table 3.15. The effect of genotypes, days of treatment and phosphorus application on fresh biomass yield and root:shoot FW ratio of selected genotypes of white clover. Plants were maintained in either P-containing (+P) or P-deprived (-P) media. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at 0.05 level according to Duncan's multiple range test.

Source of Variation	Fresh biomass yield (g/plant)			Root:Shoot FW ratio
	Root	Shoot	Total	
Genotype (G)				
43-7	0.5317^{c}	1.0577^{c}	1.5894^{c}	0.4912^{a}
43-8	1.4443^{b}	3.2602^{b}	4.7045^{b}	0.4758^{a}
45-14	2.8426^{a}	9.4245^{a}	12.2671^{a}	0.3161^{b}
45-4	1.6964^{b}	4.0326^{b}	5.7290^{b}	0.4305^{a}
47-9	1.2040^{b}	4.0363^{b}	5.2403^{b}	0.3239^{b}
Days of Treatment (D)				
D7	1.1966^{b}	3.0273^{b}	4.2239^{b}	0.4200^{a}
D14	1.4548^{b}	4.0283^{b}	5.4832^{b}	0.4242^{a}
D21	1.9799^{a}	6.0311^{a}	8.0111^{a}	0.3783^{a}
Phosphorus (P)				
0 mM P	1.7096^{a}	4.5760^{a}	6.2857^{a}	0.4178^{a}
0.5 mM P	1.3779^{a}	4.1485^{a}	5.5264^{a}	0.3972^{a}

Table 3.16. Comparison of fresh biomass yield and R:S ratio FW from each genotype grown in +P and –P at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and R:S ratio FW were determined. Values are the means from individual leaf extracts excised from three independent plants. The different letters above in the same row indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Geno-type	DOT	Fresh biomass yield (g/plant)						Root:Shoot FW ratio	
		Shoot		Root		Total			
		+P	–P	+P	–P	+P	–P	+P	–P
43-7	7	0.5353[a]	0.9503[a]	0.2790[a]	0.3957[a]	0.8143[a]	1.3460[a]	0.5119[a]	0.3955[a]
	14	1.1437[a]	1.0833[a]	0.6140[a]	0.5657[a]	1.7577[a]	1.6490[a]	0.5290[a]	0.5235[a]
	21	0.9790[a]	1.6543[a]	0.4367[b]	0.8993[a]	1.4157[a]	2.5537[a]	0.4474[a]	0.5398[a]
43-8	7	1.9107[a]	2.7713[a]	1.0367[a]	1.3020[a]	2.9473[a]	4.0733[a]	0.5454[a]	0.4888[a]
	14	3.8937[a]	2.1170[a]	1.6743[a]	0.9690[a]	5.5680[a]	3.0860[a]	0.4459[a]	0.4549[a]
	21	5.6130[a]	3.2557[a]	2.0880[a]	1.5957[a]	7.7010[a]	4.8513[a]	0.3976[a]	0.5221[a]
45-14	7	5.8377[a]	7.3477[a]	2.1360[a]	2.4090[a]	7.9737[a]	9.7567[a]	0.3676[a]	0.3464[a]
	14	8.2953[a]	12.8120[a]	2.2930[a]	3.7933[a]	10.5883[a]	16.6053[a]	0.3010[a]	0.2974[a]
	21	11.8183[a]	10.4360[a]	2.6007[a]	3.8233[a]	14.4190[a]	14.2593[a]	0.2186[b]	0.3658[a]
45-4	7	2.2107[a]	2.9337[a]	0.7907[a]	1.4683[a]	3.0014[a]	4.4020[a]	0.3310[a]	0.4816[a]
	14	2.8967[a]	4.3283[a]	1.4213[a]	2.0050[a]	4.3180[a]	6.3333[a]	0.5251[a]	0.4682[a]
	21	6.9063[a]	4.9200[a]	2.3223[a]	2.1707[a]	9.2287[a]	7.0907[a]	0.3408[b]	0.4363[a]
47-9	7	1.5087[a]	4.2670[a]	0.5617[a]	1.5870[a]	2.0703[a]	5.8540[a]	0.3417[a]	0.3899[a]
	14	1.3947[a]	2.3187[a]	0.5737[a]	0.6390[a]	1.9683[a]	2.9577[a]	0.4220[a]	0.2749[b]
	21	7.2833[a]	7.4453[a]	1.8410[a]	2.0217[a]	9.1243[a]	9.4670[a]	0.2326[a]	0.2824[a]

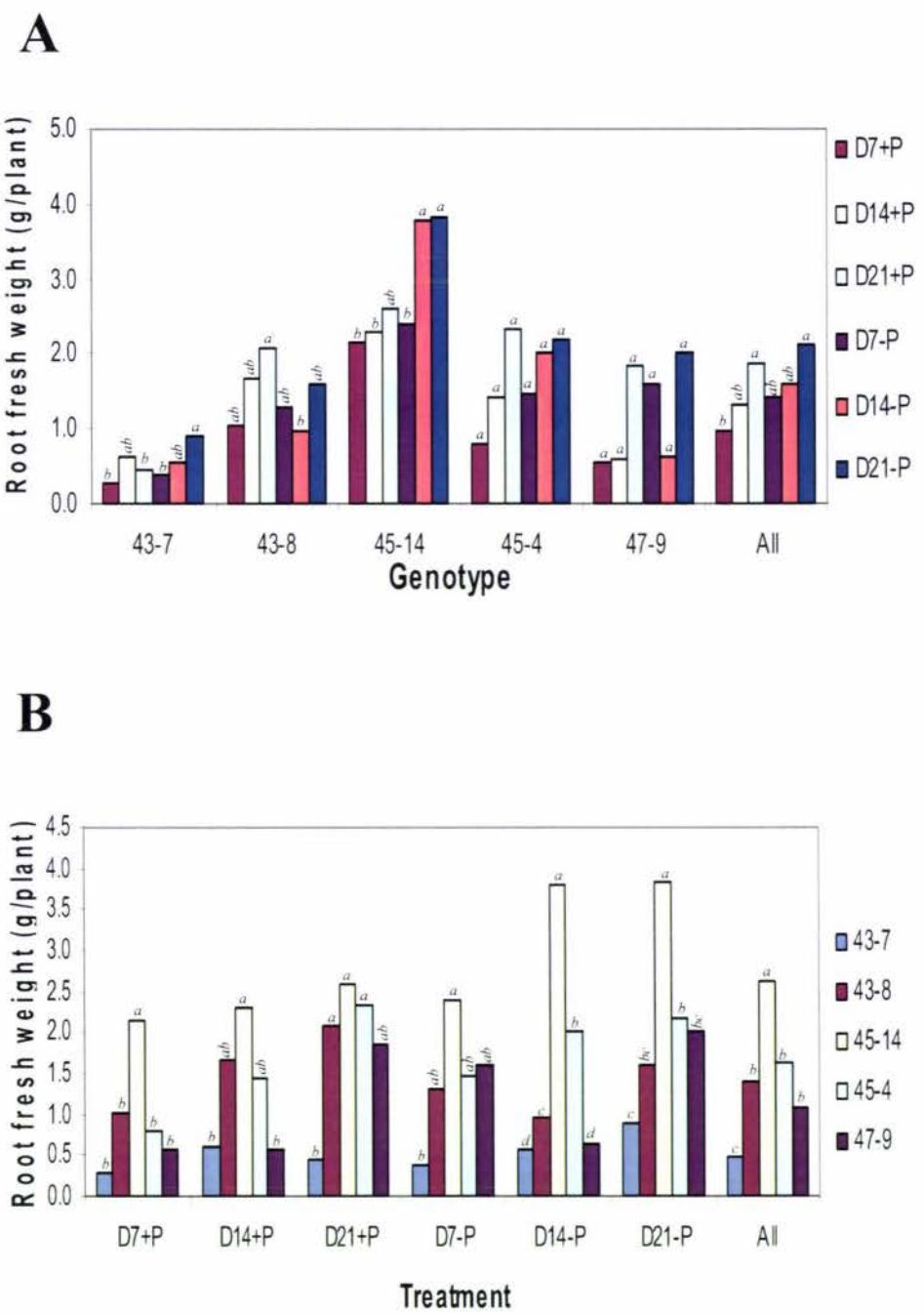


Figure 3.17. Root fresh weight from selected genotypes grown in P+ or P– media and sampled at day 7, 14, and 21. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

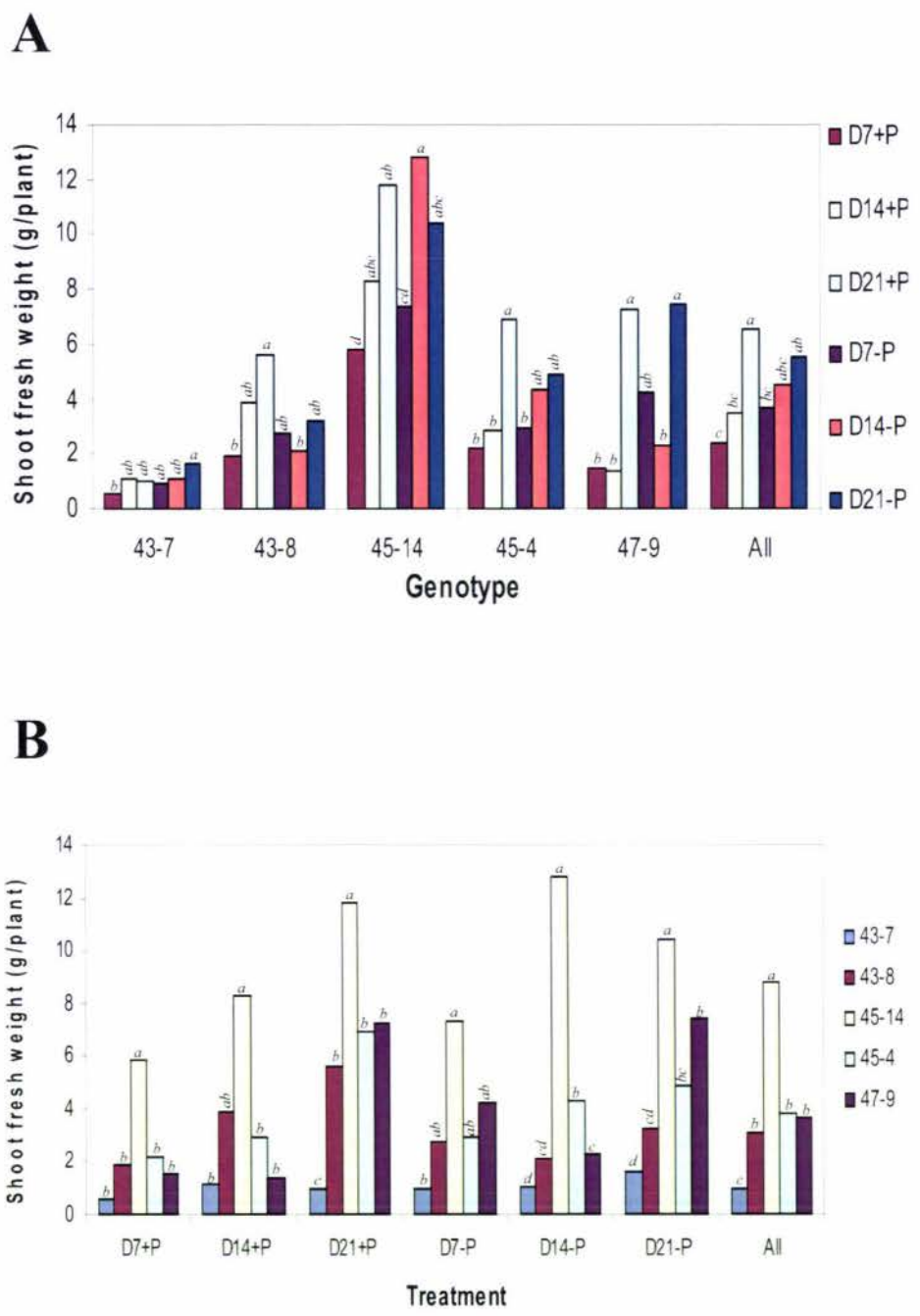
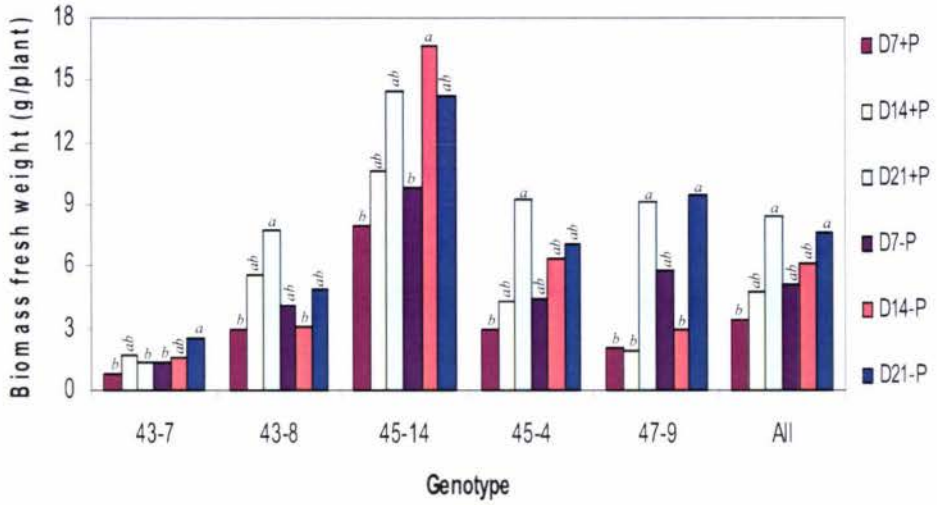


Figure 3.18. Shoot fresh weight from selected genotypes grown in P+ or P– media and sampled at day 7, 14, and 21. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

A



B

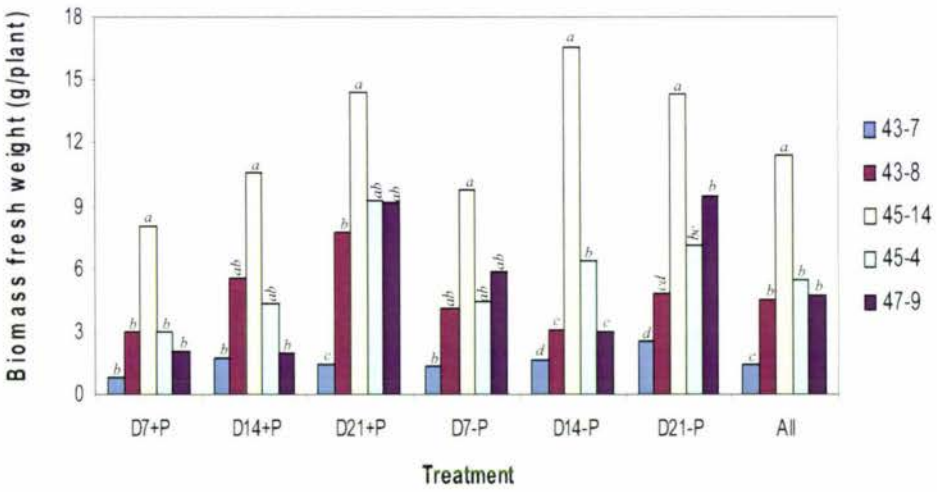


Figure 3.19. Biomass fresh weight determinations of selected genotypes of white clover grown in either P-containing (P+) or P-deprived (P–) and sampled at day 7, 14, and 21. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

