ACTIVITY OF ENZYMES INVOLVED IN PHOSPHORUS METABOLISM AS INDICATORS OF PHOSPHORUS STATUS IN WHEAT

By

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CHAPTER I

INTRODUCTION

The ultimate goal of agricultural research is to maximize crop production. In addition to control of plant diseases and environmental stresses, plant nutrition is a major theme in the improvement of crop production. Adding fertilizer to soil is the most direct way to improve crop production and has been known in agriculture for a long time (Marschner, 1986). Phosphorus is one of the most important nutrient elements in common fertilizers. Phosphorus can produce greater increases in crop yield with smaller amounts than potassium or nitrogen (Le Bot *et al.*, 1994), and is believed to be the second most important nutrient for crop production after nitrogen (Vose, 1991). The preferentially assimilated form of phosphorus is phosphate, which is a structural constituent of many important biomolecules, such as DNA, RNA (genetic information carriers), ATP (biological energy currency), and phospholipids (cell membranes). In addition, phosphorus compounds also play a regulatory role in metabolism. Although phosphorus is closely related with these vital functions in plant physiology, even extreme phosphorus deficiency does not lead to rapid death of cells and the whole plant, as would be a logical supposition.

This is because metabolism can be maintained by repeated and intensified utilization (recycling) of phosphorus in phosphorus deficient plants. In the long term, however, phosphorus deficiency may drastically reduce the yields of crops, even when it is not easily detected at an early stage. Studies of the correlation between the amount of fertilizer applied and grain yield of field-grown wheat suggest that phosphorus is certainly a limiting factor in grain yield, but the critical levels for phosphorus differ substantially by location (McLachlan, 1982; Raun and Westerman, 1991). Although crop yields are often reduced due to lack of fertilizer application, much of the fertilizer that is applied is wasted because of failure to adjust fertilizer application properly (Greenwood, 1982). Therefore, it is important and necessary in crop production improvement to diagnose phosphorus deficiency or identify the phosphorus status of crops at an early stage and apply appropriate fertilizer. In order to provide these fertilizer recommendations, plant tissue analysis and chemical soil analysis are usually employed, although neither is totally satisfactory (see next chapter). The use of enzymes that are induced or enhanced as natural responses during phosphorus deficiency as indicators may be a better alternative. Enzymes whose activities have been found to increase during phosphorus deficiency include acid phosphatase, pyrophosphate-dependent phosphofructokinase (PFP) and phosphoenolpyruvate carboxylase (PEPCase). The purpose of the present study is to examine, using enzymatic and immunological methods, the correlation between the expression of these enzymes and the physiological availability of phosphorus in hydroponically grown wheat seedlings.

Studies of these new techniques have the potential to improve diagnosis of phosphorus deficiency, and may also provide us some useful information on the mechanisms by which phosphorus deficiency affects plant growth and how plants survive under phosphorus deficiency.

CHAPTER II

REVIEW OF THE LITERATURE

Mineral Nutrition of Plants

The questions of where plants obtain their nourishment has long intrigued humans. Jan van Helmont (1577-1644) seems to be the first one to figure out this question with a well designed quantitative experiment (Hewitt and Smith, 1974; Epstein, 1972). He planted a willow branch of known weight in a tub of known weight of oven dried soil and added water as needed. After 5 years the willow increased 74 kg, while the soil had lost only about 60 g. Now we know that most of the plant biomass comes from water and carbon dioxide by photosynthesis and that the very little loss in the weight of the soil which was attributed to wastage is probably important mineral nutrients taken up by the willow.

Mineral elements have long been known to improve plant growth (Marschner, 1986), although no one knew what they are, or how they worked until the beginnings of modern experimental science. In the nineteenth century, some mineral elements such as nitrogen, sulfur, phosphorus, potassium, calcium, magnesium, and iron had been found essential for plant growth. With the improvement of analytical chemistry, additional trace essential elements including

boron, zinc, copper, molybdenum and chlorine were discovered before the mid-twentieth century (Marschner, 1986).

One direct method to determine which elements are essential to plants is to analyze which elements healthy plants contain (Salisbury and Ross, 1985), but because the uptake mechanism does not make an absolute selection between essential and other elements, not all elements detected in plant tissues are essential (Epstein, 1972; Mehra and Farago, 1994). In order to judge if a element is essential, two criteria of essentiality were proposed (described in Epstein, 1972): First, an element is essential if the plant cannot complete its life cycle without this element. Second, an element is essential if it is part of any molecule of an essential plant constituent or metabolite. Although these two criteria are enough for most cases, a third criterion was suggested by Arnon and Stout (1939) to specify that indirect beneficial effects of an element do not qualify an element as being essential. That is to say, if an element is essential, it must be acting directly within the plant.

However, a technique to grow plants in a nutrient medium, in which the amount of every elements is precisely controlled, is necessary for the test of nutrient requirements and other studies of plant nutrition. Growing plants in this way is called hydroponics, or solution culture. According to reviews of hydroponics given by Epstein (1972), Hewitt and Smith (1974), and Jones (1982), Woodward in 1699 made the earliest recorded use of a water culture method with water obtained from rivers, springs, rainfall, and so on. Then, about 1860, Sachs showed that plants can grow normally to maturity in his nutrient solution without soil. At the same time,

Knop devised another nutrition solution containing KNO_3, $Ca(NO_3)_2$, KH_2PO_4, $MgSO_4$, and $FePO_4$ based on molar ratios, and this also provided the basis for further modifications by others. The nutrient solution formula that is widely accepted and used was proposed by Arnon and Hoagland in 1940. Some essential micronutrients which had just been discovered are contained in this formula. Hydroponic technique is not only a tool for studying plant nutrition, but it also reflects the progress in the knowledge of plant nutrition.

The importance of mineral nutrition in crop production is proved by the drastic improvement of production using fertilizer. Although humans have made much progress in this kind of improvement, a better understanding of plant mineral nutrition is still necessary to improve efficiency of fertilizer use in crop production. Longnecker (1994) suggested two way to achieve this: First, improve diagnosis of nutrient deficiency and appropriate timing of fertilizer application to take maximum advantage of the added nutrient. Second, use of plant species or cultivars which are more efficient at using a given nutrient supply.

The Physiological Role of Phosphorus in Plant Growth

Phosphorus plays a vital role in the life cycle of all living things, as a component of the nucleic acids of genes and chromosomes which carry the genetic code. Phosphate forms a bridge between ribonucleoside units, in both DNA and RNA, to form very large molecules. The third hydroxyl of the bridging phosphate is still readily ionizable, with a pKa around 4, so that even as a bridge, the phosphate remains a hydrophilic, moderately acidic center (Bieleski and Ferguson, 1983).

The phosphate "bridge" is also found in the phospholipids present in membranes of all cells and subcellular organelles. In addition to serving as a constituent of membranes, the phospholipids also play an important role in the membrane transport of divalent ions, especially calcium (Seimiya and Ohki, 1973).

Phosphorus is also a constituent of the energy-rich molecule, ATP, which supplies the energy required for biosynthesis, active transport and any other reaction which needs energy. The energy is released or obtained with the phosphoryl group in a phosphorylation reaction. This kind or phosphorylation reaction occurs in almost all major metabolic pathway in plant cells. ATP is also required, especially for starch synthesis, while UTP and GTP, also containing the energy-rich phosphate bond, are required for synthesis of sucrose and cellulose respectively.

At least 50 individual phosphate esters have been identified in plants (Bieleski and Ferguson, 1983). Most of them are involved in metabolic pathways in plant cells. For example, many metabolites in glycolysis are phosphate esters, such as glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-phosphate, glyceraldehyde-3-phosphate, and so on.

In addition to its structural and metabolic role, phosphate plays a regulatory role in starch and sucrose synthesis and in partitioning carbon between the reserve polymer and disaccharide (Preiss, 1984). Inorganic phosphate, which accumulates in chloroplasts in the dark, is an inhibitor of the enzymes directly involved in the synthesis of starch, while 3-phosphoglycerate, present in illuminated chloroplasts, is

an activator of ADP-glucose synthetase, an enzyme involved in starch synthesis. Phosphate also enhances the release of photosynthate from chloroplasts to the cytosol, and activates the synthesis of fructose-2,6-bisphosphate, which inhibits fructose-1,6-bisphosphatase and stimulates glycolysis in the cytosol (Marschner, 1986; Preiss, 1984).

The Uptake, Translocation, and Metabolism of Phosphorus

Phosphorus is taken up by the plant root mainly as monovalent dihydrogen phosphate ion $H_2PO_4^-$ (Ozanne, 1980). The availability of phosphate to plants depends on the total amount of phosphorus in soil, but the availability is limited if phosphate in soils is adsorbed very strongly. The degree of adsorption is affected by many soil factor such as the soil pH and the composition of soil. Phosphate ion is adsorbed more strongly at low pH, or with aluminum and iron oxides in soil (Le Bot *et al.*, 1994). Moisture (Mackay and Barber, 1985) and temperature (Ozanne, 1980) are also important factors affecting the availability of phosphate to plants. Because phosphorus moves only limited distances from its source or location (about 5 mm), and roots of plants make up less than 1% of the total soil volume, roots make contact with less than 1% of the available phosphorus (Clark, 1991).

In addition to soil factors, many plant factors are also important in phosphorus uptake. Mechanisms by which cereal genotypes have differential abilities to grow at low or high phosphorus are not completely understood. Clark (1991) has suggested that factors such as root properties (root morphology, distribution, size, length, and

type), phosphorus translocation, distribution, accumulation, metabolic use, and interactions with other elements in plants need to be considered.

Bieleski and Ferguson (1983) compared the nutrient concentration in a typical plant tissue, its xylem sap, and the soil solution bathing the roots, and concluded the uptake of phosphate has to be a metabolically driven process, more so than for any other mineral nutrient. The effective gradient to be overcome for phosphate uptake is about 10^6-fold, if the negative membrane potential of the cell is taken into account. Phosphate is usually taken up by an active mechanism with either H^+ cotransport or HCO_3^- antiport pump (Ullrich-Eberius *et al.*, 1981).

In plant cells all phosphorus occurs as phosphate, either free as orthophosphate, or esterified to a carbon chain as in the simple phosphate esters, or attached to another phosphate by a pyrophosphate bond. However, phosphorus in phosphate is not reduced to a lower oxidation state, unlike nitrogen and sulfur in nitrate and sulfate. The phosphorus-containing material in the plant tissue was divided by Bieleski and Ferguson (1983) into five fractions: inorganic phosphate, RNA, DNA, phospholipid, and simple phosphate ester. The absolute and relative amounts of each fraction will depend on the nature of the tissue and the amount of phosphorus supplied (Marschner, 1986). Bieleski (1968) found that transferring the duckweed, *Spirodela oligorrhiza,* to a P-deficient medium decreased the organic P content by only 4-fold but the inorganic P concentration by 40-fold. Working with tobacco leaves, Kakie (cited from Marschner, 1986) also found that only the inorganic phosphate increases when phosphorus supply is increased above the

optimal level. These results reflect the ability of plants to store inorganic phosphate in vacuoles of highly vacuolated tissues. However, in grains and seeds most of the phosphate is stored as phytate (hexainositol phosphate), which is the most important phosphorus storage compound in plant seeds. In legume seeds, phytate phosphorus makes up ~50% of the total phosphorus, 60-70% in cereal grains, and 86% in wheat mill bran (Marschner, 1986).

Phosphorus Deficiency in Plants

Generally, phosphorus-deficient plants are stunted, often darker green in color than normal plants, since cell expansion is reduced more than chlorophyll synthesis and thus chlorophyll content per unit leaf area is higher. Leaves of many species often have red and purple coloration because of anthocyanin formation (Longnecker, 1994; Marschner, 1986; Salisbury and Ross, 1985; Hewitt, 1984). Because of the high mobility of phosphate in plants, phosphate is easily moved from older leaves to younger leaves. Therefore, deficiency symptoms are usually found on the oldest leaves (Salisbury and Ross, 1985).

Roots of phosphorus deficient-plants usually have longer roots per mass (Powell, 1974), and longer and more dense root hairs (Marschner, 1986). This response can be logically considered as an adaptation mechanism to increase roots surface area. In addition, like nitrogen deficiency, phosphorus deficiency also causes an increase in the ratio of roots to shoots (Moorby and Besford, 1983; Longneker, 1994). Moorby and Besford (1983) suggested that the increased ratio is

because under nutrient deficiency, the proportion of absorbed nutrients that is transported from roots to shoots is reduced. Fredeen *et al.* (1989), for example, found that there was a higher proportion of total plant phosphorus in the root system of phosphorus deficient soybean plants compared to control plants. Longnecker (1994) provided an alternative view that suggested the greater root growth in phosphorus-deficient plants is due to the feedback through shoots. This view is supported by the work of Smith *et al.* (1990) who, working with *Stylosanthes*, showed that root growth was initially stimulated in response to phosphorus deficiency.

As described in the previous section, phosphate plays an important role in starch and sucrose synthesis and in carbon partitioning. Therefore, starch accumulates (Fredeen *et al.*, 1989), and triose phosphate in chloroplast increases, in phosphorus-deficient plants (Rao *et al.*, 1990).

Diagnosis of Phosphorus Deficiency

Diagnosis by visual symptoms is the quickest method for determining the causes of crop failure due to nutrient deficiency, but there are some limitations. Because of the widespread use of fertilizer, nutrient deficiencies now are usually not severe enough to be characterized by specific visible symptoms, and similar symptoms which are induced by non-nutritional factors may be mistaken for nutrient deficiency (Hewitt, 1984; Marschner, 1986). To avoid this confusion, Marschner (1986) suggested that the typical symmetric pattern of the symptom of

nutritional disorder should be noted. Furthermore, visual diagnosis may be especially complicated when more than one mineral nutrient is deficient or there is simultaneously toxicity of another nutrient (Marschner, 1986). In the case of phosphorus, symptoms of phosphorus deficiency often resemble those of nitrogen, and the pigmentation due to phosphorus deficiency does not appear in every species. However, Marschner (1986) suggested that visual diagnosis is not a sufficient basis for making fertilizer recommendations, but it can provide a quick screening test to select possible nutrients for further precise analysis.

