PHOSPHATE ABSORPTION BY *Arabidopsis thaliana*: THE EFFECTS OF PHOSPHORUS NUTRITIONAL STATUS

A thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Plant Biology and Biotechnology at Massey University, Palmerston North, New Zealand.

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ABSTRACT

The effect of phosphorus nutritional status on phosphate uptake within the concentration range of the high affinity uptake mechanism, and subsequent translocation to the shoot was investigated in the plant species *Arabidopsis thaliana*.

Plants of different nutritional status were generated by exposure to different set phosphate concentrations throughout an aseptic hydroponic growing period. Alternatively phosphorus deficiency was induced by growth at high concentrations of phosphate followed by a period of 5 days in phosphate-free hydroponic solution. In effect these growth conditions resulted in plants of distinguishable phenotypic character with respect to phosphate absorption, phosphate translocation, arsenate sensitivity and root-shoot ratio.

To determine absorption kinetics nutrient depletion trials were carried out in which phosphate uptake was measured by monitoring the lose of phosphate from depletion solutions of set initial phosphate concentration to which the root systems of intact plants were exposed. Km and Vmax kinetic parameters were calculated from the depletion trial data using the software package "Igor Pro".

Influx and net phosphate uptake was determined by setting the initial phosphate concentration of the depletion trials using either ^{32}P labelled KH_2PO_4 or non-labelled KH_2PO_4 respectively. Radioactivity was measured by counting the Cerenkov radiation in a scintillation counter. Non-labelled phosphate depletion was measured by either spectrophotometric assay or ion chromatography.

To asses the effect of the phosphate analogue arsenate on phosphate influx, ^{32}P labelled phosphate uptake was measured with arsenate (KH_2AsO_4) present in the depletion solution at a concentration of 20 μM.

Phosphate translocation was determined by counting the Cerenkov radiation in the roots and shoots separately of plants that had been exposed to the ^{32}P labelled depletion solutions.

Under the conditions of this project, phosphorus deficient plants exhibited alterations in the kinetic parameters Km and Vmax for phosphate uptake that were dependent on how the deficiency was induced. For plants that were grown continuously at low phosphate concentrations Km was decreased without a concomitant change in Vmax. For plants that were grown at high concentrations of phosphate followed by a 5 day period of phosphate starvation, a significant increase in Vmax was recorded without an associated change to Km.

Phosphate uptake was found to be severely inhibited by the presence of arsenate in the depletion solution. Greatest inhibition however was found not to occur at the level of absorption into the plant root system but rather appeared to be at a site involved in phosphate loading into the xylem. Inhibition at this site was also found to be greatest in low phosphorus status plants. From these results it is suggested that plants of low phosphorus status possess high affinity phosphate xylem loading mechanisms, induced under conditions of phosphorus deficiency, which have a greater susceptibility to arsenate competitive inhibition and toxicity than equivalent xylem loading mechanisms in high phosphorus status plants.

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CHAPTER ONE

INTRODUCTION

1.1. PEOPLE AND PHOSPHORUS.

Phosphorus is an essential element of all known organisms.

Healthy adult humans contain approximately 800 g of this element, most of which (700 g) is found in the skeleton, with the remainder used in cellular structure, nuclei acid composition and complex processes of metabolism (Colgan 1993).

Daily phosphorus requirements are met through the animal and plant products we consume in the form of dairy foods, meats, flour, cereals, fruits and vegetables. The ultimate source however of biosphere phosphorus is a layer of the earth's crust termed the lithosphere of which phosphorus comprises approximately 0.12%. Within the lithosphere phosphorus is found predominantly as a component of apatite rock of chemical composition $Ca_{10}(PO_4,CO_3)_6(F,OH)_{2-3}$ (Cathcart 1980). Through weathering of phosphorus containing rock, phosphorus is released into the soil as soluble phosphates, and is absorbed into plants (and consequently into the food chain) as either $H_2PO_4^-$ or HPO_4^{2-} depending on the pH of the soil. At an approximate pH of 7.0 both ion species are found in the same concentrations. At lower pH levels, and those most commonly found in soils, $H_2PO_4^-$ is the predominant phosphate ion species.

1.2. PHOSPHATE AND THE LAND.

In order to maintain productive farming schemes, phosphate removed from the land as farm produce, or made unavailable through soil leaching, or immobilisation in the soil by chemical transformation into less soluble forms, must be replaced. This usually occurs by the application of commercial mineral fertilisers such as superphosphate.

Based on phosphate sorption tests, in which a quantity of soil is shaken for a short time in a solution of known phosphate concentration and the quantity of phosphate removed by the soil is determined, During (1972) classified New Zealand soils into three phosphate sorption classes, and estimated the amount of superphosphate required to produce maximal pasture yields if the soils were cultivated/virgin or almost virgin land.

Class 1. Low phosphorus sorbing soils: includes the "yellow-grey transitional to yellow-brown earths" of the Manawatu, Wairarapa, and Hawke's Bay. For maximal growth on these soils an initial dressing of 6.75 to 10.08 q/ha is suggested.[1]

Class 2. Medium phosphorus sorbing soils: includes soils derived from the Taupo and Kaharoa pumice showers about 1,700 and 800 years ago, respectively, and the large group of soils known as yellow-brown earths. For maximal growth on these soils an initial dressing of 6.75 to 17.92 q/ha is suggested.

Class 3. High phosphorus sorbing soils: these include the yellow-brown loams, soils which were formed largely on volcanic ash beds deposited more than 5,000 years ago, and soils derived from basalt. Highest pasture yields on these soils is achieved at a dressing rate of approximately 25.76 q/ha.

In recent years concern has arisen about the impact phosphatic fertilisers are having on the environment. The main reason for this concern is that not all added fertiliser is immediately consumed by the crops or pastures it was intended for, but is lost into the environment, leaching into the water table. Phosphate fertiliser runoff into streams and lakes has been linked to eutrophication where, through excessive phosphate levels in water, enhanced algal growth occurs causing a detrimental shift in the aerobic/anaerobic balance of the ecosystem.

In order to counteract the environmental and economic costs associated with fertiliser application, a greater understanding of how plants obtain nutrients from the environment is required with a view to optimising the processes involved.

[1] q/ha = hundred weight/acre * 1.12

1.3. PHOSPHORUS AND PLANTS.

In plants phosphorus is classified as a macronutrient and is one of several essential elements required in relatively large amounts for the plant to complete its life cycle. For the average representative higher plant, an adequate level of phosphorus is considered to be about 60 μmole g^{-1} of dry matter or approximately 0.2% of the plants total dry weight. This places phosphorus as the eighth most abundant element within plant dry matter behind such elements as oxygen and carbon each comprising approximately 45% (Epstein 1972).

Within plants, phosphorus is involved in a number of essential processes including energy metabolism and metabolic regulation. During energy metabolism energy trapped through photosynthesis or released during glycolysis or respiration is coupled to the synthesis of the nucleotide, adenosine triphosphate commonly referred to as ATP. On break down of this molecule by an enzyme catalysed process termed hydrolysis approximately 30 kJ of free energy is released (per mole) and harnessed to drive various cellular reactions such as protein synthesis, and cellular ion regulation. This form of free energy can also be used for the synthesis of other pyrophosphate bond containing nucleotides such as uridine triphosphate an energy rich intermediate in the sucrose biosynthetic pathway and guanosine triphosphate an intermediate in the biosynthesis of cellulose, a structural component of cell walls and the most abundant organic compound on earth.

Phosphorus is also involved in metabolic regulation by altering the activity of specific target proteins and enzymes through covalent attachment (phosphorylation) or removal of phosphate molecules (dephosphorylation). These processes are catalyzed by protein kinases, or phosphoprotein phosphatases respectively and result in either the inactivation, activation and/or changes to the allosteric properties of the target proteins (Ranjeva and Boudet 1987).

In a structural role, phosphorus is an intrinsic component in many biomolecules. As a component of phosphodiester bonds phosphorus is involved in linking ribonucleosides

into molecules of DNA and RNA. Similar bonds form the bridges connecting the hydrophobic and hydrophilic components of phospholipids conferring a number of the structural and functional properties associated with biological membranes.

1.4. PHOSPHATE UPTAKE.

In solution, ions move in response to at least two physical forces. Phosphate a negatively charged ion is acted upon by electrical gradients, being repelled from regions of negative charge and attracted to areas of positive charge. Having a specific chemical nature, phosphate ions also possess chemical potential, and diffuse from areas of high phosphate concentration to less dense regions. When the chemical and electrical potentials acting on an ion in solution are of equal and opposing strengths the solute is said to be in a state of passive flux equilibrium. For a given ion this state can be determined mathematically using the Nernst equation (Hall and Baker 1977).

In the early 1930's E. Munch a German plant physiologist introduced the concept (which has persisted to the present day) of apoplast and symplast as pathways for solute movement within plants. The apoplast he referred to as the nonliving component of plants consisting of the tracheids, vessel elements and fibers of the xylem, and the interconnecting walls of all cells. The rest of the plant he termed the symplast, consisting of the living cytoplasm of cells interconnected via plasmodesmata. In general nutrient salts enter vascular plants by direct absorption from the environment into the apoplast of the root system, driven by the electrochemical gradients that exist between the rhizosphere and the plant's root.

The first cells of a root encountered by ions during absorption are those of the epidermal layer and their associated root hairs (Fig 1.1). Within the apoplast and symplast pathways, nutrients traverse into the deeper cortical root zone, toward a tightly packed layer of cells called the endodermis. Incorporated within the radial and transverse cell walls of these specialised cells is a water resistant, suberin impregnation called the Casparian strip. In order for ions in the apoplast to penetrate beyond this point, to the vascular strands within the stele of the root, absorption into the symplast must first occur. This involves traversing the selectively permeable cytoplasmic membranes associated with each cell, overcoming actively maintained membrane electrical potentials

and for many ions moving from an area of low to high concentration, a direction that is thermodynamically not favoured.

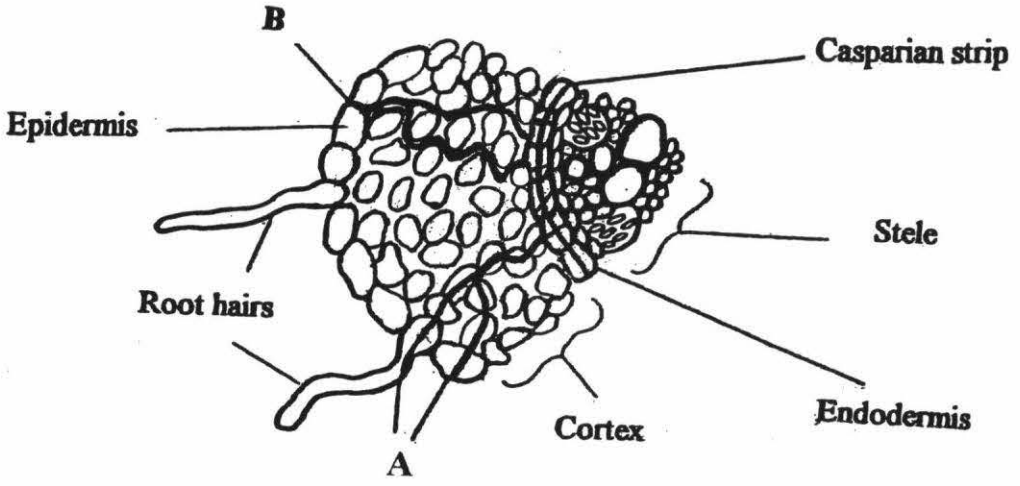


Figure 1.1 Schematic diagram of a dicotyledonous root in cross section showing the symplastic (A) and apoplastic (B) pathway of ion transport.

In the early 1950's Epstein and Hagan (1952) investigating ion absorption in barley roots, found that cation uptake proceeded in a manner similar to the kinetic patterns observed during processes of enzyme catalysis. Employing mathematical equations and terminology associated with enzyme kinetics, Epstein and Hagan pioneered the use of the Michaelis Menten kinetic parameters Vmax (the maximum rate of transport when all carriers are loaded with ion substrate) and Km (the dissociation constant of the carrier ion-complex, or ion concentration at which uptake proceeds at a rate half that of Vmax) to describe ion absorption into plant root systems. Based on this, and similar work, the present carrier concept was proposed. In carrier mediated uptake it is believed that protein sub-units within the plasma membrane selectively bind ions to be transported, forming a carrier- ion complex. Using cellular derived energy the carrier is able to shuttle the bound ion to the inner side of the membrane releasing it unmodified into the cytoplasm.

In 1977 Bowling, Graham and Dunlop (Bowling et al., 1978) discovered a direct link between cellular membrane electrical potential (PD) and phosphate uptake by the roots of *Helianthus annuus*. In their paper they outline evidence that suggests the involvement of an electrogenic pump in phosphate absorption. In a later paper Sakano and colleagues (Sakano et al., 1992) using pH dependant fluorescent dye and ^{31}P-NMR (Nuclear Magnetic Resonance) spectroscopy actually measured acidification of the cytoplasm of suspension cultured cells in direct response to proton coupled co-transport of phosphate.

Over the past few decades there have been a number of hypotheses formulated to explain carrier mediated counteraction of electrochemical forces opposing passive uptake of ions by cells. Mistrik and Ullrich in a recent paper (Mistrik and Ullrich 1996) review some of these hypotheses outlining the pros and cons established through experimentation.

Today it is widely accepted that the uptake of inorganic phosphate across plant plasma membranes proceeds via co-transport with protons along a gradient of proton motive force generated by plasma membrane H^+- ATPases (Fig 1.2). (Ullrich-Eberius et al., 1984, Dunlop 1989, Harper et al 1990, Sakano 1990, Ullrich and Novacky 1990, Lew 1991, Kim et al., 1994, Mimura 1995, Regenberg et al., 1995, Baunsgaard et al., 1996, Mistrik and Ullrich 1996).

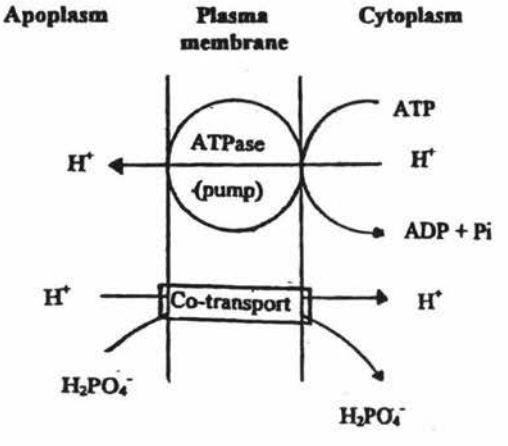


Figure 1.2 Model for the functioning and location of the electrogenic proton pump (H^+ - ATPase), and the phosphate:proton co-transport system.

1.5. UPTAKE KINETICS.

Because plants are unable to choose the mineral composition of the soils to which they are exposed they must be capable of growing under a wide and varied range of soil nutrient conditions. In investigations that have examined ion uptake over a range of concentrations (Epstein 1966, Doddema and Telkamp 1979, Furihata et al., 1992) it has been reported that absorption proceeds in a multiphasic manner with the apparent existence of several saturable kinetic classes (Fig 1.3). In a study investigating uptake of phosphate in maize root sections (Nandi et al., 1987) five such multiphasic classes were identified over concentrations ranging from 3 μM to 75 mM. Epstein (1972) refers to the recognised high affinity, low-Km (1-20 μM) absorption class, as "mechanism one" which follows simple Michaelis Menten kinetics and is highly specific for the species of ion it transports. The subsequent uptake classes of low affinity, high Km values ranging from 50 to 1,000 μM are considered by Epstein to consist of a second type of less specific uptake system, "mechanism two", containing several active binding sites for which ions compete. Such proposed dual uptake mechanisms for phosphate have been characterised for *Lemna gibba* (Ullrich-Eberius et al., 1984), *Spirodela* (McPharlin and Bieleski 1987), *Zea mays* (Nandi et al., 1987), *Catharanthus roseus* (Furihata et al., 1992), and *Holcus lanatus* (Meharg and Macnair 1992).

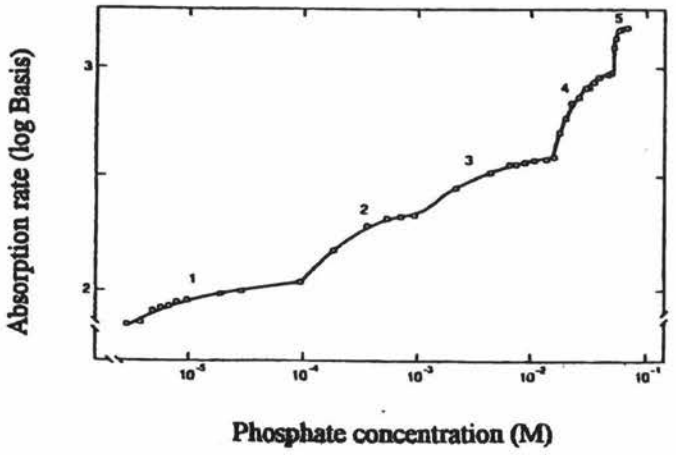


Figure 1.3 Graph of phosphate uptake rate as a function of phosphate concentration in the media surrounding maize root sections. Five separate kinetic classes are observed (numbered 1-5) over a 25,000 concentration range. Copied from Nandi et al., (1987).