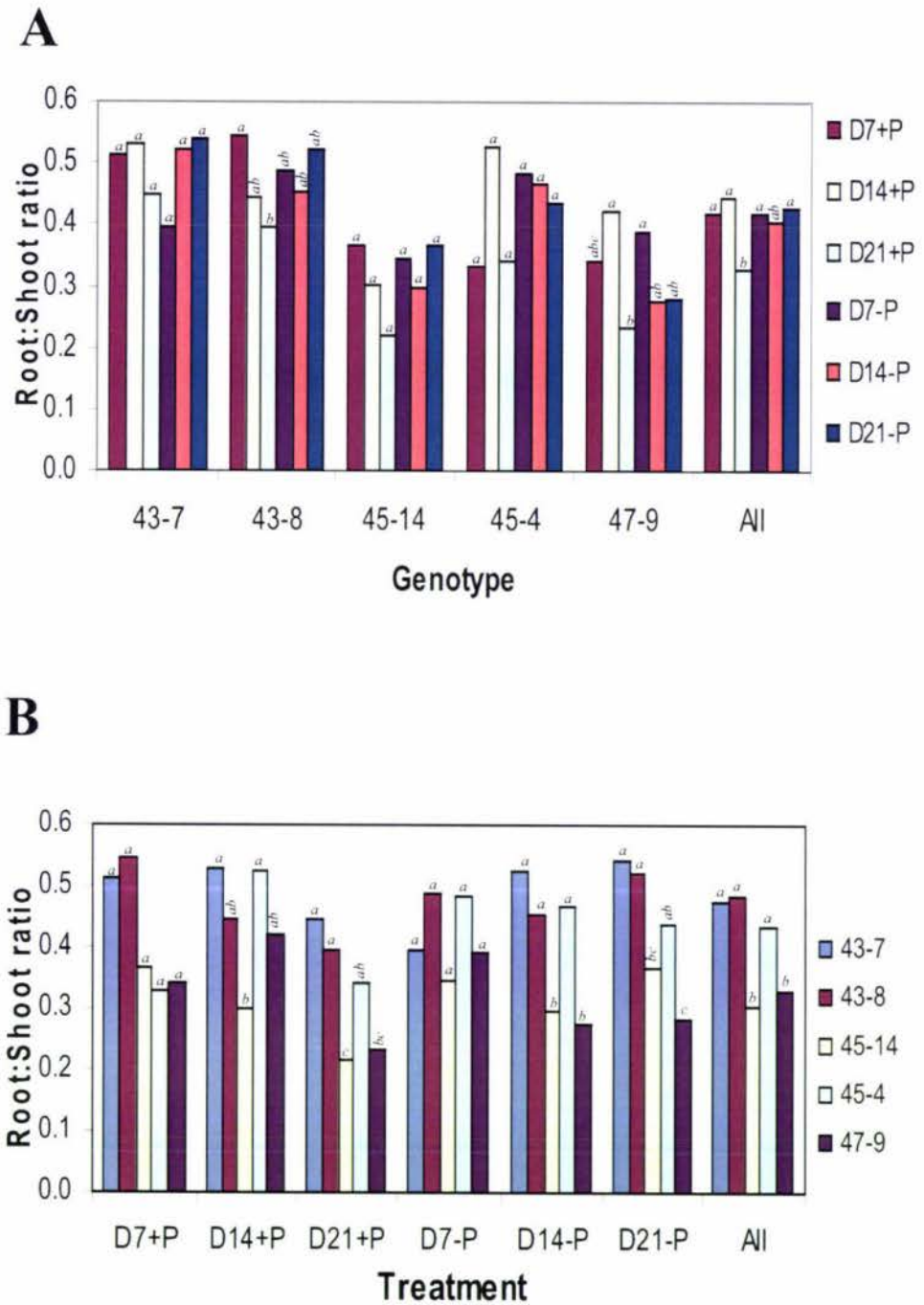


Figure 3.20. Root:Shoot fresh weight ratio from selected genotypes grown in P+ or P– media and sampled at day 7, 14, and 21. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

genotype 43-7 had the lowest root FW, shoot FW, biomass FW, but had a higher R:S ratio (Figures 3.17B, 3.18B, 3.19B, and 3.20B).

3.3.4. Comparison of acid phosphatase isoenzymes of selected genotypes grown in P-contained and P-deprived media

For the comparison of acid phosphatase isoenzymes, four genotypes were selected. These genotypes were designated 43-8, 45-14, 47-12 and 49-9, and were selected based on their performance in preliminary screenings (see Chapter 2 for a description). The profile of acid phosphatase isoforms in roots of selected genotypes grown in P-containing and P-deprived media was compared. To simplify the comparison of isoforms, root extracts were divided into an ionically-bound cell wall isoform fraction and water-soluble fraction (containing the putatively vacuolar/cytoplasmic isoforms), and resolved in native gels. In the cell wall fraction of all genotypes, the major staining acidic isoform (resolved at pH 8.8) stained as a slow migrating band present both in +P as well as in –P roots, although the activity in roots grown in –P media seem to be higher (Figure 3.21A). As well, two different isoforms appeared in roots of phosphate-deprived plants, which are not present as active forms in roots from plant grown in phosphate sufficient conditions (Figure 3.21A). Also two smaller bands can only be observed at d 7 in deprived root tissues, while in P-sufficient root extracts, only one major staining band can be observed in each the P- genotype tested (Figure 3.21A). In the cell wall extracts, clear evidence of acidic isoenzyme enhancement in the P-deprived root of all genotypes observed. For the basic isoforms (resolved at pH 4.3), only one major staining band is evident for each genotype examined in the roots grown in +P or –P media (Figure 3.21B). The only exception was for genotype 45-14, where a lightly staining higher mobility band is discernable at d 7 in –P media (Figure 3.21B).

Analysis of acidic isoenzymes (separated at pH 8.8) in the root soluble fraction revealed only one major staining band that is observed in each genotype tested (Figure 3.21C). However, for genotype 45-14, there is one large band (lower mobility) that can be seen at d 0 in P-maintained root tissue (Figure 3.21C). For the soluble fraction, only one major staining basic isoform (resolved at pH 4.3) was observed at d 0 (P-maintained) and at d 7 (P-deprived) root tissues for each genotype examined (Figure 3.21D).

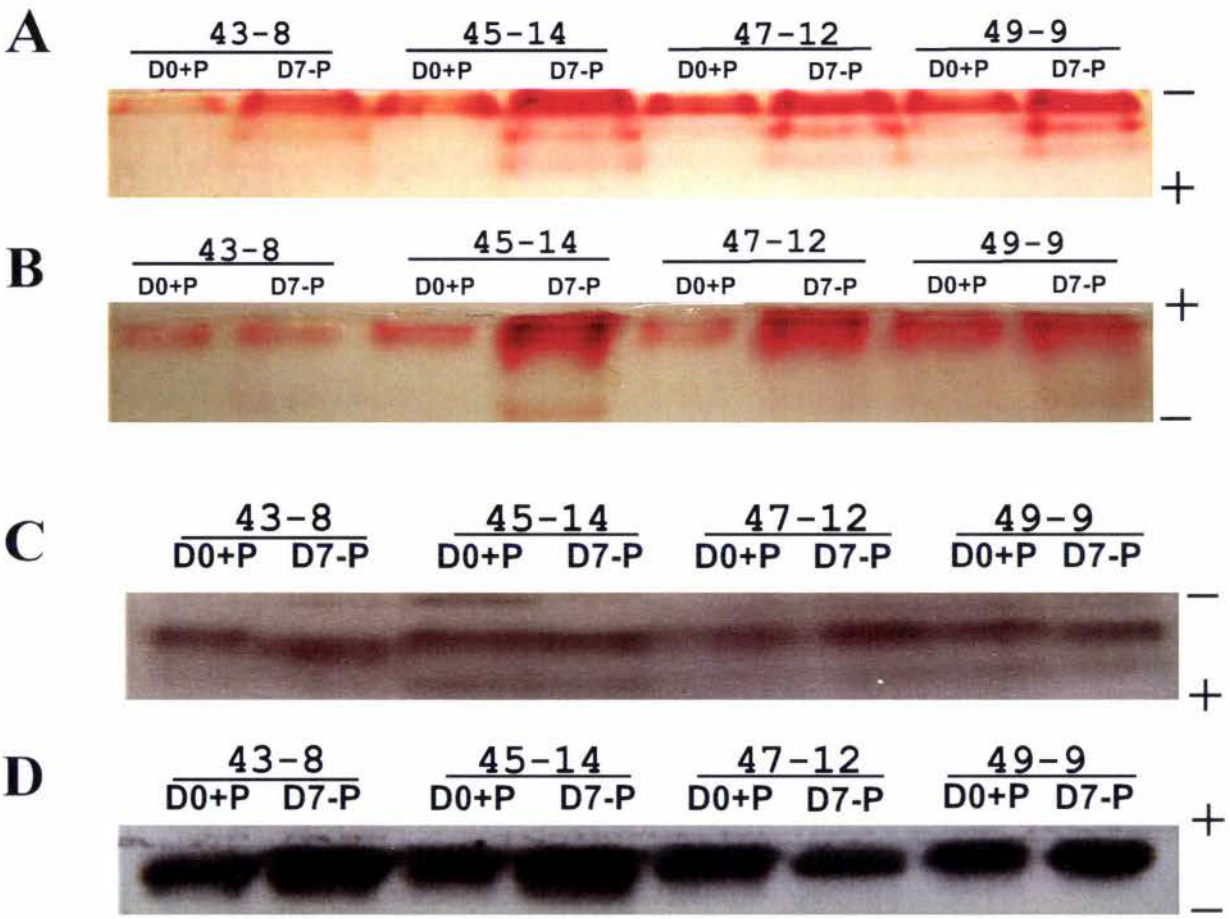


Figure 3.21. Separation of acid phosphatase isoenzymes. Roots tissue from plants maintained in either P-containing (+P) or P-deprived (-P) media were excised at 0 day and at 7 d and ionically-bound (1 M salt-extractable) cell wall proteins and water soluble whole tissue proteins were separated through 10% polyacrylamide gels at pH 8.8 to reveal acidic isoenzymes and at pH 4.3 to reveal basic isoenzymes. Isoenzymes were visualised using β-napthyl acid phosphate and Fast Garnet.

A. Ionically bound (1M salt-extractable) cell wall protein high pH discontinuous native gel
B. Ionically bound (1M salt-extractable) cell wall protein low pH discontinuous native gel
C. Water soluble whole tissue protein high pH discontinuous native gel
D. Water soluble whole tissue protein low pH discontinuous native gel

Chapter Four: Discussion

4.1. Phosphorus (Pi) levels in leaves and cell wall APase activity in roots of white clover during Pi deprivation

Phosphorus efficiency consists of acquisition efficiency (PAE), described as the ability to obtain phosphorus from the environment, and phosphorus use efficiency (PUE), defined as the ability to convert phosphorus into biomass or yield once it is obtained (Gerloff and Gabelman, 1983). PAE may be attained through efficient root morphology and root architecture (Gardner et al. 1981; Barber, 1995; Lynch, 1995) and specific phosphorus mobilizing root exudates (Marschner et al. 1987; Ae et al. 1990; Hoffland et al. 1992), while mechanisms for enhanced PUE include reduced tissue phosphorus requirement (Fawole et al. 1982; Halsted and Lynch, 1996) and efficient phosphorus remobilization from senescent or non-productive tissues to growing or productive tissues (Smith et al. 1990; Snapp and Lynch, 1996). In this thesis, four breeding lines and five selected genotypes will be characterized in terms of Pi level in leaf, root acid phosphatases, isoenzymes analyses, and relative plant growth based on their PAE and PUE in response to P deprivation.

4.1.1. Pi levels in leaves

The first part of this study was to examine the effects of P-deprivation in leaf tissue of white clover. This involved measuring the changes in Pi content in leaf tissue of white clover grown in P-sufficient and P-deficient media. A significant reduction in Pi content in the leaf has been observed in many plant species, including white clover as a result of absence or limited supply of applied P (Hay et al. 1998; and Hunter and McManus, 1999; Mollier and Pellerin, 1999; Zhang and Mc Manus, 2000; Høgh-Jensen et al. 2002). Mollier and Pellerin (1999) examined changes in P content in leaves of maize supplied with either no P (0 mM) or high P (1mM). After d 16 in P-deficient media, the leaves of maize supplied with low P contained considerably less P, with a 4.9-lower content when compared with maize grown in P-sufficient media. The researches also reported that P content was significantly lower with a 4.4-fold lower content in roots of maize after 16 d in –P solution when compared with control plants (supplied with 1mM). Another study by Hunter et al. (1999) showed that the

accumulation of P in the leaves of P-deprived white clover was 30-fold lower by d 29, relative to the plants grown in P-sufficient media.

In the experiments presented here, however, soluble P was supplied regularly and evenly to the whole root system; therefore, breeding line or genotypic differences in P uptake and utilization rather than acquisition efficiencies were assessed. This is because all BLs and genotypes have the same opportunity to obtain soluble P from the growth media, and so BLs or genotypes which can produce a higher yield have a higher ability to take up P and convert it to biomass. The study of P acquisition efficiency has been described elsewhere (Lynch et al. 1997; Ge et al. 2000; Narang et al. 2000; Nielsen et al. 2001; Liao et al. 2004). For breeding line screening, the P levels measured in the fully expanded leaves of white clover plants declined and reached consistent minimum values of 0.007% g^{-1} fresh weight for BLs 43, 45, and 47, and 0.008% g^{-1} FW for BL 49 by the end of the 14-d period maintained in P-deprived media. These values are 64, 77, 47 and 80% of the control for breeding lines 43, 45, 47 and 49, respectively. For the genotype-based screening, the Pi content decreased and reached consistent minimum values of 0.0105, 0.0106, 0.008, 0.009 and 0.009% g^{-1} fresh weight of genotypes 43-7, 43-8, 45-14, 45-4 and 47-9, respectively by the end of 21-d period maintained in P-deficient media. These values are 52, 64, 64, 55 and 58% of the control (supplied with 5 mM P) for genotypes 43-7, 43-8, 45-14, 45-4 and 47-9 respectively. Since by d 21 in –P media, all genotypes showed a considerably decrease in the –P/+P ratio, the –P/+P ratio at d 14 was used instead for easy comparison of the genotypic screening results. Relative to the control, at d 14, the –P/+P Pi level in leaves declined and reached the values of 57%, 49%, 80%, 82% and 60% for genotypes 43-7, 43-8, 45-14, 45-4, and 47-9, respectively. These reductions were most severe in genotypes 43-7 (43%), 43-8 (51%), and 47-9 (40%), while a slighter reduction was observed in genotypes 45-14 (20%) and 45-4 (18%). These results are in agreement with the other studies from several plant species in which phosphorus starvation did result in a decrease of Pi content in leaves (Freeden et al. 1989; Rao and Terry, 1995; Hay et al. 1998; Hunter and McManus 1999; Mollier and Pellerin, 1999; Zhang and McManus 2000; Høgh-Jensen et al. 2002).

