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.

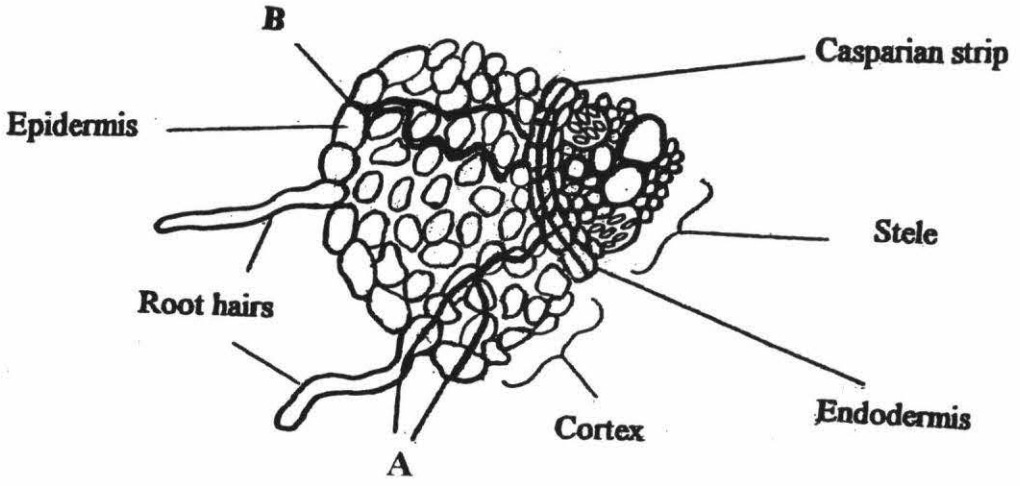

Figure 1.1 Schematic diagram of a dicotyledonous root in cross section showing the symplastic (A) and apoplastic (B) pathway of ion transport.

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).

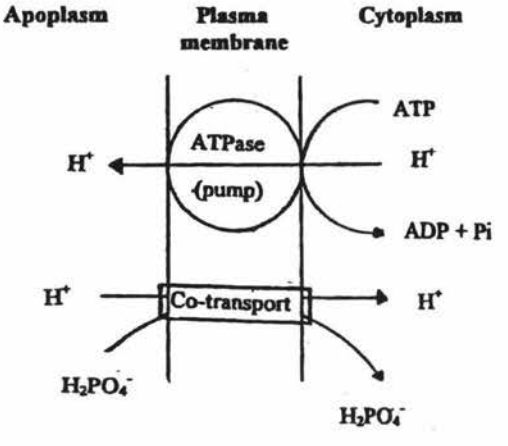

Figure 1.2 Model for the functioning and location of the electrogenic proton pump (H^+ - ATPase), and the phosphate:proton co-transport system.

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).

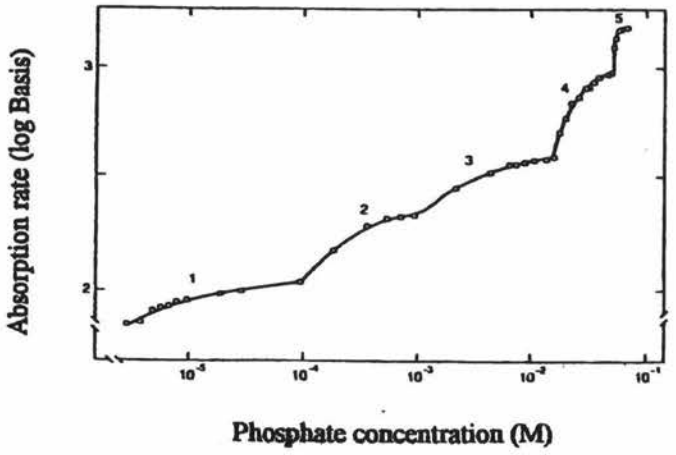

Figure 1.3 Graph of phosphate uptake rate as a function of phosphate concentration in the media surrounding maize root sections. Five separate kinetic classes are observed (numbered 1-5) over a 25,000 concentration range. Copied from Nandi et al., (1987).

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.