In opposition to Epstein's two mechanism uptake system, is the proposal that only a single mechanism for each ion is in operation with multiphasic uptake the result of an increase in the concentration of specific carriers in the plasma membrane rather than a second mechanism with different kinetic characteristics. Shimogawara and Usuda (1995) have proposed this to be the case for phosphate uptake in suspension-cultured tobacco cells. In their investigations they found that phosphate uptake profiles by both phosphate starved and non-starved cells could be explained by assuming the existence of only one kind of Michaelis-Menten type phosphate transport system. They also reported that apparent increases in the maximum rate of phosphate uptake (Vmax) induced by phosphate starvation was completely preventable by the protein synthesis inhibitor cycloheximide. Thus it was concluded that the induced Vmax shift was not in response to the operation of a second mechanism but rather an increase in the concentration of uptake mechanisms of the type already present in the plasma membrane. Phosphate deficiency induced increases in Vmax without concurrent changes in Km have also been reported for other plant species and tissue types (Anghinoni and Barber 1980, Drew et al 1984).

1.6. Cmin.

Just as plant species vary in their respective kinetic uptake parameters, Km and Vmax, in relation to phosphate absorption, different species also vary in the minimum concentration to which they are able to deplete phosphate to in solution. This concentration is termed Cmin. Mouat (1983) investigating phosphate uptake in New Zealand pasture plants, recorded lowest Cmin values for the grass species New Zealand browntop (*Agrostis tenuis Sibth*) and Ruanui ryegrass (*Lolium perenne L.*) of 0.04 μM and 0.08 μM respectively. Highest Cmin values were recorded for the legumes 'Grasslands Huia' white clover (*Trifolium repens L.*) and Kent wild white clover (*Trifolium repens L.*) of 0.54 μM and 0.22 μM respectively.

Other studies have revealed that Cmin values not only vary between species but also vary within species depending on plant age. In experiments carried out by Jungk and Barber (1975), a three fold difference in Cmin value was recorded between 14-day-old corn plants and 52-day-old corn plants, with the older plants exhibiting the lower Cmin

capability of 0.1 μM. For soybean plants, Cmin values were found to increase with plant age, from 0.04 μM recorded for 14-day-old plants shifting to 0.17 μM for 75 day-old-plants (Edwards and Barber 1976).

1.7. *Arabidopsis* A MODEL PLANT.

For investigation of the physiological processes involved in phosphate uptake, it is often useful to have a model plant system that can be applied to the wider range of commercially important plant species. In recent years *Arabidopsis thaliana* has been afforded this role. *Arabidopsis* belongs to a class of plants called the Angiosperms, a group of approximately 250,000 plant species, hypothesised to have evolved from a common ancestor within the last 150 million years. Of the plant species used in agriculture and horticulture today the overwhelming majority, like *Arabidopsis* are angiosperms.

Apart from *Arabidopsis'* close evolutionary relationship to the plants it models, it possesses a number of other useful characteristics. *Arabidopsis* has a relatively short life-cycle of approximately 1.5 months, this coupled with high seed production make it an ideal subject for classical mendelian type studies involving intergeneration genetic comparisons and back-crossings. Of greater importance to molecular biologists is the simple design of the plant's genomic construct, with small amounts of "junk" DNA and most genes present as single copy DNA sequences. Because of this, *Arabidopsis* genetic sequences have been used successfully as genetic probes to isolate and identify homologous genes in other angiosperms (Meyerowitz 1994).

1.8. *Arabidopsis* AND PHOSPHATE.

Many aspects of phosphate uptake by *Arabidopsis thaliana* have already been investigated. Krannitz, Aarssen and Lefebvre (1991b) have characterised a number of physiological and morphological characters relating to phosphate utilisation in inbred homozygous lines of *Arabidopsis thaliana*. They report on initial uptake rates, Cmin levels, root to shoot ratios, and specific root length in plants grown aseptically for sixteen days, with seed reserves the only source of phosphate.

Dunlop et al., (1997) have reported on the kinetic properties of phosphate absorption in 3-day-old *Arabidopsis* plants of different phosphorus status, and offer support for a dual mechanism phosphate uptake system.

Arabidopsis mutants have been used to identify and clone genetic sequences that code for plasma membrane proton pumps. These pumps are involved in the maintenance of the proton motive gradients across plasma membranes that are the driving force behind membrane transport processes such as phosphate uptake (Harper et al., 1990, Regenberg et al., 1995, Baunsgaard et al., 1996).

An *Arabidopsis* mutant has been isolated that can only transfer 3 to 10 % the amount of phosphate to the shoot that wildtype plants translocate when grown in media of low phosphate concentrations (< 200 μM). At high media phosphate concentrations (> 200 μM) both wildtype and mutant plants are found to transport the same amounts of phosphate to their shoots. These results suggest that the mutant plant is deficient in activity of a high affinity phosphate transfer protein at the site of xylem phosphate loading (Poirier et al., 1991).

Two high affinity phosphate transporters have been genetically sequenced from *Arabidopsis* plants, that show a high degree of amino acid sequence similarity with high affinity phosphate transporters of *Saccharomyces cerevisiae*, *Neurospora crassa*, and the mycorrhizal fungus *Glomus versiforme*. The number of mRNA transcripts coding for the two transport mechanisms, are reported to increase as phosphate starvation increases and are found to be localised in the root system. (Muchhal et al., 1996).

1.9. PHOSPATE ABSORPTION BY *Arabidopsis thaliana*: THE EFFECTS OF PHOSPHORUS NUTRITIONAL STATUS.

In this study I have set out to add to the rapidly growing knowledge, being collected on *Arabidopsis thaliana* by investigating the relationship between phosphorus nutritional status and phosphate absorption and translocation.

This work has focused on phosphate uptake within the concentration range favoured by the high affinity uptake system (Epstein 1972) and equivalent to concentrations present in soil solutions (Marschner 1995). Plants of different nutritional status were generated by exposure to different set phosphate levels throughout most of their growth period. In effect this resulted in plants of distinguishable phenotypic character with respect to phosphate absorption kinetics, phosphate translocation, arsenate sensitivity, and certain morphological characteristics associated with adequate and inadequate levels of phosphorus nutrition (Salisbury and Ross 1985, Krannitz et al., 1991b, Garcia and Ascencio 1992). To determine absorption kinetics, nutrient depletion trials were carried out in which phosphate uptake was measured by monitoring the lose of phosphate from depletion solutions of set initial phosphate concentration to which the root systems of intact plants were exposed. Km and Vmax kinetic parameters were calculated from depletion trial data using the software package "Igor Pro" (Wavemetrics Inc., Lake Oswego, Oregon).

Influx and net phosphate uptake were determined in separate depletion trials by setting the initial phosphate concentration of the depletion solution with either ^{32}P labelled KH_2PO_4 or non-labelled KH_2PO_4 respectively. Non-labelled phosphate depletion was measured by either spectrophotometric assay or ion chromatography. Radioactivity was measured by counting Cerenkov radioactivity in a scintillation counter.

To determine the effect of arsenate on phosphate influx, 200 nmol of arsenate (KH_2AsO_4) was added to the ^{32}P labelled KH_2PO_4 depletion solution to give an arsenate concentration of 20 μM. Phosphate influx was measured as previously, by counting the Cerenkov radiation in each sample aliquot removed at regular time intervals from the depletion solution.

Phosphate translocation was determined by counting the Cerenkov radiation in the roots and shoots separately of plants that had been exposed to the ^{32}P labelled depletion solutions.

This study also compares phosphate absorption and translocation in *Arabidopsis* plants that have been made phosphorus deficient using two different techniques. In one technique phosphorus deficiency was induced by growing plants continuously at low phosphate solution concentrations (KH_2PO_4 = 10 μM) throughout the growing period. Alternatively plants were grown at high phosphate concentrations (KH_2PO_4 = 250 μM) over the initial stages of growth, followed by complete removal of phosphate from the nutrient solution for the final 5 days of growth prior to carrying out the depletion trials. Both these techniques are commonly reported in the materials and methods of literature reporting studies in phosphorus deficiency. However seldom are different growing methods considered when comparing uptake kinetic parameters sited from different studies. In this study the validity of such comparisons is examined.

CHAPTER TWO

MATERIALS AND METHODS.

2.1. PLANT MATERIAL

Arabidopsis thaliana (L) Heynh c.v. Columbia seeds were germinated in potting mix and the resulting plants grown to maturity under glass house conditions. The seed harvested from these plants was used to generate all experimental plant material. All collected seed was stored in an air tight container , at room temperature, in the dark until required.

2.2. ASEPTIC GROWING METHODS.

In order to eliminate nutrient uptake interference from bacteria and fungus that under natural conditions cohabit in the root rhizosphere, all experimental plant material was grown under sterile, hydroponic conditions. The initial step of aseptic growth, seed surface sterilisation, was carried out as described by Griffiths (1996), adapted from the method of Valvekens et al., (1988) and was as follows:- in a laminar flow cabinet, a quantity of seed was placed in an eppendorf tube containing 1.5 ml of 70% ethanol. After a two minute vortexing period the ethanol was siphoned off using a sterile glass pipette, and replaced by a wash solution consisting of 0.5% w/v sodium dodecyl sulphate (BDH) and 5% v/v Janola (a commercial bleach with sodium hypochlorite (31.5 g/L) as the active ingredient). Following a 20 minute wash period the seeds were rinsed 6 times in sterile (autoclaved) Milli Q water (reagent grade water), and spread, using a sterile wire loop onto the surface of germination medium. The germination medium was composed of 0.8% agar w/v (Bacto-agar by Difco) supplemented with Murashige and Skoog (M.S.) nutrient solution (Table 2.1) and contained within sealed sterile petri dishes. After germination, 2 - 3 days, plants were transferred to surface sterilised (70% ethanol wash for 30 minutes followed by 6 rinses in sterile Milli Q water), polystyrene floats and placed on sterile liquid nutrient solution maintained in Sigma Plant Cell Culture Pots.

Throughout the experiments all plants were germinated and grown in a Temperzone Controlled Environment Growth Cabinet. The light intensity of the cabinet was 500 microeinsteins m^{-2} sec^{-1} and the temperature was 24°C or 25°C as specified for each experiment. All sterilisation procedures were carried out using sterilised equipment, in a sterile Laminar air flow hood. Germination media and nutrient solutions were made up in Milli Q water and autoclaved before experimental use.

2.3. NUTRIENT SOLUTIONS.

The nutrient solutions used throughout this project (Table 2.1) were based on the inorganic components of the basal medium used by Murashige and Skoog (1962), a modification of White's nutrient solution (White 1943) designed originally for the culturing of excised roots.

2.4. MODIFICATION OF THE MURASHIGE AND SKOOG SOLUTION.

In order to obtain plants of different phosphorus status but equal in all other nutritional aspects one of the M.S. stocks was modified. Instead of potassium dihydrogen orthophosphate being added as part of stock solution C, a stock containing several nutrient components, the phosphate ion was added separately as a 50 mM KH_2PO_4 solution (stock G, Table 2.1). Depending on the specifications of each experiment, the nutrient solutions were at either full, half or one tenth strength of those used in Murashige and Skoog's work. The basal nutrient solution (consisting of stocks A to F) was made up as required in 10 litre volumes. Stock G was made by supplementing a litre of the basal solution with 6.805 g of KH_2PO_4. Both the basal solution and stock G were adjusted to pH 6.0 through the addition of 2 M KOH or 2 M HCL as required. All chemicals used were of either reagent or analytical grade. Stock solutions were refrigerated until required.

2.5. NUTRIENT DEPLETION TECHNIQUE.

After the *Arabidopsis* plants had been grown hydroponically for a period of time, as outlined for each experiment, all plants were removed from their nutrient solutions, washed twice in phosphate free nutrient solution and placed in separate identical experimental solutions of common phosphate concentration. Continuos stirring of the solutions was maintained by an orbital shaker (Gyrotory Shaker model G2) operating at 80 RPM. Phosphate uptake by plants was determined by measuring the phosphate concentration in aliquots removed from the experimental solutions at regular time intervals (Drew et al 1984).

2.6. PHOSPHATE ANALYSES.

Three methods to determine phosphate concentration in aliquots of experimental solutions and plant material were adopted at different stages over the course of this project. These were ion chromatography, spectrophotometric assay and scintillation counting of radioactively labelled phosphate. Each of these methods is outlined in detail under the heading of the particular experiment in which they were used.

2.7. STATISTICAL ANALYSES .

P-values for the experimental data were determined by one way analyses of variance (ANOVA) using the statistical computer programme 'Vital' version 3.05, created by Robert Fletcher, AgResearch Grasslands, Palmerston North.

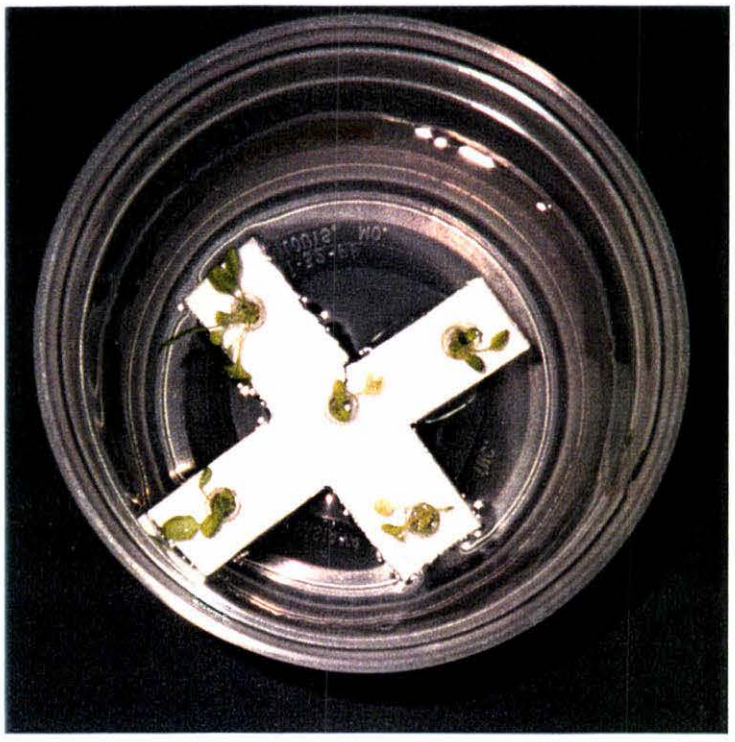
Table 2.1. Composition of stock, and nutrient solutions.

stock	constituents	concentration of stock solution (g/l)	volume of stock in final ½ strength medium (ml/l)	volume of stock in final 1/10 strength medium (ml/l)
A	NH_4NO_3	82.5	10	2
B	KNO_3	95	10	2
C	H_3BO_3	1.24	2.5	0.5
	KI	0.165		
	$Na_2MoO_4.2H_2O$	0.05		
	$CoCl_2.2H_2O$	0.007		
D	$CaCl_2.2H_2O$	58.8	2.5	0.5
E	$MgSO_4.7H_2O$	74.0	2.5	0.5
	$MnSO_4.4H_2O$	4.46		
	$ZnSO_4.7H_2O$	1.71		
	$CuSO_4.5H_2O$	0.005		
F	Ferric EDTA	8.0	2.5	0.5
G	diluted stock A-F			
	+ KH_2PO_4	6.805 (50 mM)		

2.8. EXPERIMENT 1.

Approximately eighty *Arabidopsis* seeds were spread onto ½ strength M.S. gel plates and placed in a growth cabinet running on a 14 hour photo period at 24°C. Under these conditions most seeds germinated within three days. After eight days of growth, young plants were plucked from the plates using sterile tweezers and gently inserted into half length (the top half) of 1 ml Gilson pipette tips (1 plant per tip). The tips were then fixed into polystyrene floats (5 tips per float), made from disposable drinking cups, and placed in Sigma Plant Cell Culture Pots (Fig 2.1). Each culture pot contained 500 ml of full strength M.S. solution supplemented to one of five different phosphate treatment concentrations:- 0.5, 2, 8, 32, or 128 μM. Eight replicate pots were set up for each phosphate treatment. The plants were then returned to the growth cabinet and grown under these conditions for 38 days (Fig 2.2). Nutrient replenishment was carried out weekly by renewing the solutions with fresh sterile M.S solution of appropriate phosphate level. At the conclusion of the hydroponic growing period the *Arabidopsis* plants were removed from the pots and blotted dry with paper towels. Fresh weights for root and shoot material per pot was recorded as were the dry weights after an 18 hour period at 80°C in a drying oven.

A



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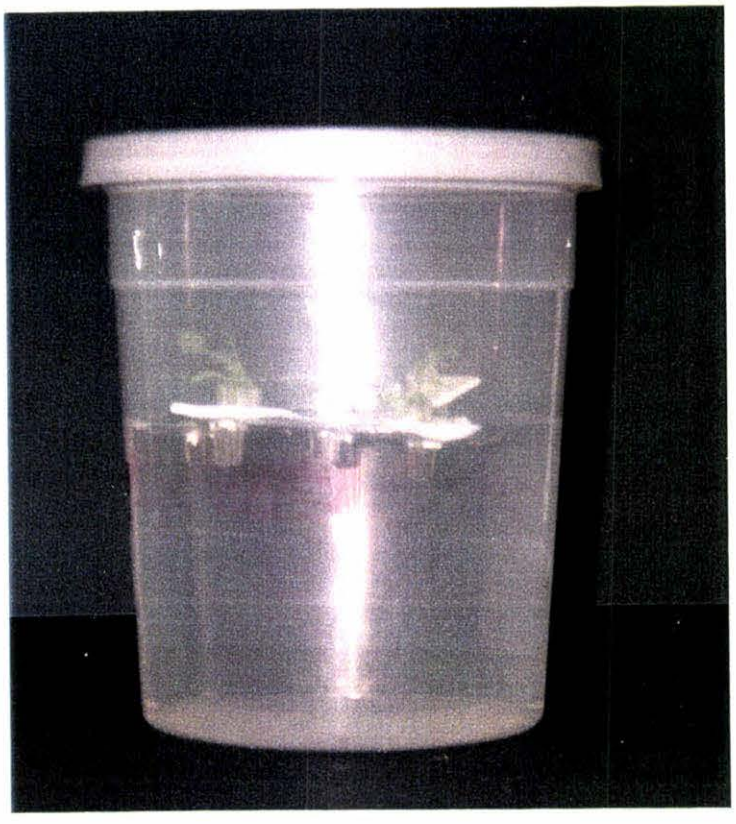


Figure 2.1. View from above (A) and side (B) of plant growing method employed in experiments1-3.