Using chemical methods to analyze the nutrient content in plants tissue is a direct way to determine if plants are under nutrient deficiency. Actually, plant analysis has been employed to assess the soil fertility for a long time: it was used by the German chemist Liebig in last century (Bould, 1984). To correct or prevent phosphorus deficiency through plant analysis, a highly correlated relationship between plant growth and phosphorus concentration in plant tissue is required. The critical concentration of phosphorus in tissue, which is defined as those levels at which growth or yield is 5 to 10% below maximum (Bouma, 1983), must be determined for use as the boundary between phosphorus sufficiency and deficiency. Unfortunately, in addition to phosphorus status, the phosphorus content of plant is affected by many other factor including developmental stage of plants, age of leaves (if leaves are used as sample), plant species, the interactions with other nutrient elements, and some environmental factors (Marschner, 1986). Kamprath and Watson (1980) suggested that in order to improve the use of plant tissue analysis as

a tool for diagnosing the phosphorus status of soils, more information is needed on how various growth factors affect the correlation between phosphorus content in the tissue and plant growth, as well as studies to determine the critical concentration of phosphorus in plant tissue when other factors have been adequately supplied.

Like plant analysis, soil testing is also a conventional method to determine the phosphorus status for plants, and the relationship between phosphorus levels in soil and the corresponding growth or yield curves must be determined. It is, however, difficult to accomplish this because soil analysis can only provide the potential availability of phosphorus, and as described in the previous section, the uptake of phosphorus as well as other elements is affected by numerous soil and plant factors. Without sufficient information and understanding of such factors, it is difficult to interpret the results of soil tests. Marschner (1986) stated that a combination of both methods provides a better basis for fertilizer recommendation than one method alone.

Acid Phosphatase and Phosphorus Deficiency

Acid phosphatase (EC 3.1.3.2.), functions to hydrolyze inorganic orthophosphate from orthophosphate monoesters with optimal pH below 7.0. It is widely distributed among plants, with multiple forms differing in size, substrate specificity and electrophoretic behavior (Pan, 1987).

Under phosphorus deficiency, acid phosphatase activity in tissue extract was reported to increase in algae (Price, 1961), moss (Press and Lee, 1983), duckweed

(Bieleski and Johnson, 1972), tomato (Besford, 1979a), barley (Besford 1979b), cucumber (Besford 1979b), maize (Besford 1979b), oat (Besford 1979b), wheat (Besford 1979b; Barrett-Lennard *et al.*, 1982), and karri tree, (O'Connell and Grove, 1985). However, a significant increase was not found in bean and cowpea under mild phosphorus deficiency (Fernandez and Ascencio, 1994). Increased secretion of acid phosphatase activity was also found on root surfaces (Barrett-Lennard *et al.*, 1993; Antibus and Lesica, 1990; Hedley *et al*, 1982) or in the medium of suspension cells (Goldstein *et al*, 1988; Lefebvre *et al*, 1990). Working with field-grown wheat, McLachlan (1982) found a significant inverse relationship between acid phosphatase activity and both phosphorus content during the early stage, as well as final grain yield.

p-Nitrophenyl phosphate (pNPP) is usually used as substrate for acid phosphatase activity assay, because the yellow color of the product, *p*-nitrophenol, can be detected spectrophotometrically very conveniently. Other substrates are occasionally used, if the assay for some specialize acid phosphatase is necessary, such as PEP for PEP phosphatase (Duff et al., 1991).

Electrophoretic analysis is sometimes used for the studies of acid phosphatase (Pan, 1987; McLachlan, 1984; Barret-Lennard *et al.*, 1982), because its multiple forms (isozymes) can be easily visualized by staining for enzymatic activity. Barret-Lennard *et al.* (1982) using cellulose acetate electrophoresis showed that there are two enhanced isozymes of acid phosphatase of phosphorus-deficient wheat seedling. Working with field-grown wheat, McLachlan (1984) also found one enhanced

isozyme of acid phosphatase under phosphorus deficiency using starch gel electrophoresis. McLachlan *et al.* (1987) showed that the grain yield of wheat is highly correlated to a special leaf acid phosphatase isozyme which was observed in the previous work (McLachlan, 1984).

Although acid phosphatase was reported not to be affected by imbalances of nutrient elements other than phosphorus (Besford, 1979c), an increase in acid phosphatase activity was reported in wheat due to aging (McLachlan, 1984), in spinach plants experiencing salt stress (Pan, 1987), as well as water deficit stress, but with an electrophoretic pattern different from phosphorus deficiency (Barret-Lennard *et al.*, 1982). Nonetheless, acid phosphatase has been suggested as a useful indicator of phosphorus stress in crops by Besford (1979b), and McLachlan (1982).

PEPCase and Phosphorus Deficiency

PEPCase (EC 4.1.1.31), which catalyzes the irreversible β– carboxylation of PEP by HCO_3^- to yield oxalacetate and Pi, is apparently present in all living plant cells (O'Leary, 1982). Although most abundant in leaves of C-4 species, other isozymes of PEPCase are present in leaves of C-3 species and in root, fruit, and other tissues that lack chlorophyll, regardless of the species (Salisbury and Ross, 1985).

Activity of PEPCase was found to increase in response to phosphorus deficiency in the green alga *Selenastrum minutum* (Theodouou *et al.*, 1991), black mustard suspension cells (Duff *et al.*, 1989) and leaves and roots of hydroponically

grown tomato (Pilbeam *et al.*, 1993). The increase in PEPCase activity was suggested by Theodouou *et al.* (1991) as a means of releasing phosphate from PEP, thus allowing inorganic phosphate to be recycled during plant metabolism. However, Pilbeam *et al.* (1993) suggested that the increase in PEPCase activity was for maintenance of the cytosolic ion balance in plants, because PEPCase plays a key role in the "biochemical pH-stat" proposed by Davies (1986). In the "biochemical pH-stat" model, pH control in plant tissues is achieved by the net production or consumption of organic acids. An increase of PEPCase results in net synthesis of organic acid (malate), and a decrease of PEPCase causes net malate consumption to take place, the $-COO^-$ anion charge of the organic acid being lost as CO_2.

PFP and Phosphorus Deficiency

Pyrophosphate-dependent phosphofructokinase (PFP, officially named pyrophosphate-D-fructose-6-phophate 1-phosphotransferase, EC 2.7.1.90), which occurs in higher plants and some microorganisms, catalyzes the conversion of fructose-6-phosphate and PPi to fructose-1,6-bisphosphate and Pi (Cseke *et al.*, 1982). Most plant PFPs display potent activation by nanomolar concentrations of fructose-2,6-bisphosphate (Stitt, 1990). The PFP of many plants consists of two pairs of subunits of 66 (α-subunit) and 60 (β-subunit) kDa (Theodorou, 1992). The α-subunit was suggested to be involved in regulation of catalytic activity by fructose-2,6-bisphosphate (Theodorou, 1992).

PFP was found to increase in activity during phosphate starvation in black mustard suspension cells, and suggested to work as a bypass for phosphofructo-kinase in glycolysis under phosphorus deficiency by Duff *et al.* (1989), since the latter enzyme requires ATP. Working with the same black mustard suspension cells, Theodorou (1992) found that only the α-subunit, and not the β–subunit of PFP was enhanced in phosphorus-deficient cells, and that the activity of PFP increased in phosphorus-deficient cells only in the presence of fructose-2,6-bisphosphate. Similar results were found in black mustard seedlings by Theodorou and Plaxton (1994) except that no β-subunit was observed on western blots of leaf extracts of both phosphorus-deficient and -sufficient seedlings.

CHAPTER III

MATERIALS AND METHODS

Plant Material

Seeds of wheat (cultivars Pioneer 2180 or TAM-W101) were surface sterilized in 0.5% sodium hypochloride for 20 min, thoroughly washed, and placed in petri dishes containing two layers of filter paper wetted with distilled water. After germination in the dark for three days, selected seedlings were transferred to hydroponic culture containing 1/4 strength Hoagland's solution [1.5 mM KNO_3, 1.0 mM $Ca(NO_3)_24H_2O$, 0.5 mM $MgSO_47H_2O$, 0.00025% (w/v) Fe-EDTA, 11.5 μM H_3BO_3, 2.25 μM $MnCl_24H_2O$, 75 nM $CuSO_45H_2O$, 200 nM $ZnSO_47H_2O$, 25 nM H_2MoO_4, and various level of $NH_4H_2PO_4$, from 0 to 500 μM], on a 16 h light, (illumination of 350 μmole m^{-2} s^{-1}), and 8 h dark period at 25°C day and 20°C night temperature in a reach-in growth chamber. For hydroponic growth, six seedlings were placed on a cork supporter on a 1.8 L glass jar which was covered with aluminum foil to exclude light. The roots in the solution were aerated continuously. The water lost due to transpiration was replaced every day, and the nutrition solution was replaced every 7 days. The seedlings were usually harvested on the 21st day after transfer (24-days-old). After measuring the fresh weight, seedlings were

either stored in a -80°C freezer for enzyme analysis or placed in a 70°C oven for dry weight determination.

In another time course study, the wheat seedlings were harvested for fresh weight determination and enzyme analysis every 3 days after transfer until they were 24 days old.

Extraction of Soluble Enzymes

The frozen plant tissue was ground with mortar and pestle in cold 50 mM Tris-HCl buffer, pH 7.6 (1:3 w/v in leaf tissue, 1:1 w/v in root tissue) using a small amount of white quartz sand (Sigma Chemical Co., St. Louis, USA) to assist in breaking the tissue. This extract was centrifuged at 14,000 x g for 15 min at 4°C, and the supernatant was centrifuged again for 30 min to remove unbroken cells, cell walls, and other insoluble material.

Determination of Total Protein

Protein concentrations of the extracts were determined by the method of Bradford (1976) using Bio-Rad's protein assay reagent with bovine serum albumin (Fraction V, Sigma Chemical Co., St. Louis, USA) as standard. To 2.5 ml of diluted protein assay reagent was added 50 µl of appropriately diluted enzyme sample (0.2-0.8 mg protein/ml). After thoroughly mixing and incubating for about 5-10 min, the absorbance at 595 nm was measured. The protein concentration was then

calculated using a calibration curve constructed with 5 standard points (0.2, 0.4, 0.6, 0.8, and 1.0 mg protein/ml).

Assay of APase Activity

The 5 ml assay solution contained 100 mM sodium acetate buffer, pH 5.0, 1 mM *p*-nitrophenylphosphate (pNPP) as substrate and 0.1% BSA in a 25 ml test tube. Assay tubes were placed in a 37°C water bath 5 min before adding plant extract. After appropriate amount of extract (15 µl of leaf extract, or 25 µl of root extract) was added to the assay solution and thoroughly mixed, a 0.5-ml aliquot from the reaction mixture was immediately pipeted into a dilution tube containing 2.5 ml of "reaction-stop" solution (0.2 N NaOH), as blank solution. After 15 min, another 0.50-ml aliquot of reaction mixture was pipeted into another dilution tube. The amount of *p*-nitrophenol (pNP) liberated in the reaction solution was determined by measuring absorbance at 405 nm against the blank solution, taking 18.8 $mM^{-1}cm^{-1}$ as the extinction coefficient of pNP. One activity unit of APase was defined as one µmol of pNP released per min. Product formation was linear with time over 20 min, and the reaction velocity was proportional to enzyme concentration.

Assay of PEPCase Activity

The assay method described by Duff et al. (1989) was used to determine activity of phosphoenolpyruvate carboxylase (PEPCase). The activity was measured

at room temperature by following the oxidation of NADH at 340 nm using the "time scan" mode of a SHIMADZU UV-160A UV-VIS recording spectrophotometer. The 1 ml of assay solution contained 50 mM Bis-Tris-Propane-HCl (pH 8.0), 4 mM $NaHCO_3$, 4 mM $MgCl_2$, 1 mM DTT, 0.15 mM NADH, 1 mM glucose-6-phosphate, 4 units of porcine heart malate dehydrogenase, and 4 mM PEP (substrate). The reaction was initiated by adding plant extract. The PEPCase-dependent oxidation of NADH was monitored and calculated by using an extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1}. One activity unit of PEPCase was defined as the amount of enzyme that produced one μmole of oxaloacetate (OAA) per min. One mole of OAA converted one mole of NADH to NAD^+ during its reduction to malate.