The BL screening results showed that the –P/+P Pi ratio reached a maximum at d 5 for BLs 43 and 45 and then decreased thereafter at d 7 and d 14. A rapid decrease in –P/+P Pi ratio at d 14 was observed in BL 47. BL 49 had the highest –P/+P Pi ratio and also it had a constant –P/+P Pi ratio throughout the experimental period. For the

breeding line screening, at d 14 in P-deprived media, the Pi content in BL 49 was only reduced 20% relative to the control, while this reduction was more severe in BL 47 (53%) and a slight reduction was observed in BL 45 with 23% reduction, while a medium reduction was observed in BL 43 with 36% reduction in Pi content relative to the control. Using –P/+P ratio as a criteria, this suggests that BLs 45 and 49 are more efficient at accumulating P in the leaf tissues during Pi deprivation, BL 43 is a medium accumulator, while BL 47 is a poor P accumulator. For the genotype-based screening, the –P/+P Pi ratio in leaves was similar in genotypes 43-7 and 43-8. A similar pattern can also be seen in genotype 45-14 and 45-4. The rapid reduction of the –P/+P Pi ratio started after d 7 in genotypes 43-7 and 43-8. For genotypes 45-14 and 45-4, no difference in the –P/+P Pi ratio was observed at d 7 and d 14. Although genotype 47-9 showed no change in –P/+P Pi ratio throughout the experimental period, it had a lower –P/+P Pi ratio when compared with genotypes 45-14 and 45-4. For the five genotypes of white clover tested, there is some diversity among genotypes of white clover. For example, genotypes 45-14 and 45-4 performed particularly well at both levels of P supply (in terms of an ability to accumulate a high Pi level in the leaves and also an ability to maintain a constant Pi level throughout the experimental period), whereas genotype 43-7 performed poorly (in terms of its poor ability to accumulate Pi level in leaves and a rapid reduction of –P/+P Pi ratio at d 14) at all levels of P supply.

In this study, the degree of the reduction of Pi content in leaves is dependent on BLs and genotypes. Based on the level of Pi content in leaves, these results showed that BLs 45 and 49 have a higher ability to accumulate P in the leaves relative to other BLs (in terms of their ability to maintain a similar –P/+P ratio of Pi content throughout the experimental period). The same observation can also be made for genotypes 45-14 and 45-4. This suggests that during Pi deprivation, BLs 45 and 49, and genotypes 45-14 and 45-4 may mobilize their phosphate from senescent or non-productive tissues to growing or productive tissues. This suggestion is in accordance with the results from Smith et al. (1990) and Snapp and Lynch, (1996).

The next question that was addressed was whether the similar –P/+P ratio of Pi content in leaves observed in BLs 45 and 49, and genotypes 45-14 and 45-4 had a direct link with the APase activity in the roots. To answer this question, this following experiment was performed.

4.1.2. Acid phosphatase activity in root cell walls of white clover

Acid phosphatases (APases) are ubiquitous enzymes that have been studied in many plant species and tissues (Duff et al. 1994). The existence of acid phosphatases in cell walls of plants has been demonstrated in tobacco (Suzuki and Sato, 1976; Kaneko et al. 1983; 1990; 1998; Kaida et al. 2003; Sano et al. 2003), white clover (Hay et al. 1998; Hunter et al. 1999; Zhang and McManus 2000), soybean (Ferte et al. 1993), rice (Igaue et al. 1976), sycamore (Crasnier et al. 1980; Ricard et al. 1981; Crasnier et al. 1985; Crasnier and Giordani, 1995), potato (Sugawara et al. 1981; Tu et al. 1988), and maize (Tu et al. 1988). Numerous reports document the induction of APases in P-stressed plants and the significance of changes in intracellular APases may have a role in phosphate recycling (Smyth and Chevalier, 1984; Duff et al. 1994). Therefore, increased intracellular APases can contribute to enhancing PAE and PUE under P-limited conditions.

In this thesis, acid phosphatases from roots maintained in both P-deprived and P-containing media were extracted as a water-soluble whole tissue extract and an ionically-bound (1M salt-extractable) cell wall extract, and the activity in both fractions determined. This study aimed to compare the activity of the high salt-extractable (ionically-bound) cell wall APase from roots of selected breeding lines and genotypes grown in P-deprived media with those grown in P-sufficient media. In both the cell wall and total soluble APase extracts from roots, the highest activity was observed in tissues excised from plants maintained in P-deprived media. Similar increases in APase activity in P-deficient roots have been observed in Arabidopsis (Trull et al. 1997), rice (Ni et al. 1996), and white clover (Hunter et al. 1999; Zhang and McManus, 2000)

The response of APase to P deficiency can also be expressed as the –P/+P ratio of APase activity. BLs 43 and 47 showed an increase in the –P/+P ratio over the time period indicated, while BL 45 showed a slight decrease in this ratio at d 14. BL 49 showed a rapid increase at d 7 in terms of the –P/+P ratio which then decreased at d 14 to the same level at d 5. No significant difference in the –P/+P ratio for the total soluble APase activity was observed in all BLs tested. Based on the –P/+P cell wall APase ratio, it can be observed that each BL showed a different degree of induction in enzyme activity. BLs 49 had the highest enzyme activity ratio at d 7 (2.5-fold), while BL 47 had its highest enzyme activity ratio at d 14 (2.18-fold). For breeding lines at d 14, the –P/+P ratio of cell wall APase was the highest in BL 47 (2.1), followed by BL 43 (1.96),

BL 45 (1.5), and BL 49 (1.32). These results are in agreement with a steady decrease in –P/+P ratio of Pi content at d 14 in BL 49, followed by BL 45, BL 43 and a rapid reduction in BL 47.

For genotype screening, the results up to d 14 only will be discussed for easy comparison with BL screening since all plants showed symptoms of Pi deficiency and reduction in plant growths at d 21. However, this thesis does show that there are genotypic differences in the responses of APase to Pi deficiency in white clover. There was an increase in both APase activities in roots as a result of P deprivation. At d 14, the –P/+P ratio of cell wall activity in the roots was different in four genotypes at the specific time indicated, but significantly higher in genotype 45-14 (2.62), followed by genotypes 43-8 (1.96), 45-4 (1.73), 43-7 (1.70) and 47-9 (1.41). Also the –P/+P ratio of total soluble APase activity was higher in genotype 45-14 at d 14 (1.93). A statistical difference in cell wall and total soluble APase activity in roots was observed as early as d 5 for BLs 45 and 47 and at d 7 in genotype 45-14 after P deprivation. This time period is late when compared to the responses of APases in other plant species. Yun and Kaeppler (2001) reported increased APase activity in maize roots under P deficiency occurred as early as d 3 in P efficient (Mo17) and P inefficient (B73) cultivars. This suggests that plants sense the reduction in leaf Pi content by increase APase activity in the cell wall. The magnitude of the induction of cell wall APase is dependent on the level of Pi content in the leaves.

The next question that needed to be addressed was whether there is correlation between the highest enzymes activities in roots observed in BLs 47, 49 and genotype 45-14 with the level of Pi content in leaves. A further question was whether the induction of cell wall APase activity occurred before or after the reduction in leaf Pi content during Pi-deprivation. To answer these questions the next experiment was performed.

4.1.3. Effect of Pi deprivation on Pi levels and cell wall APase activity in roots of white clover

To understand whether P treatment induced increases in APase activity in roots by a low level of Pi or Pi deficiency, the effect of P treatment on the level of APase activities was determined at different time intervals. The results showed that by comparison with the control, the Pi level of shoots was strongly reduced after 14 d in

BL 47, coupled with a significant increase in APase activities. In contrast to earlier reported results, indicating that APase activities are independent of phosphate levels (Szabo-Nagy et al. 1992; Fernandez and Ascencio, 1994), the induction of APase activity in BL 47 is likely to be caused by the low level of Pi. These results suggest a dependence of the enzyme level on Pi availability as a signal for induction of APase activity in white clover. Similar reports on the increase in APase activities in inverse proportion to the low level of Pi has been demonstrated in numerous plant species (Hunter and Leung, 2001; Yun and Kaeppler, 2001; Ming et al. 2002; Lim et al. 2003, Raposo et al. 2004; Sharma et al. 2005; Playsted et al. 2006). The expression of higher APase activities in roots suggests its global role in enhancing Pi availability and possibility recycling of organic Pi compounds.

There are two prevailing hypotheses about the role of acid phosphatases in plants and its relationship with plant nutritional P status. The first is that plants adapted to low P conditions (efficient in P uptake and utilization) would present high leaf or root APase activity as a sign of the ability to hydrolyze and remobilize P (root secretion and/or leaf synthesis). This would make P available to the plant from soil or other older plant parts (Lee, 1988; Lefebvre et al. 1990; Barrett-Lennard et al. 1993). For the present study, therefore, BLs or genotypes would be expected to have different abilities to hydrolize and mobilize P. The second hypothesis is that plants adapted to low P conditions (efficient in P uptake and utilization) would present a lower P demand and, consequently, a lower leaf or root APase activity would then be a chemical indicator of the severity P deficiency. The more the plant is stressed in relation to P, the higher the APase activity and the less adapted the plant would be (McLachlan, 1980a; 1980b; Silberbush et al. 1981; Helal, 1990; Tadano et al. 1993).

The first hypothesis anticipates a direct role for APases on plant P uptake and P efficiency use mechanisms, while the second assumes an indirect relationship – that is, an indication of a lower or higher P demand by the plants. Clark and Brown (1974) observed differences for intact root APase activity between two maize inbred lines. Using young plants, the highest activity inbred line presented also had a higher P content. In the present study, BL 47 had the highest –P/+P cell wall APase activity at d 14 and also had the lowest –P/+P Pi content ratio at d 14. This showed a negative correlation between cell wall APase activity and Pi content in BL 47. The results also showed that the reduction in Pi content in leaves occurred at the same time as the reduction in root cell wall APase (based on –P/+P ratio) or the induction in cell wall

APase activity occurred after reduction in leaf Pi content was observed (based on the results of statistical analyses). Unlike BL 47, BL 49 can maintain a constant –P/+P Pi content ratio in leaves throughout the experimental period while displaying a reduction in the cell wall APase activity ratio by 50% at d 14. For BL 49, based on the –P/+P ratio, the result showed no correlation between cell wall APase activity and Pi content, and the reduction of cell wall APase activity occurred before any reduction in Pi content, in leaves was observed. Based on statistical analyses, the induction of cell wall APase activity in BL 49 occurred after the reduction in leaf Pi content was observed. For BLs 43 and 45, the statistical analyses result showed that the induction of cell wall APase activity in roots occurred after the reduction in leaf Pi content was observed.

Genotype studies showed that genotypes 45-14 and 45-4 had a constant –P/+P Pi ratio throughout the experimental period. The result also showed they had different –P/+P cell wall APase ratio at d 14 (2.62 for genotype 45-14 and 1.73 for genotype 45-4). For genotypes 43-8 and 45-4, statistical analyses showed that the induction of cell wall APase activity in roots occurred at the same time as the reduction of Pi content in leaves, while for genotypes 43-7 and 47-9 the decrease in Pi content had no significant effect on the induction of APase activity. Only genotype 45-14 showed that the induction of cell wall APase activity in roots occurred after the reduction of leaf Pi content was observed. Zhang and McManus (2000), using white clover, showed that plants showed that the first significant difference in cell wall APase activity between the +P and –P media was observed after 1 week, while for leaf Pi content the first significant difference in leaf Pi content were observed after two week. In the current study, statistical analysis showed each BL and genotype showed a different timing of the induction of leaf Pi content and APase activity and the induction of cell wall APase activity in roots occurred after a reduction in leaf Pi content was observed. This may be in common with a more widely observed phenomenon of a preferential accumulation or phosphate in leaf tissue at the expense of roots (Plaxton and Carswell, 1999). Since only these two parameters (Pi content in leaves and cell wall root acid phosphatase activity) were statistically significant in the breeding line and genotype screening, they can be categorized as the plant parameters most sensitive to P deprivation, suggesting that these two parameters may be the most suitable for screening white clover breeding lines/genotypes for P-uptake efficiency.

Whether the rapid induction of cell wall APase activity and rapid or constant reduction in leaf Pi content also had a significant effect on the biomass production were addressed in the next experiment.

4.1.4. Effect of Pi deprivation on APase isoenzymes of selected white clover genotypes

The induction of APase enzyme activity has been shown to be accompanied by the induction of a specific isoenzymes in P-deprived wheat leaves (Barrett-Lennard et al. 1982; McLachlan, 1984). This thesis also extended the examination of APase with a comparison of isoforms. The root surface activity measured enzymatically can be derived from ionically-bound cell wall enzymes that have been shown in white clover roots (Hunter et al. 1999, Hunter and McManus, 1999) and /or the (covalently) cell-wall-bound enzymes that have been shown in the roots of other species (Lamport and Northcote, 1960; Woolhouse, 1969), including subterranean clover (Dracup et al. 1984). Staining of the major cell wall basic isoform was enhanced in the P-deprived root extracts from all genotypes, and was especially more abundant in genotypes 45-14. This is in agreement with the cell wall APase activity data, where the highest cell wall APase activity was observed at d 7 in –P root extracts in this genotype. The differences in the isoform staining were apparent specifically to genotype 45-14 at d 7, with an additional minor higher mobility isoform. This finding is similar to the previous report showing the induction of novel APases under P deficient conditions in many other plant species (Goldstein et al. 1988b; Duff et al. 1994; Lim et al. 2003). A similar study using high and low phosphorus responders of white clover, Hunter and McManus (1999) showed that although several APase isoforms were enhanced in P-deprived roots, no induction of novel APases was observed. However, without further purification of the cell wall basic isoform, we cannot determine the contribution made to cell wall APase activity by this isoform. For the cell wall acidic isoforms, two additional minor higher mobility isoforms were discernable in the P-deprived root extracts from all genotypes. Again, a strong induction of an acidic isoform was observed in genotype 45-14 at d 7 in P-deprived roots and this is also in agreement with the cell wall APase induction at d 7 in P-deprived roots. Differences in isoform staining were apparent at d 7 when cell wall APase activity had a significant difference in –P relative to +P root extracts.

This study also examined the soluble isoforms (putatively the vacuolar/cytoplasmic acid phosphatases). Again, enhancement of major basic isoforms was observed in the P-deprived root extracts in all genotypes, except for genotype 47-12, in which the major basic isoform was enhanced in P-sufficient root extract at pH 4.3. Also at d 7 in P-deprived roots, a minor low mobility acidic isoform was observed only in P-sufficient root extracts of genotype 45-14. In this study, enhancement of specific isoforms was observed in all genotypes in the P-deprived treatment and there was a genotype-specific isoforms detected. Further, the complement of basic and acidic isoforms revealed in these genotypes is not identical to other white clover genotypes studied. So far, in this study, the genotypes were compared in terms of their responses to P deprivation. This study has also shown that there are differences in the root APase activity or isoform complement between the high P-response and low P-response genotypes examined. The identification of distinct soluble and wall-associated APases in white clover can aid in understanding the significance of the induction of acid phosphatase activity particularly in response to P-deprivation.

4.2. Effect of Pi deprivation on several growth parameters of white clover

The possibility of screening breeding lines or genotypes for differences in P absorption and utilization to improve the efficiency of P fertilizer use or to obtain higher productivity in P-poor or P-deprived soils has received significant attention (Clark and Brown, 1975; Nielsen and Barber, 1978; Baligar and Barber, 1979; Fageria and Barbosa, 1982; Fageria et al. 1988). Thus, the objective of the study in this part was to evaluate white clover breeding lines and genotypes for phosphorus use efficiency. Efficient breeding lines and genotypes with other characteristics can be used directly in advanced field trials or in breeding programs.