Figure 2.2. Set up of pots in growth cabinet.

2.9. EXPERIMENT 2.

Seeds were spread on to full strength M.S. gel plates and placed in a growth cabinet set to run on a 14 hour photoperiod at 24°C. Seven days later seedlings were transferred from the plates, as described in experiment 1, to 500 ml volumes of half strength M.S solution supplemented to one of four different phosphate concentrations:- 10, 25, 100, or 250 μM. Over the next 21 days the phosphate, and M.S nutrient levels in each pot were maintained through complete solution renewal every 2 days. On the 22nd day of hydroponic growth a phosphate depletion experiment was carried out as described in the following schedule.

t = -530 minutes	all solutions changed to 100 ml of 0.5 μM $CaSO_4$ adjusted to pH 6 with 0.1 M KOH
t = -120 minutes	solutions renewed to 18 ml with fresh 0.5 μM $CaSO_4$ adjusted to pH 6 with 0.1 M KOH
t = 0	2 ml of 150 μM KH_2PO_4, 0.5 μM $CaSO_4$ solution of pH 6 added to give a phosphate concentration of 15 μM and total volume of 20 ml
t = 2 minutes	a 200 μl aliquot of solution was removed, placed in an eppendorf tube, sealed and frozen until analyses the following day.
t = 20 minutes	at 20 minute intervals, from t = 0 to t = 60 minutes 200 μl aliquots were removed and stored as above
t = 120 minutes	at t = 120, 180 and 360 minutes respectively 200 μl samples were removed and stored as above.

At the completion of the depletion experiment, fresh and dry weights for all plant material were recorded as per experiment 1.

2.9.1 PHOSPHATE ANALYSIS.

Phosphate analysis in this experiment was carried out by High Performance Ion Chromatography using a DIONEX BIO - LC ion chromatograph. In this process the collected depletion samples are injected on to an HPIC - AS4A anion exchange separator column. Separation of the various anion components is achieved through their different ionic affinities for the ion exchange resin of the column to which they bind. Following separation, the effluent flows into a second column which functions to chemically suppress conductivity of the eluant, enhancing the detection of anion species in the injected sample which takes place in a conductivity detector.

2.9.2. ION CHROMATOGRAPHY SET UP, AND CONDITIONS OF ANALYSIS.

Method file:-	PHOS.MET (Appendix 1)
Separator column:-	Ionpac AS4A with Ionpac AG4A guard column
Eluant:-	3.6 mM Na_2CO_3 / 3.4 mM $NaHCO_3$
Eluant flow rate:-	2.0 ml/minute
Regenerant:-	25 mM H_2SO_4
Regenerant flow rate:-	3 to 5 ml/minute
Detection:-	Conductivity (range 30 μS)
Standard:-	15 μM PO_4 / 0.5 μM $CaSO_4$ of pH 6

For each sample injection, 80 μl of depletion solution was injected manually using a 100 μl gastight Hamilton syringe. After each injection the syringe was flushed three times with Milli Q water. At the beginning of each analysis period and after every eight sample runs an auto calibration run using the phosphate standard was carried out.

2.10. EXPERIMENT 3.

The materials and methods used in experiment 3 were identical to those of experiment 2 with the following exceptions:-

1) 2 ml of 150 μM phosphate added at time 0 was radioactively labelled with ^{32}P at a rate of 1.0 μCi/ml

2) the depletion period was for 1 hour.

3) 0.1 ml samples were collected every 5 minutes during the course of depletion.

4) $^{32}PO_4$ concentrations were determined by counting the Cerenkov radiation in a scintillation counter.

2.10.1.CERENKOV RADIATION COUNTING.

Cerenkov radiation is the light emitted by an electrically charged particle as it moves through a substance at a speed exceeding that of light moving in the same substance (Beiser 1967). The Cerenkov radiation produced from the radioactive decay of ^{32}P in water can be used to determine the concentration of radioactively labelled phosphate in solution. In Experiment 3, $^{32}PO_4$ depletion was measured by adding 10 ml of water to each collected sample and quantifying the Cerenkov radiation released from the $^{32}PO_4$ in a LKB Wallac RackBeta 'Spectral' liquid scintillation counter.

Duplicate standards of 50 μl taken from the 10 μM ^{32}P labelled depletion solution were counted at the beginning and end of each detection period and were used to calculate phosphate concentration from the detected radioactivity of each sample.

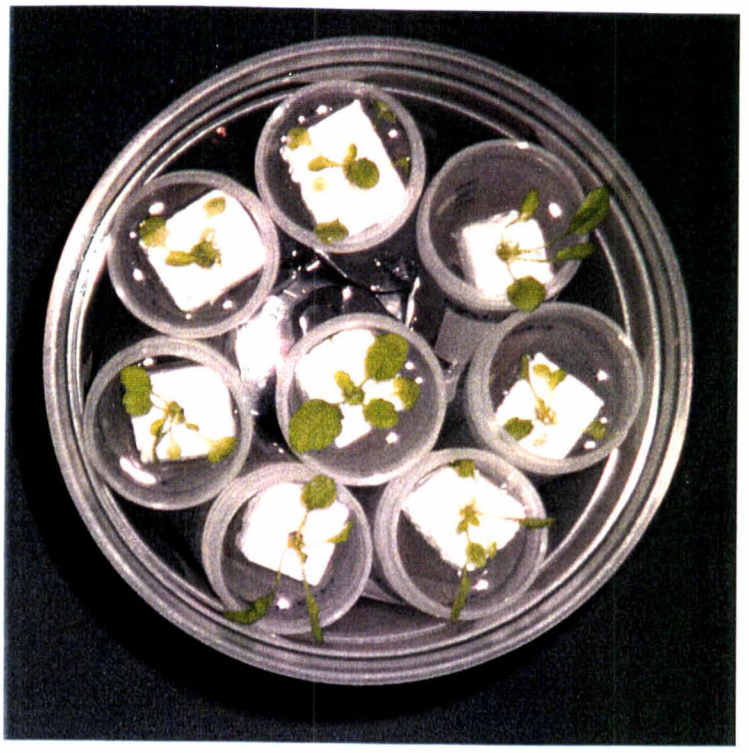
2.11. EXPERIMENT 4.

In experiment 4 the *Arabidopsis* seeds were surface sterilised and spread on agar gel plates of 1/10 strength M.S and 250 μM phosphate concentration. The plates were placed in a growth cabinet running on a 16 hour photoperiod at a constant temperature of 25°C. Seven days after planting, seedlings were removed from the plates, inserted into 1 cm cubic polystyrene floats and placed in falcon tubes contained in Sigma Plant Cell Culture pots (8 tubes per pot) (Fig 2.3). Each tube contained 50 ml of 1/10 strength M.S. of either:- 10, 25, 100 or 250 μM phosphate concentration. The total solution volume was renewed every 48 hours for the duration of the 14 day growing period. For each of the 10, 25 and 100 μM phosphate treatments there were two replicate pots while for the 250 μM phosphate treatment four replicate pots were set up.

After nine days of nutrient renewal, replenishment of the phosphate component in two of the 250 μM pots was stopped giving the plants of these pots 5 days exposure to phosphate free nutrient solutions. These plants were designated P250→0.

On the fifteenth day of hydroponic growth, three separate depletion experiments were carried out on replicate plants to establish uptake rates for $^{32}PO_4$, PO_4, and $^{32}PO_4$ in the presence of 20 μM KH_2AsO_4.

A



B

Figure 2.3. View from above (A) and side (B) of plant growing method employed in experiment 4.

2.11.1. DEPLETION EXPERIMENTS.

t = -30 mins first wash, all solutions changed to 50 ml of 1/10 strength M.S. pH = 6 Phosphate concentration = 0

t = -10 mins repeat of first wash

t = 0 three separate depletion trials initiated :-

1) four replicate plants from each phosphate treatment placed in separate 10 ml volumes of 1/10 strength M.S. solution of 10 μM phosphate concentration.

2) four replicate plants from each phosphate treatment placed in separate 10 ml volumes of 1/10 strength M.S. of 10 μM ^{32}P labelled phosphate.

3) four replicate plants from each treatment, excluding P250→0 plants, placed in 1/10 strength M.S. of 10 μM ^{32}P labelled phosphate and 20 μM arsenate.

t = 1 hr at hourly intervals, from t = 1 to t = 8hours, 0.7 ml aliquots collected

t = 8 hr at 2 hourly intervals, from t = 8 to t = 12 hours, 0.7 ml aliquots collected

t = 12 hr depletion experiment terminated.

At the end of the depletion period, all non radioactively labelled samples were sealed in eppendorf tubes and frozen. All radioactively labelled samples, collected in scintillation vials had 10 ml of water added, and were loaded on to the scintillation counter, and counted over night as per experiment 3. All plants were dissected into root and shoot segments. The roots and shoots of those plants that were exposed to ^{32}P were placed in separate scintillation vials containing 10 ml of water and were measured for radioactivity in the scintillation counter. Dry weights were recorded after an overnight drying period in an oven at 80°C.

2.11.2. SPECTROPHOTOMETRIC QUANTIFICATION OF PHOSPHATE.

In experiment 4, the phosphate concentration of non-radioactive solution samples was determined colourimetrically by spectrophotometric quantification of the malachite green/phosphomolybdate complex by the method of Shimogawara and Usuda (1995).

The working reagent of this assay was made from three stock solutions:-

stock 1. 14.3 g ammonium molybdate dissolved in 250 ml of 6.0 M HCl
stock 2. 5.8 g polyvinyl alcohol dissolved in 250 ml of hot H_2O
stock 3. 1.22 g of malachite green dissolved in 100 ml of H_2O.

Stocks 1, 2 and 3 were mixed at a ratio of 5:5:2 (v/v) respectively to produce the working reagent, and kept at room temperature for at least 12 hours before use. The reagent was stored at room temperature in a polypropylene bottle.

To measure the concentration of phosphate in each sample aliquot, 300 µl of the working reagent was added to each of the 700 µl samples and incubated at 30°C for a period of 30 minutes allowing the colour to develop completely. At the end of this period the absorbance was measured at 630 nm in a U-2000 Hitachi spectrophotometer. Standards were made from the same 10 µM non-labelled phosphate solution as used in the depletion trial. 1/10 strength M.S. solution was added to produce standard phosphate solutions of 0, 1.0, 2.5, 5.0, 7.5 and 10 µM respectively. A new standard curve was generated after every 10 samples were measured. Lowest R^2 values recorded for the standard curves was 0.979.

2.11.3. DEPLETION TRIAL CONTROLS.

In experiment 4, solutions identical to those used in the depletion trials, but without plants, were sampled over the time course of the depletion trials to determine non-plant effected phosphate removal from the depletion solutions.

To determine actual plant phosphate absorption at any time interval over the depletion course, best fit exponential equations characterising the non-plant phosphate removal,

were calculated for the three depletion trials of experiment 4, and included in all uptake calculations.

For depletion trial 1. Apparent non-plant phosphate uptake approximated the exponential equation : $y = 106.77e^{-0.1159x}$ ($R^2 = 0.9879$)

For depletion trial 2. Apparent non-plant phosphate uptake approximated the exponential equation : $y = 88.972e^{-0.1379x}$ ($R^2 = 0..9718$)

For depletion trial 3. Apparent non-plant phosphate uptake approximated the exponential equation : $y = 92.238e^{-0.1452x}$ ($R^2 = 0.9859$)

For these equations y = depletion solution concentration (nmol)
x = time.

2.11.4. Km AND Vmax DETERMINATION.

The kinetic parameters Km and Vmax were determined simultaneously for phosphate uptake by individual plants, by fitting the time course data of phosphate depletion with the theoretically expected curve described by the theory (Appendix 2) of Shimogawara and Usuda (1995). Curve fitting was performed by a non-linear least-squares method without weights. The actual calculation was performed by application of the Levenberg-Marquardt algorithm using the software package "Igor Pro" (Wavemetrics Inc., Lake Oswego, Oregon).

2.11.5. PRELIMINARY WORK ON CELL MEMBRANE ELECTRICAL POTENTIAL.

Using plants that had been grown under the conditions of experiment 4, but were not used in the depletion trials of that experiment, a preliminary investigation was carried out looking at the relationship between root cell membrane electrical potential (PD) and the uptake of phosphate by *Arabidopsis*.

Phosphate uptake into cells has been found to occur via a proton-phosphate symport mechanism which harnesses the H^+-ATPase generated proton motive force across the plasma membrane to bring the negatively charged phosphate ions through the membrane and into the actively maintained electrically negative environment of the cytoplasm (Mistrik and Ullrich 1996).

Following a method adapted from Bowling and Dunlop (1978), a single, intact lateral root was extended across the surface of a perspex chamber fixed to the bottom of a petri dish, and was secured in place by two drops of super glue (cyano acrylic adhesive) at opposite ends of the root. The root was positioned so that the interval between the two glued fixture points was pulled as tight and straight as possible without signs of visible damage to the root. The petri dish was then placed on the stage of a dissection microscope for viewing, and filled with 1/10 strength M.S. solution pumped in by a "Gilson" peristalic pump (model Minipuls 3).

The electropotential between the inside of root cells and the solution bathing the root was determined by inserting a microelectrode into the root using an "Inchworm" micromanipulator (Burleigh Instruments Inc). The electrodes were made from micropipettes pulled from glass capillaries using a micro electrode puller (model 8104 C.F.Palmer Ltd), and were filled with 3 M KCl. The tip diameter was estimated to be less than 1μm. The microelectodes were connected via a Ag/AgCl electrode to a voltage follower (World Precession Instruments) the output of which was recorded by a MacLab using Chart data interface software (ADInstruments). The circuit was completed by a 3M KCL salt bridge between a Ag/AgCl electrode and the solution bathing the root (Fig 2.4).

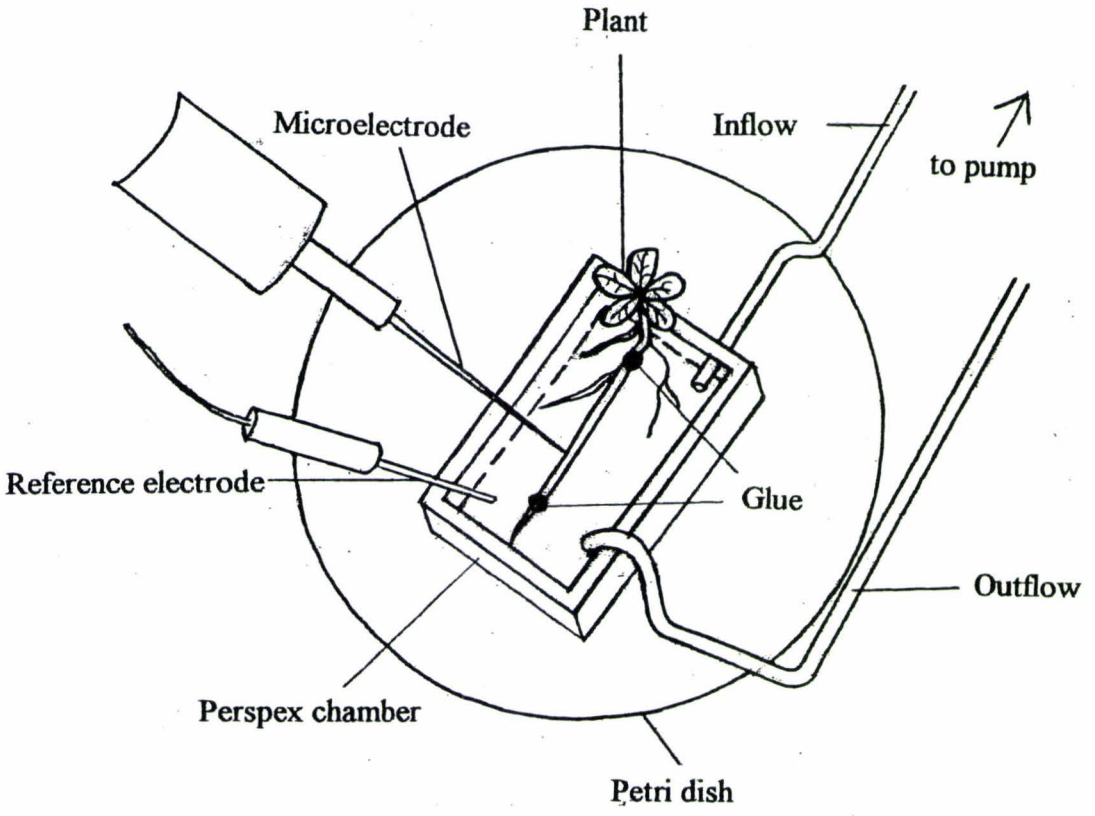


Figure 2.4 Experimental arrangement for measurement of root cell PD as effected by phosphate absorption.