Assay of PFP Activity

Assay of this enzyme was modified from the method of Theodorou et al. (1992). The same strategy as the PEPCase assay was used here, namely, following the oxidation of NADH at 340 nm. The 1 ml assay solution contained 50 mM Tris-HCl, pH 7.5, 5 mM fructose -6-phosphate, 0.4 mM pyrophosphate, 0.15 mM NADH, 5 mM $MgCl_2$, 1 unit of aldolase, 10 units of triose-phosphate isomerase, and 1 unit of glycerol-3-phosphate dehydrogenase. In addition, 2 μM fructose-2,6-bisphosphate was added to the solution to enhance the difference in PFP activity between phosphorus deficient and phosphorus sufficient plants (Theodorou et al., 1992). Assays were initiated by mixing appropriate amount of plant extract into the assay solution, and the change in absorbance at 340 nm was monitored for 3 min. The

oxidation rate of NADH was calculated using 6.22 $mM^{-1}cm^{-1}$ as the extinction coefficient of NADH. The PFP activity was expressed as μmol of product (fructose-1,6-phosphate) produced per min. It should be noted that one mole of the product of the PFP reaction requires the oxidation of two moles of NADH.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a Bio-Rad Mini-PROTEAN II cell and the discontinuous system of Laemmli (1970). The glass plates, spacers, and combs were cleaned with ethanol and wiped dry before casting the gel. The 7.5%T SDS separating gel (10 ml) was prepared by mixing 1.88 ml of 40% acrylamide/ bisacrylamide , 37.5:1 mixture, 2.5 ml of 1.5 M Tris-HCl buffer, pH 8.8, 5.47 ml of distilled water, 100 μl of 10% SDS, and 50 μl of 10% (w/v) ammonium persulfate. After the solution was degassed for about 20 min, 5 μl of TEMED was added, and the solution was gently mixed and poured into the gel sandwich to about 1 cm below the level to be occupied by the comb. The monomer solution was immediately overlaid with distilled water and allowed to polymerize for at least one hour. The water was then drained off, and the separating gel was overlaid with a stacking gel consisting of 125 mM Tris-HCl, pH 6.8, 0.1% of SDS and acrylamide concentration of 4% T. The well-forming comb was inserted into the stacking gel before it polymerized. Samples for SDS-PAGE were prepared by mixing enzyme extract with equal volume of SDS-PAGE sample buffer containing 10% (w/v) SDS, 20% (v/v) glycerol, 0.02% (w/v) Bromophenol Blue tracking dye, 10% (v/v) β-mercaptoethanol

and 125 mM Tris-HCl, pH 6.8, and heating this mixture in a water bath at 100°C for 5 min. In addition to the enzyme samples, 5 µl or 10 µl of low range, pre-stained SDS-PAGE standards (Bio-Rad, Richmond, CA) was also loaded as molecular weight markers. For Western blot analysis, ripened banana fruit extract or potato tuber extract was loaded as a positive control for PEPCase or PFP respectively. The extracts were prepared identically to the wheat tissue extract preparation described in the previous section. The gels were run under constant voltage conditions at 4°C, with usually 100 volt during the first 10 min, and then 150 volt until the tracking dye reaching the bottom of the gel. For Western blots, the gels were put into the transfer buffer (see next section) after electrophoresis was completed.

Western Blots

After electrophoresis, the proteins was transferred to nitrocellulose membranes by electroblotting using a Mini Trans-Blot Electrophoresis Transfer Cell (Bio-Rad, Richmond, CA) and then probed with appropriately diluted anti-(wheat germ APase) immune serum. Immune serum was obtained from rabbits that had been bled one month after injection with commercial wheat germ acid phosphatase (Sigma Chem. Co., St. Louis, USA). The Sigma APase had been partially purified by sequential NH_4SO_4 precipitation prior to its use as an antigen. Anti-(potato tuber PFP) immune serum was provided by Dr. William Plaxton (Moorhead and Plaxton 1991), and anti-(banana fruit PEPCase) IgG also provided by Dr. Plaxton. The transfer was performed for 3 h at 90 volt in a transfer buffer containing 25 mM Tris,

192 mM glycine, and 20% (v/v) methanol, pH 8.3. The nitrocellulose membranes were then incubated in 5% non-fat milk in TBS (50 mM Tris and 200 mM NaCl, pH 7.5) as a blocking reagent at 4°C overnight, and then washed with TBST (0.05% v/v Tween 20 in TBS) three times and once in TBS one time, at least 10 min each time. The membranes were then incubated in anti-serum solution diluted in TBS containing 0.5% BSA for 2 h at room temperature, and washed with TBST and TBS as described above. After soaking the membranes in 5% non-fat milk again for 5 min at room temperature, and washing with TBST and TBS, the membranes were incubated in 1/20,000 diluted secondary antibody (IgG Fraction of goat-antiserum to rabbit IgG, labeled with alkaline phosphatase, Sigma Chem. Co., St. Louis, USA) in TBS with 0.5% BSA for 2 h at room temperature. Membranes were next washed with TBST and TBS, rinsed with distilled water, placed into color developing solution [one tablet of SIGMA FAST BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablet dissolved in 10 ml of distilled water] until sufficient color had developed. Color development was then stopped by transferring membranes to distilled water.

Periodate Oxidation (modified from Laine and Faye, 1988)

In order to avoid the interference of the binding of anti-(glycosyl group) antibodies to carbohydrate moieties of glycoproteins (like acid phosphatase in this study), mild periodate oxidation was used during Western blot analysis for acid phosphatase. After incubating in blocking reagent and washing with TBST and

TBS, the nitrocellulose membrane used to detect proteins cross-reacting with acid phosphatase antibody was incubated for 2 hours in the dark at room temperature in 0.1 M sodium acetate buffer, pH 4.5, containing 10 mM sodium metaperiodate. Membranes were then treated for 30 min with 50 mM sodium borohydride in TBS. The subsequent steps were the same as described in the previous section.

Characterization of APase Isoenzymes by Native PAGE

Native PAGE was performed similarly to the SDS-PAGE described above, except that the buffer system did not contain any SDS or β-mercaptoethanol. In addition, the sample mixtures were not heated, and the total acrylamide concentration was usually used 6% T instead of 7.5% T. After electrophoresis, the gels were put into the APase activity staining solution, which was 0.1% (w/v) $MgCl_2$, 0.1% (w/v) naphthyl phosphate, 0.1% (w/v) fast red TR salt in 200 mM sodium acetate buffer, pH 5.0 at room temperature overnight, and then transferred to distilled water.

CHAPTER IV

RESULTS

Critical Concentration of Inorganic Phosphate in Nutrient Solution

Figure 1 (p.32) shows the growth of wheat shoots of two cultivars (Pioneer 2180 and TAM W101) 24 days after germination in the nutrient solution containing inorganic phosphate at levels of 0, 25, 100, 250 and 500 μM. Although the absolute growth of cultivar Pioneer 2180 was higher than that of cultivar TAM W101, both cultivars showed the same level of growth reduction due to phosphorus deficiency. Therefore, we cannot conclude that there is any difference in phosphorus deficiency tolerance between theses two cultivars under these growth conditions. The "critical concentration" of inorganic phosphate in the nutrient solution was about 150 μM to 200 μM, in which there is 5 to 10% reduction in growth (according to the definition suggested by Bouma, 1983), for both cultivars. Although "critical concentration" is usually used to indicate the critical amount of nutrient in tissue, the "critical concentration" in this study refers to the critical amount of phosphate in the nutrient solution.

Response of Wheat Seedlings to Phosphorus Deficiency

In addition to the reduction of growth mentioned above, the 24-day-old seedlings showed withering on almost all the oldest leaves under conditions of severe phosphorus deficiency (0 and 25 μM Pi). Purple pigmentation was observed at the leaf bases of the seedlings under the most severe phosphorus deficiency (0 μM Pi) for cultivar Pioneer 2180 only. Roots of the severely phosphorus-deficient seedlings (0 and 25 μM Pi) looked more slender, had more dense root hairs, and appeared yellow compared to the roots of phosphorus-sufficient seedlings. Although the seedlings grown in 100 μM Pi were under mild phosphorus deficiency, it was difficult to tell the difference visually between them and the phosphorus-sufficient seedlings (250 and 500 μM Pi). The water contents in wheat tissue, shown in Figure 2 (p.33), seemed to correlate to phosphorus status on both shoots and roots, and the patterns of the correlation of the two cultivars were almost identical. The water content, which was calculated from the ratio of dry weight to fresh weight of seedlings, was significantly lower in phosphorus-deficient seedlings, but not in those grown in 100 μM Pi.

Figure 3 (p.34) shows the ratio of root fresh weight to shoot fresh weight of phosphorus-sufficient and phosphorus-deficient seedlings. There was a strong correlation between the ratio and the relative growth, which indicated the phosphorus status of the seedlings, for both Pioneer 2180 ($r = 0.9814$) and TAM W101 ($r = 0.9995$). Although the regression curves of these two cultivar do not completely match each other, they are not significantly different.

Expression of Enzymes of Phosphorus Nutrition

The basal level of specific activity of both APase and PEPCase in phosphorus-sufficient seedlings was higher in roots than in leaves. It was 2.5 and 4-fold higher for APase and PEPCase, respectively, (Figure 4 and 5, p.35-36). Activities of APase and PEPCase from both leaf and root extracts increased under phosphorus deficiency (Figure 4 and 5). The increases of activity was greater in roots than leaves for APase, but for PEPCase the increase in leaves was greater than roots. However, the activity of both enzymes increased significantly at mild phosphorus deficiency in root, but not in leaves (note the values at 100 μM).

Like the other two enzymes, PFP specific activity was much higher, about 10-fold, in roots compared to leaves under phosphorus-sufficient conditions (Figure 6, p. 37). The increase in PFP specific activity in leaves was similar to those of APase and PEPCase. However, a contrary pattern was found in roots: activity decreased slightly under phosphorus deficiency (Figure 6).

Native PAGE gels stained for APase activity (Figure 7 and 8, p. 38-39) show the expression of APase isozymes in leaves and roots of plants under different levels of phosphate supply. Because of the limitation of resolution of the electrophoresis and the sensitivity of the staining, it is difficult to conclude how many isozymes occur in wheat tissue or if isozymes are newly induced or only enhanced by phosphorus deficiency. However, there were at least four APase isozymes on the gel for leaves (Figure 7), and five APase isozymes on the gel for roots (Figure 8). The

four isozymes from leaves had the same mobility (Rf) as the first four isozymes from roots, i.e. they were probably the same isozymes. In the case of leaf, the activity of some of the APase isozymes were highly enhanced (bands 2 and 4 in Figure 7) while the others were not very different (band 1 in Figure 7) from that in phosphorus-sufficient plants. This suggests that phosphorus deficiency not only causes APase activity to increase, but also induces or activates specific APase isoenzymes. In the gel for the root extract (Figure 8), every isoenzyme seemed to change evenly, except for a smear on the upper portion of the 0 μM Pi lane that may represent an isozyme of APase newly induced by phosphorus deficiency.

Western blots probed with anti-(wheat germ APase) immune serum are shown in Figure 9 and 10 (p. 40 and p. 41). Treating the membrane with periodate did get rid of some unspecifically binding bands and make the bands on the membranes more clear, but none of the proteins which reacted with the antibody were enhanced in plants experiencing phosphorus deficiency. Western blots probed with anti-(banana fruit PEPCase) IgG are shown in Figure 11 (p. 42). For leaf extracts, two bands (116 kD and 112 kD) on the membrane showed an increase as phosphorus supply decreased, while a 125 kD band decreased as phosphorus supply decreased and three bands (81, 72 and 69 kD) remained unchanged. In addition, a group of proteins on the higher molecular weight position also increased, although their density were not as strong as the 116 kD and 112 kD bands. There were also 116 ,112, 72, and 69 kD proteins in the membrane for root extract. Unlike the leaf, there appeared to be little induction of 116 and 112 kD proteins under phosphorus-

limiting conditions. Western blots probed with anti-(potato tuber PFP) immune serum are shown on Figure 12 (p.43). There were two proteins (67 kD and 63 kD) detected in the wheat leaf extract. The 63 kD protein was more abundant than the 67 kD protein in each lane. The 63 kD protein was also detected in root extract, while the 67 kD protein appeared only on the 500 μM Pi lane. Expression of PFP monomers increased in leaf extract from phosphorus-deficient seedlings. However, in root extracts, contrary to the leaf extract, there was a positive correlation between phosphorus supply and synthesis of PFP protein which decreased in plants experiencing phosphorus deficiency.

Time Course of Response to Phosphorus Deficiency

Figure 13 and 14 (p. 44 and 45) present the growth curves of wheat seedlings grown in the nutrient solution containing sufficient phosphate (250 μM) and deficient phosphate (25 μM). Under phosphorus sufficiency, the shoot fresh weight of the seedlings increased, and exhibited a faster growth rate, than those under phosphorus deficiency beginning at 12 days. Roots of these two groups exhibited a similar growth rate up to 24 days. The change in ratio of root fresh weight to shoot fresh weight is given in Figure 15 (p. 46). The root/shoot ratios of both phosphorus-sufficient and phosphorus-deficient seedlings tended to increase with time, although there was no increase between the 15th day and the 20th day. The ratios of the seedlings under phosphorus deficiency were always higher than those under sufficiency, and the significant difference in the ratios between them could be found

($P < 0.05$) when seedlings were only 6 days old, which represents only 3 days of differential phosphate treatment. Figures 16, 17 and 18 (p. 47, 48 and 49) present the changes in activity over time of APase, PEPCase, and PFP from leaves and roots under the two different phosphorus treatments. APase activities from leaf extracts of phosphorus-deficient plants were significantly higher than those under sufficiency as early as the 9th day after germination, while increased PEPCase and PFP activity was not detected until the 18th day. In the case of roots, activities of APase and PEPCase under phosphorus deficiency was higher beginning on the 9th day and 15th day respectively, but activity of PFP under the two different phosphorus treatments was the same up to 24 days.