The adaptive response of white clover plants to continuously low P-supply depends on the breeding lines and the genotypes. The first adaptive response is a change in plant morphology resulting in a proportional reduction in shoot dry weight for BL 49 (Figure 3.16B), root fresh weight (Figure 3.17B), and shoot fresh weight (Figure 3.18B). It should be noted, however, that this study did focus on short-term changes,

particularly the timing of any increase in APase activity and changes in Pi content. Thus, changes in growth parameters are also examined over this short term frame.

4.2.1. Effect of Pi deprivation on leaf area, weight of an individual leaf and shoot DW

4.2.1.1. Leaf area and weight of an individual leaf

The observed effect of P starvation on leaf area that is observed in plants in this thesis during P deprivation is not consistent with the results of other authors who have reported a rapid and severe effect of P deficiency on leaf growth (Atkinson, 1973; Radin and Eidenbock, 1984; Sicher and Kremer, 1988; Freeden et al. 1989; Rao and Terry, 1989; Lynch et al. 1991; Qui and Israel, 1992). Studies by Mollier and Pellerin (1999), for example, using maize showed that after 3 d in P-deficient media, the total leaf area per plant was 80.4% of that of control. After 16 d, the total leaf area was only 20% of that of control plants. They suggested that this was the consequence of a slower rate of leaf appearance and of a reduced final size of individual leaves. In the experiment reported in this thesis, no significant reduction of leaf area was observed for plants grown in +P or –P media for both genotypic screening and breeding line screening. Although BL 47 has less leaf area than BL 43 in +P and –P media, both have the same weight of an individual leaf. When the –P/+P ratio of leaf area and weight of an individual leaf at d 14 were compared, it was observed that BL 47 had higher ratios (1.24, 1.26, respectively) relative to BL 43 (1.01 and 1.11, respectively). A rapid reduction of Pi content and a rapid increase in cell wall APase activity at d 14 might could explain the higher ratios on leaf area and weight of an individual leaf in BL 47 which is not observed in BL 43. BL 45 had the highest –P/+P leaf area ratio (1.37) at d 14 in –P media relative to that of +P media. This suggests that Pi deprivation alone was independent of cell division and cell expansion. However, reduction in leaf area was not only influenced by phosphorus, but also by breeding lines/genotypes and days of treatments. When these traits were included, BL 45 had the highest leaf area in P-deprived media at d 14. Although at d 14 the –P/+P ratio of Pi content in BL 45 and BL 49 was quite similar, BL 45 had a higher cell wall APase activity compared to BL 49. Also BL 45 has a higher leaf area ratio relative to BL 49 and a higher weight of an individual leaf ratio relative to BL 49. This suggests that during Pi deprivation, BL 45

with the same Pi level in leaves, performed relatively well as defined by having a higher leaf area and weight of an individual leaf when compared with BL 49. Genotypes 45-14 and 45-4 had the same –P/+P ratio, but 45-4 had a higher –P/+P ratio in terms of leaf area and weight of an individual leaf. This implies that genotype 45-4 performed better in –P media relative to genotype 45-14. However, statistical analyses showed that genotype 45-14 had the highest leaf area and weight of an individual leaf and this was significantly different than genotype 45-4 in –P and in +P media at d 14. Although genotype 45-14 and 45-4 had the same –P/+P ratio in terms of Pi content, genotype 45-14 performed relatively well by having a higher cell wall APase activity, leaf area and weight of an individual leaf. Statistical analyses also showed that genotypes 43-7, 43-8, and 47-9 had the lowest leaf area and weight of an individual leaf in –P and +P at d 14. Whether higher Pi content and cell wall APase activity had a significant effect on other growth parameters was assessed in the next experiments.

4.2.1.2. Shoot DW

Significant variation in the ability of white clover genotypes to take up and utilize P under P-deprived conditions has been demonstrated previously in the field where all the genotypes were compared. BLs 47 and 49 showed greater yield at deficient P supply when similar growing conditions were employed. In this thesis, a higher –P/+P shoot DW ratio was observed for BLs 43 and 45, while for BL 47, the ratio was 1.15 and BL 49 had the lowest ratio. This suggests that only BLs 43 and 45 performed relatively well in terms of their high shoot DW in –P media, while these lines performed poorly in +P media. BL 47 on the other hand performed moderately well such that in the –P or +P media, BL 47 had the same shoot DW. Although BL 49 had a lower –P/+P ratio compared to BL 47, they both had similar shoot DW production in –P media. BLs 45 and 49 produced considerably greater shoot growth in P deficient media while having a similar shoot tissue P concentration in P-deprived media, but a different in cell wall APase activity (BL 45 had the highest enzyme activity while BL 49 had the lowest enzyme activity). This translates to a higher –P/+P ratio in terms of shoot DW and a considerably larger accumulation of P in shoots of BL 45. Although BL 45 had the highest shoot DW at d 14 in –P media, the statistical analyses showed that BL 45 was not significantly different from BL 49 or other BLs. However, when the performance of these two BLs in +P media was compared, the results showed that BL 49 was significantly different from BL 45. This suggests a lower level P utilization in

BL 45 compared with BL 49. It should be recognized that higher uptake of P under deficient conditions, even when combined with lower utilization efficiency, is still a desirable trait that would ensure good growth under P-limiting conditions. BL 49 therefore has some physiological mechanisms that are superior to those in terms of P uptake from the medium at deficient P concentrations. However, the nature of these mechanisms is not clear at present. Overall, BL 49 showed the highest performance in terms of shoot DW in –P media and +P media.

When the effect of P-deprivation on the accumulation of shoot dry weight in selected genotypes was compared, the results were slightly different. The shoot dry weight of plants growing in a –P solution was comparable with that of control plants grown in +P solution for selected genotypes. However, for genotype 47-9, the shoot DW of plants grown without P was significantly higher than plants grown in a complete nutrient solution at 14 d after P was withdrawn. A decline in P concentration was observed in selected genotypes of white clover grown in –P solution. When shoot DW of genotypes 43-7 and 43-8 were compared, both genotypes had a similar Pi shoot tissue ratio but genotype 43-8 had more cell wall APase activity relative to genotypes 43-7. Genotype 43-7 also had a higher –P/+P shoot DW ratio when compared with genotype 43-8. It suggests that genotype 43-7 produced more shoot DW in –P media, while genotype 43-8 produced more shoot DW in +P media. Genotype 47-9 maintained a constant –P/+P Pi ratio up to 14 days, but had the lowest cell wall APase activity ratio when compared with other genotypes. Genotype 47-9 also had the highest –P/+P shoot DW ratio suggesting that genotype 47-9 produced more shoot DW in the –P media. There was no significant difference in the shoot P concentrations of plants growing in complete and –P solutions 7 d after P was withdrawn (genotype 43-7). However, the shoot P concentrations of plants grown in a –P solution for 14 d were significantly less than those of plants growing in complete nutrient solutions. Thus between 7 d and 14 d after P withdrawal, genotype 43-7, when grown in a –P solution, had a significantly lower shoot P concentration but retained comparable growth to plants grown in a complete nutrient solutions. One application of such observations could be to use promoters from genes with up-regulated expression between 7 and 14 d after P withdrawal to drive the expression of a marker gene in smart plants because they would allow the plant to respond to P deficiency before growth was affected (eg. genotype 43-7). Genotypes 45-14 and 45-4 produced a considerably different shoot DW in response to P-deficiency while having similar shoot tissue P concentration, but with a higher cell

wall APase activity in genotype 45-14. That translates into a considerably larger shoot DW in genotype 45-14 with a high level of Pi utilization compared to genotype 45-4. Genotype 45-14 showed a higher P efficiency than other genotypes according to all criteria. Genotype 45-14 has also been proven to be an excellent performer in liquid media from previous experiment due to its ability to grow more roots in response to P-deficiency (data not shown). In addition, relative to other genotypes, genotype 45-14 has the longest tap root, more dispersed and longer lateral roots and more dispersed shallower basal roots (data not shown). Genotype 45-14 was superior to all genotype in P uptake under P deficiency as well as under P sufficiency, suggesting that higher affinity of transporters and/or low P minimum P concentration at which uptake starts may exist in genotype 45-14. Callus cultures have now been established from this genotype which may allow some of these possibilities to be examined.

4.2.2. Effect of Pi deprivation on fresh biomass yield and R:S ratio

4.2.2.1. Root FW

Contradictory results are found in the literature about the effect of phosphorus deficiency on root growth. Several authors have observed an enhanced root growth on P deficient plants (Narayanan and Reddy, 1982; Anuradha and Narayanan, 1991; Rychter and Randall, 1994), whereas other authors have reported a reduction of root growth under P deficiency (Radin and Eidenbock, 1984; Hajabbasi and Schumacher, 1994; Kondracka and Rychter, 1997; Mollier and Pellerin, 1999). Such apparent contradictions probably reflect differences between experimental approaches used (individual root versus whole root systems), the variable under consideration (root biomass or root elongation), time scale of the experiment (short term versus long term) and other experimental conditions which may interfere with the measured process (Mollier and Pellerin, 1999).

Wissuwa (2003) proposed three parameters to measure the tolerance to P deficiency, which are (i) a P partitioning factor, (ii) internal efficiency in dry matter production and (iii) specific root surface area of P uptake per unit root size. This author showed that by raising these parameters by 10%, an improvement of 62-64% in P uptake was observed. Furthermore, the results showed that a 22% increase in root-growth related parameters will triple the P uptake in a Near Isogenic Line (NIL-C443) from rice. This study showed that improving root growth would be the most efficient

way to enhance tolerance to P deficiency. Other similar results using different plant species were also demonstrated by Teo et al. (1992) using lowland rice and by Ermani et al. (1994) using maize grown in P-deficient soil. However, Wissuwa (2003) argued that there may be several limitations as to which of the root-growth related parameters would be most suitable for genotypic improvements. Greater P partitioning to roots may not be useful if plants have already optimized P partitioning. Moreover, reduction of P supply to shoots would cause insufficient photosynthesis and assimilate supply for root growth with a subsequent negative effect on P uptake. Therefore, selection of genotypes with a low internal P requirement for dry matter production may be an efficient way to improve tolerance to P deficiency. BL 49 can be placed in this category. This BL showed a constant –P/+P Pi content ratio in leaves during the experimental period. Despite having the lowest root FW ratio, the BL has the highest shoot DW in +P. This may suggest that BL 49 has already optimized the balance in P partitioning therefore greater P partitioning to the root may not be useful.

Reduction in root growth as a consequence of P deficiency has been reported by several authors (Atkinson, 1973; Tennant, 1976; Amijee et al. 1989; Bruce et al., 1994; Hajabbasi and Schumacher, 1994; Rosolem et al. 1994). Unlike other BLs, a reduction in root growth was not observed in BL 45 at d 14 in –P media. In fact, only BL 45 showed an increase in root FW in P-deprived media when compared to the other BLs. Genotype 45-14 also showed a similar result to BL 45. In contrast, an increase in root fresh weight in genotype 45-14 was observed at d 14 in –P media and then was significantly different with d 7 and 14 in +P media. Similar findings were also reported by Anghinoni and Barber (1980) who observed an increased root dry weight in 12-d-old maize plants. BL 47 and genotype 47-9 showed that root FW was not affected by P-deficiency as late as d 14. These findings are similar to what has been reported by Radin and Eidenbock (1984). These authors showed that root dry weight in cotton was unaffected by low P as late as 13 d after transfer to the low P nutrient solution. The explanation for the high rates of root growth in low-P plants is that low-P plants diverted more of their photosynthate toward roots because less was utilized in shoot growth (Freeden et al. 1989; Mollier and Pellerin, 1999; Vance et al. 2003). Studies showed that this was because of much higher levels of starch and sucrose in roots when compared with leaves in low-P plants, indicating an active transport of photosynthate to the roots. In contrast, the results in this study also showed that some BLs and genotypes display a reduction in root FW as a result of P-deficiency. For example, BL 49 had a

27% reduction in root FW, while genotype 43-8 had a 43% reduction in its root FW. Similar findings were reported by other workers who showed a reduction in root FW in P-deficient plant species (Radin and Eidenbock, 1984; Hajabbasi and Schumacher, 1994; Kondracka and Rychter, 1997; Mollier and Pellerin, 1999). In P-sufficient media at d 14, statistical analyses showed that BL 49 had a significantly different root FW relative to BL 45, while there was no difference in root FW in –P media. This suggests that BL 49 grew more roots in +P media, while having the same root FW in –P media compared to BL 45. The highest shoot DW in +P media can also be observed in genotype 45-14.

Plants under phosphorus stress cannot simply grow more roots throughout the soil profile without slowing plant growth by diverting too many resources from photosynthesis. An ideal root architecture for phosphorus acquisition is the one that enhances phosphorus acquisition at minimum carbon cost, or optimizes the value of phosphorus uptake with respect to the relative value of the resources required for root growth. The phosphorus costs of root growth may be relatively greater than the phosphorus costs of leaf growth because, unlike leaves, roots appear to be unable to effectively remobilize phosphorus to the rest of the plants through programmed organ senescence (Snapp and Lynch, 1996).

4.2.2.2. Shoot FW

The effect of removing P supply on growth was determined at the specific time indicated in selected breeding lines and genotypes of white clover. The shoot fresh weight of plants growing in a solution lacking P (-P solution) was comparable with that of control plants grown in a complete nutrient solution throughout the experimental period.

At d 14, BLs 45 and 49 had the same –P/+P ratio in terms of Pi content but BL 45 had a higher cell wall APase activity ratio, the highest –P/+P shoot FW ratio and root FW ratio. Although BL 45 had the highest shoot FW ratio relative to BL 49, statistical analyses showed that they were not statistically significant. However in the +P media, BL 49 was statistically significant in terms of shoot FW when compared with BL 45 (BL 49 produced twice as much shoot FW as BL 45). Statistical analyses showed that BLs 43 and 47 had no difference in all parameters measured.

Further, genotypes 43-7 and 47-9 had a similar Pi content, a slight difference in shoot FW, while genotype 47-9 had a higher shoot FW when compared with genotype

43-7. This suggests that during Pi deprivation, genotype 47-9, with similar Pi content and a slight different root FW grew more shoot FW relative to genotype 43-7. Genotype 45-14 and 45-4 had the same –P/+P ratio in terms of Pi content, but genotype 45-14 had a higher APase activity ratio, root FW ratio and shoot FW ratio. Genotype 43-8 had more shoot in –P media, while genotype 43-7 had the same shoot FW in +P or –P media. Genotypes 45-14, 45-4 and 47-9 had more shoot FW in –P media. Overall, when the average performance was compared, BL 49 and genotype 45-14 were more desirable because they had higher growth performances in both media.