The effect of phosphate absorption on PD was determined by recording the change in voltage that accompanied the exchange of the phosphate free solution bathing the root into which the electrode had been inserted, for an identical solution to which KH_2PO_4 had been added.

CHAPTER THREE

RESULTS

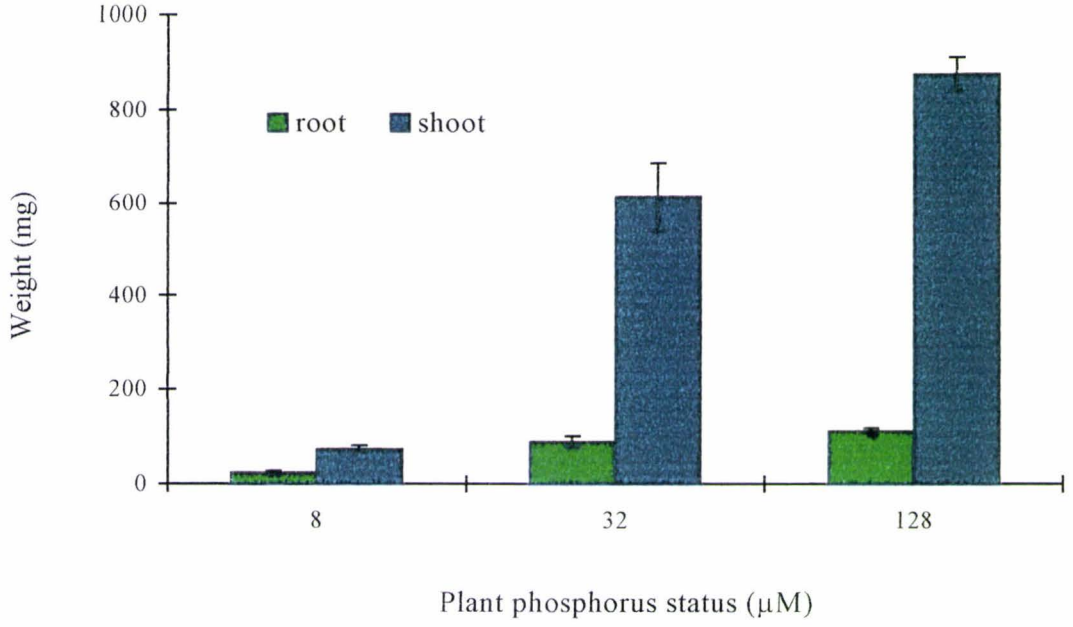
3.1. EXPERIMENT 1.

The purpose of this experiment, was to establish the phosphate supply necessary to produce viable and experimentally useful plants. Originally five phosphate levels (0.5, 2.0, 8.0, 32, and 128 μM respectively) were selected to produce plants ranging from severely phosphorus deficient (0.5 μM) through to adequate phosphorus nutritional status (128 μM). All hydroponic growth solutions of experiment 1 were of 500 ml.

During the first week of hydroponic growth it became evident that the lowest levels of phosphate concentrations (0.5 and 2.0 μM) combined with the non renewal of solution over this period were not sufficient to sustain plant growth. All plants at these phosphate levels died within the first week. Of the remaining treatments, only 4 pots of plant material per treatment survived to the end of the intended 38 day growing period, each one consisting of 5 plants relocated from the eight replicate pots, over the course of the experiment.

To assess what effect the different phosphate treatments had on plant growth, shoot and root weights (both fresh and dried) for each pot of plant material were recorded (Fig 3.1a and 3.1b). The mean fresh root weight of 8 μM grown plants was significantly less ($p<0.01$) than those of the 32 μM and 128 μM PO_4 treatments, corresponding to a reduction in size of 73.75% and 79.03% respectively. With oven drying, the weight differences were reduced to 50% and 60%, still significant ($p<0.01\%$). There were no significant weight differences between roots of the 32 and 128 μM PO_4 plants. For the shoot material, significant weight differences ($p<0.01$) of 88.01% and 91.58% were recorded between the 8 μM PO_4 grown plants and those of the 32 and 128 μM treatments respectively. On drying, the weight margins reduced slightly to 83.6% and 87.1%.

a.



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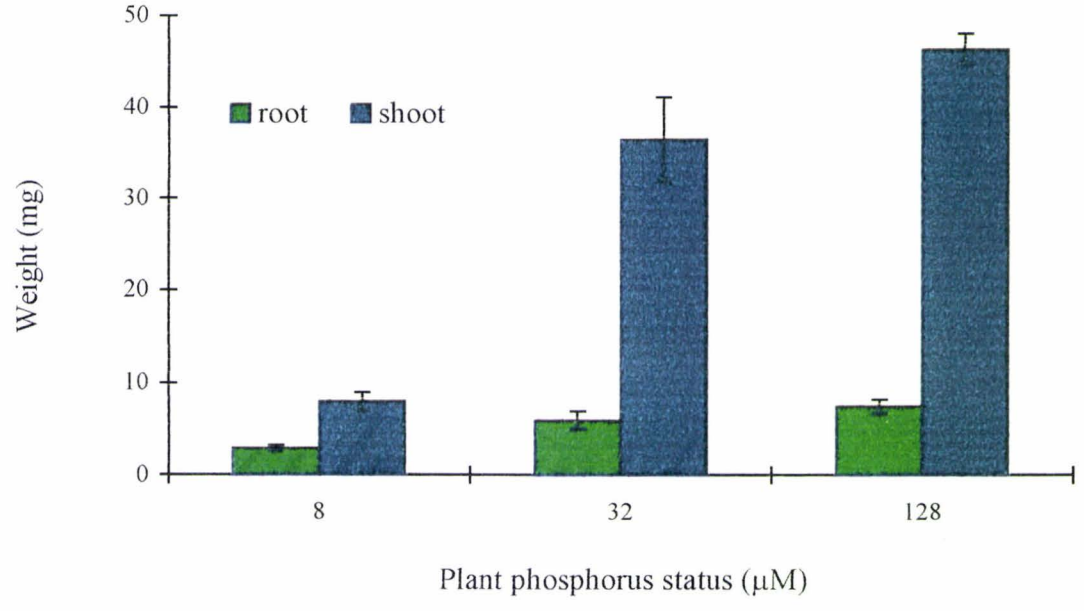


Figure 3.1 Root and shoot weights for experiment 1. Fresh weight, (a); Dry weight, (b); Each column represents the mean of 4 pots of 5 plants each. Bars represent ± standard errors.

3.2. EXPERIMENT 2.

In experiment 2 the first nutrient depletion experiments were carried out to determine if plants of different phosphorus status exhibited differences in absorption rates for phosphate uptake at the low phosphate concentration of 15 μM.

Based on the results from experiment 1 the set phosphate levels in which plants were grown for the remaining experiments of this project were 10 μM (P10 plants), 25 μM (P25 plants), 100 μM (P100 plants), and 250 μM (P250 plants). All growth solutions for experiment 2 were of 500 ml volumes and were completely renewed every two days. Under these conditions, differences in both root and shoot weights (Fig 3.2) were less pronounced than for experiment 1.

For plants grown at 10 μM phosphate concentrations the average dry root weight of 5.0 mg was 47.9% smaller than corresponding 250 μM grown plants ($p<0.05$). There was no significant difference in root weight between plants of 25 μM, 100 μM or 250 μM phosphorus status plants.

The shoots of 10 μM grown plants (Fig. 3.2), were smaller than in the other three treatments ($p<0.05$ for 25 μM and 100 μM plants) with greatest size difference evident as a 56.7% decrease in shoot mass relative to 250 μM plants ($p<0.01$). There was no significance to the difference in shoot size between plants of 25 μM, 100 μM or 250 μM phosphorus status.

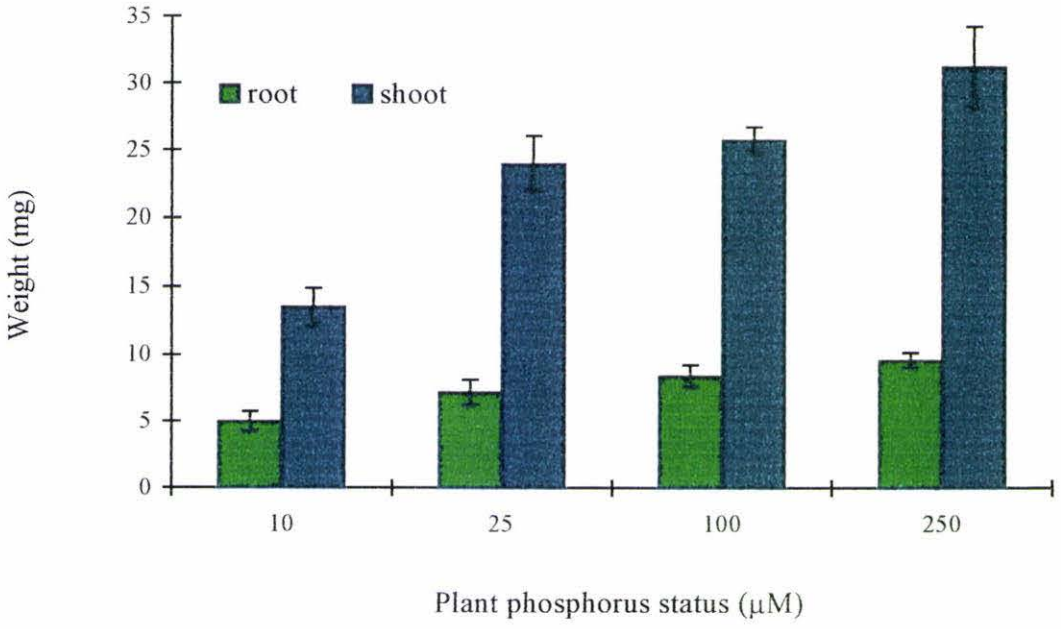


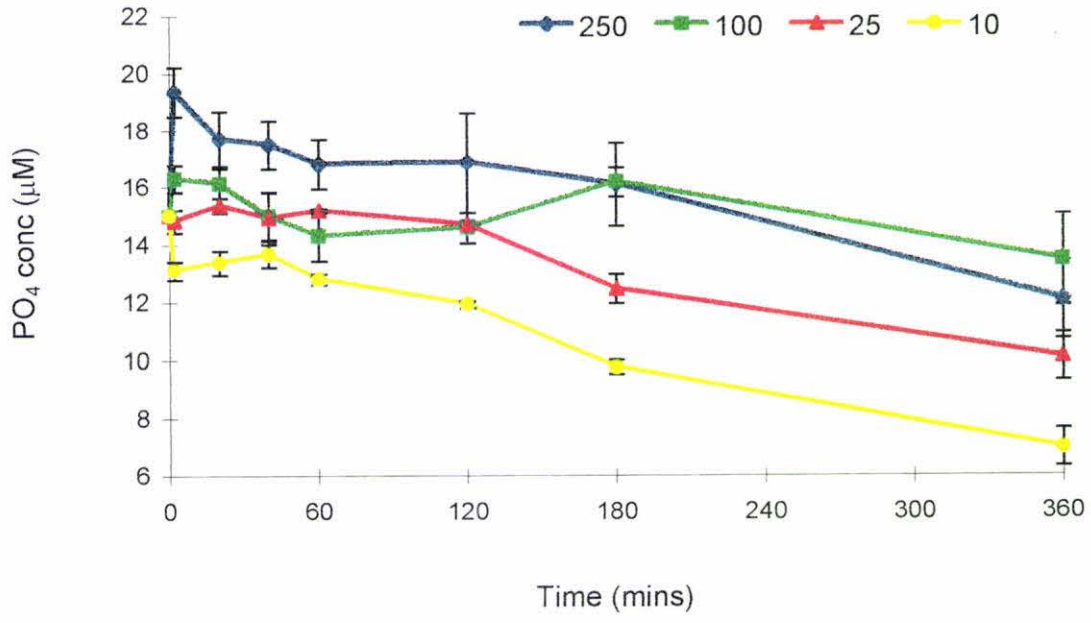
Figure 3.2 Root and shoot dry weights for experiment 2 plants. Number of replicates used: 10 μM- 4 ; 25 μM- 6; 100 μM- 5; 250 μM- 5. Bars represent ± standard errors.

3.2.1. PHOSPHATE DEPLETION.

Prior to exposure to the depletion solution, all plants were removed from the growing solutions and washed twice over an 8 hour 50 minute period, in phosphate free 0.5 μM $CaSO_4$ solution. The purpose of these washes was to remove any non-bound phosphate from the root systems of the experimental plants, and to accustom the plants to the depletion solution. To begin the depletion period plants were placed in individual 20 ml volumes of fresh wash solution, set to an initial phosphate concentration of 15 μM. Periodically over a 6 hour time course 200 μl aliquots were removed from each depletion solution and analysed for phosphate concentration by High Performance Ion Chromatography. From the data collected at each sample time, depletion curves were generated (Fig 3.3a) representing phosphate absorption by the plants over the depletion period.

Over the early parts of the depletion curves (Fig 3.3b) plants of 10 μM and 25 μM phosphorus nutritional status effect a net removal of PO_4 while in contrast, 100 μM and 250 μM plants lose or efflux phosphate from their roots into the depletion solution.By the end of the depletion period all plants had achieved a net absorption of phosphate, however a solution concentration difference of no less than 5 μM ($p<0.001$) between 10 μM and 250 μM plants was maintained throughout the length of the depletion period.

a.



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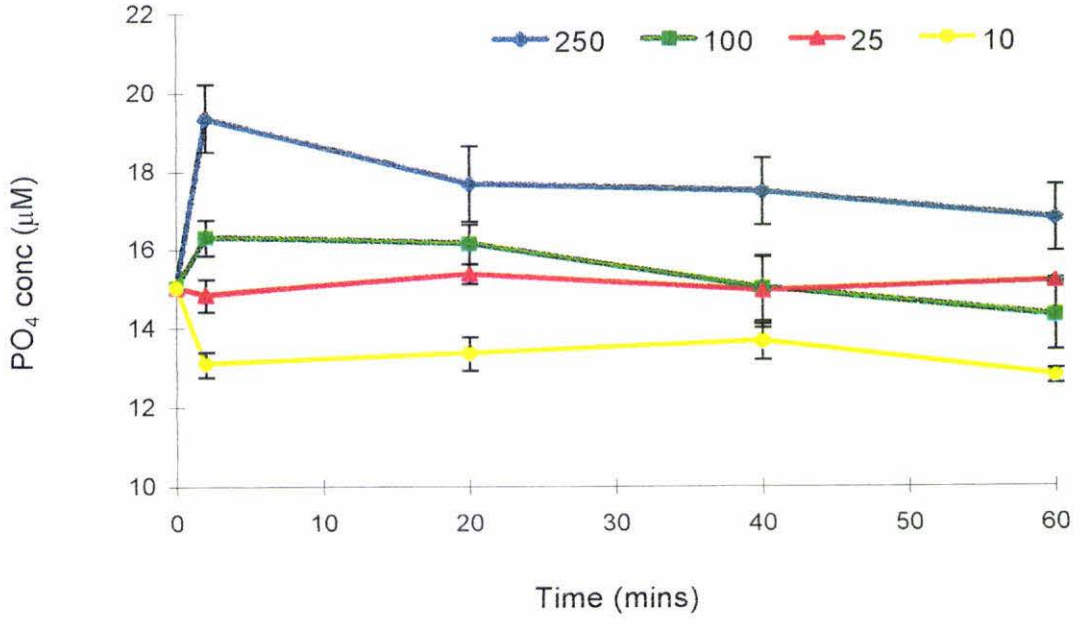


Figure 3.3 Depletion of a 15 µM KH_2PO_4 solution by plants of different phosphorus nutritional status. (a): 360 minute depletion period; (b): detail of the first 60 minutes of the same depletion trial. Number of replicates (pots of 5 plants) used: 10 µM- 4 ; 25 µM- 6; 100 µM- 5; 250 µM- 5. Bars represent ± standard errors.

3.3. EXPERIMENT 3.

In experiment 2, P25, P100 and P250 plants, over the first 2 hours of exposure to the uptake solution, had net movement of phosphate from their roots into the depletion solution. The aim of experiment 3 was to determine if phosphate influx was taking place concurrently with phosphate efflux, and if so was the rate of influx dependent on plant phosphorus nutritional status. This was achieved, by labelling the 15 μM PO_4 depletion solution with ^{32}P and monitoring depletion of radioactivity from the solution by measuring the Cerenkov radioactivity in solution aliquots removed over the depletion time course.

From the curves generated over the sixty minute depletion period (Fig 3.4) it appears that all plant roots removed labelled PO_4 from the solution, with no significant differences in depletion rates relative to differences in plant phosphorus nutritional status.