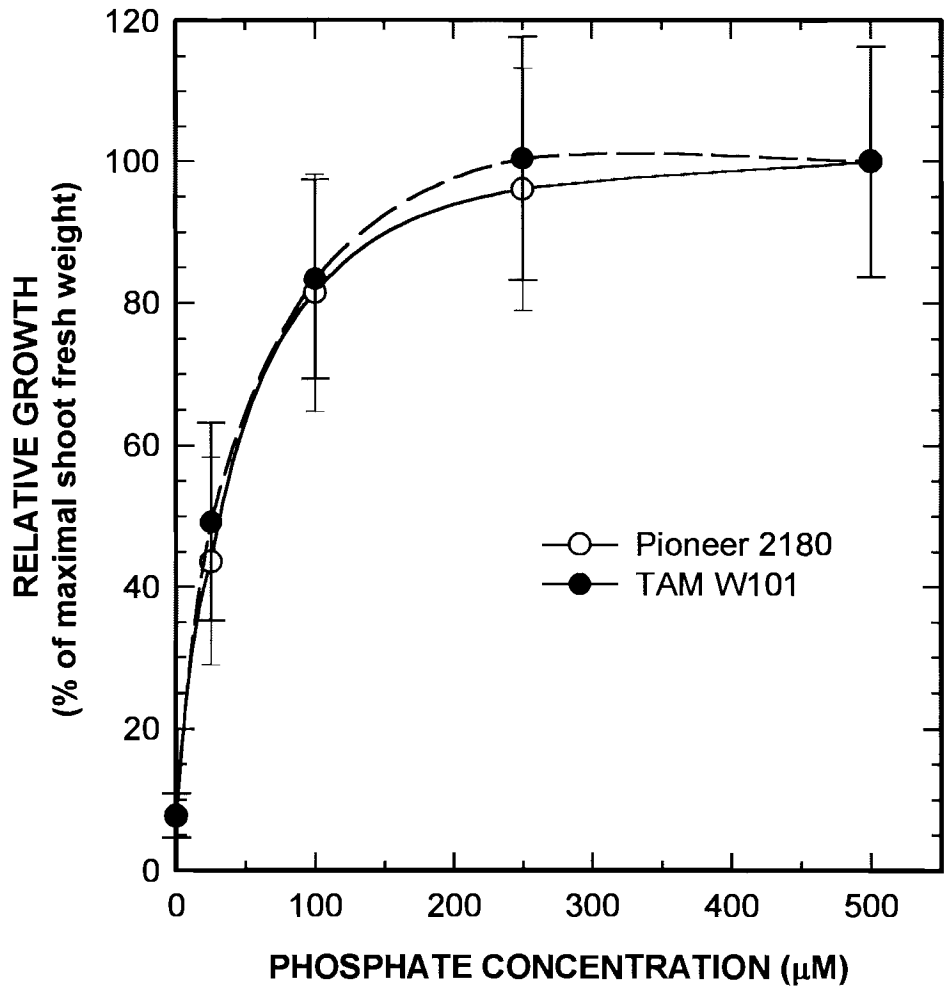

Figure 1. Relationship between inorganic phosphate concentration in nutrient solution and relative shoot growth of 24-day-old wheat seedlings (cv. Pioneer 2180 and TAM W101). All values represent the mean of 30 seedlings. Bars indicate standard deviations. (maximum shoot fresh weight: Pioneer 2180, 4.49 g; TAM W101, 3.45 g)

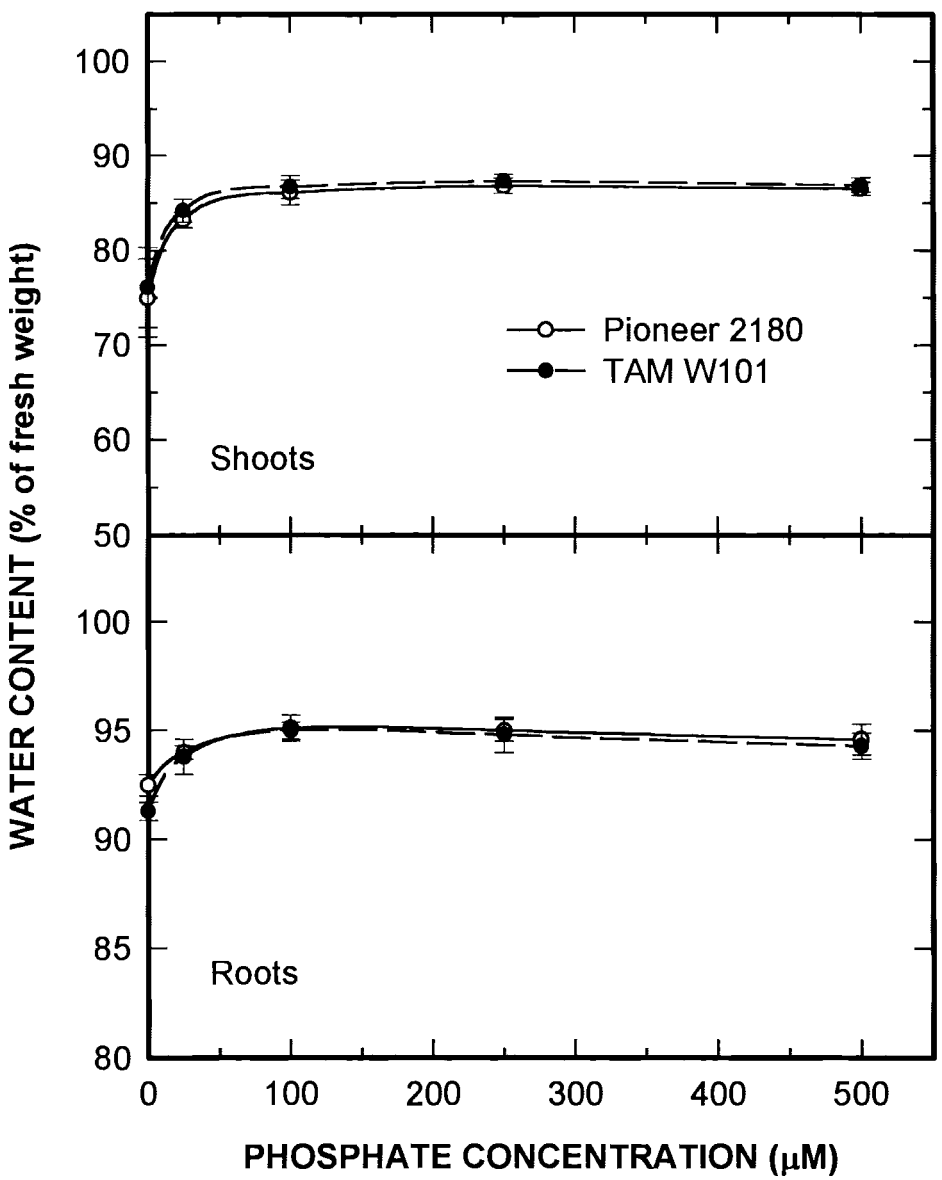

Figure 2. Relationship between inorganic phosphate concentration in nutrient solution and water content of shoots and roots from 24-day-old wheat seedlings (cv. Pioneer 2180 and TAM W101). All values represent the mean of 18 seedlings. Bars indicate standard deviations.

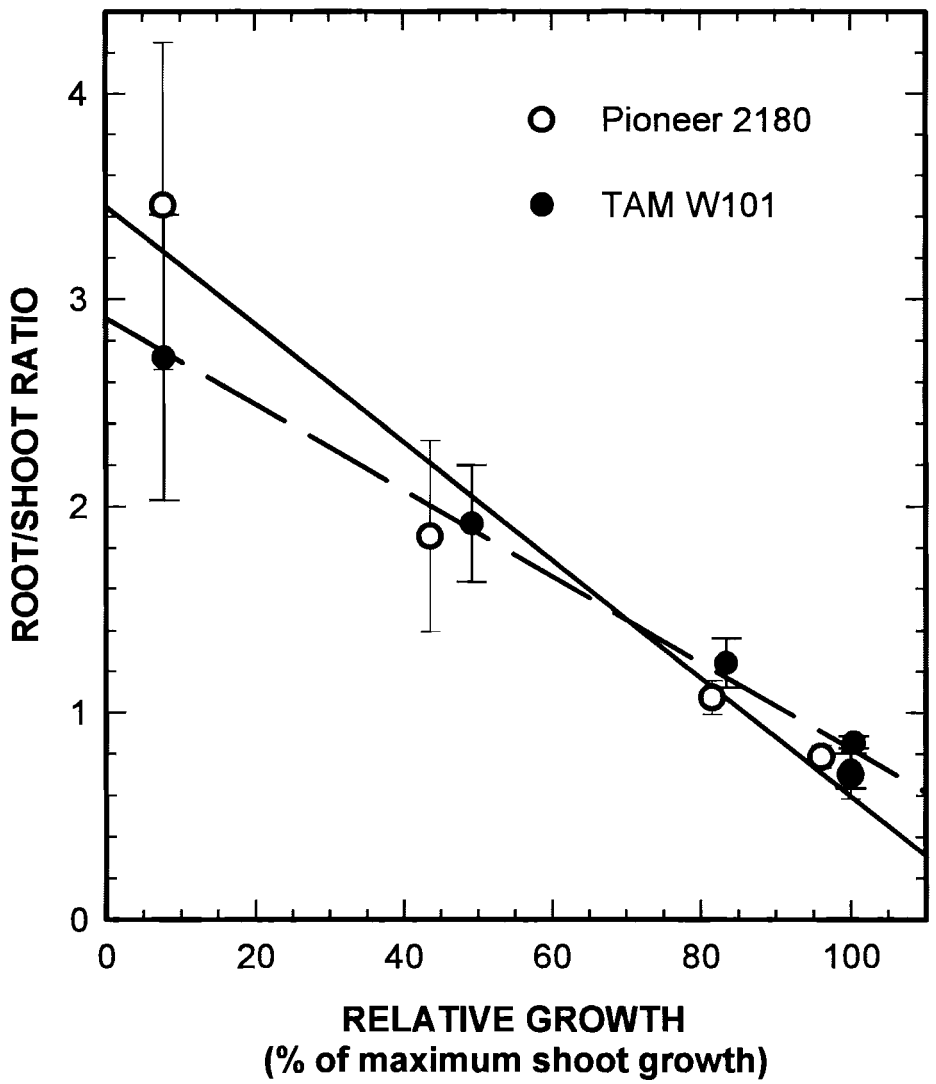

Figure 3. Relationship between shoot fresh weight and root / shoot ratio of 24-day-old wheat seedlings (cv. Pioneer 2180 and TAM W101). All values represent the mean of 30 seedlings. Bars indicate standard deviations.

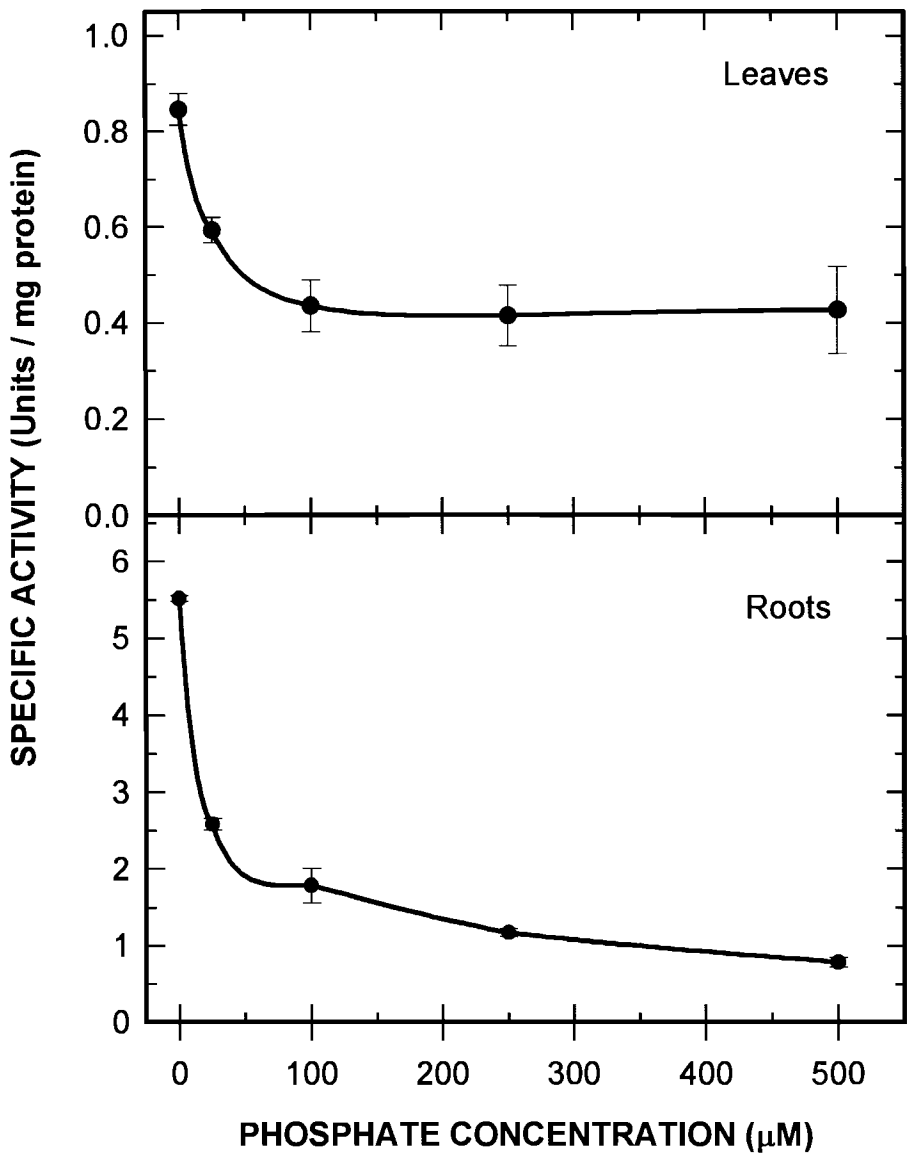

Figure 4. Relationship between inorganic phosphate concentration in nutrient solution and APase activity from leaves shoots and roots from 24-day-old wheat seedlings (cv. Pioneer 2180). All values represent the mean of 3 determinations. Bars indicate standard deviations.