4.2.2.3. Total biomass (BM) FW

Reduction in growth and yield are the commonly agronomic responses of plants to Pi deficiency (Nielsen at al, 2001). However, this reduction in growth and yield is determined by the many physiological and biochemical changes in plants under P deficiency. In common bean, the reduction of growth caused by low P availability has been associated with relatively increased below-ground biomass and reduction of leaf appearance rate and not by decreased carbon assimilation (Lynch et al. 1991; Lynch and Beebe, 1995; Nielsen et al. 2001). In the present study, a higher total BM FW for genotype 45-14 at low P was correlated with its higher activity of root acid phosphatase and root fresh weight, which contributed to its apparent higher ability in mobilizing and taking up P. When these genotypes or BLs were cultured in the same container, they competed for limited P in the solution. The one that had the greater P uptake ability should have an advantage over the other. In this study, BL 49 and genotype 45-14 have an ability to taking up P when it is available and mobilizing it from its sink when P is unavailable. On the contrary, BL 43 and genotype 43-7 suffered from P treatment due to their lower ability in terms of P uptake. This situation limited the ability of these plants to develop roots as indicated by a decreased root FW.

Studies have shown that genotypic variation existed for DM production and that older varieties generally required less P per unit dry matter compared with modern, improved varieties. For example, Wang et al. (2004) showed that when compared with the conventional cultivar, the wild soybean genotype XM6 has a relatively higher amount of biomass and P content despite its smaller root size and shorter total root length when grown in the low P soil. In the present study, BLs 43 and 47 had no difference in leaf area and weight of individual leaf in +P or –P media, shoot DW, root FW, and shoot FW. They also had no difference in biomass FW although BL 47 had

70% more biomass FW relative to BL 43 in +P media. This suggests that BL 47 performed relatively well in +P media (in terms of its biomass production in +P media and its response to available P) and is more efficient at utilizing P when compared with BL 43. The results also showed that BL 47 had the same fresh biomass when maintained in the +P and in –P media. The highest total biomass of BL 45 at –P was correlated with its steady decrease of Pi content ratio in leaf, medium cell wall APase activity, the highest root FW ratio, the highest leaf area ratio, the highest weight of an individual leaf, the highest of shoot DW, the highest root FW, and the highest shoot DW. This suggests that BL 45 performed relatively well in –P media and performed poorly in +P media. In contrast, BL 49 had a constant –P/+P ratio in terms of Pi content, the lowest cell wall APase activity ratio, the same leaf area ratio in –P and +P media, the lowest weight of an individual leaf ratio, the lowest shoot DW and shoot FW ratio, the lowest root FW, and the lowest total biomass ratio. This suggests that BL 49 performed poorly in –P media but performed well in +P media. Although BL 49 had the lowest –P/+P ratio of almost all parameters observed, it still had a higher biomass FW in –P media (not significantly different with BL 45), but in +P media, BL 49 was significantly different from BL 45. BL 49 (as determined by its performance in the +P media) is therefore chosen as a good performer because it can respond well to added P and its ability to maintain a higher Pi content in leaves during Pi deprivation and it performed relatively well in –P media.

For genotype screening, genotype 43-7 had a higher –P/+P total biomass FW relative to genotype 43-8. This suggests that genotype 43-7 performed relatively well in –P media. Although genotype 43-7 had a higher biomass ratio when compared with genotype 43-8, statistical analyses showed that it had the lowest biomass FW. Both genotypes had no difference in cell wall APase and total soluble APases, leaf area, weight of an individual leaf, shoot DW, shoot FW in +P and –P media. However differences were observed in the root FW in –P media. Therefore, a higher biomass FW observed in genotype 43-8 at d 14 in –P media was due to its ability to grow more roots during Pi deprivation. Genotypes 45-14 and 45-4 had the same –P/+P Pi content ratio, relative to genotype 45-4, while genotype 45-14 had the highest cell wall APase ratio, a lower leaf area ratio, a lower weight of an individual leaf, the highest shoot DW ratio but had same shoot FW ratio. Genotype 45-14 also had the highest root FW and the highest total biomass FW. This suggests that genotype 45-14 performed relatively well in –P media with high cell wall APase activity and an ability to grow more roots in –P

media hence its highest shoot DW. The same shoot FW ratio showed that both genotypes 45-14 and 45-4 performed relatively well in –P media. Statistical analyses showed that genotype 45-14 was statistically significant compared to genotype 45-4 in shoot DW and in total biomass FW. This suggests that genotype 45-14 performed relatively well in +P and –P media.

The results also indicate that large differences in shoot dry weight response to applied P exist within the range of BLs studied. Some broad differences in the pattern of shoot dry matter response are apparent. BL 45 is clearly distinguishable as having the highest dry weight but is not as responsive as BL 49 to applied P. Based on these responses to yield potential and P response, the four breeding lines can be separated into two classes. BL 45 has a response typified by a high yield potential and a low responsive to P, indicating that it performs relatively well under extremely low P conditions and performs very poorly under moderate to high P conditions. BL 43 can also be categorized in this class. BLs 47 and 49 had a high yield potential and were highly responsive to added P, and both BLs also showed a high yield potential in low or high fertility. From a practical point of view, BLs/genotypes which produce high shoot dry matter in a low P level and respond well to added P are the most desirable because they are able to express their high yield potential in a wide range of P environments. BL 49 and genotype 45-14 fall into this group. These are often referred to as widely adapted BL/genotypes, in contrast to other BLs/genotypes which can be referred to as more specifically adapted BLs/genotypes. These results also indicate changes in the performance of the plants grown in the field when compared with the plants grown in the temperature-controlled growth room. This can be observed in BLs 43, 45 and 49. The performance of BL 47 was similar to the performance of the BL 47 populations collected at Ballantrae Hill Country. This suggests that some adaptation to low P status may have occurred (Caradus et al. 1980). BL 47 also has a smaller leaf area when compared with other BLs. This adaptation appears to be toward a smaller plant form rather than an ability to utilize P more efficiently than the other accessions. These results also suggest that clover plants that are adapted to low phosphorus situations in the field tended to maintain the same response when grown in liquid media. These low responsive types tend to be small-leaved prostrate types (Caradus et al. 1980).

As mentioned previously, the time course study of the cell wall APase showed that the induction of APase in respond to P stress occurred at the same time or after reduction of the Pi level in the leaf for selected breeding lines and genotypes. However,

less is known about whether phosphate regulates APase activity in whole plants before any reduction in biomass accumulation. In this study, for both breeding line and genotypic screenings, the result showed that the reduction in Pi content and acid phosphatase activity occurred before inhibition of biomass accumulation which meant that the response is more likely to be in response to phosphate level rather than 'global' stress response which occurs after the reduction of biomass accumulation (Goldstein et al. 1988a, 1988b). This finding is similar to that reported by Hunter and Leung (2001) for *Capiscum annum*.

4.2.2.4. Root:Shoot FW ratio

An increase in root-to-shoot ratio is often observed in plants in response to P deprivation (Atkinson, 1973; Anghinoni and Barber, 1980; Sicher and Kremer, 1988; Freeden et al. 1989; Smith et al.1990; Lynch et al. 1991; Paul and Stitt, 1993; Cakmak et al. 1994; Rosolem et al. 1994; Rychter and Randall, 1994; Ciereszko et al. 1996; Horst et al. 1996; Zhu and Lynch, 2004). Zhu and Lynch (2004) reported an increased root:shoot ratio of approximately 39% in maize plants grown in low P media. The higher root:shoot ratio observed for P-deficient plants is associated with a higher proportion of carbohydrates being partitioned to the roots and a higher sugar concentration in the roots (Cakmak et al. 1994; Ciereszko et al. 1996; Freeden et al. 1989; Khamis et al. 1990; Paul and Stitt, 1993, Rychter and Randal, 1994, Trull et al. 1997). Furthermore, several authors have observed that the increased root:shoot ratio in phosphate-deficient plants was also associated with higher carbohydrate content in the roots (Freeden et al. 1989; Khamis et al. 1990; Paul and Stitt, 1993; Cakmak et al. 1994; Rychter and Randall, 1994; Ciereszko et al. 1996).

The study presented here shows that the increase in root:shoot ratio which is commonly observed in P-deficient plants was not observed in the BLs and genotypes screened. Average performance of all BLs in +P and –P media showed that a higher root:shoot ratio was recorded in BL 49 and BL 43, and that this was significantly different from BL 45 and BL 47 (Table 9). Statistical analyses showed that P deprivation had no effect on root:shoot ratio except at d 14 in +P media where BL 43 had the highest R:S ratio, BLs 45 and 47 with medium R:S ratio, and BL 49 with the lowest R:S ratio. In P-sufficient condition, BL 43 was the least efficient in terms of total biomass production but had a higher root:shoot ratio, while BL 49 was the most efficient BL but had the lowest R:S ratio, and BL 43 the least efficient BL but had the

highest R:S ratio. Similar findings can also be observed in genotypic screenings. Statistical analyses showed that genotypes 43-7, 43-8 and 45-4 had a higher R:S ratio and were statistically different from genotypes 45-14 and 47-9. Genotype 45-14, the most efficient genotype at utilizing P, and had the highest total BM in P-sufficient and P-deficient media, also had the lowest R:S ratio in +P or in –P media. The least efficient genotypes 43-7 and 43-8, had the highest R:S ratio in +P and in –P media.

Overall, based on the Pi content level, APase activity and growth parameter results, several adjustments on previous studies of these breeding lines in the field had been made. Table 4.1 summarized the comparison of the performance of these breeding lines in the field and in the climatic-controlled room.

Table 4. 1. Conclusion of comparison BLs performance in the field and in liquid media

	Performance	
BL	**Field**	**Liquid Media**
43	Low performance in low fertility High performance in high fertility **LLHH**	Low performance in low fertility Low performance in high fertility **HLLH**
45	Low performance in low fertility Low performance in high fertility **LLLH**	High performance in low fertility Low performance in high fertility **HLLH**
47	High performance in low fertility High performance in high fertility **HLHH**	High performance in low fertility High performance in high fertility **HLHH**
49	High performance in low fertility Low performance in high fertility **LHHL**	High performance in low fertility High performance in high fertility **HLHH**

Chapter Five: Summary

White clover breeding lines were rooted in vermiculite in half strength Hoagland's solution for two weeks, then were transferred to the half strength Hoagland's liquid media for 6 weeks. Plants then were subjected in P-containing or P-deprived media for two weeks (BL screening) and three weeks (genotype screening). Phosphate levels were measured in the first fully expanded mature leaf. The deprivation of exogenous phosphate influenced P status of BLs and genotypes of white clover markedly with a decrease in P content of –P plants when compared with +P plants. A significant consistent difference in leaf Pi content was first observed at d 3 in –P media for BLs 45 and 49 (designated as fast response). For BL 47, this difference was first observed at d 5 (medium response), and for BL 43 this difference was first observed at d 7 (slow response). For genotype screening, a significant consistent difference was observed at d 7 in genotypes 45-14, 45-4 and 47-9 (designated as fast response), and at d 14 for genotypes 43-7 and 43-8 (slow response). The –P/+P ratio also showed that BL 49 maintained the highest and a constant –P/+P throughout the experimental period. Similar results can also be observed in genotypes 45-15 and 45-4. In contrast, BL 47 showed a rapid reduction in leaf Pi content at d 14 in –P media. This rapid reduction in leaf Pi content can also be observed in genotypes 43-7 and 43-8. In concert with a decrease in phosphate content in leaves, an increase in cell wall APase activity was observed in roots of white clover plants maintained in –P media. A significant consistent difference in cell wall APase activity was observed first in BLs 45 and 49 at d 5 (designated as a fast response), BL 47 at d 7 (a medium response), BL 43 at d 14 (a slow response). For genotype screening, the difference occurred at d 7 for genotype 45-4 (the fast response) and at d 14 for genotypes 43-8 and 45-14 (slow response), while no significant difference was observed in genotypes 43-7 and 47-9. The results showed that the induction of the cell wall APase activity occurred after the reduction of leaf Pi content was observed. The –P/+P ratio of cell wall APase activity also showed that BL 49 had the lowest APase activity at d 14, while BL 47 had the highest APase activity. While the lowest –P/+P ratio of enzyme activity in BL 49 occurred at d 14, the –P/P ratio of Pi in leaves did not change throughout the experimental period. This suggests that there is no correlation between Pi content and cell wall APase activity in BL 49, while for BL 47 the highest enzyme activity ratio was negatively correlated with the

lowest ratio of Pi content in leaves. For genotype screening, genotype 45-14 maintained the highest and most constant ratio of Pi content in leaves, but also had the highest–P/+P ratio of cell wall APase activity in roots. Therefore genotype 45-14 showed a positive correlation between APase activity and Pi content, while other genotypes showed no correlation between Pi content in leaves and the cell wall APase activity in roots. The study here showed that the rate of phosphate uptake varied widely among breeding lines and genotypes. This is in accordance with the result by Ryle et al. (1981) which showed that white clover is a strong outbreeding species and that individual genotypes show wide variation of physiological characteristics. Another study by Burdon and Harper (1980) showed that many white clover genotypes with different growth rates could occur within a small area of established pasture (Burdon, 1980). Therefore, the variation observed here may have resulted from genotypic differences or from differences in the previous history of plants. In the field fluctuations in temperature, moisture and oxygen concentrations can be limiting factors and often root growth is affected by defoliation events (Evans, 1973), the degree of mechanical impedance of the soil (Russell, 1977), soil flora and fauna (Russell, 1973) and variations in nutrient availability in soil (Russell, 1977). All these factors may contribute to the large differences between the previous studies in the field and this hydroponic study in the control room.

There were genotypic differences in the responses of APases to P deficiency in white clover. The –P/+P ratio in terms of APase activity was lower or similar in other genotypes, but significantly higher in the roots of genotype 45-14. Cell wall and total soluble APase were significantly higher in the roots of genotype 45-14 relative to other genotypes. The higher level of APase activity in genotype 45-14 may have been contributed to by one minor high mobility basic isoform was observed only in genotype 45-14. APase isoform studies showed that the induction of the major cell wall basic isoform was enhanced in –P root extracts from all genotypes. For the cell wall acidic isoforms, two additional minor high mobility isoforms were observed in –P root extracts from all genotypes. A strong induction of a major low mobility acidic isoform in genotype 45-14 at d 7 in –P media was in accordance with the induction of cell wall APase observed in genotype 45-14 at d 7 in –P media. Similar enhancement of a major basic soluble isoform was also observed in –P root extracts from all genotypes with the exception for genotype 47-12. In addition, a minor low mobility acidic soluble isoform

was observed only in +P roots extracts in genotype 45-14. This suggests that genotypic differences in the level of APase activity are facilitated by differences in the profiles and activities of isoforms. Furthermore, the differences in the root APase isoforms between high-response and low P-response genotypes can aid in understanding the significance of the induction of APase activity particularly in response to P-deprivation.

BL and genotype screening results showed that BL 49 and genotype 45-14 are the most efficient white clover ecotypes. Both performed relative well in +P and –P media. The highest shoot DW and shoot FW in BL 49 and genotype 45-14 are as a result of their ability to maintain the highest ratio of –P/+P ratio of Pi content in leaves as well as the ability to grow more roots (determined by a higher root FW) in –P media. Further, changes in the performance of plants grown in the field and in the temperature control room have been observed. BL 49 for example, based on the field performance showed low responsive to P fertilizer but in this experiment it showed that BL 49 has a high performance in high fertility. Moreover, BL 45 showed low performance in either high or low fertility soil in the field, while in the laboratory, BL 43 showed low performance in either high or low fertility soil. On the other hand, BL 47 showed the same performance in the field as in the laboratory. The results also showed that BL 45 is more adaptable to low fertility soils, while BL 49 and genotype 45-14 are adaptable to low and high fertility soils. Gourley et al. (1994) proposed that germplasm can be categorized to differ in P efficiency only when similar yields are obtained. Studies by Gahoonia and Nielsen (1996) using wheat and barley genotypes showed that the relative growth rates of the genotypes did not differ and the spring barley genotypes Etna and Riga originating from the same parental germplasm did not differ in P depletion profile, suggesting that the observed variation in P depletion may be associated with the ability of the germplasms to acquire P from the rhizosphere soil. The present investigation was carried out under a precisely controlled environment. The results showed significant variation in their efficiency to acquire P from the liquid media among selected breeding lines and genotypes. However, it should be noted that changes in the levels of Pi in the media were not measured. In conclusion, screening of genotypes for efficiency of P uptake and utilization can effectively performed on a large number of genotypes in nutrient solution. Selected genotypes can then be re-screened in soil, with only a relatively small number of most promising genotypes to proceed to the field testing.