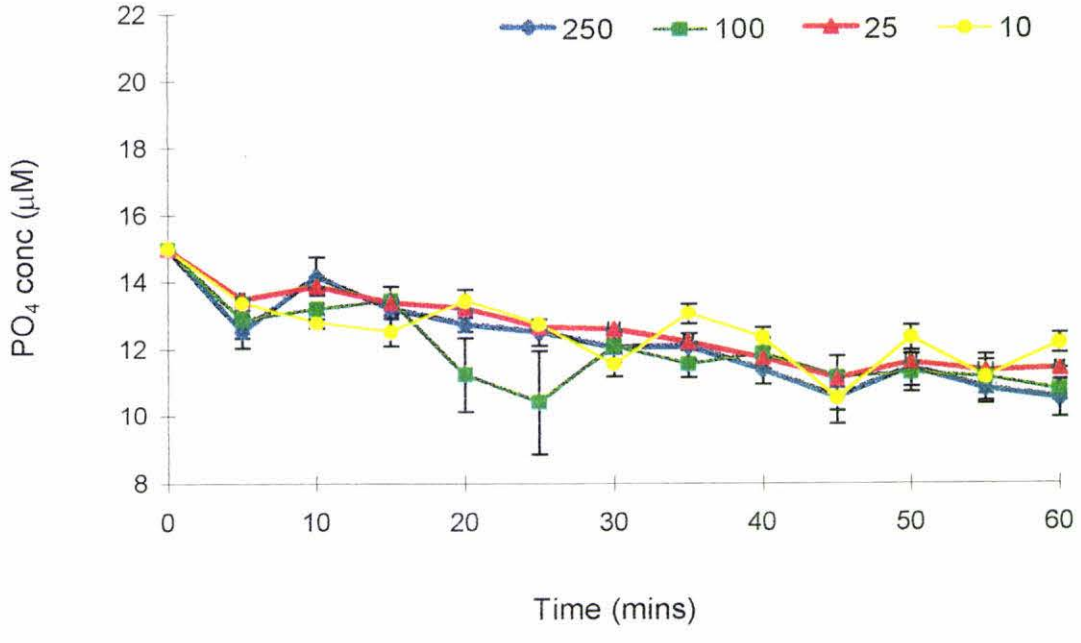


Figure 3.4 Depletion of a 15 μM P^{32} labelled KH_2PO_4 solution by plants of different phosphorus nutritional status. Each line represents the mean of 4 pots. Bars represent ± standard errors.

In experiment 3, plant root weights (Fig 3.5) were on average smaller than those of equivalent plants grown under similar conditions in experiment 2 (compare Fig 3.2 and Fig 3.5). As both lots of experimental plants were grown under identical conditions but at different times, these results indicate a degree of experimental error that should be considered when comparing results from separate experiments.

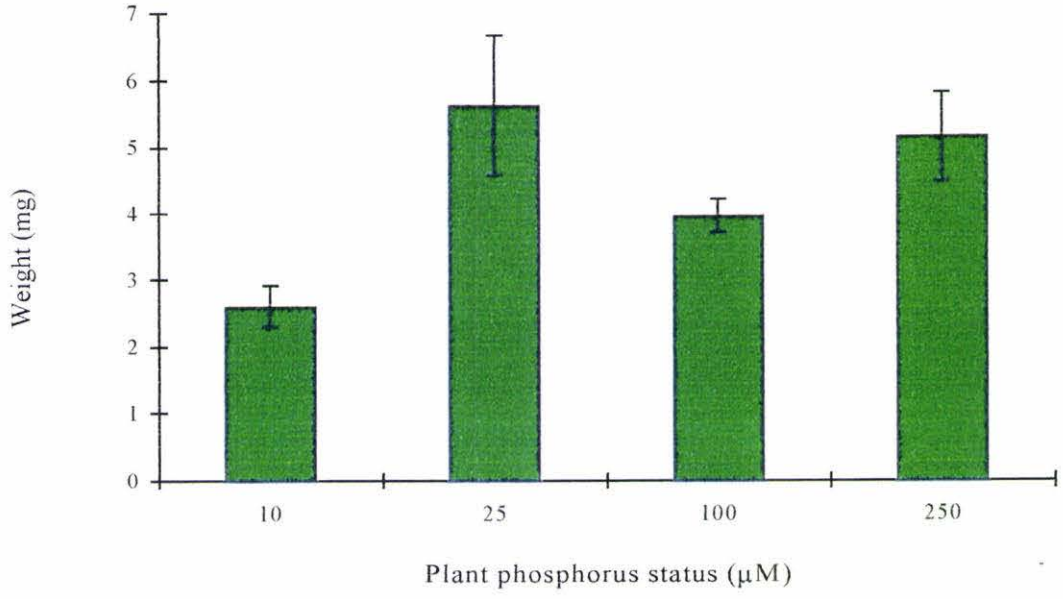


Figure 3.5 Root dry weights for experiment 3 plants grown at different phosphate concentrations. Each line represents the mean of 4 pots of 5 plants each. Bars represent ± standard errors.

3.4. EXPERIMENT 4.

Phosphorus deficiency in higher plants induces physiological changes that help plants to survive under conditions of limited phosphate availability. Such changes include reductions in the levels of phosphate effluxed from the plant root system into the environment (Schjorring and Jensen 1984, Lee et al., 1990), and increases in the root systems affinity for phosphate ions through the operation of a phosphorus deficiency induced high affinity uptake mechanism (Epstein 1972). For some plant species, suppression of this high affinity mechanism is found to confer tolerance against the metabolic poison arsenate, permitting growth in soils high in arsenate concentration (Meharg and Macnair 1991,1992).

In this final experiment three different depletion trials were carried out to quantify net phosphate uptake (influx - efflux), phosphate influx, and the effect of arsenate on phosphate influx within the range of the high affinity phosphate uptake mechanism (Epstein 1972) in plants of different phosphorus nutritional status. These trials were undertaken to determine whether the processes involved in phosphate uptake in *Arabidopsis*, were modified in response to phosphorus deficiency and if arsenate could be used as a tool to identify such modifications.

In experiment 4, a fifth plant phosphorus nutritional status was created by with holding the phosphate supply from a group of P250 plants for the last 5 days of their growth period. These plants were designated P250→0.

Net uptake was measured by monitoring the change in total phosphate concentration in the depletion solution, set at an initial phosphate concentration of 10 μM with non-radioactive phosphate. Phosphate was determined by spectrophotometry as described in the materials and methods for experiment 4.

In figure 3.6 it can be seen that no net uptake of PO_4 was recorded for any plants over the first hour, with P25 and P250 plants showing a net PO_4 loss. In the following hour, all plants showed signs of net uptake, with the exception of P250 plants, which did not show a net depletion until after hour 3.

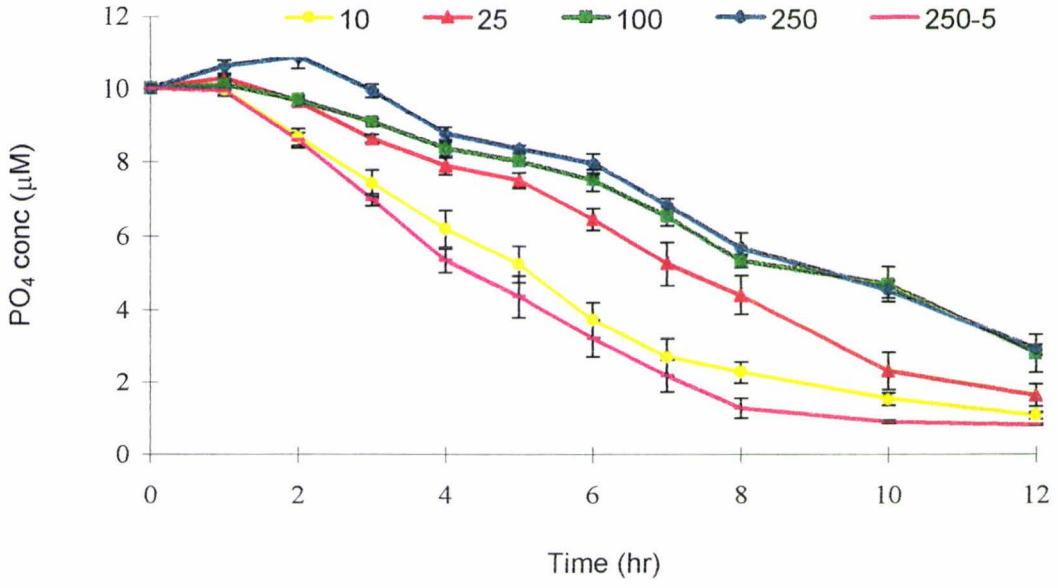


Figure 3.6 Depletion of a 10 µM KH_2PO_4 solution by plants of different phosphorus status. Each line represents the mean of 4 plants. Bars represent ± standard errors.

Phosphate influx was measured by monitoring the removal of $^{32}PO_4$ from the depletion solution by the plant root systems. ^{32}P was determined by Cerenkov counting. Over the first hour of depletion (Fig 3.7), all plants regardless of phosphorus status, exhibited a rapid initial rate of uptake. Over the rest of the depletion period, P250→0 plants showed the greatest capacity to deplete PO_4 from the nutrient solution, with depletion curves closest in resemblance to P10 plants rather than P250 plants from which they were derived.

As had been evident for non labelled PO_4 depletion, slowest depletion rates were recorded for P100 and P250 plants respectively.

The presence of arsenate, in the uptake solution (Fig 3.8) effected depletion by reducing $^{32}PO_4$ uptake in all plants, regardless of phosphorus status, to the same absorption rate.

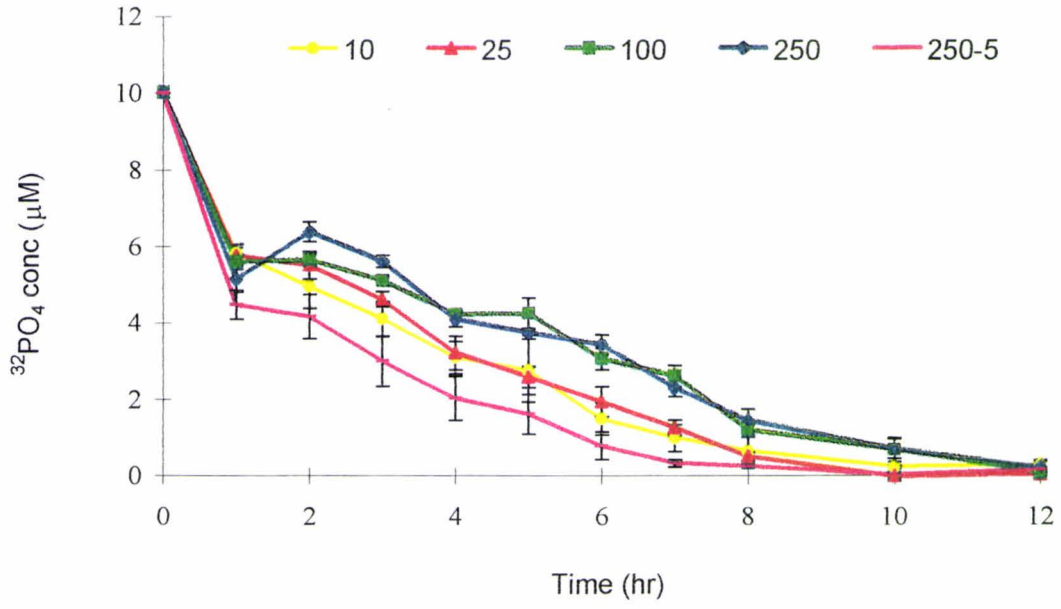


Figure 3.7 Depletion of 10 μM ^{32}P labelled KH_2PO_4 solution by plants of different phosphorus status. Each line represents the mean of 4 plants. Bars represent ± standard errors.

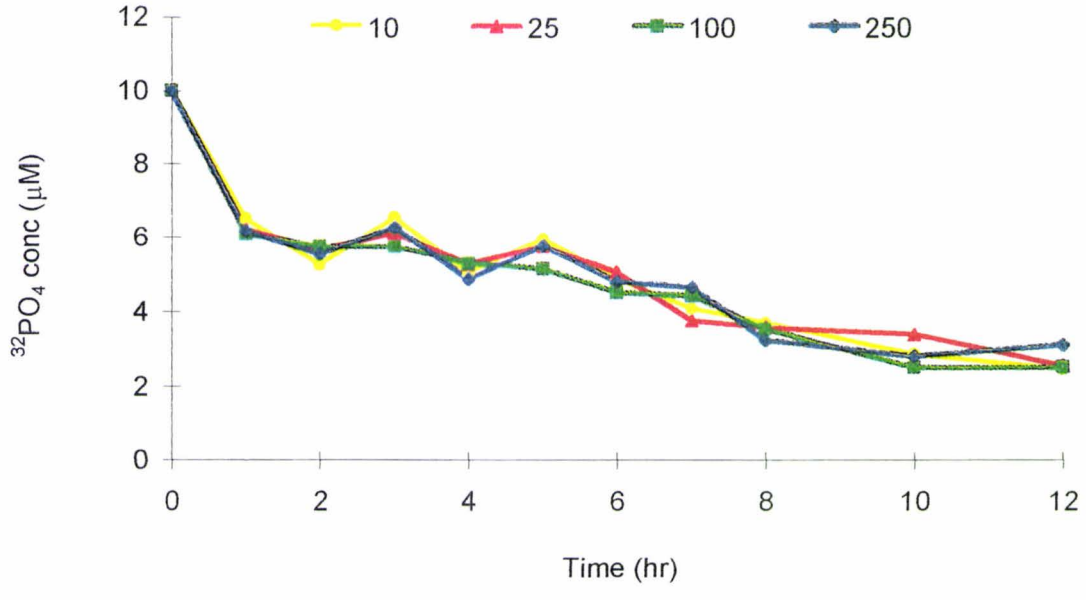


Figure 3.8 Depletion of a 10 μM ^{32}P labelled KH_2PO_4, 20 μM KH_2AsO_4 solution by plants of different phosphorus status. Each line represents the mean of 4 plants. ± standard error bars have been included but are smaller than the markers used for each data point.

P status	Km (μM) -As	Km (μM) +As	Vmax (nmol/hr/plant) -As	Vmax (nmol/hr/plant) +As
P10	24.8 ± 4.4	74.1 ± 7.9	99 ± 16	55 ± 3.6
P25	15.4 ± 4.3	75.0 ± 5.9	62 ± 23	52 ± 2.4
P100	11.0 ± 1.4	74.1 ± 3.6	40 ± 1	55 ± 3.2
P250	37.0 ± 6.9	74.4 ± 5.8	90 ± 19	54 ± 2.8
P250-0	46.0 ± 15.8	NA	241 ± 91	NA

Table 3.1 Effects of phosphorus status on the kinetic parameters Km and Vmax. Number of replicates used: P10 -4; P25 -4; P100- 4; P250- 4; P250→0- 4. Kinetic parameters were derived from the depletion curves as described in materials and methods for experiment 4.

When Michaelis Menten kinetic parameters are derived from the depletion curves of figure 8, lowest Km values were obtained for P100 and P25 plants with only a slight significant increase ($p<0.05$) between these values and those obtained for P10 plants. Significantly larger ($p<0.001$) Km values were obtained for the P250 and P250→0 plants (Table 3.1). Vmax was significantly greater($p<0.001$) in P250→0 plants relative to all other phosphate treatments, but was not significantly different between the other treatments. The addition of arsenate to the depletion solution resulted in an increase in Km for all treatments, without significant alteration to Vmax.

With arsenate present in the depletion solution there were no significant differences in Km and Vmax values between plants of different phosphorus status.

Vmax values have been expressed on a per plant bases due to the non-proportional relationship between root size and phosphate absorption within and between plant phosphorus status (Krannitz et al., 1991a). Expressed this way, as opposed to on a per mg of root basis, standard deviation for Vmax values for replicate plants are lower (Fig 3.9). Expression of Vmax on a per unit weight basis implies a linear relationship between root weight and phosphate absorption, with a line of regression for the two variables having a Y intercept at 0. This was not the case for plants grown under these experimental conditions (Fig 3.10).

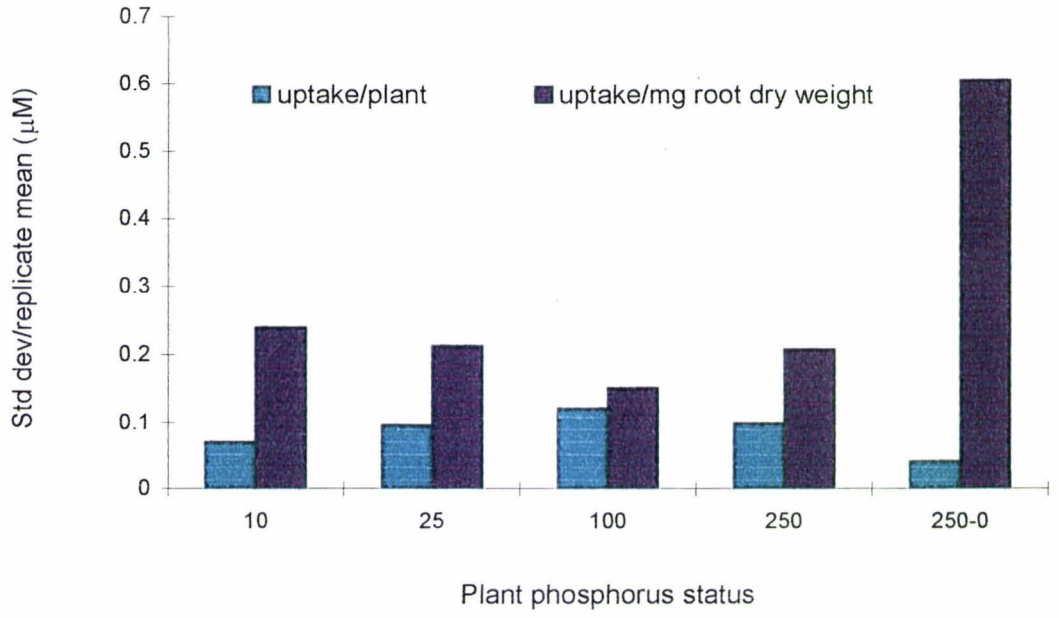


Figure 3.9 Graph comparing the standard deviation from the mean between replicate plants, associated with phosphate uptake expressed on a per plant basis, and a per mg of root dry weight basis. Each column represents the mean of 4 plants.