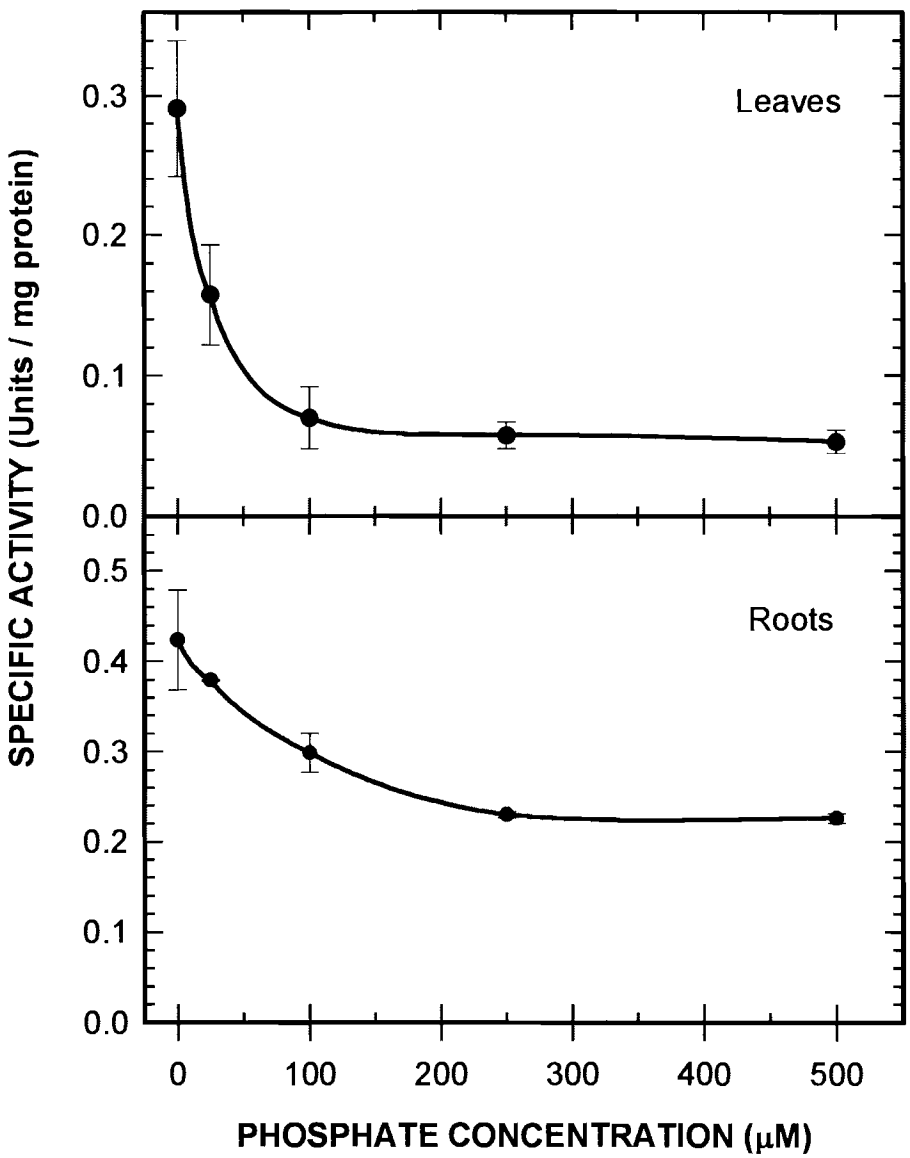

Figure 5. Relationship between inorganic phosphate concentration in nutrient solution and PEPCase activity from leaves shoots and roots from 24-day-old wheat seedlings (cv. Pioneer 2180). All values represent the mean of 3 determinations. Bars indicate standard deviations.

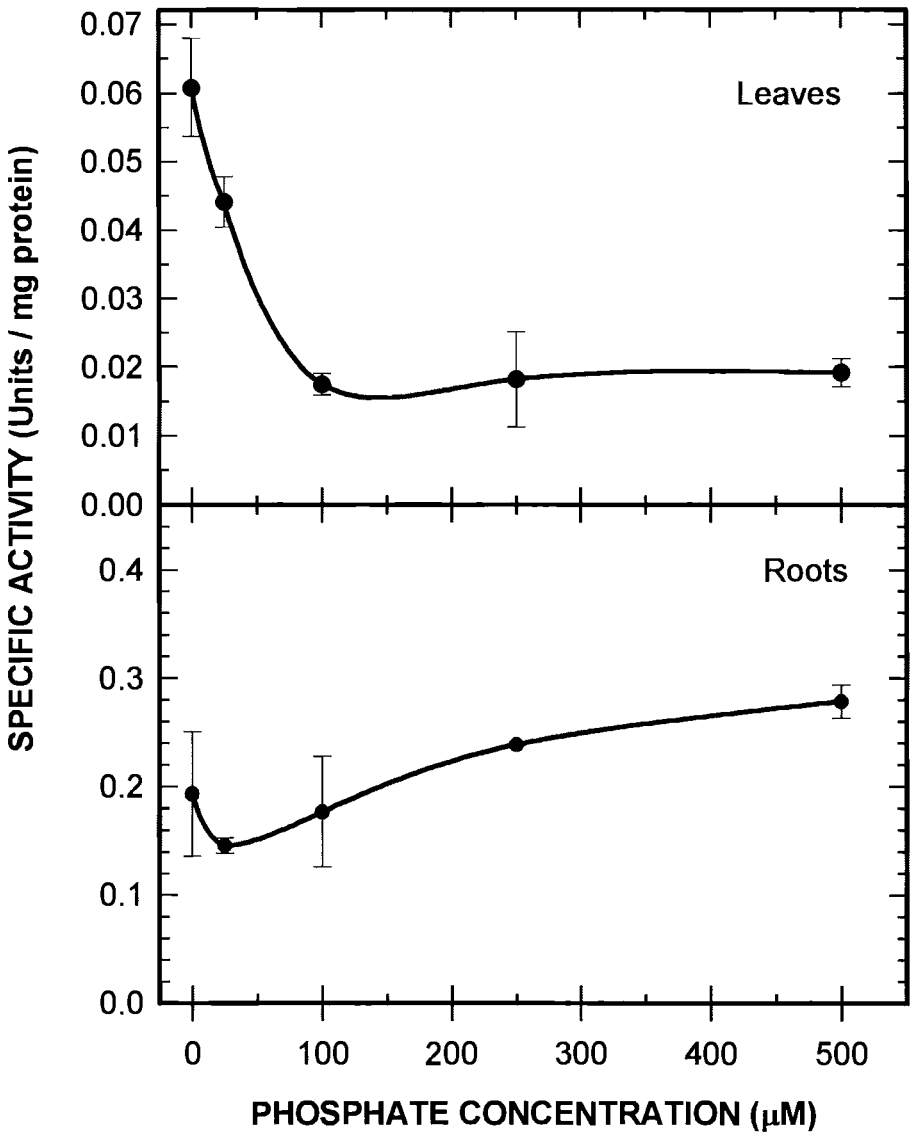

Figure 6. Relationship between inorganic phosphate concentration in nutrient solution and PFP activity from leaves shoots and roots from 24-day-old wheat seedlings (cv. Pioneer 2180). All values represent the mean of 3 determinations. Bars indicate standard deviations.

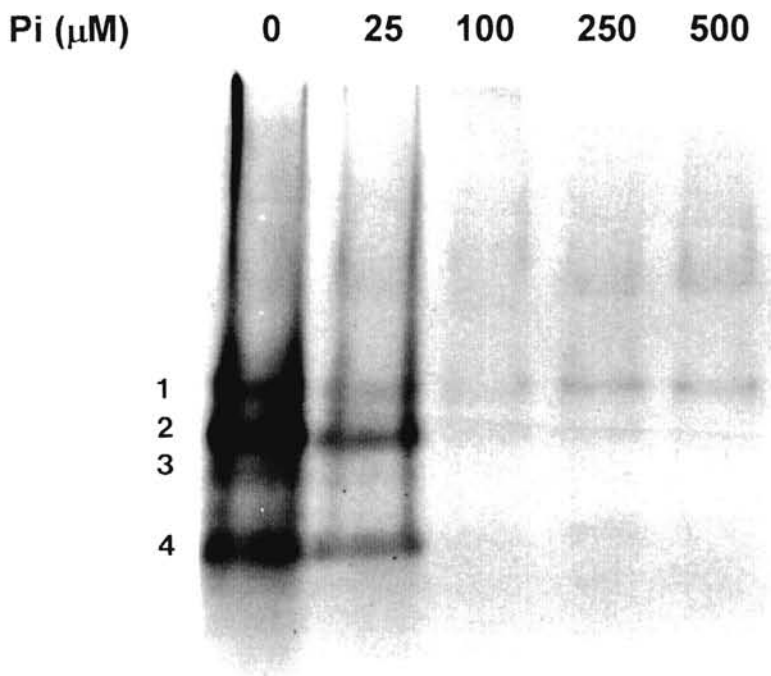

Figure 7. Characterization of wheat APase isozymes by native PAGE. Proteins were extracted from leaves of 24-day-old wheat (cv. Pioneer 2180) seedlings grown in different levels of nutrient phosphate as indicated for each lane. Protein loaded into each lane was 75 μg.

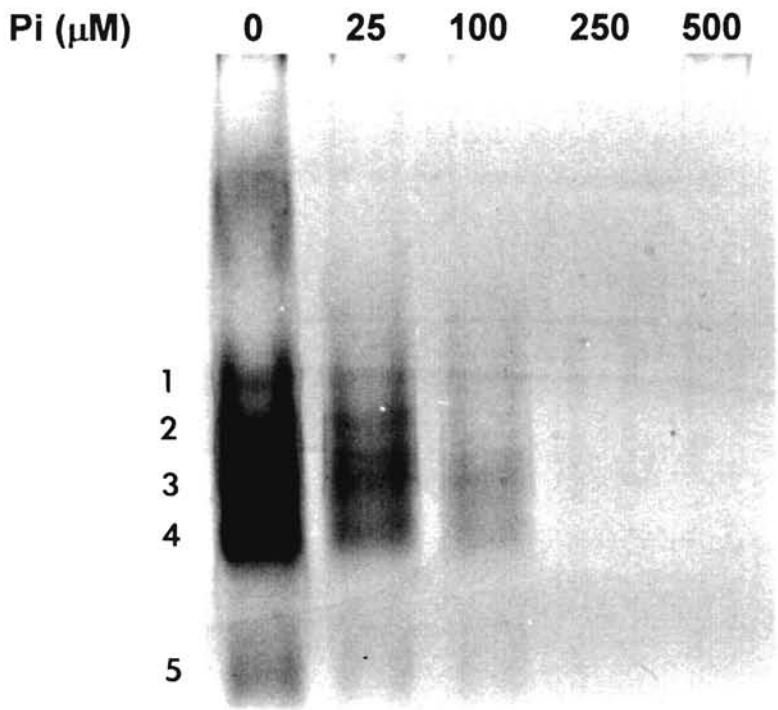

Figure 8. Characterization of wheat APase isozymes by native PAGE. Proteins were extracted from roots of 24-day-old wheat (cv. Pioneer 2180) seedlings grown in different levels of nutrient phosphate as indicated for each lane. Protein loaded into each lane was 12 μg.

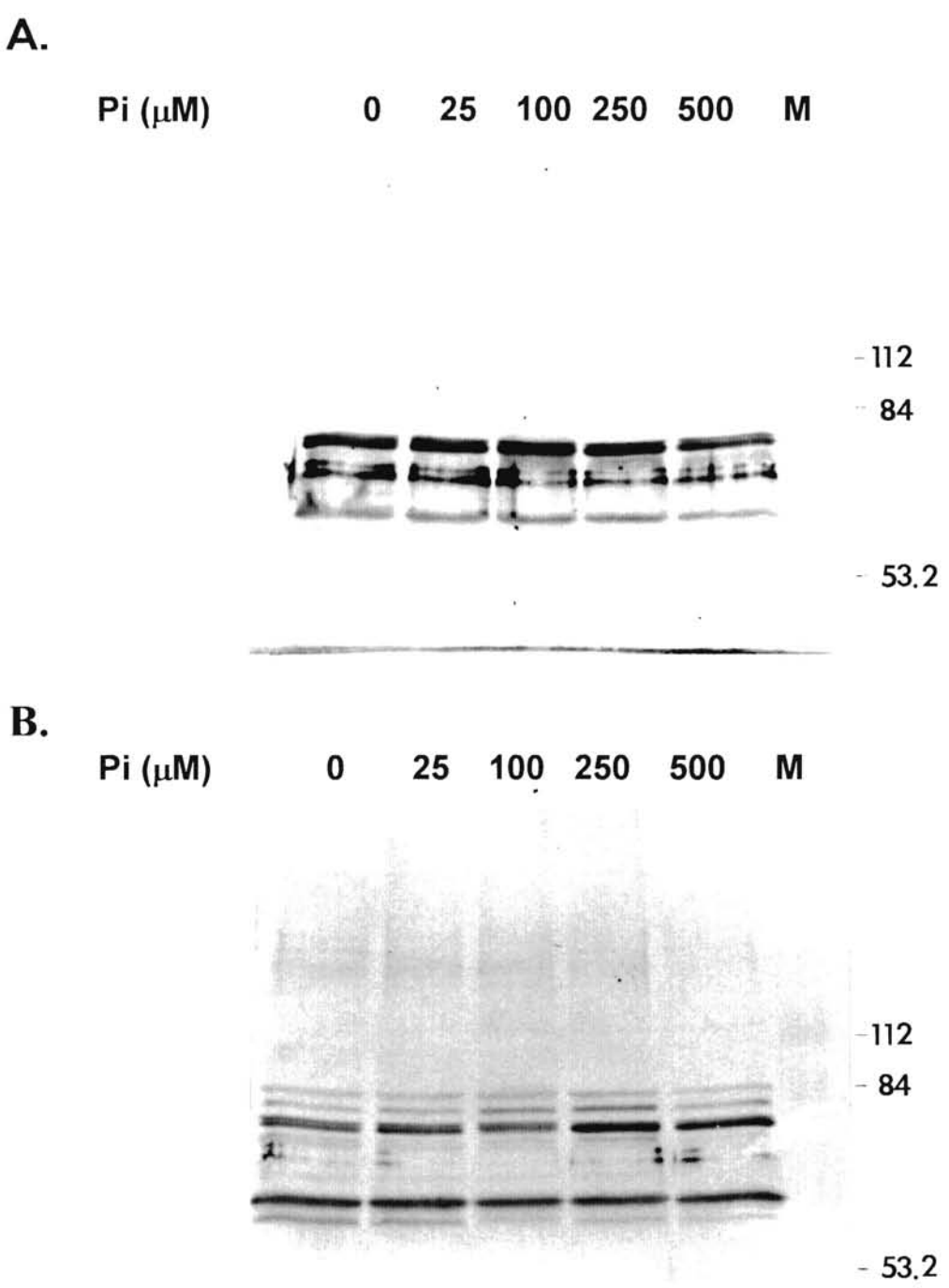

Figure 9. Western blot analysis of APase of leaf extracts from 24-day-old wheat (cv. Pioneer 2180) seedlings grown in different levels of nutrient phosphate as indicated for each lane. (A) without periodate treatment, or (B) following treatment with 10 mM sodium periodate (see Materials and Methods for details) Protein loaded into each lane was 60 μg. M, molecular weight standards in kD.

