

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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

**A thesis presented in partial fulfilment of the requirements
for the degree of**

**Master of Philosophy
in Plant Biology**

**at
Massey University
Palmerston North
New Zealand**

JOLLANDA EFFENDY

2007

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 (P_i) 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.

P_i 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 P_i deprivation.

In terms of individual BLs and genotypes, the screening results showed that BL 49 and genotype 45-14 displayed a constant P_i 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.

Acknowledgments

I would like to thank my senior supervisor, Prof. Michael T. McManus, for his excellent supervision, patience, and encouragement throughout my study and during the preparation of this thesis.

I would like to thank my co-supervisor, Dr. Derek Woodfield, for sharing his knowledge and expertise in white clover, in addition to his guidance, advice, discussion and construction comments.

Other very important contributions were made by the member of the lab, both past and present. They are Dr. Balance Chen, Dr. Ning, Dr. Sarah Dorling, Jan, Susanna, Ludivine, Marissa, Aluh, Elizabeth, Rachel, Fiona, Mathew, Matthew, Lena, and Cait.

I wish to thank all of my friends for their wonderful companionship and support throughout my study at Massey University.

I wish to thank Rosemary Knowles and Mark Herrings for their wonderful friendship during the first two years I was in New Zealand.

I wish to express my sincere gratitude to the NZAID for funding my study in New Zealand, and Pattimura University (UNPATTI) Ambon, Indonesia for granting permission to study here. Similar appreciations go to the Institute of Molecular Biosciences and New Zealand Society of Plant Physiologists for providing generous travel and accommodation assistance grant to make it possible for me to go to a conference in Christchurch and in Australia.

Finally, I would like to thank my parents and Ulf who always support me through their love, encouragement and understanding.

Table of Contents

Abstract	i
Acknowledgments	iii
List of Figures	viii
List of Tables	x
List of Appendices	xii
Abbreviations	xiv
Chapter One: Introduction	1
<i>Overview</i>	<i>1</i>
<i>1.1. Mineral nutrition in plants</i>	<i>1</i>
<i>1.2. Phosphorus and plant growth</i>	<i>2</i>
<i>1.2.1. Effect of Pi deprivation on root growth</i>	<i>4</i>
<i>1.2.2. Effect of Pi deprivation on shoot growth</i>	<i>6</i>
<i>1.2.3. Effect of Pi deprivation on Root:Shoot Ratio</i>	<i>6</i>
<i>1.2.4. Effect of Pi deprivation on leaf area and weight of individual leaf</i>	<i>7</i>
<i>1.2.5. Effect of Pi deprivation on biomass accumulation</i>	<i>8</i>
<i>1.3. Strategies adopted by plants to withstand Pi-deprivation</i>	<i>9</i>
<i>1.4. Pi deprivation induced changes in gene expression</i>	<i>9</i>
<i>1.5. Pi homeostasis and signal transduction during Pi deprivation</i>	<i>10</i>
<i>1.6. Pi starvation and secondary metabolism</i>	<i>11</i>
<i>1.7. Pi deprivation and root architecture</i>	<i>12</i>
<i>1.8. Pi deprivation and Pi transporters</i>	<i>12</i>
<i>1.9. Pi deprivation and the role of ethylene</i>	<i>14</i>
<i>1.10. The role played by APase in P-deprived roots</i>	<i>15</i>

1.10.1. <i>Acid phosphatase biochemistry and terminology</i>	15
1.10.2. <i>Distribution, localization and function of plant APases</i>	16
1.10.2.1. <i>Intracellular APases</i>	17
1.10.2.2. <i>Extracellular APases</i>	18
1.10.3. <i>Acid phosphatases and changes in gene expression during Pi deprivation</i>	18
1.10.4. <i>Correlation between APase activity and Pi content during Pi deprivation</i>	19
2.0. <i>White clover</i>	21
2.1. <i>White clover in New Zealand</i>	21
2.2. <i>White clover and phosphate uptake and utilization</i>	22
3.0 <i>Thesis Aims</i>	24
Chapter Two: Materials and Methods	26
2.1. <i>Plant Material</i>	26
2.2. <i>Growth of white clover plants</i>	27
2.2.1. <i>Stock Plants</i>	27
2.2.2. <i>Growth of white clover in liquid media</i>	31
2.3. <i>Chemicals</i>	32
2.4. <i>Measurement of leaf phosphate</i>	32
2.5. <i>Extraction of soluble and cell wall proteins</i>	34
2.6. <i>Acid phosphatase assays</i>	34
2.7. <i>Acid phosphatase isoenzyme analyses</i>	36
2.7.1. <i>High pH non-dissociating discontinuous buffer system (Davis Method)</i>	36
2.7.2. <i>Low pH non-dissociating discontinuous buffer systems (Reisfeld Method)</i>	37
2.7.3. <i>Staining high and low non-dissociating discontinuous native gels</i>	37
2.8. <i>Statistical Analyses</i>	38