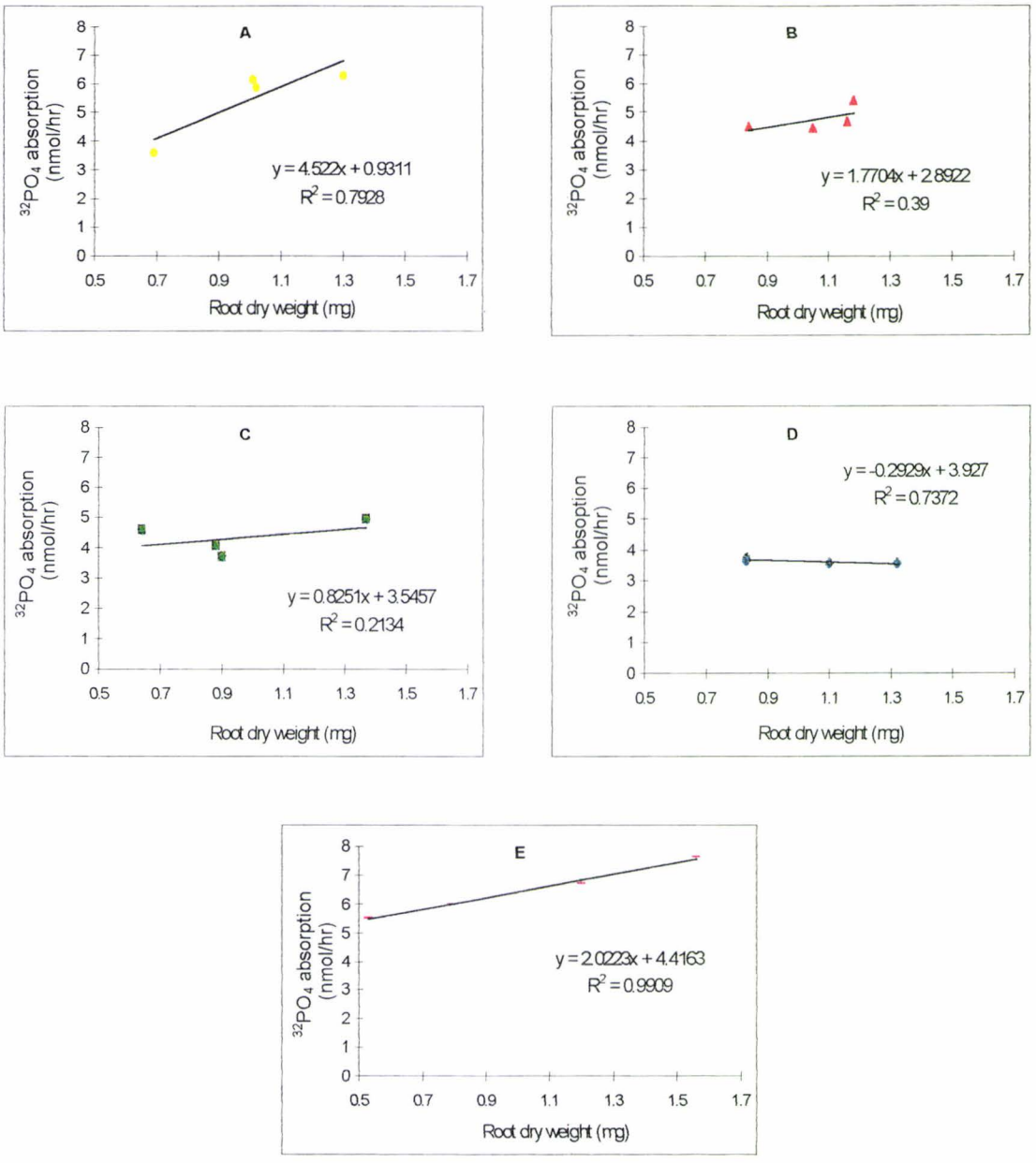


Figure 3.10 Linear regressions relating the variables root dry weight and ^{32}P labelled phosphate absorption for (A) P10 plants; (B) P25 plants; (C) P100 plants; (D) P250 plants; (E) 250→0 plants.

Differences in plant weight in relation to phosphorus status were manifest in shoot weight differences (Fig 3.11), with an increase in phosphorus status from 10 μM to 250 μM resulting in a significant gain in shoot weight of 56.25% ($p<0.001$) and no apparent differences in the root weights. There was no distinction in shoot size between P250→0 and P250 plants.

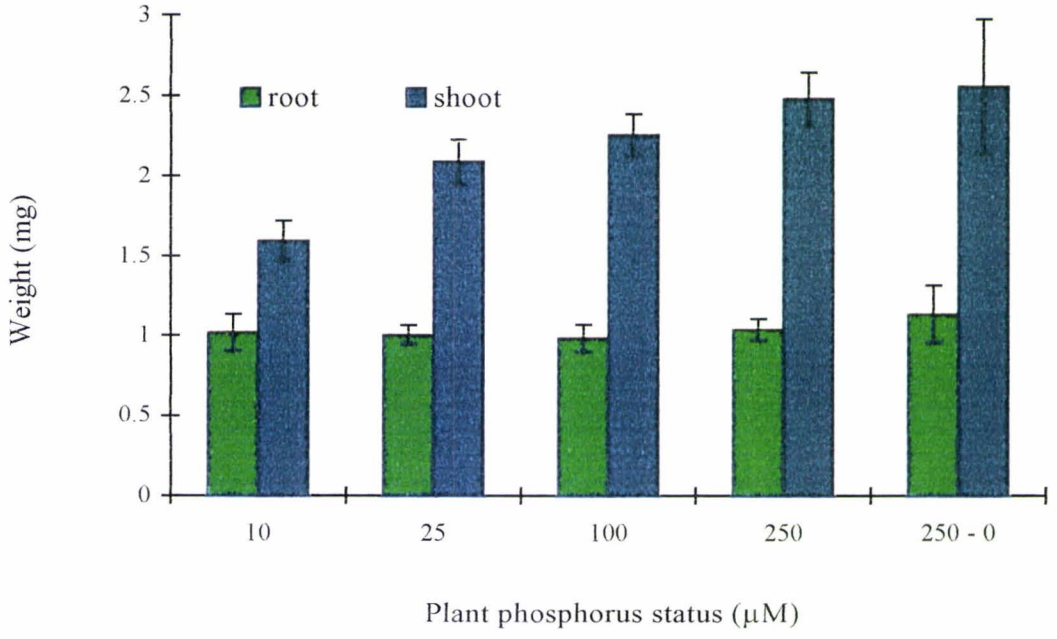
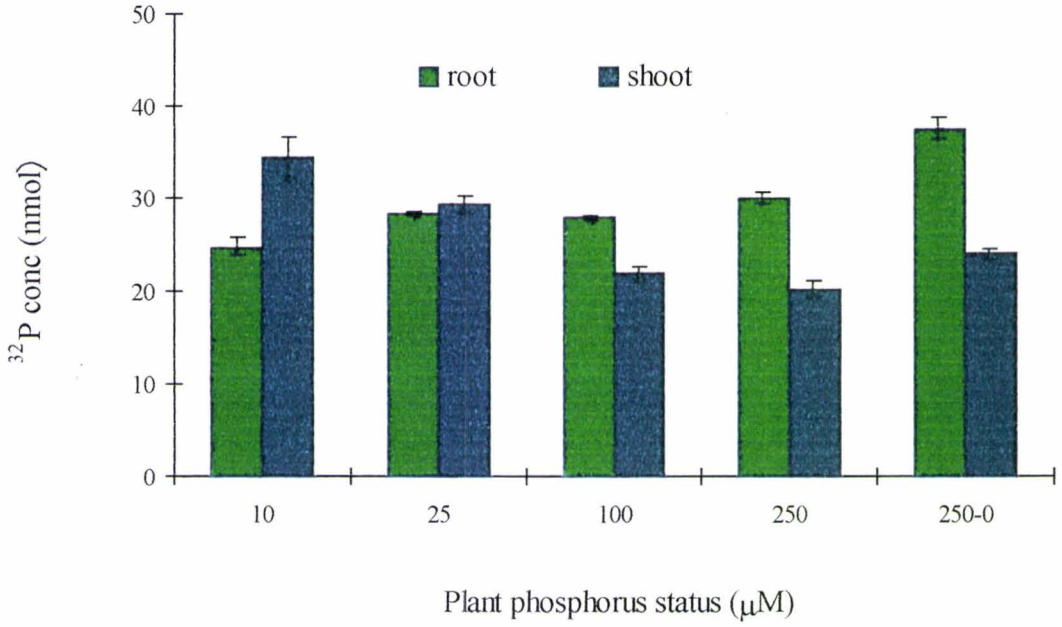


Figure 3.11 Root and shoot dry weights for plants grown at different phosphate concentrations. Each column represents the mean of 12 plants. Bars represent ± standard errors.

Translocation of $^{32}PO_4$ to the shoot was significantly greater ($p<0.001$) in P10 and P25 plants with respect to P250 plants (Fig. 3.12a). No significant difference was observed between P100, P250 or P250→0 plants. When arsenate was present in the depletion solution a reduction in the total amount of $^{32}PO_4$ absorbed was recorded for all plants (compare Figs 3.12a and 3.12b). Arsenate also effected $^{32}PO_4$ translocation to the shoots, with a reduction of 92.2% for P10 plants relative to a 67.0% decrease for P250 plants (Fig 3.13).

a.



b.

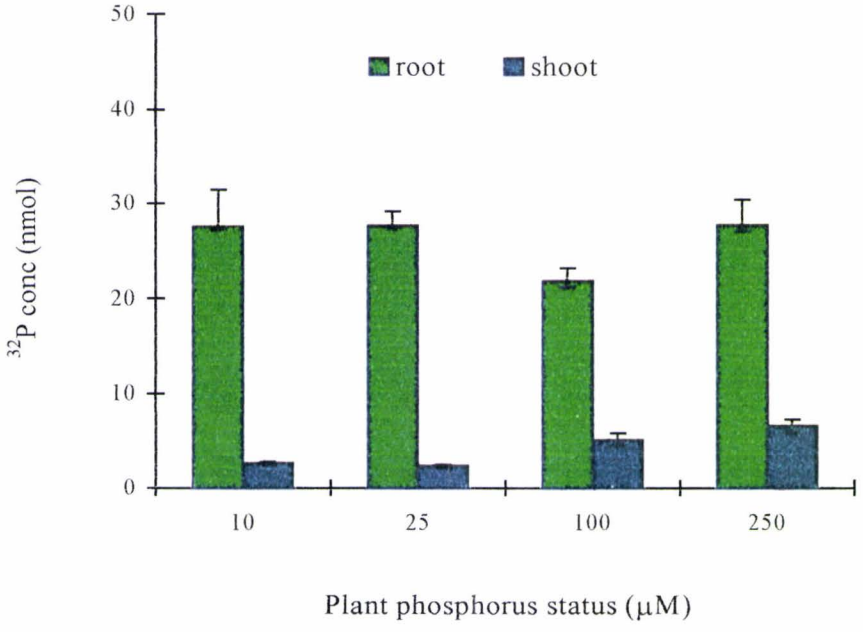


Figure 3.12 Location of P^{32} labelled phosphate absorbed over a 12 hour depletion period from arsenate free solution (a), or in 20 μM arsenate solution (b). Each column represents the mean of 4 plants. Bars represent ± standard errors.

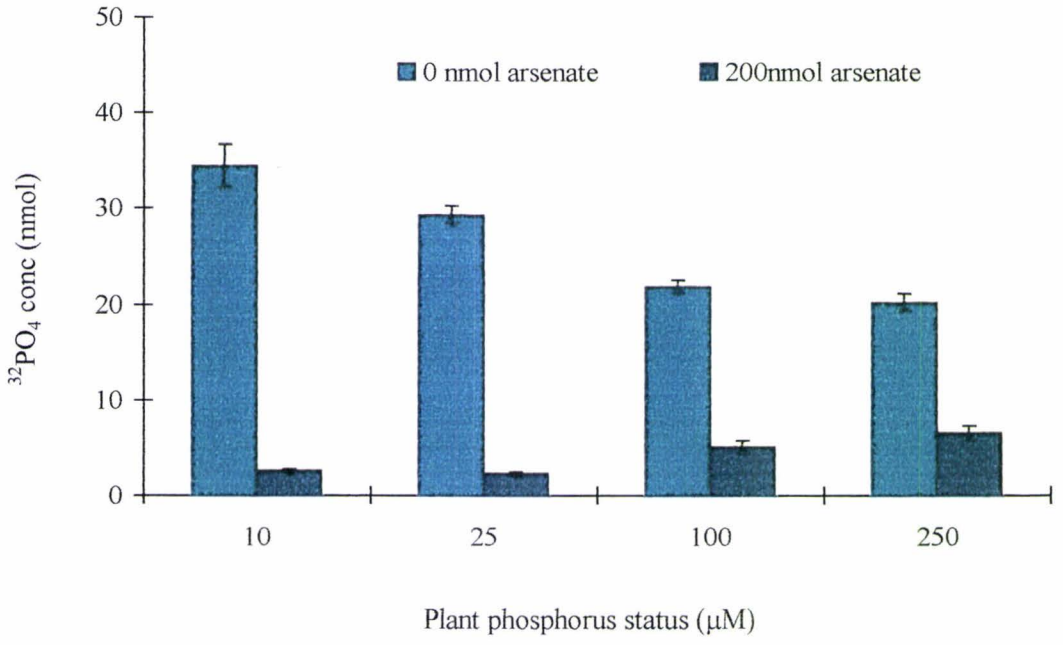


Figure 3.13 Shoot content of ^{32}P labelled phosphate absorbed over a 12 hour period from arsenate free depletion solution, or from depletion solution supplemented to 20 μM KH_2AsO_4. Each column represents the mean of 4 plants. Bars represent ± standard errors.

3.5. MEMBRANE ELECTROPOTENTIAL.

Following the depletion trials of experiment 4, surplus replicate P250 plants were used for a preliminary investigation into the effects of phosphate absorption on cell membrane electropotential. This work was carried out over a period of approximately a month. During this time the hydroponic solutions in which the plants were maintained were not renewed. Because of this the plants used in this work were considered to be phosphorus deficient.

For most plants analysed, strongly negative initial transmembrane potentials exceeding -200 mV were recorded prior to exposure to phosphate. Figures 3.14 and 3.15 are traces representing the effect on membrane electropotential of the addition of KH_2PO_4 to the external solution to which the plant root system was exposed.

In Figure 3.14 an immediate membrane depolarisation occurred following exposure of the root to 250 μM KH_2PO_4. Similar responses have been recorded for root cells of *Trifolium repens* (Dunlop 1989, Dunlop and Gardiner 1993) and the photosynthetic tissues of *Lemna gibba* (Ullrich-Eberius et al., 1984). For P sufficient *Trifolium repens* both KH_2PO_4 and KSO_4 were found to cause similar degrees of membrane depolarisation, while H_3PO_4 was found to have no effect. From these results it was suggested that the K^+ counter ion was responsible for the membrane depolarisation and that phosphate uptake in this situation could best be explained by a H^+/H_2PO_4 symport system operating with a stoichiometry of 1:1. For P deficient *Trifolium repens* H_3PO_4 was found to cause an immediate but transient membrane electropotential depolarisation suggesting the operation of H^+/H_2PO_4 symport systems of different stoichiometry to those operating in P sufficient plants. In *Lemna gibba* membrane depolarisation was found to be in direct response to phosphate uptake, which was suggested to proceed via a $2H^+/H_2PO_4$ symport system.

In this present study, without the inclusion of controls such as H_3PO_4 and K_2SO_4 in the external solution, it has not been determined if the recorded depolarisation in figure 3.14 is in response to K^+ or H_2PO_4 uptake or both.

The trace of transmembrane potential in figure 3.15 indicates no immediate change in the membrane electropotential in response to exposure to KH_2PO_4. Approximately 8 minutes after solution change though, and at later stages in the trace, the membrane potential was found to alternate between a strongly negative electropotential of greater than -200 mV and weaker potentials ranging between -50 and -100 mV respectively. Rapid switching between membrane electropotentials as reported here is often more commonly reported in relation to the operation of ion channels investigated using the technique of patch clamping rather than the slower symport systems studied by this current method.

In future work, less restrained by time I aim to investigate the relationship between phosphorus nutritional status in *Arabidopsis* and membrane electropotentials as effected by phosphate absorption and hope to characterise the stoichiometry of the H^+/H_2PO_4 symport system.