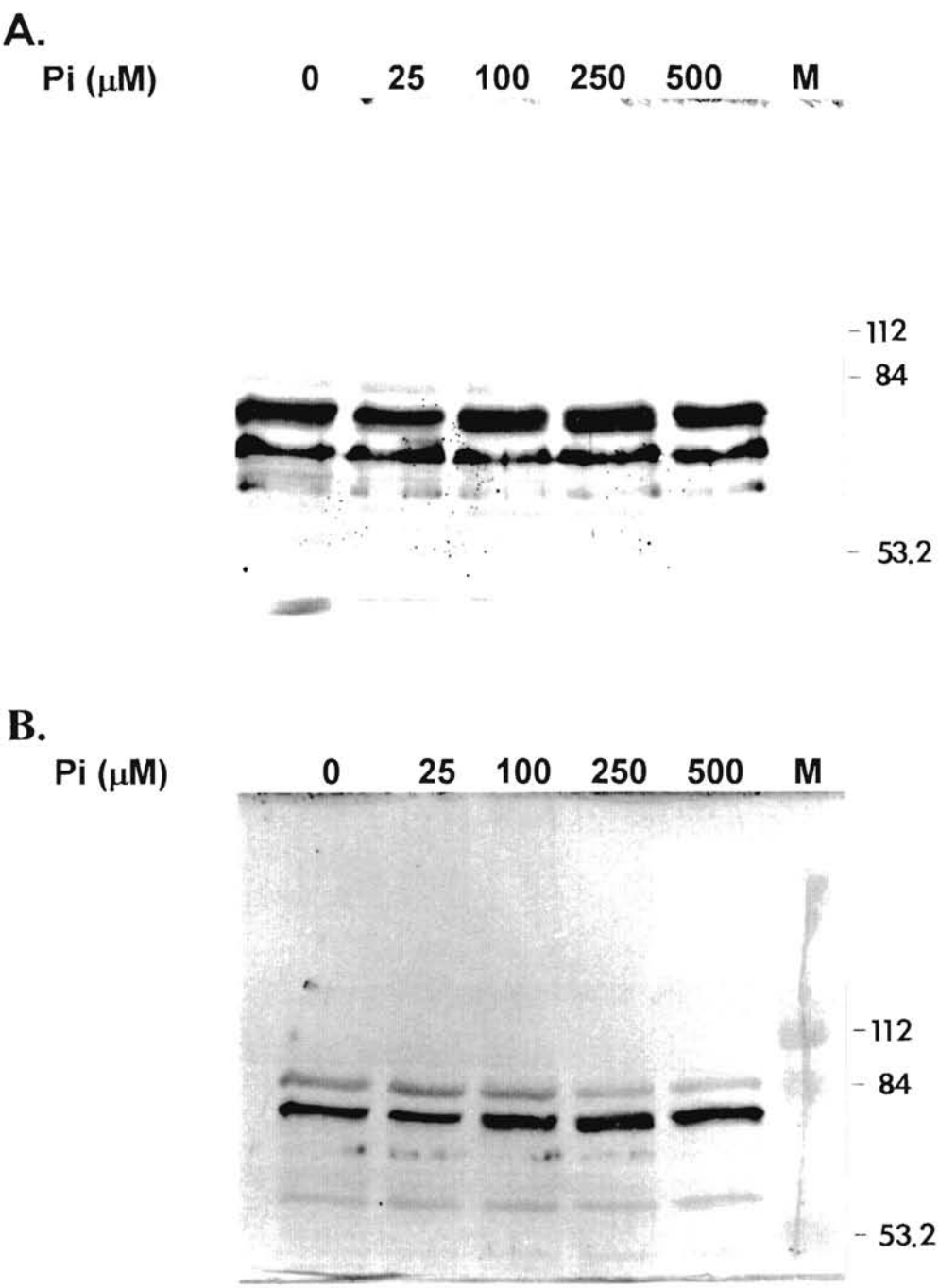

Figure 10. Western blot analysis of APase of root extracts from 24-day-old wheat (cv. Pioneer 2180) seedlings grown in different levels of nutrient phosphate as indicated for each lane. (A) without periodate treatment, or (B) following treatment with 10 mM sodium periodate (see Materials and Methods for details). Protein loaded into each lane was 10 μg. M, molecular weight standards in kD.

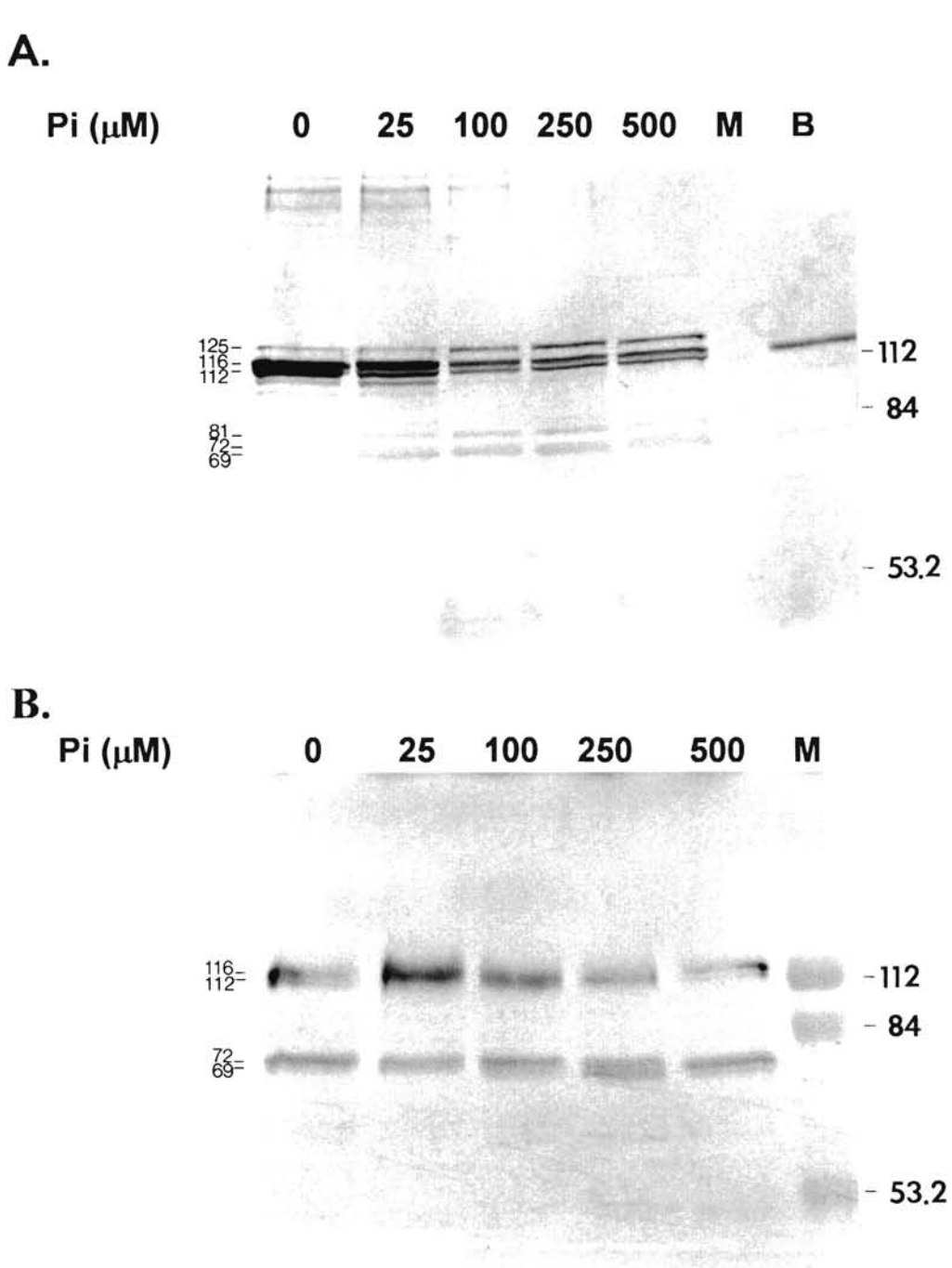

Figure 11. Western blot analysis of PEPCase from (A) leaf or (B) root extracts of 24-day-old wheat (cv. Pioneer 2180) seedlings grown in different levels of nutrient phosphate as indicated for each lane. Protein loaded into each lane was 60 μg for leaf extract and 10 μg for root extract. M, molecular weight standards; B, banana fruit crude extract (see Materials and Methods for details). The sizes (kD) of major protein bands detected by the antibody are labeled on the left side.

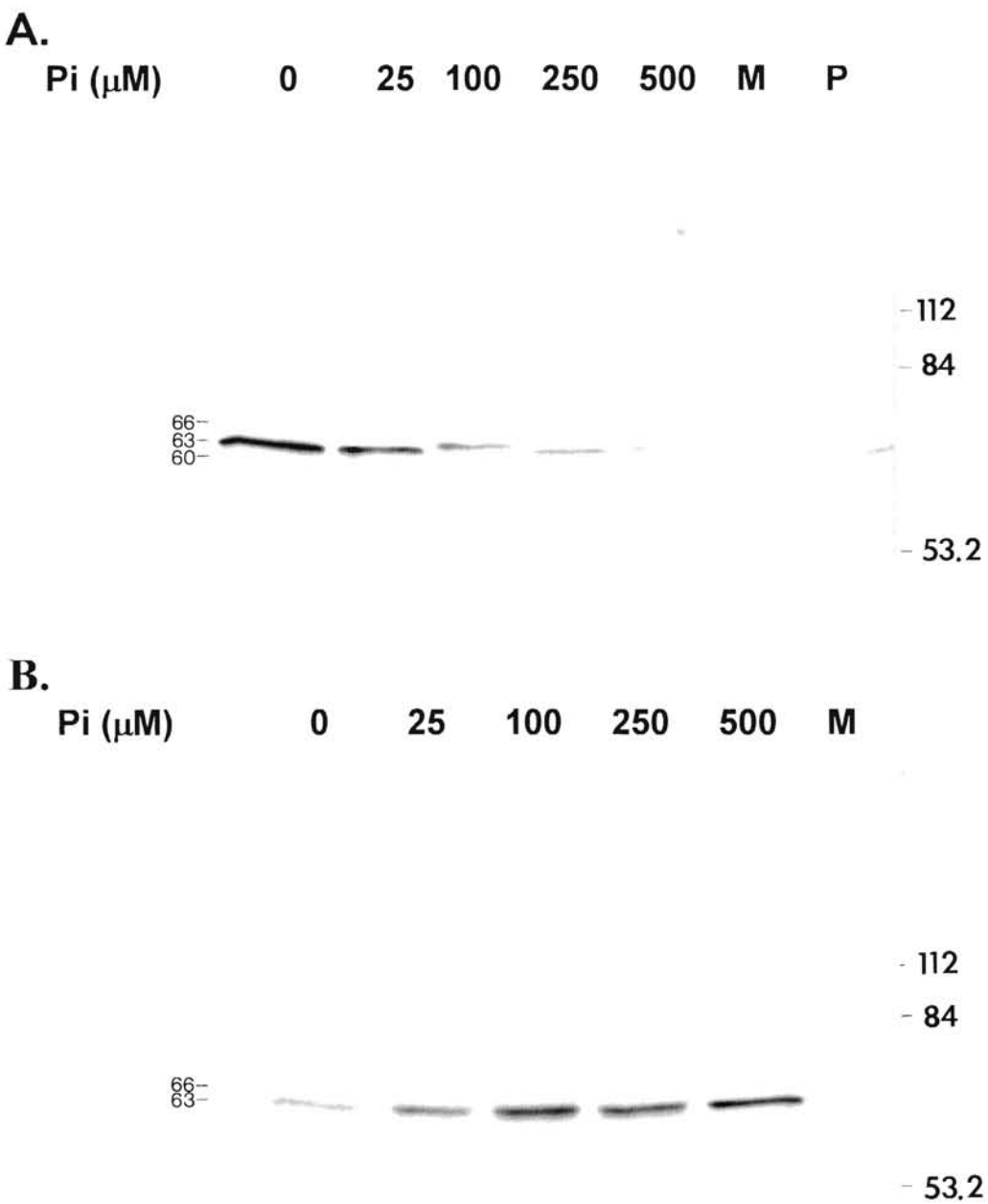

Figure 12. Western blot analysis of PFP from (A) leaf or (B) root extracts of 24-day-old wheat (cv. Pioneer 2180) seedlings grown in different levels of nutrient phosphate as indicated for each lane. Protein loaded into each lane was 60 μg for leaf extract and 10 μg for root extract. M, molecular weight standards; P, potato tuber crude extract (see Materials and Methods for details). The sizes (kD) of major protein bands detected by the antibody are labeled on the left side.