Future Work:

The results reported in this studies described the effect of Pi deprivation on the cell wall APase activity in roots of white clover, Pi content and several growth parameters have opened up some interesting aspects that can be pursued further.

Results from plants grown in liquid media in the Ecology Glasshouse (Genotype screening) or in the climatic control room (BL screening) confirmed the previous findings in term of reduction level of inorganic phosphate (Pi) in the leaves and changes in APase activity in roots in P-contained and P-deprived plants. Genotype 45-14 and BL 45 have shown consistent results regarding their slower reduction in Pi content in leaves upon prolonged Pi withdrawal and steady induction in APase activity in P-deprived media. Genotype 45-14 and BL 45 also showed a remarkable performance in Pi-deficient media by producing the largest fresh biomass, fresh root weight, fresh total biomass, shoot dry weight, the largest leaf area and the heaviest individual leaf. Also genotype 45-14 produced the lowest root:shoot ratio compared with other genotypes. Questions need to be answered such as, whether the superior performance of genotype 45-14 and BL 45 grown in liquid media is truly related to the specific mechanism to enhance P uptake or utilization during P deficiency? It has been suggested that enhanced Pi uptake during Pi deprivation is correlated with an increase number of high-affinity Pi transporters specifically expressed in roots. It would be interesting to determine if the superior performance of genotype 45-14 also occurs at the transcriptional level. Degenerate primer design can be used to identify the high-affinity Pi transporter gene family in white clover. To design degenerate primers for this transporter, the nucleotide sequence surrounding the conserved amino acid sequence region typical of high-affinity Pi transporters can be aligned using the genomic sequences of high affinity Pi transporters from several species such as *Arabidopsis*□ rose, *Medicago truncatula*□tomato, potato, barley. These primers can then be used for RT-PCR or northern hybridization analyses to study the expression of this high-affinity Pi transporter during Pi deprivation in white clover. The second question that needs to be answered regards the root morphology of genotype 45-14 which is quite distinct compared with other genotypes (data not shown). Studies showed that a shallower root system may be advantageous for phosphorus acquisition in many soils. Is the efficiency P uptake observed in genotype 45-14 related to its distinct root morphology?

Changes in specific isoforms of phosphatase under Pi deprivation are commonly observed. Results from the selected genotypic screening using genotype 45-14 showed an interesting finding regarding the accumulation of one specific lightly staining high mobility acid phosphatase isoenzymes observed at d 7 in P-deprived media using discontinuous native gel from ionically bound (1 M salt-extractable). Whether this specific isoenzyme is only expressed in genotype 45-14 in Pi-deficient media needs to be confirmed. Also, it would be interesting to know when the accumulation of this specific isoenzyme occurs. Does it occur earlier than d 7 or does it occur later?

Appendices

Appendix 1. The effect of interaction between breeding lines and days of treatment on Pi content and APase activity sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi content and APase were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction BLxD	Pi (%P.g^{-1}FW)	Acid phosphatase activity	
		Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
BL43D3	0.01095^{ab}	1.520^{cd}	8.3300^{e}
BL45D3	0.01082^{abc}	1.314^{d}	8.2200^{e}
BL47D3	0.01081^{abc}	1.262^{d}	7.8900^{e}
BL49D3	0.01078^{abc}	1.265^{d}	8.7300^{de}
BL43D5	0.01181^{a}	1.909^{bcd}	9.4000^{cde}
BL45D5	0.01004^{abcd}	1.625^{cd}	10.1600^{bcde}
BL47D5	0.01169^{a}	1.692^{bcd}	8.8400^{de}
BL49D5	0.01017^{abcd}	1.590^{cd}	10.0600^{bcde}
BL43D7	0.01008^{abcd}	2.072^{bcd}	13.5300^{ab}
BL45D7	0.01011^{abcd}	1.739^{bcd}	12.6500^{abcd}
BL47D7	0.00994^{abcd}	1.570^{cd}	10.4400^{abcde}
BL49D7	0.00971^{bcde}	1.821^{bcd}	12.4600^{abcd}
BL43D14	0.00905^{cde}	2.490^{ab}	13.8100^{a}
BL45D14	0.00812^{e}	2.262^{abc}	11.1400^{abcde}
BL47D14	0.01100^{ab}	2.856^{a}	14.2500^{a}
BL49D14	0.00857^{de}	1.807^{bcd}	13.1200^{abc}

Appendix 2. The effect of interaction between breeding lines and phosphorus treatment on Pi content and APase activity sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi content and APase activity were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction BL x P	Pi content (%P.g^{-1}FW)	Acid phosphatase activity	
		Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
BL43+P	0.01205^{ab}	1.553^{bc}	10.310^{a}
BL45+P	0.01086^{c}	1.329^{c}	10.140^{a}
BL47+P	0.01283^{a}	1.301^{c}	9.280^{a}
BL49+P	0.01102^{bc}	1.233^{c}	10.320^{a}
BL43-P	0.00889^{d}	2.443^{a}	12.230^{a}
BL45-P	0.00869^{d}	2.140^{a}	10.950^{a}
BL47-P	0.00889^{d}	2.389^{a}	11.430^{a}
BL49-P	0.00859^{d}	2.008^{ab}	11.860^{a}

Appendix 3. The effect of interaction between days of treatment (D) and phosphorus (P) application on Pi content and APase activity sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi content and APase activity were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction DxP	Pi content (%P.g^{-1}FW)	Acid phosphatase activity	
		Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
D3+P	0.01221^{a}	1.1090^{f}	8.7800^{cd}
D5+P	0.01195^{a}	1.4040^{def}	9.0900^{cd}
D7+P	0.01132^{a}	1.1930^{ef}	11.0800^{bc}
D14+P	0.01128^{a}	1.7100^{cd}	11.1100^{bc}
D3-P	0.00947^{bc}	1.5720^{cde}	7.8100^{d}
D5-P	0.00990^{b}	2.0040^{bc}	10.1500^{cd}
D7-P	0.00860^{c}	2.4080^{b}	13.4600^{ab}
D14-P	0.00709^{d}	2.9970^{a}	15.0500^{a}

Appendix 4. The effect of interaction between genotypes, days of treatment (D) and phosphorus (P) application on Pi content and APase activity sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi level, leaf area and weight of an individual leaf were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Breeding Line	Days and P treatment	Pi content (%P.g^{-1}FW)	Acid phosphatase activity	
			Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
BL 43	D3+P	0.01245^{bcd}	1.445^{efgh}	9.040^{ef}
	D5+P	0.01243^{bcd}	1.704^{defgh}	8.590^{ef}
	D7+P	0.01221^{bcde}	1.381^{efgh}	11.730^{abcdef}
	D14+P	0.01111^{cdefg}	1.681^{defgh}	11.890^{abcdef}
	D3-P	0.00945^{fghij}	1.595^{efgh}	7.630^{f}
	D5-P	0.01119^{cdefg}	2.114^{cdef}	10.220^{bcdef}
	D7-P	0.00794^{ijkl}	2.764^{bc}	15.330^{abc}
	D14-P	0.00699^{kl}	3.298^{ab}	15.730^{ab}
BL 45	D3+P	0.01261^{bc}	1.043^{gh}	8.910^{ef}
	D5+P	$0.01019^{cdefghi}$	1.253^{efgh}	9.020^{ef}
	D7+P	0.01127^{cdefg}	1.216^{fgh}	12.840^{abcdef}
	D14+P	0.00936^{fghijk}	1.806^{cdefgh}	9.780^{cdef}
	D3-P	0.00902^{ghijkl}	1.585^{efgh}	7.540^{f}
	D5-P	0.00989^{efghij}	1.997^{cdefg}	11.300^{abcdef}
	D7-P	0.00895^{ghijkl}	2.261^{cde}	12.460^{abcdef}
	D14-P	0.00688^{l}	2.717^{bc}	12.500^{abcdef}
BL47	D3+P	0.01154^{bcdef}	0.969^{h}	8.197^{f}
	D5+P	0.01378^{ab}	1.303^{efgh}	8.291^{f}
	D7+P	0.01095^{cdefgh}	1.135^{fgh}	9.007^{ef}
	D14+P	0.01506^{a}	1.796^{cdefgh}	11.620^{abcdef}
	D3-P	0.01008^{defghi}	1.555^{efgh}	7.582^{f}
	D5-P	0.00960^{fghij}	2.081^{cdef}	9.385^{def}
	D7-P	0.00892^{ghijkl}	2.005^{cdefg}	11.876^{abcdef}
	D14-P	0.00695^{kl}	3.916^{a}	16.873^{a}
BL49	D3+P	0.01222^{bcde}	0.980^{h}	8.960^{ef}
	D5+P	0.01142^{cdefg}	1.356^{efgh}	10.440^{bcdef}
	D7+P	0.01084^{cdefgh}	1.041^{gh}	10.730^{bcdef}
	D14+P	0.00961^{fghij}	1.557^{efgh}	11.150^{bcdef}
	D3-P	0.00934^{fghijk}	1.550^{efgh}	8.510^{ef}
	D5-P	0.00892^{ghijkl}	1.823^{cdefgh}	9.680^{cdef}
	D7-P	0.00858^{hijkl}	2.602^{bcd}	14.190^{abcde}
	D14-P	0.00753^{jkl}	2.057^{cdefg}	15.080^{abcd}

Appendix 5. The effect of interaction between breeding lines and days of treatment on leaf area, weight of an individual leaf, and shoot DW sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf, and shoot DW were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction BLxD	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)
BL43D3	412.4^{bcd}	0.1002^{cde}	1.1500^{de}
BL45D3	557.0^{a}	0.1102^{bcde}	1.6410^{de}
BL47D3	320.4^{def}	0.0896^{e}	1.3790^{de}
BL49D3	499.8^{ab}	0.1277^{abcde}	1.2750^{de}
BL43D5	324.7^{def}	0.1081^{bcde}	0.9510^{e}
BL45D5	386.0^{cde}	0.1365^{abcd}	1.4290^{de}
BL47D5	268.8^{f}	0.0973^{de}	1.2580^{de}
BL49D5	442.1^{bc}	0.1456^{ab}	1.3950^{de}
BL43D7	366.3^{cdef}	0.1131^{bcde}	0.9510^{e}
BL45D7	470.3^{abc}	0.1620^{a}	1.8730^{cd}
BL47D7	286.9^{ef}	0.0917^{e}	1.8300^{cde}
BL49D7	462.2^{abc}	0.1557^{a}	1.7740^{cde}
BL43D14	322.9^{def}	0.0985^{cde}	1.4880^{de}
BL45D14	397.8^{bcd}	0.1379^{abc}	2.7460^{ab}
BL47D14	290.5^{ef}	0.0905^{e}	2.5570^{bc}
BL49D14	402.3^{bcd}	0.1262^{abcde}	3.3780^{a}

Appendix 6. The effect of interaction between breeding lines and phosphorus treatment on leaf area, weight of an individual leaf and shoot DW sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf and shoot DW were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction BL x P	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)
BL43+P	355.0^{b}	0.1104^{bc}	1.080^{c}
BL45+P	441.0^{a}	0.1397^{a}	1.749^{ab}
BL47+P	281.3^{c}	0.0891^{c}	1.798^{ab}
BL49+P	444.4^{a}	0.1519^{a}	2.089^{a}
BL47-P	358.1^{b}	0.0995^{c}	1.189^{bc}
BL43-P	464.5^{a}	0.1336^{ab}	2.095^{a}
BL45-P	302.0^{c}	0.0955^{c}	1.714^{abc}
BL49-P	458.7^{a}	0.1258^{ab}	1.823^{ab}

Appendix 7. Effect of interaction between days of treatment (D) and phosphorus (P) application on leaf area, weight of an individual leaf and shoot DW sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf and shoot DW were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction DxP	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)
D3+P	459.2^{a}	0.1176^{ab}	1.3830^{b}
D5+P	333.1^{b}	0.1310^{a}	1.2590^{b}
D7+P	402.6^{ab}	0.1358^{a}	1.7150^{b}
D14+P	327.0^{b}	0.1066^{ab}	2.3590^{a}
D3-P	435.6^{a}	0.0963^{b}	1.3400^{b}
D5-P	377.7^{ab}	0.1128^{ab}	1.2570^{b}
D7-P	390.3^{ab}	0.1254^{ab}	1.4990^{b}
D14-P	379.8^{ab}	0.1200^{ab}	2.7250^{a}

Appendix 8. Effect of interaction between BL, days of treatment (D) and phosphorus (P) application on leaf area, weight of an individual leaf and shoot DW sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf and shoot DW were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Breeding Line	Days and P treatment	Leaf area (mm)	Weight of an individual leaf (g)	Shoot DW (g)
BL 43	D3+P	426.0^{abcdef}	0.1062^{bcdef}	1.208^{de}
	D5+P	317.3^{defg}	0.1276^{abcdef}	0.912^{e}
	D7+P	356.0^{cdefg}	0.1149^{abcdef}	1.125^{de}
	D14+P	320.7^{defg}	0.0928^{cdef}	1.077^{de}
	D3-P	$398.8^{abcdefg}$	0.0941^{cdef}	1.092^{de}
	D5-P	332.0^{defg}	0.0887^{def}	0.991^{e}
	D7-P	376.5^{bcdefg}	0.1112^{bcdef}	0.777^{e}
	D14-P	325.2^{defg}	0.1042^{cdef}	1.898^{cde}
BL 45	D3+P	559.3^{a}	0.1234^{abcdef}	1.604^{cde}
	D5+P	356.8^{cdefg}	0.1507^{abc}	1.506^{cde}
	D7+P	513.0^{abc}	0.1721^{a}	1.882^{cde}
	D14+P	335.0^{defg}	0.1125^{bcdef}	2.004^{cde}
	D3-P	554.7^{a}	0.0970^{cdef}	1.678^{cde}
	D5-P	$415.2^{abcdefg}$	0.1224^{abcdef}	1.351^{de}
	D7-P	427.7^{abcdef}	0.1518^{abc}	1.865^{cde}
	D14-P	460.5^{abcde}	0.1633^{ab}	3.487^{ab}
BL47	D3+P	331.0^{defg}	0.0959^{cdef}	1.428^{de}
	D5+P	253.5^{g}	0.0953^{cdef}	1.401^{de}
	D7+P	281.7^{fg}	0.0855^{def}	1.986^{cde}
	D14+P	259.2^{g}	0.0796^{f}	2.377^{bcd}
	D3-P	309.8^{efg}	0.0834^{ef}	1.330^{de}
	D5-P	284.0^{fg}	0.0994^{cdef}	1.115^{de}
	D7-P	292.2^{fg}	0.0978^{cdef}	1.674^{cde}
	D14-P	321.8^{defg}	0.1013^{cdef}	2.736^{bc}
BL49	D3+P	520.3^{ab}	0.1449^{abcd}	1.291^{de}
	D5+P	$404.7^{abcdefg}$	0.1505^{abc}	1.218^{de}
	D7+P	459.7^{abcde}	0.1707^{a}	1.868^{cde}
	D14+P	393.0^{bcdefg}	0.1414^{abcde}	3.978^{a}
	D3-P	479.2^{abcd}	0.1106^{bcdef}	1.259^{de}
	D5-P	479.5^{abcd}	0.1407^{abcde}	1.573^{cde}
	D7-P	464.7^{abcde}	0.1408^{abcde}	1.681^{cde}
	D14-P	$411.5^{abcdefg}$	0.1111^{cdef}	2.778^{bc}