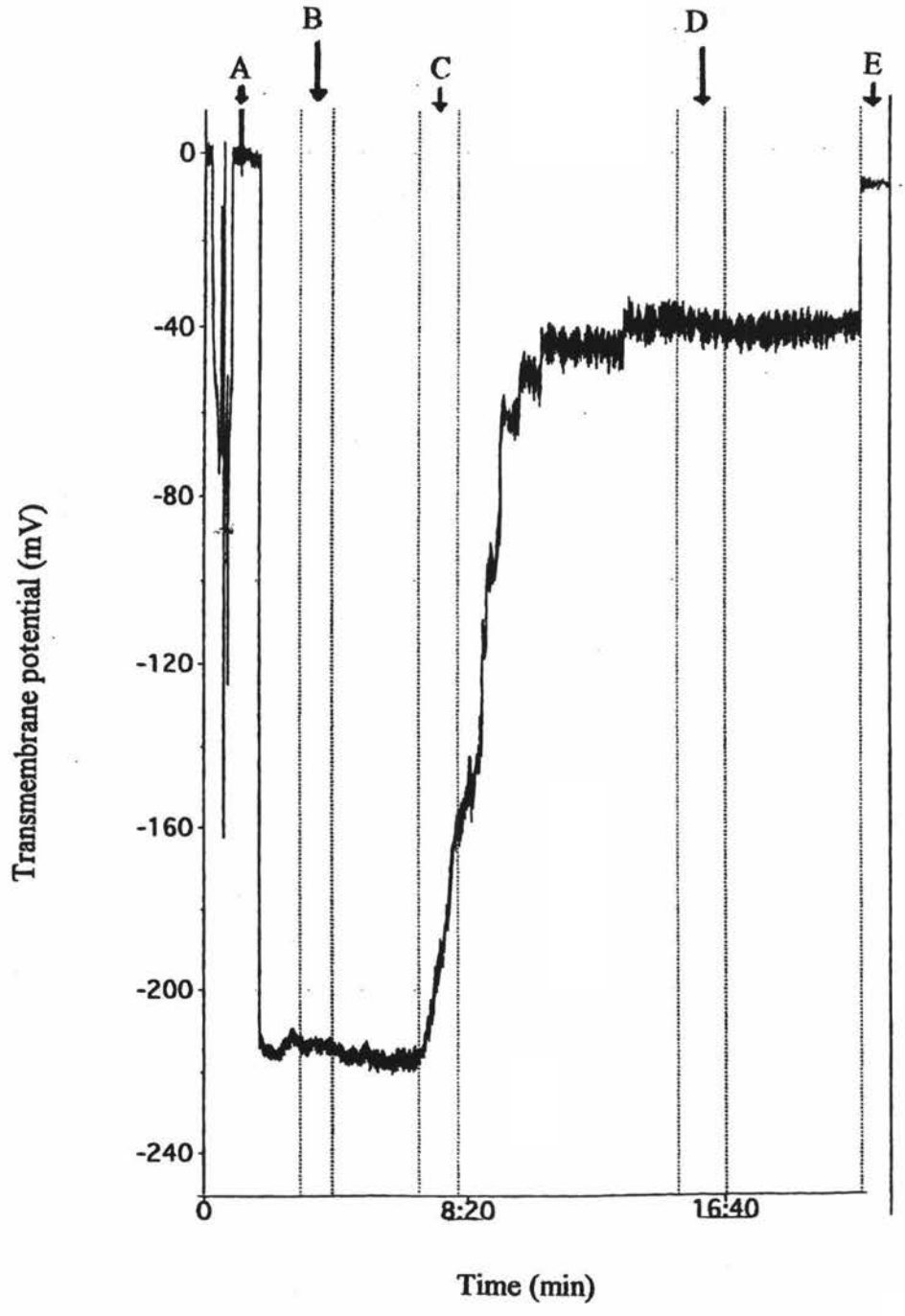


Figure 3.14. The effect of adding phosphate to the external solution on the root cell membrane electropotential. (A) electrode inserted into root cell, (B) 10 ml solution renewal, KH_2PO_4 concentration = 0, (C) 10 ml solution renewal, KH_2PO_4 concentration = 250 μM, (D) 10 ml solution renewal, KH_2PO_4 concentration = 0, (E) electrode removed from root.

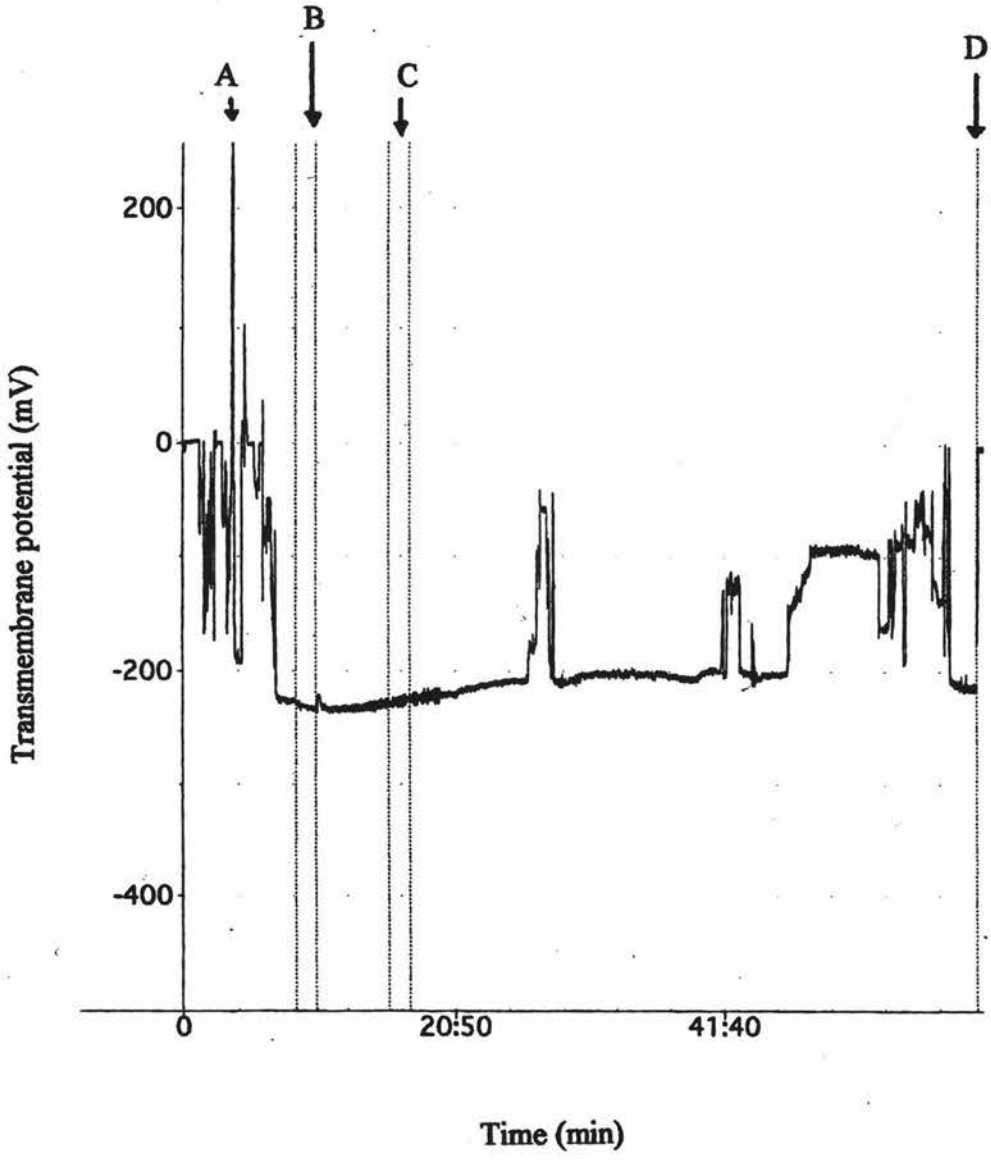


Figure 3.15 Transients in membrane electropotential following the addition of phosphate to the external solution. (A) electrode inserted into root cell, (B) 10 ml solution renewal, KH_2PO_4 concentration = 0, (C)10 ml solution renewal, KH_2PO_4 concentration = 250 μM, (D) electrode removed from root.

CHAPTER FOUR

DISCUSSION

The three early experiments carried out in this project were to develop the various growing methods and analytical techniques that would be used to investigate phosphate uptake in *Arabidopsis thaliana*.

Throughout the experimental work, all plants were grown under aseptic hydroponic conditions to eliminate interference with phosphate absorption, at the plant root surface, associated with mycorrhizal infection (Bieleski 1973), and bacterial contamination (Tate 1984). Any hydroponic solutions and plant material that showed signs of non-sterility, evident as algal growth and greening of the nutrient solution, were rejected from the experiment. In the first two experiments approximately 5% of all plants were rejected under this criteria. in the final experiment all plant material was accepted as contaminant free.

In experiment 1 plants were not able to grow at solution phosphate concentrations of 2 μM or less. In work investigating growth under conditions of low soil phosphate concentrations, healthy plant growth has been reported at much lower concentrations (Pierre and Parker 1927). Asher and Loneragan (1967) were able to grow 8 different temperate annual pasture species including subterranean clover (*Trifolium subterranean*, L.) and silver grass (*Vulpia (Festuca) myuros*, (L.) Gmel.) in sand culture, at a phosphate concentration of 0.2 μM with no apparent symptoms of phosphorus deficiency. Mouat (1983) grew 6 pastoral species 'Grasslands Huia' white clover (*Trifolium repens* L.), New Zealand brown top (*Agrostis tenuis* Sibth.), 'Grasslands Maku' tetraploid lotus (*Lotus pedunculatus* Cav), crested dogstail (*Cynosurus cristatus* L.), 'Grasslands Ruanui' ryegrass (*Lolium perenne* L.) and Kent wild white clover (*T.repens* L.) at phosphate concentrations ranging from 0.2 μM to 5.0 μM by percolating the growth solutions through 15 kg columns of soil. Growth in soils of low phosphate concentrations appears to be possible due to the buffering nature of soils resulting from

the reversible binding of phosphate to the surface of the soil particles (Bieleski 1973). When plants are grown in a natural soil environment, a depletion zone 1-2mm in radial diameter from the surface of the root axis (Jungk 1987), aids in the desorption of phosphate from the solid phase (by diffusion) into the soil solution, providing a relatively constant supply of phosphate to the root. Without the buffering strength of soil particles, phosphate in water possess a diffusion constant 200 times greater than that in soils high in soluble inorganic phosphate but remarkably 200,000 times greater than that for phosphate in deficient soils (Bieleski 1973). Under conditions of experiment 1 it is probable that the rate of phosphate diffusion between plant roots and the 500 ml hydroponic solution, was so rapid as to lead to the total exhaustion of available phosphate in solutions of 2 μM and less, and subsequent plant death at these levels through phosphate starvation.

For plants grown in 500 ml nutrient solutions at phosphate concentrations of 8 μM, 32 μM and 128 μM, there was a lack of developmental uniformity between plants grown in the same container with a communal nutrient source. The differences in plant development however averaged out to give distinct root and shoot weights for each phosphate treatment (Fig. 3.1 and b). Under these conditions it was evident that small differences in plant size prior to transfer from the agar plates on which the plants were germinated were compounded under the conditions of communal, hydroponic growth. In experiment 4 each plant was grown with its own individual nutrient supply.

In experiment 2 the first nutrient depletion trial was conducted. This involved exposing plants to a solution of set phosphate concentration and quantifying net absorption of the ion over a 6 hour time course. To do this 200 μl sample aliquots were removed at various time intervals, from the depletion solution over a 6 hour period and analysed for phosphate content by ion chromatography. For phosphate analysis using this technique, it was necessary to use a depletion solution of vastly different chemical composition to the nutrient solution in which the plants had been grown, to avoid saturation of the ion separator column by the nitrate component of the growth solution which consequently blocks phosphate detection. Even at 1/10 the strength of the growing solution, nitrate levels were still sufficiently high as to mask phosphate detection at the low levels found

in the depletion solution. To avoid this interference, the depletion solution was composed of 0.5 μM $CaSO_4$ and was kept free of all other anion components in the growing solution except for phosphate which was added as KH_2PO_4. The pH of the solution was adjusted to 6.0 by the addition of 0.1 M KOH.

From the depletion curves generated in experiment two (Figure 3.3a), it can be seen that plants of highest phosphorus status increase the phosphate concentration of the depletion solution by approximately 4 μM at the time of the removal of the first sample. This was not expected as the plants had been washed in phosphate free solution for over 8 hours prior to the depletion period to remove any non-bound phosphate from the root systems. Similar results have been reported by Mouat (1983) for clover plants transferred from a complete nutrient solution to a single salt solution of $CaH_4(PO_4)_2$. He refers to this phenomenon as transfer shock and noted similar effects in plants that had been subjected to drying, shaking, or to changes in solution temperature, total salt concentration, or a change in salt species.

In experiment 3 the phosphate component of the depletion solution was radioactively labelled and depletion was measured by recording the difference in radioactivity in sample aliquots collected at different times over the depletion period. Because prior to exposure of the plants to ^{32}P during the depletion period, they did not contain any radioactively labelled phosphate, a measure of the loss of radioactivity from the depletion solution gives a measure of phosphate influx into the plant. In figure 3.4 it can be seen that there was no significant difference in influx between plants of different phosphorus status.

The difference between net uptake as measured in experiment 3 and the gross uptake of experiment 2 is apparent efflux. By comparing figures 3.4 and 3.3 it can be seen that under the conditions of these two experiments, that it was not increased phosphate influx that was responsible for the increase in uptake rate in low phosphorus status plants but rather a reduction in phosphate efflux from the root system of these plants. When comparing these results however it must be noted that these two experiments were conducted on different days using different sets of plants. This issue was addressed in experiment 4.

In work in which phosphate efflux has been measured (Lee et al., 1990), it is reported for maize plants, recovering from phosphorus deficiency, that efflux rate is increased relative to phosphate influx as the phosphorus status of the plant increases. In non P-stressed barley and rape plants (Schjorring and Jensen 1984), efflux is also reported to be much higher relative to influx than for plants of low P-status in which net uptake shows maximum enhancement.

In experiment 4, three different depletion trials were conducted to identify characteristics of phosphate uptake in *Arabidopsis thaliana* that are modified in response to phosphate deficiency. The three characteristics investigated were :-

a) net uptake (influx minus efflux); determined by monitoring the change in total phosphate (initial set10 μM concentration plus phosphate effluxed from the plant minus absorption) concentration of the depletion solution.

b) influx; determined by monitoring the depletion of an initial 10 μM ^{32}P labelled phosphate concentration (excluding effluxed phosphate).

c) the effect of arsenate on influx; determined by monitoring the depletion of an initial 10 μM ^{32}P labelled phosphate concentration in a depletion solution containing 20 μM KH_2AsO_4.

To eliminate the effects of communal growth, and error associated with comparison between experiments carried out on different days (experiments 1, 2 and 3), all plants used in the three depletion trials of experiment 4 were grown as replicates within each phosphorus status, under the same conditions, at the same time with each plant having its own individual nutrient solution.

To reduce the effects that may have arisen from transfer shock in experiments 2 and 3, associated with gross differences in chemical composition between the growth solution

and depletion solution, phosphate was measured by a spectrophotometric assay instead of ion chromatography. Using this technique it was not necessary to alter the chemical nature of the depletion solution with respect to the growing solution (other than standardising the phosphate concentration to 10 μM) because the other components of the nutrient solution did not interfere with the assay. With this technique there was no loss in sensitivity of phosphate detection in relation to ion chromatography.

For depletion experiments b and c, ^{32}P labelled PO_4 was determined by Cerenkov counting.

In this final experiment two different methods were used to develop phosphorus deficiency in the *Arabidopsis* plants. In the first method, plants of low phosphorus status were generated by keeping the concentration of phosphate in the hydroponic growth solution at set, low concentrations of either 10 μM or 25 μM for the duration of the growing period as in the earlier experiments. High phosphorus status plants were generated through growth at phosphate concentrations of 100 μM or 250 μM. Under these conditions root size was not significantly different between different plants with respect to phosphorus status. In the shoot though, there were visual signs of phosphorus deficiency in P10 and P25 plants such as a reduction in shoot size, 56% for P10 plants and 20% for P25 plants compared to P250 plants, and also a noticeable accumulation of red anthocyanin pigment in the leaf tissue of 10 μM grown plants. These symptoms have also been reported for *Arabidopsis* plants containing the *pho1* mutation which not only causes stunted shoot growth at low phosphate levels, but also results in a 95% reduction of inorganic phosphate transported to, and accumulated in the leaf tissue of inflected plants (Poirier et al., 1991). Loneragan and Asher (1967) also report stunted growth in several species of pasture plants grown at phosphate concentrations of 0.04 μM as well as the retention of most of the absorbed phosphate within the root systems of these plants. In plants that are grown continuously at low phosphate levels shoot development is greatly reduced with respect to the root and contains a much smaller concentration of the plant's total phosphate (Loneragan and Asher 1967).

The alternative method used to induce phosphate stress, was to grow plants at the highest concentration of phosphate used in the experiment (250 μM) over the initial stages of development, followed by transfer to phosphate free nutrient solution for the last 5 days of experimental growth. These plants were designated P250→0, and unlike P10 and P25 plants exhibited no obvious external signs of phosphorus deficiency relative to the P250 plants. Lee et al (1990), using a similar method to induce phosphorus deficiency, noted a negligible effect on growth in maize plants after an 8 day period without phosphate but also reported a significant reduction in the concentration of total phosphate in the affected roots of over 50%, and a decrease in vacuolar Pi concentration of over 90% after just 2 days in phosphate free solution. In plants that are induced to become phosphate deficient following a period of non-stressed growth it appears that the shoot is able to act as a phosphate source while the root becomes a strong sink. In corn plants after 4 days exposure to phosphate-free solutions (Anghinoni and Barber 1980) a 50% reduction in shoot phosphate levels was recorded while root phosphate concentrations dropped by only 21%. Root morphology was also altered as the phosphate stress was prolonged with both an increase in root weight and length reported. There was no significant change in the shoot weight. Smith et al (1990) reports a net flow of inorganic phosphate from the shoot to the root in *Stylosanthes hamata* plants at the onset of phosphate deficiency.