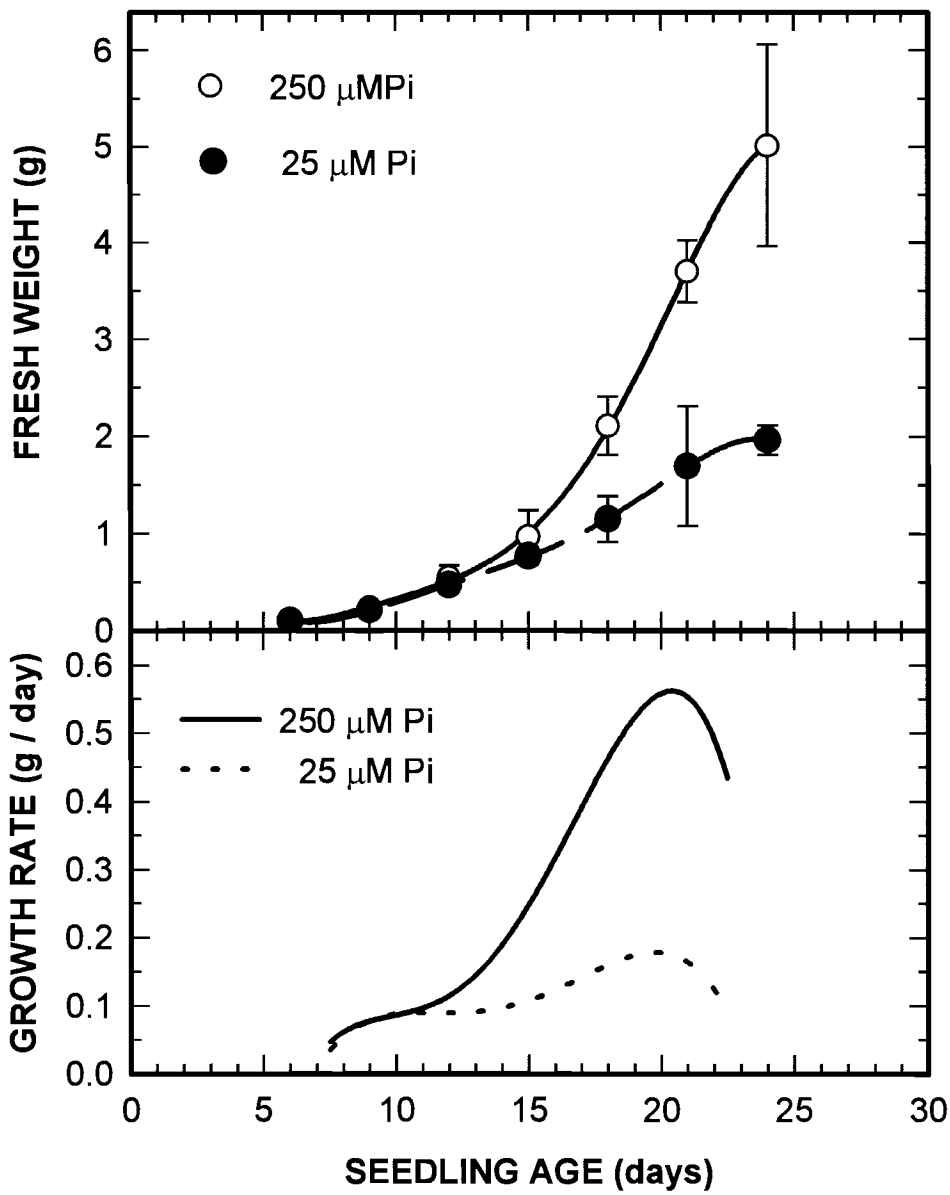

Figure 13. Growth curve and growth rate curve using leaf fresh weight of wheat (cv. Pioneer 2180) seedlings grown in nutrient solution containing 250 μM or 25 μM inorganic phosphate. The growth rate curve is the first derivative of the growth curve. All values represent the mean of 12 (before 12 days of growth) or 6 seedlings (12-24 days of growth). Bars indicate standard deviations.

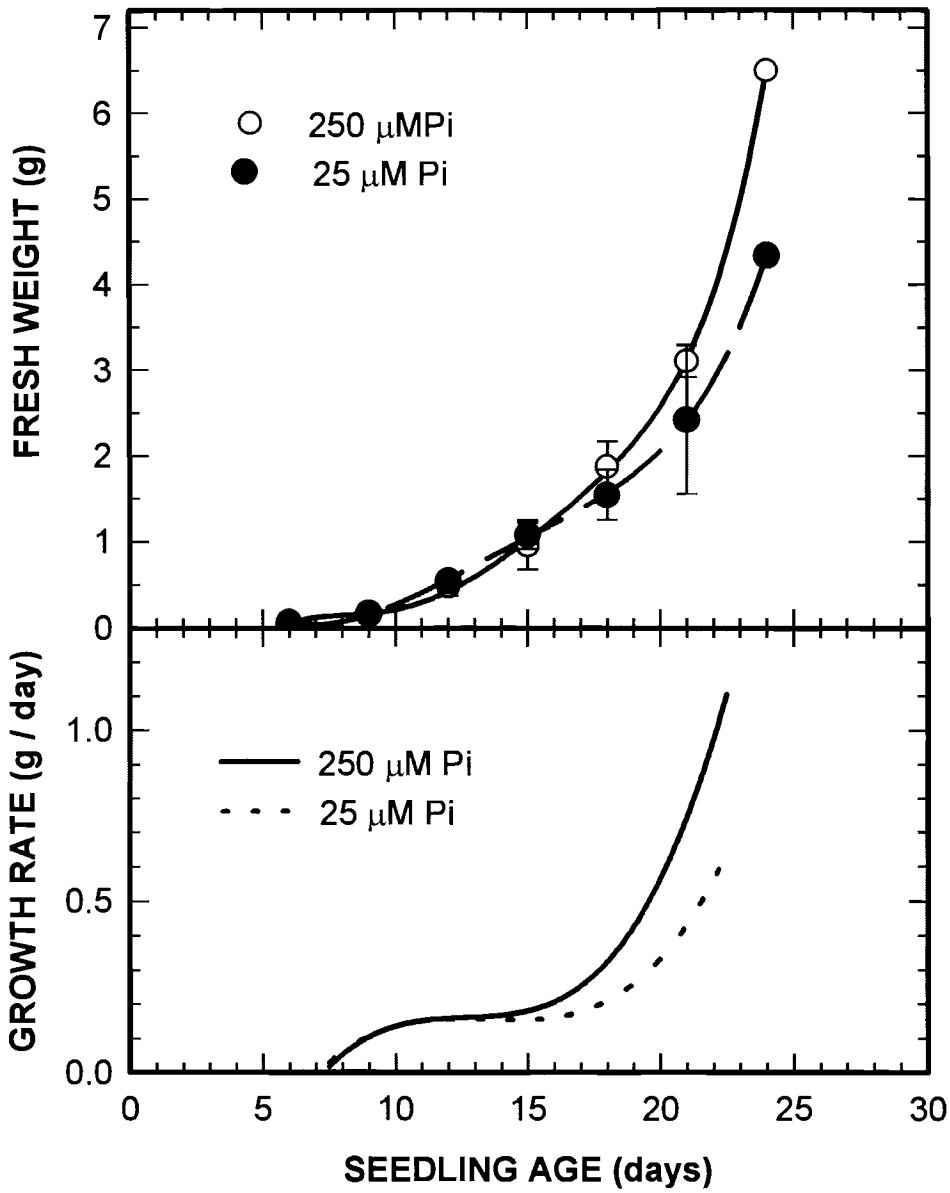

Figure 14. Growth curve and growth rate curve using root fresh weight of wheat (cv. Pioneer 2180) seedlings grown in nutrient solution containing 250 μM or 25 μM inorganic phosphate. The growth rate curve is the first derivative of the growth curve. All values represent the mean of 12 (before 12 days of growth) or 6 seedlings (12-24 days of growth). Bars indicate standard deviations.

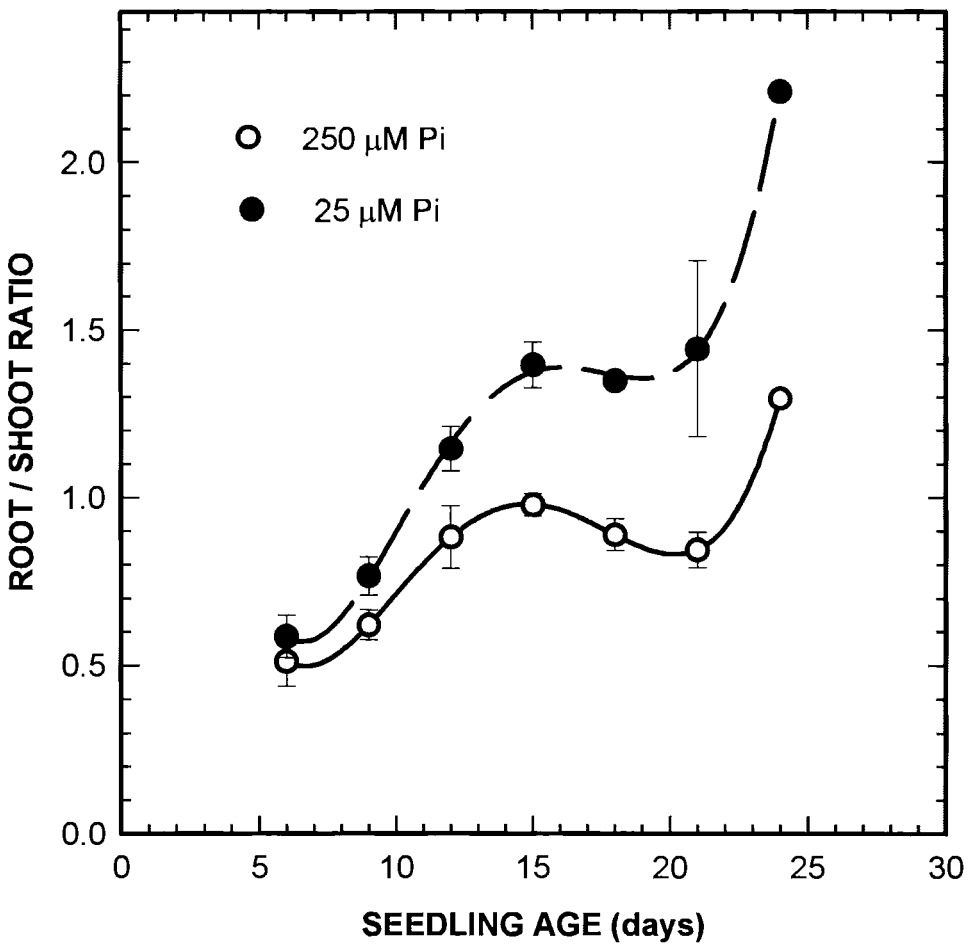

Figure 15. Root / shoot fresh weight ratio of wheat (cv. Pioneer 2180) seedlings grown in nutrient solution containing 250 μM or 25 μM inorganic phosphate. All values represent the mean of 12 (before 12 days of growth) or 6 seedlings (12-24 days of growth). Bars indicate standard deviations.

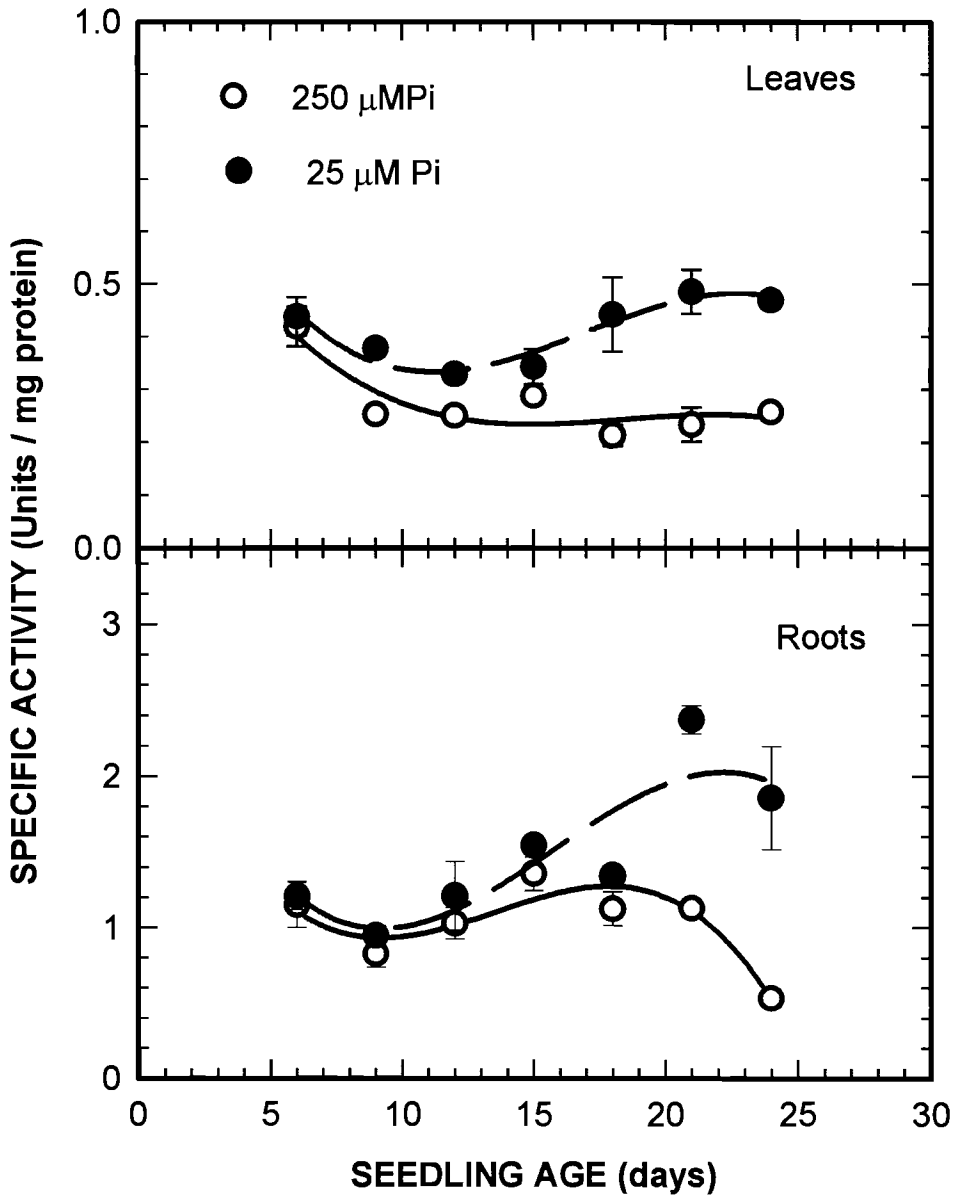

Figure 16. Acid phosphatase activity in leaves and roots from wheat (cv. Pioneer 2180) seedlings grown in nutrient solution containing 250 μM or 25 μM inorganic phosphate. All values represent the mean of three separate determinations. Bars indicate standard deviations.