Chapter Three: Results	39
3.1. <i>White clover growth in phosphate deprived conditions.....</i>	39
3.1.1. <i>Visual changes in white clover plants maintained in –P conditions</i>	39
3.1.2. <i>Selection of root material for the extraction and characterization of acid phosphatase</i>	39
3.2. <i>Selected breeding line screening.....</i>	40
3.2.1. <i>Onset of P-deficiency in selected breeding lines of white clover leaf tissue</i>	40
3.2.2. <i>Comparison of acid phosphatase (APase) activity of selected breeding lines of white clover grown in P-containing and P-deprived media.....</i>	45
3.2.3. <i>Relative plant growth in response to Pi deprivation</i>	46
3.3. <i>Selected Genotypic Screening.....</i>	63
3.3.1. <i>Onset of P-deficiency in selected genotypes of white clover leaf tissue</i>	63
3.3.2. <i>Comparison of acid phosphatase (APase) activity in white clover grown in P-containing and P-deprived media</i>	68
3.3.3. <i>Relative plant growth in response to Pi deprivation</i>	71
3.3.4. <i>Comparison of acid phosphatase isoenzymes of selected genotypes grown in P-contained and P-deprived media.....</i>	87
 Chapter Four: Discussion	 89
4.1. <i>Phosphorus (Pi) levels in leaves and cell wall APase activity in roots of white clover during Pi deprivation</i>	89
4.1.1. <i>Pi levels in leaves.....</i>	89
4.1.2. <i>Acid phosphatase activity in root cell walls of white clover ...</i>	92
4.1.3. <i>Effect of Pi deprivation on Pi levels and cell wall APase activity in roots of white clover</i>	93
4.1.4. <i>Effect of Pi deprivation on APase isoenzymes of selected white clover genotypes</i>	96
4.2. <i>Effect of Pi deprivation on several growth parameters of white clover.....</i>	97

4.2.1. <i>Effect of Pi deprivation on leaf area, weight of an individual leaf and shoot DW</i>	98
4.2.1.1. <i>Leaf area and weight of an individual leaf</i>	98
4.2.1.2. <i>Shoot DW</i>	99
4.2.2. <i>Effect of Pi deprivation on fresh biomass yield and R:S ratio</i>	101
4.2.2.1. <i>Root FW</i>	101
4.2.2.2. <i>Shoot FW</i>	103
4.2.2.3. <i>Total biomass (BM) FW</i>	104
4.2.2.4. <i>Root:Shoot FW ratio</i>	107
Chapter Five: Summary	109
<i>Future Work:</i>	<i>112</i>
Appendices	114
Bibliography	138

List of Figures

Figure 2.1.	The growth of five selected white clover genotypes grown in P-containing and P-deprived media at day 21:	28
Figure 2.2.	Phosphate standard curve for leaf phosphate content determination.	33
Figure 2.3.	Nitrophenol (ρ NP) standard curve for the determination of the amount of ρ NP liberated by hydrolysis of acid phosphatase.....	35
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.....	44
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.....	47
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.....	48
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.....	53
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.....	54
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.....	55
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.	59
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.	60
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.....	61
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.	62
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.....	67

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.....	70
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.....	72
Figure 3.14. Leaf area of selected genotypes grown in P+ or P- media and sampled at day 7, 14, and 21.....	77
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.	78
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	79
Figure 3.17. Root fresh weight from selected genotypes grown in P+ or P- media and sampled at day 7, 14, and 21	83
Figure 3.18. Shoot fresh weight from selected genotypes grown in P+ or P- media and sampled at day 7, 14, and 21	84
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	85
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	86
Figure 3.21. Separation of acid phosphatase isoenzymes.....	88

List of Tables

Table 2.1.	Five selected genotypes used in genotypic screening. Genotypes selected over replicates of all genotypes using Screening 1 (Breeding line screening).....	27
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).....	30
Table 2.3.	Composition of resolving and stacking gels used for high pH discontinuous native gel.	37
Table 2.4.	Composition of resolving and stacking gels used for low pH discontinuous native gel.	38
Table 3.1.	Probability of F for Pi content and APase activity of selected breeding lines of white clover.....	41
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....	42
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).....	43
Table 3.4.	Probability of F for leaf area, weight of an individual leaf, shoot DW, fresh biomass yield and R:S FW ratio.....	49
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.....	50
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	52
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	57
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	58
Table 3.9.	Probability of F for Pi content and APase activity of selected genotypes of white clover.....	64
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.....	65

Table 3.11. Comparison of Pi content and acid phosphatase activity from each genotype grown in +P and -P at the specific time indicated	66
Table 3.12. Probability of F for several growth parameters of selected genotypes of white clover.....	73
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.....	74
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	76
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	81
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	82

List of 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....	114
Appendix 2.	The effect of interaction between breeding lines and phosphorus treatment on Pi content and APase activity sampled at the the specific time indicated	115
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	116
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.....	117
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.	118
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	119
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	120
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	121
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	122
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	123
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.....	124
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	125

Appendix 13.	The effect of interaction between genotypes and days of treatment on Pi content and APase activity sampled at the specific time indicated....	126
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.....	127
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.....	128
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.	129
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.....	130
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.....	131
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.....	132
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.....	133
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.....	134
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.....	135
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.....	136
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.....	137

Abbreviations

$A_{405\text{ nm}}$	absorbance [$\log(I_0/I)$] in a 1 cm light path at 405 nm
3-PGA	3-phosphoglyceric acid
APase	acid phosphatase
APS	ammonium persulphate
<i>AtACP5</i>	<i>Arabidopsis thaliana</i> acid phosphatase type 5
<i>AtPAP12</i>	<i>Arabidopsis thaliana</i> purple acid phosphatase type 12
AVG	aminoethoxyvinylglycine
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
<i>LePS2</i>	<i>Lycopersicon esculentum</i> 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
$^{\circ}\text{C}$	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
<i>Pht</i>	phosphate transporter
Pi	inorganic phosphate
<i>psr1</i>	phosphate starvation response type 1
PUE	phosphorus utilization efficiency
<i>pup1</i>	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
<i>SPT2</i>	<i>Saccharomyces cerevisiae</i> phosphate transporter type 2
TEMED	N,N,N',N'-Tetramethylethylenediamine
Tris	tris (hydroxymethyl) methylamine
V	Volt ($\text{kg m}^2 \text{s}^{-3} \text{A}^{-1}$)
v/v	volume per volume
W	Watt ($\text{kg m}^2 \text{s}^{-3}$)
w/v	weight per volume
ρNPP	ρ -nitrophenyl phosphate