Appendix 9. The effect of interaction between breeding lines and days of treatment on fresh biomass yield and root:shoot FW ratio sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and root:shoot FW ratio were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction BLxD	Fresh biomass yield (g/plant)			Root:Shoot FW ratio
	Root	Shoot	Total	
BL43D3	2.0850^{bc}	7.9690^{cd}	10.050^{cd}	0.2640^{ab}
BL45D3	2.3450^{bc}	9.9800^{bcd}	12.320^{bcd}	0.2509^{ab}
BL47D3	2.2930^{bc}	9.2430^{bcd}	11.540^{bcd}	0.2751^{ab}
BL49D3	2.0620^{bc}	7.9050^{cd}	9.970^{cd}	0.2757^{ab}
BL43D5	1.5860^{c}	5.6040^{d}	7.190^{d}	0.2992^{a}
BL45D5	1.8090^{c}	7.7140^{cd}	9.520^{cd}	0.2485^{ab}
BL47D5	1.9170^{bc}	7.4970^{cd}	9.410^{cd}	0.2705^{ab}
BL49D5	2.2080^{bc}	7.7950^{cd}	10.000^{cd}	0.2858^{ab}
BL43D7	1.4910^{c}	5.9840^{d}	7.480^{d}	0.2612^{ab}
BL45D7	2.3410^{bc}	10.6070^{bcd}	12.950^{bcd}	0.2425^{ab}
BL47D7	2.6220^{bc}	12.2440^{abc}	14.870^{bc}	0.2212^{b}
BL49D7	2.7450^{bc}	10.0270^{bcd}	12.770^{bcd}	0.2874^{ab}
BL43D14	2.3030^{bc}	8.2490^{cd}	10.550^{cd}	0.2990^{a}
BL45D14	3.1500^{b}	13.6450^{ab}	16.800^{ab}	0.2357^{ab}
BL47D14	3.0910^{b}	14.2790^{ab}	17.370^{ab}	0.2195^{b}
BL49D14	4.2490^{a}	16.8450^{a}	21.090^{a}	0.2585^{ab}

Appendix 10. The effect of interaction between breeding lines and phosphorus treatment on fresh biomass yield and root:shoot FW ratio sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and root:shoot FW ratio were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction BL x P	Fresh biomass yield (g/plant)			Root:Shoot FW ratio
	Root	Shoot	Total	
BL43+P	1.8300^{c}	6.9070^{b}	8.740^{b}	0.2776^{ab}
BL45+P	2.2170^{abc}	9.9460^{ab}	12.160^{ab}	0.2379^{b}
BL47+P	2.4920^{abc}	11.2880^{a}	13.780^{a}	0.2330^{b}
BL49+P	2.8820^{a}	11.5380^{a}	14.420^{a}	0.2636^{ab}
BL43-P	1.9020^{bc}	6.9960^{b}	8.900^{b}	0.2841^{a}
BL45-P	2.6050^{abc}	11.0270^{a}	13.630^{a}	0.2509^{ab}
BL47-P	2.4700^{abc}	10.3440^{ab}	12.810^{ab}	0.2602^{ab}
BL49-P	2.7500^{ab}	9.7480^{ab}	12.500^{ab}	0.2900^{a}

Appendix 11. The effect of interaction between days of treatment and phosphorus application on fresh biomass yield and root:shoot FW ratio, sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and root:shoot FW ratio were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction DxP	Fresh biomass yield (g/plant)			Root:Shoot FW ratio
	Root	Shoot	Total	
D3+P	2.0310^{c}	9.1120^{bcd}	11.140^{b}	0.2282^{d}
D5+P	1.7810^{c}	7.2840^{cd}	9.060^{b}	0.2669^{abcd}
D7+P	2.4780^{abc}	10.8360^{abc}	13.310^{ab}	0.2429^{bcd}
D14+P	3.1310^{ab}	12.4470^{ab}	15.580^{a}	0.2741^{abc}
D3-P	2.3610^{bc}	8.4360^{cd}	10.800^{b}	0.3047^{a}
D5-P	1.9790^{c}	7.0210^{d}	9.000^{b}	0.2850^{ab}
D7-P	2.1220^{c}	8.5950^{cd}	10.720^{b}	0.2633^{abcd}
D14-P	3.2650^{a}	14.0630^{a}	17.330^{a}	0.2322^{cd}

Appendix 12. The effect of interaction between breeding lines, days of treatment and phosphorus application on fresh biomass yield and root:shoot FW ratio sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and root:shoot FW ratio were determined. Values are the means from six independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Breeding Line		Fresh biomass yield (g/plant)			Root:Shoot FW ratio
		Root	Shoot	Total	
BL 43	D3+P	2.0920^{bcd}	8.5550^{cdef}	10.646^{cdef}	0.2367^{bcde}
	D5+P	1.3820^{d}	5.5040^{ef}	6.886^{ef}	0.2763^{abcde}
	D7+P	1.7330^{cd}	7.1700^{cdef}	8.903^{cdef}	0.2480^{bcde}
	D14+P	2.1150^{bcd}	6.3980^{def}	8.512^{cdef}	0.3493^{a}
	D3-P	2.0780^{bcd}	7.3830^{cdef}	9.461^{cdef}	0.2913^{abcde}
	D5-P	1.7900^{cd}	5.7030^{ef}	7.493^{def}	0.3220^{abc}
	D7-P	1.2500^{d}	4.7980^{f}	6.047^{f}	0.2744^{abcde}
	D14-P	2.4910^{bcd}	10.1000^{bcdef}	12.591^{bcdef}	0.2487^{bcde}
BL 45	D3+P	1.9940^{bcd}	10.2690^{bcdef}	12.260^{bcdef}	0.2073^{e}
	D5+P	1.9010^{cd}	8.3240^{cdef}	10.220^{cdef}	0.2467^{bcde}
	D7+P	2.4970^{bcd}	11.2790^{bcdef}	13.780^{bcdef}	0.2422^{bcde}
	D14+P	2.4750^{bcd}	9.9120^{bcdef}	12.390^{bcdef}	0.2555^{abcde}
	D3-P	2.6960^{bcd}	9.6900^{cdef}	12.390^{bcdef}	0.2946^{abcde}
	D5-P	1.7170^{cd}	7.1050^{cdef}	8.820^{cdef}	0.2504^{bcde}
	D7-P	2.1840^{bcd}	9.9350^{bcdef}	12.120^{bcdef}	0.2429^{bcde}
	D14-P	3.8250^{ab}	17.3790^{ab}	21.200^{ab}	0.2159^{de}
BL47	D3+P	2.1380^{bcd}	9.5500^{cdef}	11.690^{cdef}	0.2247^{de}
	D5+P	2.1170^{bcd}	8.2100^{cdef}	10.330^{cdef}	0.2779^{abcde}
	D7+P	2.6800^{bcd}	13.4200^{abcde}	16.100^{abcde}	0.2013^{e}
	D14+P	3.0310^{bcd}	13.9700^{abcd}	17.000^{abcd}	0.2281^{cde}
	D3-P	2.4490^{bcd}	8.9300^{cdef}	11.380^{cdef}	0.3255^{ab}
	D5-P	1.7160^{cd}	6.7800^{cdef}	8.500^{cdef}	0.2631^{abcde}
	D7-P	2.5630^{bcd}	11.0700^{bcdef}	13.630^{bcdef}	0.2411^{bcde}
	D14-P	3.1510^{bcd}	14.5900^{abc}	17.740^{abc}	0.2110^{de}
BL49	D3+P	1.9020^{cd}	8.0720^{cdef}	9.970^{cdef}	0.2440^{bcde}
	D5+P	1.7230^{cd}	7.0950^{cdef}	8.820^{cdef}	0.2669^{abcde}
	D7+P	3.0000^{bcd}	11.4780^{bcdef}	14.480^{bcdef}	0.2800^{abcde}
	D14+P	4.9030^{a}	19.5070^{a}	24.410^{a}	0.2637^{abcde}
	D3-P	2.2230^{bcd}	7.7380^{cdef}	9.960^{cdef}	0.3075^{abcd}
	D5-P	2.6930^{bcd}	8.4940^{cdef}	11.190^{cdef}	0.3047^{abcd}
	D7-P	2.4910^{bcd}	8.5760^{cdef}	11.070^{cdef}	0.2947^{abcde}
	D14-P	3.5950^{abc}	14.1830^{abcd}	17.780^{abc}	0.2533^{bcde}

Appendix 13. The effect of interaction between genotypes and days of treatment on Pi content and APase activity sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi content and APase activity were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction GxD	Pi content (%P.g^{-1}FW)	Acid phosphatase activity	
		Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
43-7D7	0.021583^{a}	1.5054^{ab}	12.2761^{ab}
43-8D7	0.014667^{bcde}	1.9067^{ab}	9.4923^{bcd}
45-14D7	0.013623^{cde}	1.9200^{ab}	9.4166^{bcd}
45-4D7	0.014225^{cde}	1.3102^{b}	9.8422^{abcd}
47-9D7	0.017819^{b}	1.4010^{ab}	12.9855^{ab}
43-7D14	0.017000^{bc}	1.6737^{ab}	8.2627^{cde}
43-8D14	0.018137^{b}	2.2740^{a}	9.7256^{abcd}
45-14D14	0.012343^{de}	1.7951^{ab}	8.3920^{cde}
45-4D14	0.012662^{de}	1.6460^{ab}	11.3335^{abc}
47-9D14	0.014319^{cde}	1.7954^{ab}	13.3859^{a}
43-7D21	0.015353^{bcd}	1.4943^{ab}	6.9228^{de}
43-8D21	0.013593^{cde}	1.8869^{ab}	5.0848^{e}
45-14D21	0.011127^{e}	1.2907^{b}	6.4404^{de}
45-4D21	0.011613^{e}	1.4956^{ab}	7.4934^{de}
47-9D21	0.012917^{de}	1.3969^{ab}	6.4310^{de}

Appendix 14. The effect of interaction between genotypes and phosphorus treatment on Pi content and APase activity of plants sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi content and APase activity were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction GxP	Pi content (%P.g^{-1}FW)	Acid phosphatase activity	
		Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
43-7+P	0.021565^{a}	1.1379^{d}	8.7892^{bc}
43-8+P	0.018588^{b}	1.4071^{cd}	7.2759^{bc}
45-14+P	0.014121^{c}	1.3033^{cd}	6.3448^{c}
45-4+P	0.014565^{c}	1.1541^{d}	8.2617^{bc}
47-9+P	0.018618^{b}	1.2919^{cd}	8.8187^{bc}
43-7-P	0.014392^{c}	1.9778^{b}	9.5185^{bc}
43-8-P	0.012343^{cd}	2.6379^{a}	8.9258^{bc}
45-14-P	0.010608^{d}	2.0339^{b}	9.8212^{abc}
45-4-P	0.011101^{d}	1.8138^{bc}	10.8511^{ab}
47-9-P	0.011418^{d}	1.7703^{bc}	13.0496^{a}

Appendix 15. Effect of interaction between days of treatment (D) and phosphorus (P) treatment on Pi content and APase activity sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi content and APase activity were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction DxP	Pi content (%P.g^{-1}FW)	Acid phosphatase activity	
		Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
D7+P	0.018047^{a}	1.2846^{c}	9.3870^{b}
D14+P	0.018322^{a}	1.2913^{c}	8.5736^{bc}
D21+P	0.016106^{b}	1.2006^{c}	5.7336^{d}
D7-P	0.014720^{b}	1.9327^{b}	12.2181^{a}
D14-P	0.011463^{c}	2.3824^{a}	11.8663^{a}
D21-P	0.009735^{c}	1.8251^{b}	7.2154^{cd}

Appendix 16. The effect of interaction between genotypes, days of treatment and phosphorus application on Pi content and APase activity sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and Pi content and APase activity were determined. Values are the means from individual leaf extracts excised from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction GxDxP	Pi content (%P.g^{-1}FW)	Acid phosphatase activity	
		Cell wall fraction OD_{405} (min^{-1}g^{-1}FW)	Total soluble fraction OD_{405} (min^{-1}g^{-1}FW)
43-7D7+P	0.022775^{a}	1.0542^{ef}	12.1437^{abc}
43-7D14+P	0.021716^{ab}	1.2396^{ef}	$8.1209^{cdefghi}$
43-7D21+P	0.020206^{abcd}	1.1198^{ef}	6.1031^{efghi}
43-7D7-P	0.020392^{abc}	1.9565^{bcdef}	12.4085^{abc}
43-7D14-P	$0.012284^{fghijklm}$	2.1079^{abcde}	$8.4046^{cdefghi}$
43-7D21-P	0.010500^{jklm}	1.8689^{bcdef}	$7.7425^{cdefghi}$
43-8D7+P	0.014941^{efghij}	1.4124^{def}	$8.4235^{cdefghi}$
43-8D14+P	0.024275^{a}	1.5372^{cdef}	$8.6316^{cdefghi}$
43-8D21+P	0.016549^{cdef}	1.2718^{ef}	4.7726^{i}
43-8D7-P	0.014392^{efghij}	2.4011^{abcd}	10.5611^{cdef}
43-8D14-P	$0.012000^{ghijklm}$	3.0108^{a}	10.8196^{cdef}
43-8D21-P	0.010637^{ijklm}	2.5020^{abc}	5.3969^{ghi}
45-14D7+P	0.015088^{efghi}	1.7686^{bcdef}	$8.3541^{cdefghi}$
45-14D14+P	$0.013765^{efghijk}$	0.9905^{f}	5.7122^{fghi}
45-14D21+P	$0.013510^{fghijkl}$	1.1507^{ef}	4.9681^{hi}
45-14D7-P	$0.012157^{fghijklm}$	2.0713^{abcde}	10.4791^{cdefg}
45-14D14-P	0.010922^{ijklm}	2.5997^{ab}	11.0718^{cde}
45-14D21-P	0.008745^{m}	1.4307^{def}	$7.9128^{cdefghi}$
45-4D7+P	0.015824^{efgh}	0.9792^{f}	$8.1460^{cdefghi}$
45-4D14+P	$0.013892^{efghijk}$	1.2024^{ef}	9.8864^{cdefgh}
45-4D21+P	$0.013980^{efghijk}$	1.2806^{ef}	6.7525^{defghi}
45-4D7-P	$0.012627^{fghijklm}$	1.6413^{bcdef}	11.5384^{bcd}
45-4D14-P	0.011431^{hijklm}	2.0896^{abcde}	12.7806^{abc}
45-4D21-P	0.009245^{lm}	1.7106^{bcdef}	$8.2343^{cdefghi}$
47-9D7+P	0.021608^{ab}	1.2087^{ef}	9.8675^{cdefgh}
47-9D14+P	0.017961^{bcde}	1.4868^{cdef}	10.5169^{cdef}
47-9D21+P	0.016284^{defg}	1.1803^{ef}	6.0716^{efghi}
47-9D7-P	0.014029^{efghij}	1.5933^{bcdef}	16.1036^{ab}
47-9D14-P	0.010676^{ijklm}	2.1041^{abcde}	16.2549^{a}
47-9D21-P	0.009549^{klm}	1.6135^{bcdef}	6.7904^{defghi}