Under phosphate stress, increase in the rate of phosphate uptake is achieved by shifts in the kinetic parameters by either decreasing Km, by increasing Vmax or by alteration of both parameters (Meharg and Macnair 1992). Both methods employed to induce phosphate deficiency in this study have resulted in difference from the kinetic uptake parameters that characterise phosphate absorption by non-phosphate stressed plants.

P10 and P25 plants that had been grown continuously in low phosphate concentration solutions, exhibited a decrease in Km for phosphate without a concomitant alteration in Vmax (Table 3.1). Similar findings have been reported for other plant species grown continuously at sub-optimal phosphate levels. Mouat (1983) reported a phosphorus deficiency induced Km decrease in the pasture species Brown top. In barley plants grown in media deficient in phosphate, a Km value decrease of 3 fold was reported without an

associated change in Vmax (Cartwright 1972). Jungk et al (1990) report Km decreases for both maize and soybean plants grown at low phosphate concentrations.

In P250→0 plants, in which phosphate was withheld from the nutrient solution for the last 5 days of growth, Vmax values were significantly increased without changes in Km. Anghinoni and Barber (1980) using a similar regime to induce phosphate stress, report a 55% increase in Vmax for phosphate starved maize roots without an accompanying change in Km. Vmax increases without significant change to Km are also reported for *Lemna gibba* plants (Ullrich-Eberius et al 1984), and barley roots (Drew et al., 1984) when phosphate nutrition is stopped.

Comparison of the respective depletion curves for each phosphorus status (Fig. 3.7) indicate that P10 and P250→0 plants have an enhanced capability to absorb phosphate in the concentration range of the high affinity uptake mechanism, part of this enhancement being due to an apparent reduction in the level of phosphate efflux by the respective root systems (Fig.3.6).

By dissecting the plants used in the two $^{32}PO_4$ depletion experiments of experiment 4, into roots and shoots, and measuring the radioactivity of each segment by Cerenkov counting, the amount of $^{32}PO_4$ translocated from the root to the shoot of each plant over the depletion period was determined. Greatest translocation of the $^{32}PO_4$ was found to have occurred in P10 and P25 plants respectively, with over half the absorbed $^{32}PO_4$ being translocated to the shoots. For P100, P250 and P250→0 plants the majority of $^{32}PO_4$ absorbed remained in the root tissue with the level of the $^{32}PO_4$ retained in the roots of P250→0 plants significantly higher ($p<0.001$) than for any other treatment. There was no significant difference in concentrations of $^{32}PO_4$ in the roots of P10, P25, P100 or P250 plants.

Just as the concentration of $^{32}PO_4$ within the roots and shoots of these plants is not representative of the total concentration of inorganic phosphate in these tissues, nor is the amount of $^{32}PO_4$ translocated between the root and the shoot representative of the total amount of inorganic phosphate under transport in these plants. Upon absorption,

$^{32}PO_4$ entering into the plant root system mixes with a non-labelled pool of phosphate accumulated in the plant over the course of its life time. Because the $^{32}PO_4$ forms only a fraction of this pool, the amount of $^{32}PO_4$ translocated to the shoot represents only a fraction of the total phosphate translocated. The size of this fraction is dependant on the size of the non-labelled inorganic pool of phosphate in the root system.

For plants grown under conditions of phosphorus deficiency it is likely that they will contain a smaller pool of phosphate in their root system than plants grown at much higher phosphate concentrations. If this is assumed for P10 plants with respect to P250 plants then this may explain the differences in $^{32}PO_4$ translocation, with the greater amount of $^{32}PO_4$ in the shoots of P10 plants being a consequence of a smaller pool of non-labelled phosphate in their root system with respect to P250 plants.

For P250→0 plants, in which phosphorus deficiency (evident as increases in phosphate uptake (Fig. 3.7) and Vmax (Table 3.1) with respect to P250 plants) was established by withholding phosphate from the nutrient solution for the last 5 days of their growth period, $^{32}PO_4$ translocation patterns (Fig. 3.12a) show greater similarity to those observed for P250 plants than the $^{32}PO_4$ distribution patterns of the phosphorus deficient P10 plants. This may be because the amount of $^{32}PO_4$ translocated in P250→0 plants is representative of a larger fraction of a smaller total amount of translocated phosphate, with respect to P250 plants, due to a dwindling phosphate pool in the root system under these conditions of phosphorus deficiency (Lee et al., 1990). This would support Bieleski's (1973) statement of "the roots of a P-deficient plant usually retain more P and transport much less. By pre-empting Pi, root growth is maintained at the expense of shoot growth, leading to the decreased shoot/root ratio that is a feature of P-deficiency" which may also explain the significant increase ($P<0.001$) in the amount of $^{32}PO_4$ accumulated in the root system of P250→0 plants with respect to P250 plants. Without knowing the size of the total amount of phosphate translocated over the course of the depletion experiments these ideas remain speculative only, and await further investigation.

Alterations in Km with or without changes to Vmax are believed to indicate the functioning of a second type of transport system operating with a different affinity for the same ion under concern (Epstein 1972, Lee 1982). In *Holcus lanatus*, arsenate tolerance is due to suppression of the high affinity phosphate uptake system (Meharg and Macnair 1992). This also appears to be the case in the arsenate tolerant grasses *Agrostis capillaris* and *Deschapsia cespitosa* (Meharg and Macnair 1991).

In the final trial of experiment 4, 200 nmol of arsenate (to give a 20 μM concentration), was added to the final 10 μM $^{32}PO_4$ depletion solution, to determine if plants of different phosphorus status exhibited differences in phosphate uptake in the presence of arsenate. In higher plants arsenate is absorbed by the same uptake systems as phosphate (Marschner 1995) and is therefore a competitive inhibitor of phosphate uptake. Arsenate has also been found to act as a phosphate analogue during phosphorylation processes and the synthesis of compounds such as ATP. In these situations arsenate becomes an energy uncoupler as it does not form the energy rich bonds associated with phosphate compounds, and hence can not be used as an energy source in active metabolic processes (Luttge and Higinbotham 1979).

The Vmax values recorded for phosphate uptake by all plants, in the presence of arsenate were not significantly different from the values recorded for phosphate uptake in replica plants exposed to arsenate free solution. This indicates that the nature of the arsenate inhibition on phosphate uptake is competitive (Ullrich-Eberius et al., 1989, Asher and Reay 1979). Mild competitive inhibition has also been reported for 3 day old *Arabidopsis* plants grown at phosphate concentrations ranging from 0.0 μM to 5 mM (Dunlop et al., 1997).

Comparison of figures 3.7 and 3.8 indicate that $^{32}PO_4$ uptake was severely reduced in the presence of arsenate, regardless of phosphorus status. The degree of reduction was such as to eliminate the differences in phosphate absorption rates between different phosphorus status plants, that were evident for phosphate uptake in arsenate free depletion solution. Total phosphate absorption by P10 plants was reduced by 48.9% while a reduction of 31.5% was recorded for P250 plants when arsenate was added to

the depletion solution. Figures 3.12a and 3.12b indicate that approximately the same amount of $^{32}PO_4$ was accumulated in the root systems of each plant with or without arsenate present in the depletion solution. Comparison of the amounts of phosphate transported to the shoots of these same plants indicates that arsenate has severely inhibited phosphate translocation, evident as a reduction in the amount of $^{32}PO_4$ present in the shoots of P10 plants of 92% and a 67% reduction in shoot accumulated $^{32}PO_4$ recorded for P250 plants.

These results indicate that transport of phosphate into the xylem in *Arabidopsis* plants is more severely inhibited by arsenate than absorption of phosphate into the root system. These results could be explained by the existence of a high affinity xylem loading phosphate transporter that is induced under conditions of phosphate deficiency, which is of higher susceptibility to arsenate competitive inhibition and toxicity than the equivalent xylem loading mechanisms of high phosphorus status plants.

The absence of such a high affinity transport mechanism as suggested here, is believed responsible for the decreased ability of the *pho1 Arabidopsis* mutant to transport phosphate to its shoot when grown in media containing 200 μM phosphate or less. This defect resulting in only 3 to 10% of the amount of phosphate being transferred to the shoot in mutant plants as occurs in wild type plants grown under identical conditions, is found to be overcome when the plants are grown in the presence of an abundant phosphate supply (Poirier et al.,1991). These results also suggest that the shoot may have a regulatory role in governing the amount of phosphate absorbed by the plants, as $^{32}PO_4$ accumulation within plant roots in the presence or absence of arsenate was not significantly different. However the total amount of phosphate absorbed was severely reduced when phosphate was blocked from exiting the root into the shoot by arsenate inhibition as too were the differences in phosphate absorption rates associated with phosphorus status. It is also clear from these results that phosphate translocation in low phosphorus status plants is much more inhibited than in high phosphorus status plants (Fig. 3.13).

In literature reporting ion influx into plants e.g Epstein 1972, Anghinoni and Barber 1980, Drew et al., 1984, and Ullrich-Eberius èt al., 1984, uptake rates are usually expressed on a per root size basis such as weight or length. This is done in order to provide a common basisfor ion uptake comparisons to be made between plants of different size. Krannitz et al (1991a) found though, that this form of standardisation implies that the two variables uptake rate and root size are linearly related to influx such that an increase in root size results in a proportional increase in ion absorption. This relationship would be true if a line of regression relating the two variables root size and absorption had an intercept at zero. For the *Arabidopsis* plants grown under the conditions of this study no such relationship was found to exist (Fig. 3.10). As a consequence, to avoid spurious correlation between treatment effects and uptake rate based on plant size, Vmax values in this report have been expressed on a per plant basis. Uptake rates expressed in this manner were found to have less standard deviations between replicate plants than if uptake was expressed on a per mg of dry root basis (Fig. 3.9).

CHAPTER FIVE

CONCLUSIONS

This study reports the findings on the effects of different levels of phosphorus nutrition on phosphate absorption by *Arabidopsis thaliana*, a plant widely used as a model system for the study of developmental and physiological processes in higher plants.

As recorded for other plant species, *Arabidopsis* responds to prolonged phosphorus deficiency by maintenance of root growth at the expense of the shoot (Figs. 3.1b, 3.2 and 3.11) resulting in a difference in shoot to root dry weight ratio of 2.4 for high phosphorus status plants (P250) to 1.6 for low phosphorus status plants (P10).

On re-supply of phosphate in the low concentration range of the high affinity mechanism, phosphorus deficient plants were found to exhibit alterations in the kinetic parameters Km and Vmax for phosphate absorption, that were dependent on how the phosphorus deficiency state had been induced. In plants that were grown continuously at low phosphate concentrations (P10 and P25 plants), there was a decrease in Km (higher affinity for the phosphate ion) without a concomitant change in Vmax. However in plants in which phosphorus deficiency had been induced by stopping the supply of phosphate to high phosphorus status plants (P250) for a period of 5 days, a large increase in Vmax was recorded but no alteration in Km. These results highlight a degree of phenotypic plasticity in response to diversities in environmental phosphate availability that perhaps need to be considered for other species when evaluating shifts in kinetic uptake parameters associated with phosphorus deficiency.

Another notable effect of phosphorus deficiency in *Arabidopsis* plants was a reduction in the level of phosphate efflux with respect to high phosphorus status plants. This may simply be a consequence of the reduced levels of phosphate contained in low phosphorus status plants, but if future research proves this to be a physiological adaptation to

phosphorus deficiency, then altering plant physiology to reduce phosphate efflux could be a prime research area that facilitates the development of phosphorus efficient plants.

Finally, *Arabidopsis* plants grown under the conditions of this project were severely inhibited in their ability to actively absorb 10 μM phosphate in the presence of 20 μM arsenate. Greatest inhibition was found not to occur at the level of absorption into the plant root system but rather appeared to be at a site involved in phosphate loading into the xylem. As inhibition was greatest in low phosphorus status plants, it is suggested that these plants possess high affinity phosphorus deficiency induced phosphate xylem loading mechanisms that are of greater susceptibility to arsenate competitive inhibition and toxicity than equivalent xylem loading mechanisms of high phosphorus status plants.

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APPENDIX 1

DIONEX METHOD PARAMETERS - PHOS.MET

System Parameters

System Name: Anion/cation system	
Number of Detectors	1
Detector 1 Type	CDM-2
Detector 1 real time plot scale (nS)	30000.00
Run time (mins)	8.00
Sampling rate (seconds)	0.20

-- DETECTOR 1 PARAMETERS --

Report Options

Save Data File	Yes
Data File Name: C:\DX\DATA\DATA0681.D02	
Create ASCII Report File	No
Print Report	Yes
List Peaks Not Found in this run	Yes
Report Unknowns Found in this run	Yes
Print Chromatogram	Yes
AutoScale Chromatogram to Highest Peak	No
Fill Peaks with Colour	No
Draw Grid Lines on Chromatogram	No
Label with Peak Number	No
Label with Retention Times on Chromatogram	No
Label with Component Name	No
Format File Name: C:\DX\METHOD\DEFAULT.PRF	

Integration Parameters

Starting Peak Width (seconds)	10.0
Peak Threshold (mV or uS/data pt interval)	0.500
Peak Area Reject	1000
Are Reject for Reference Peaks	1000
Percent Retention Time Window for Reference Peaks	5.0

Data Events

Time	Description
1.00	Start peak detection
1.90	Force baseline at start of all peaks
8.00	Stop peak detection

Calibration Parameters

Number of Levels for Calibration	1
Calibration Fit Type	Linear
Replace Or Average Calibrations	Replace
External or Internal Calibration	External
Calibrate by Area or Height	Area
Default Injection Volume	1.0
Default Dilution Factor	1.0
Response Factor for Unknown Peaks	0.0
Calibration Standard Volume	1.0
Internal Standard Volume	1.0
Sample Unit	ppm

Component # 1 chloride — Retention Time 1.45
Reference Peak phosphate — Window Size 3.00%
Amount = K0 + K1*Area
K0 = 0.00000E+000
K1 = 6.36266E-008

Level	Amount	Area	Height
1	2.80000E+000	44006734	8332330

Component # 2 nitrate — Retention Time 2.67
Reference Peak chloride — Window Size 3.00%
Amount = K0 + K1*Area
K0 = 0.00000E+000
K1 = 1.29336E-007

Level	Amount	Area	Height
1	1.00000E+001	77318256	8664374

Component # 3 phosphate — Retention Time 4.20
Reference Peak sulphate — Window Size 3.00%
Amount = K0 + K1*Area
K0 = 0.00000E+000
K1 = 2.49757E-007

Level	Amount	Area	Height
1	1.50000E+001	60058267	3849627

Component # 4 sulphate — Retention Time 5.53
Reference Peak sulphate — Window Size 3.00%
Amount = K0 + K1*Area
K0 = 0.00000E+000
K1 = 1.07072E-007

Level	Amount	Area	Height
1	1.00000E+001	93394775	4784832

APPENDIX 2

THEORY

(copied from Shimogawara, K. and Usada, H. 1995).

The Michaelis-Menten rate equation (Eq. 1)

$$\frac{-\mathrm{d}[\mathrm{S}]}{\mathrm{d}t} = \frac{V_{\max}[\mathrm{S}]}{K_m + [\mathrm{S}]} \tag{1}$$

can be solved by regarding it as a differential equation with two variables, [S] and t. In this way, we obtain

$$V_{\max} t + K_{\mathrm{m}} \ln\left(\frac{[\mathrm{S}]}{[\mathrm{S}_0]}\right) + [\mathrm{S}] - [\mathrm{S}_0] = 0, \tag{2}$$

where $[S_0]$ is the initial concentration of the substrate.

Dividing by K_m and regrouping, we obtain

$$\frac{V_{\max} t}{K_m} + \ln[\mathrm{S}] + \frac{[\mathrm{S}]}{K_m} = \ln[\mathrm{S}_0] + \frac{[\mathrm{S}_0]}{K_m} \tag{3}$$

By subtracting $\ln(K_{\mathrm{m}})$ from both sides of the equation, we obtain

$$\frac{V_{\max} t}{K_m} + \ln[\mathrm{S}] - \ln(K_m) + \frac{[\mathrm{S}]}{K_m} = \ln[\mathrm{S}_0] - \ln[K_m) + \frac{[\mathrm{S}_0]}{K_m} \tag{4}$$

Hence,

$$\frac{V_{\max} t}{K_m} + \ln\left(\frac{[\mathrm{S}]}{K_m}\right) + \frac{[\mathrm{S}]}{K_m} = \ln\left(\frac{[\mathrm{S}_0]}{K_m}\right) + \frac{[\mathrm{S}_0 1]}{K_m} \tag{5}$$

Let us define the function $L(x)$ as

$$L(x) = -\ln(x) - x \ (\because x>0). \tag{6}$$

Substituting Eq. 6 into Eq. 5, we have

$$\frac{V_{\max} t}{K_m} - L\left(\frac{[\mathrm{S}]}{K_m}\right) + L\left(\frac{[\mathrm{S}_0 1]}{K_m}\right). \tag{7}$$

Let us now define L^{-1} as the inverse function of the function L, so that Eq. 7 can be solved for [S] as

$$[S] = K_m L^{-1}\left\{\frac{V_{max} t}{K_m} + L\left(\frac{[S_0]}{K_m}\right)\right\} \quad (8)$$

Thus, the concentration of phosphate remaining in solution after each sample collection is expressed explicitly by a variable t (time) and three parameters (K_m, V_{max} and S_0). This function is best-fitted to the series of data points on the curve for depletion of substrate (Pi) by the non-linear least-squares method. Then K_m, V_{max} and S_0 (with their standard errors) can be obtained as outputs.