Appendix 17. The effect of interaction between genotypes and days of treatment on leaf area, weight of an individual leaf and shoot DW sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf and shoot DW were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction GxD	Leaf Area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)
43-7D7	114.67^{e}	0.04217^{e}	0.1370^{e}
43-8D7	161.33^{de}	0.05483^{de}	0.3537^{de}
45-14D7	477.67^{ab}	0.17750^{a}	1.1178^{c}
45-4D7	387.17^{c}	0.10383^{bc}	0.4740^{de}
47-9D7	189.00^{de}	0.08200^{cd}	0.5657^{de}
43-7D14	135.33^{e}	0.05550^{de}	0.2153^{de}
43-8D14	161.17^{de}	0.06033^{de}	0.4525^{de}
45-14D14	493.17^{a}	0.20017^{a}	2.0927^{ab}
45-4D14	372.33^{c}	0.11500^{b}	0.7330^{cd}
47-9D14	187.00^{de}	0.07200^{cde}	0.3862^{de}
43-7D21	165.00^{de}	0.06000^{de}	0.2400^{de}
43-8D21	159.00^{de}	0.06633^{de}	0.7603^{cd}
45-14D21	413.17^{bc}	0.20100^{a}	2.3863^{a}
45-4D21	469.33^{ab}	0.17150^{a}	1.2040^{c}
47-9D21	223.83^{d}	0.10117^{bc}	1.7050^{b}

Appendix 18. The effect of interaction between genotypes and phosphorus treatment on leaf area, weight of an individual leaf and shoot DW sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf and shoot DW were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction GxP	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)
43-7+P	131.89^{d}	0.04956^{e}	0.1558^{f}
43-8+P	163.44^{cd}	0.05911^{e}	0.5882^{cdef}
45-14+P	454.78^{ab}	0.20267^{a}	1.5738^{b}
45-4+P	398.33^{b}	0.11956^{bc}	0.7516^{cde}
47-9+P	203.00^{c}	0.07633^{de}	0.6466^{cdef}
43-7-P	144.78^{cd}	0.05556^{e}	0.2391^{ef}
43-8-P	157.56^{cd}	0.06189^{e}	0.4561^{def}
45-14-P	467.89^{a}	0.18311^{a}	2.1574^{a}
45-4-P	420.89^{ab}	0.14067^{b}	0.8558^{cd}
47-9-P	196.89^{cd}	0.09378^{cd}	1.1247^{bc}

Appendix 19. Effect of interaction between days of treatment (D) and phosphorus (P) treatment on leaf area, weight of an individual leaf and shoot DW sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf and shoot DW were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction DxP	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)
D7+P	261.07^{a}	0.08893^{a}	0.4007^{c}
D14+P	242.47^{a}	0.08787^{a}	0.6303^{bc}
D21+P	307.33^{a}	0.12753^{a}	1.1985^{ab}
D7-P	270.87^{a}	0.09520^{a}	0.6585^{bc}
D14-P	297.13^{a}	0.11333^{a}	0.9216^{abc}
D21-P	264.80^{a}	0.11247^{a}	1.3197^{a}

Appendix 20. The effect of interaction between genotypes, days of treatment and phosphorus application on leaf area, weight of an individual leaf and shoot DW sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and leaf area, weight of an individual leaf and shoot DW were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction GxDxP	Leaf area (mm^2)	Weight of an individual leaf (g)	Shoot DW (g)
43-7D7+P	118.67^{g}	0.04367^{ij}	0.1050^{g}
43-7D14+P	120.33^{g}	0.04433^{ij}	0.2127^{fg}
43-7D21+P	156.67^{fg}	0.06067^{hij}	0.1497^{fg}
43-7D7-P	110.67^{g}	0.04067^{j}	0.1690^{fg}
43-7D14-P	150.33^{fg}	0.06667^{ghij}	0.2180^{fg}
43-7D21-P	173.33^{fg}	0.05933^{hij}	0.3303^{fg}
43-8D7+P	151.33^{fg}	0.05867^{hij}	0.2890^{fg}
43-8D14+P	157.67^{fg}	0.05333^{hij}	0.5813^{defg}
43-8D21+P	181.33^{fg}	0.06533^{ghij}	0.8943^{cdefg}
43-8D7-P	171.33^{fg}	0.05100^{hij}	0.4183^{efg}
43-8D14-P	164.67^{fg}	0.06733^{ghij}	0.3237^{fg}
43-8D21-P	136.67^{fg}	0.06733^{ghij}	0.6263^{defg}
45-14D7+P	463.00^{abc}	0.18833^{bc}	0.9277^{cdef}
45-14D14+P	438.00^{abc}	0.18600^{bc}	1.5417^{bc}
45-14D21+P	463.33^{abc}	0.23367^{a}	2.2520^{a}
45-14D7-P	492.33^{ab}	0.16667^{cd}	1.3080^{bcd}
45-14D14-P	548.33^{a}	0.21433^{ab}	2.6437^{a}
45-14D21-P	363.00^{cd}	0.16833^{cd}	2.5207^{a}
45-4D7+P	393.33^{bcd}	0.08033^{ghij}	0.3957^{efg}
45-4D14+P	317.33^{de}	0.09233^{fgh}	0.5790^{defg}
45-4D21+P	484.33^{ab}	0.18600^{bc}	1.2800^{bcd}
45-4D7-P	381.00^{bcd}	0.12733^{def}	0.5523^{defg}
45-4D14-P	427.33^{bc}	0.13767^{de}	0.8870^{cdefg}
45-4D21-P	454.33^{abc}	0.15700^{cd}	1.1280^{cde}
47-9D7+P	179.00^{fg}	0.07367^{ghij}	0.2863^{fg}
47-9D14+P	179.00^{fg}	0.06333^{hij}	0.2367^{fg}
47-9D21+P	251.00^{ef}	0.09200^{fgh}	1.4167^{bc}
47-9D7-P	199.00^{fg}	0.09033^{fghi}	0.8450^{cdefg}
47-9D14-P	195.00^{fg}	0.08067^{ghij}	0.5357^{defg}
47-9D21-P	196.67^{fg}	0.11033^{efg}	1.9933^{ab}

Appendix 21. The effect of interaction between genotypes and days of treatment on fresh biomass yield, and root:shoot FW ratio sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and root:shoot FW ratio were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction GxD	Fresh biomass yield (g/plant)			Root:Shoot FW ratio
	Root	Shoot	Total	
43-7D7	0.3373^{f}	0.7428^{g}	1.0802^{f}	0.4537^{abc}
43-8D7	1.1693^{def}	2.3410^{efg}	3.5103^{def}	0.5171^{a}
45-14D7	2.2725^{bc}	6.5927^{bc}	8.8652^{b}	0.3570^{cde}
45-4D7	1.1295^{def}	2.5722^{efg}	3.7017^{def}	0.4063^{abcd}
47-9D7	1.0743^{def}	2.8878^{efg}	3.9622^{def}	0.3658^{cde}
43-7D14	0.5898^{ef}	1.1135^{fg}	1.7033^{f}	0.5263^{a}
43-8D14	1.3217^{de}	3.0053^{efg}	4.3270^{def}	0.4504^{abc}
45-14D14	3.0432^{ab}	10.5537^{a}	13.5968^{a}	$0.2992d^{e}$
45-4D14	1.7132^{cd}	3.6125^{def}	5.3257^{cde}	0.4966^{ab}
47-9D14	0.6063^{ef}	1.8567^{efg}	2.4630^{ef}	0.3485^{cde}
43-7D21	0.6680^{ef}	1.3167^{fg}	1.9847^{ef}	0.4936^{ab}
43-8D21	1.8418^{cd}	4.4343^{cde}	6.2762^{bcd}	0.4599^{abc}
45-14D21	3.2120^{a}	11.1272^{a}	14.3392^{a}	0.2922^{de}
45-4D21	2.2465^{bc}	5.9132^{bcd}	8.1597^{bc}	0.3885^{bcd}
47-9D21	1.9313^{cd}	7.3643^{b}	9.2957^{b}	0.2575^{e}

Appendix 22. The effect of interaction between genotypes and phosphorus treatment on fresh biomass yield and root:shoot FW ratio sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and root:shoot FW ratio were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction G x P	Fresh biomass yield (g/plant)			Root:Shoot FW ratio
	Root	Shoot	Total	
43-7+P	0.4432^{f}	0.8860^{c}	1.3292^{c}	0.4961^{a}
43-8+P	1.5997^{cd}	3.8058^{b}	5.4054^{b}	0.4630^{a}
45-14+P	2.3432^{b}	8.6504^{a}	10.9937^{a}	0.2957^{c}
45-4+P	1.5115^{cd}	4.0046^{b}	5.5160^{b}	0.3990^{ab}
47-9+P	0.9921^{def}	3.3956^{bc}	4.3877^{bc}	0.3321^{bc}
43-7-P	0.6202^{ef}	1.2293^{c}	1.8496^{c}	0.4863^{a}
43-8-P	1.2889^{cde}	2.7147^{bc}	4.0036^{bc}	0.4886^{a}
45-14-P	3.3419^{a}	10.1986^{a}	13.5404^{a}	0.3365^{bc}
45-4-P	1.8813^{bc}	4.0607^{b}	5.9420^{b}	0.4620^{a}
47-9-P	1.4159^{cd}	4.6770^{b}	6.0929^{b}	0.3157^{bc}

Appendix 23. The effect of interaction between days of treatment and phosphorus application on fresh biomass yield and root:shoot FW ratio sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and root:shoot FW ratio were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction DxP	Fresh biomass yield (g/plant)			Root:Shoot FW ratio
	Root	Shoot	Total	
D7+P	0.9608^{b}	2.4006^{c}	3.3614^{b}	0.4195^{a}
D14+P	1.3153^{ab}	3.5248^{bc}	4.8401^{ab}	0.4446^{a}
D21+P	1.8577^{a}	6.5200^{a}	8.3777^{a}	0.3274^{b}
D7-P	1.4324^{ab}	3.6540^{bc}	5.0864^{ab}	0.4204^{a}
D14-P	1.5944^{ab}	4.5319^{abc}	6.1263^{ab}	0.4038^{ab}
D21-P	2.1021^{a}	5.5423^{ab}	7.6444^{a}	0.4293^{a}

Appendix 24. The effect of interaction between genotypes, days of treatment and phosphorus application on fresh biomass yield and root:shoot FW ratio, sampled at the specific time indicated. Plants were maintained in either P-containing or P-deprived (-P) media, harvested at the time indicated and fresh biomass yield and root:shoot FW ratio were determined. Values are the means from three independent plants. The different letters above in the same column indicate that the values are significantly different at the 0.05 level, according to Duncan's multiple range test.

Interaction GxDxP	Fresh biomass yield (g/plant)			Root:Shoot FW ratio
	Root	Shoot	Total	
43-7D7+P	0.2790^{j}	0.5353^{h}	0.8143^{l}	0.5119^{ab}
43-7D14+P	0.6140^{hij}	1.1437^{h}	1.7577^{jkl}	0.5290^{ab}
43-7D21+P	0.4367^{ij}	0.9790^{h}	1.4157^{jkl}	0.4474^{abcde}
43-7D7-P	0.3957^{ij}	0.9503^{h}	1.3460^{kl}	$0.3955^{abcdefg}$
43-7D14-P	0.5657^{ij}	1.0833^{h}	1.6490^{jkl}	0.5235^{ab}
43-7D21-P	0.8993^{fghij}	1.6543^{gh}	2.5537^{ijkl}	0.5398^{a}
43-8D7+P	$1.0367^{defghij}$	1.9107^{fgh}	2.9473^{hijkl}	0.5454^{a}
43-8D14+P	$1.6743^{bcdefghi}$	3.8937^{defgh}	$5.5680^{defghijkl}$	0.4459^{abcde}
43-8D21+P	2.0880^{bcdef}	5.6130^{cdef}	7.7010^{cdefgh}	0.3976^{abcdef}
43-8D7-P	$1.3020^{cdefghij}$	2.7713^{efgh}	4.0733^{ghijkl}	0.4888^{abc}
43-8D14-P	0.9690^{efghij}	2.1170^{efgh}	3.0860^{hijkl}	0.4549^{abcd}
43-8D21-P	$1.5957^{bcdefghi}$	3.2557^{efgh}	$4.8513^{efghijkl}$	0.5221^{ab}
45-14D7+P	2.1360^{bcdef}	5.8377^{cde}	7.9737^{cdefg}	$0.3676^{bcdefgh}$
45-14D14+P	2.2930^{bcd}	8.2953^{bc}	10.5883^{bc}	0.3010^{defgh}
45-14D21+P	2.6007^{b}	11.8183^{a}	14.4190^{ab}	0.2186^{h}
45-14D7-P	2.4090^{bc}	7.3477^{bcd}	9.7567^{cd}	0.3464^{cdefgh}
45-14D14-P	3.7933^{a}	12.8120^{a}	16.6053^{a}	0.2974^{defgh}
45-14D21-P	3.8233^{a}	10.4360^{ab}	14.2593^{ab}	$0.3658^{bcdefgh}$
45-4D7+P	0.7907^{ghij}	2.2107^{efgh}	3.0014^{hijkl}	0.3310^{cdefgh}
45-4D14+P	$1.4213^{bcdefghij}$	2.8967^{efgh}	4.3180^{ghijkl}	0.5251^{ab}
45-4D21+P	2.3223^{bc}	6.9063^{cd}	9.2287^{cde}	0.3408^{cdefgh}
45-4D7-P	$1.4683^{bcdefghij}$	2.9337^{efgh}	$4.4020^{fghijkl}$	0.4816^{abc}
45-4D14-P	2.0050^{bcdefg}	4.3283^{defgh}	$6.3333^{cdefghij}$	0.4682^{abc}
45-4D21-P	2.1707^{bcde}	4.9200^{cdefg}	$7.0907^{cdefghi}$	0.4363^{abcdef}
47-9D7+P	0.5617^{ij}	1.5087^{gh}	2.0703^{jkl}	0.3417^{cdefgh}
47-9D14+P	0.5737^{ij}	1.3947^{gh}	1.9683^{jkl}	0.4220^{abcdef}
47-9D21+P	$1.8410^{bcdefgh}$	7.2833^{bcd}	9.1243^{cdef}	0.2326^{gh}
47-9D7-P	$1.5870^{bcdefghi}$	4.2670^{defgh}	$5.8540^{cdefghijk}$	$0.3899^{abcdefg}$
47-9D14-P	0.6390^{hij}	2.3187^{efgh}	2.9577^{hijkl}	0.2749^{fgh}
47-9D21-P	2.0217^{bcdefg}	7.4453^{bcd}	9.4670^{cde}	0.2824^{efgh}

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