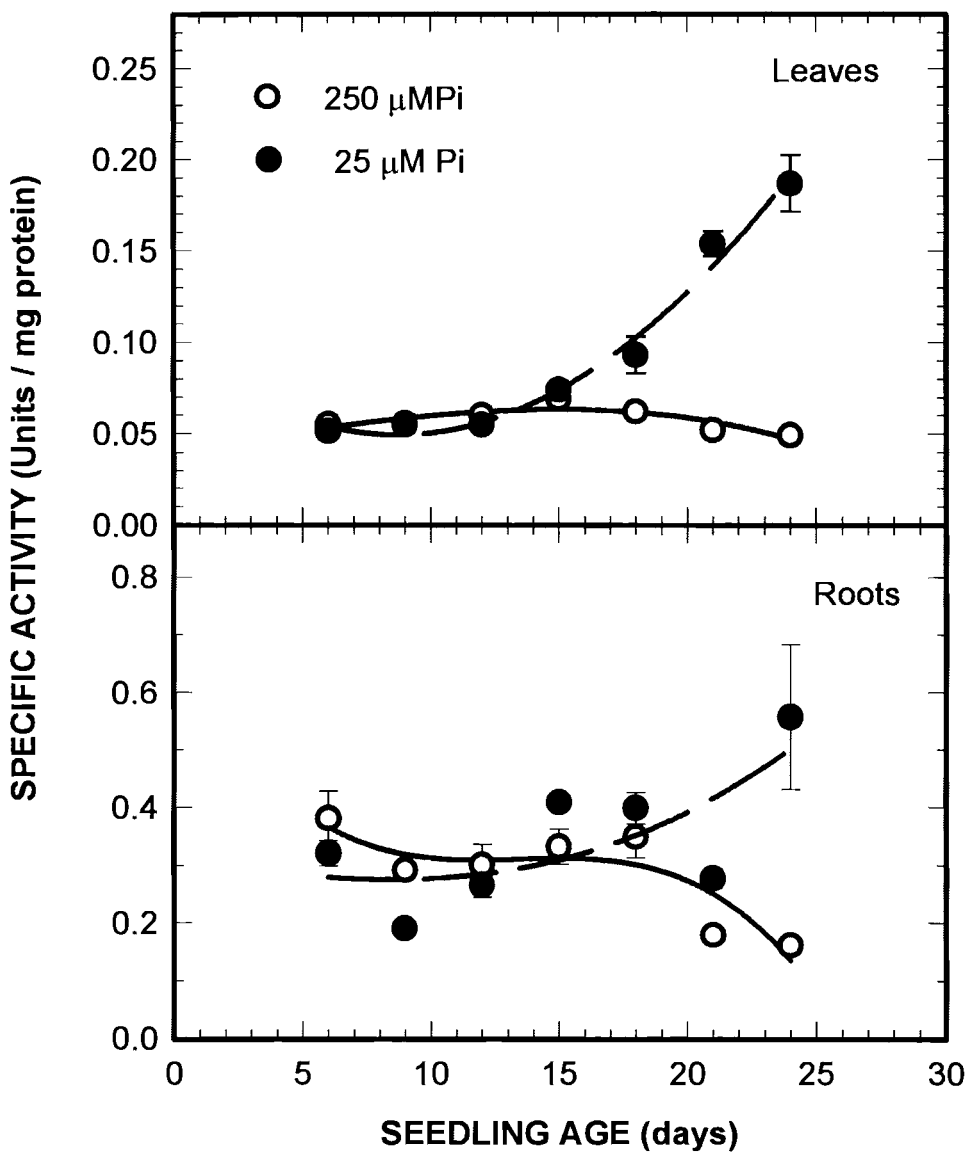

Figure 17. PEPCase activity in leaves and roots from wheat (cv. Pioneer 2180) seedlings grown in nutrient solution containing 250 μM or 25 μM inorganic phosphate. All values represent the mean of three separate determinations. Bars indicate standard deviations.

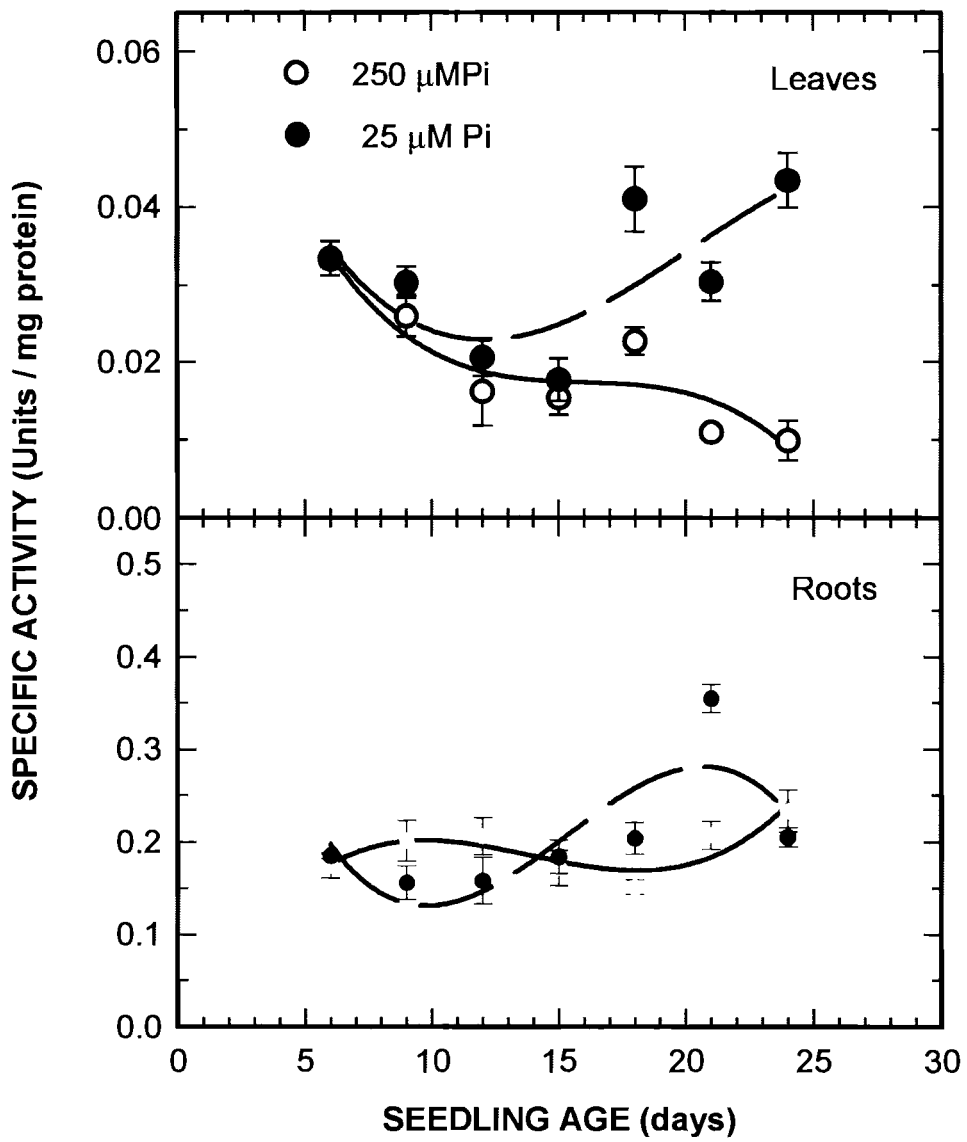

Figure 18. PFP activity in leaves and roots from wheat (cv. Pioneer 2180) seedlings grown in nutrient solution containing 250 μM or 25 μM inorganic phosphate. All values represent the mean of three separate determinations. Bars indicate standard deviations.

CHAPTER V

DISCUSSION

Wheat cultivar Pioneer 2180 exhibited slightly fast growth than cultivar TAM W101 in this study; this cultivar was also suggested to have higher phosphorus-efficiency (W. R. Raun, personal communication). However, both Pioneer 2180 and TAM W101 showed the same extent of growth reduction during reduction of phosphorus supply (Figure 1). There are two types of mechanisms that may confer phosphorus-efficiency, as suggested by Hedley *et al.* (1994): first, 'internal' mechanisms, which allow high yield per unit P in the crop, and second, 'external' mechanisms, which involve greater phosphorus extraction from the soil. Because hydroponic culture was used in this study and the mobility of phosphate was high in solution compared to soil, the 'external' mechanism is not an important factor. Therefore, the similar response to phosphorus deprivation suggests that these two cultivars have the same level of 'internal' phosphorus-efficiency, and may differ in the 'external' adaptation for phosphorus deficiency. To examine this supposition, experiments using soil culture would be necessary.

The activity of an enzyme is usually expressed either on a fresh weight basis or on a protein basis. Presenting the enzyme activity in either way has its specific physiological meaning. In the present study, however, enzyme activity expressed on a protein basis was used, since doing so can provide more accurate data than

measuring the activity on a fresh weight basis in the small scale (below 1 ml) enzyme preparations used in this study, and can reflect the real proportion of total protein in tissues. The total protein concentration (protein/g fresh weight) was reported to decrease in phosphorus-deficient black mustard seedlings (Theodorou and Plaxton, 1994). Therefore, the increase of enzyme activity reported in the present study may reflect not only the increase in absolute amount of the enzyme, but the decrease of total protein as well.

A problem in this study was the difficulty in getting consistent results from different batches of plant material. For example, the average acid phosphatase activities in leaf tissue of 24-day-old wheat seedlings grown in 25 and 250 μM Pi shown in Figure 4 were about 0.6 and 0.4 unit/mg protein respectively, but those of another separate experiment shown in Figure 16 were only 0.5 and 0.25 unit/mg protein. This phenomenon may be caused by minor differences between two separate experiments in growth condition, harvesting, or storing, although every effort was made to hold conditions as identical as possible.

Acid phosphatase has been studied extensively in relation to phosphorus deficiency in plants. The acid phosphatase activity (units/mg of protein) of phosphorus-sufficient and phosphorus-deficient black mustard reported by Duff *et al.* (1991) by assaying in pH 4.6, 25°C, with PEP as substrate was 0.026, 0.047 in leaves, 0.07, 0.12 in stems, 0.038, 0.174 in roots, and 0.045, 0.53 in suspension cells. The data listed above are generally lower than those in the present study: 0.43 and 0.85 in leaves and 0.79, 5.50 in roots (Figure 4) by assaying in pH 5.0, 37°C, with

pNPP as substrate. In both of these studies, however, the increase in acid phosphatase activity in roots was greater than that in leaves. Working with the leaf extract of seven plant species, Besford (1979b) showed the acid phosphatase activity (units/mg of protein) of phosphorus-sufficient and phosphorus-deficient was 1.01, 1.63 in barley, 0.53, 1.20 in cucumber, 0.41, 0.71 in maize, 0.98, 0.99 in oats, 0.23, 0.15 in rice, 0.2, 2.77 in tomato, and 0.73, 2.24 in wheat when assayed at pH 5.8, 30°C, with pNPP as substrate. These data show that there is a large range in acid phosphatase activity among different species, and the activity of all species is greater under phosphorus deficiency, except for oats and rice. In addition, the activity from wheat leaves was higher than that shown in the present study under either phosphorus sufficiency or deficiency. This higher activity may result from the effect of aging, because all plants used by Besford (1979b) were 11 weeks old, and acid phosphatase activity was reported to increase with wheat age by McLachlan (1984). The pH value, temperature, and substrate should be reported when the acid phosphatase activity data are compared. Basically, the data on acid phosphatase activity in the present study agree with most of previous studies.

The APase isozymes observed to increase under phosphorus deficiency using native PAGE in the present study (bands 2 and 4 in Figure 7) appear to be similar to those seen in acid phosphatase zymogram analysis of phosphorus-deficient wheat leaves by Barrett-Lennard (1982) with cellulose acetate electrophoresis or McLachlan (1984) with starch electrophoresis.

As an indicator of phosphorus deficiency, acid phosphatase activity has one main drawback, namely that the activity does not increase until severe deficiency. Another problem in the use of acid phosphatase activity as an indicator of phosphorus status is that the activity is also affected by other factors in addition to phosphorus status. McLachlan (1984) suggested that the complications introduced by other factors can be overcome by developing an acid phosphatase zymogram which is similar to Figure 7 in this study. However, a possible better alternative is immunological method by using antibodies raised against a individual acid phosphatase isozyme which is highly correlated with phosphorus status.

Western blots for acid phosphatase (Figure 8 and 9) in the present study, surprising, did not show induction of new acid phosphatases as phosphate supply decreased. One possible reason is that the antibody, raised from wheat germ acid phosphatase, did not react with all wheat leaf and root acid phosphatases. Another supposition is that the increase of acid phsophatase activity is not due to the increase of amount of APase proteins, but due to the increase of an activator of some acid phosphatase isozymes. In black mustard seedlings, however, Duff *et al.* (1991) concluded that the activity of acid phosphatase was governed by the synthesis and degradation of acid phosphatase proteins. This was based on results of immunoblots using polyclonal antibodies raised against purified PEP-degrading APase. I did confirm, however, that periodate oxidation is a powerful tool for cleaning the background when working with Western blots of glycoproteins like acid phosphatase.

An interesting finding in this study is that phosphorus deficiency may reduce tissue water content (Figure 2). Because acid phosphatase activity, which is normally associated with phosphorus deficient plants, is also found in water-stressed wheat leaves (Barrett-Lennard *et al.*, 1982), and in salt-stressed spinach leaves (Pan, 1987), the physiological function of acid phosphatase during phosphorus deficiency may be more than that of releasing inorganic phosphate.

In previous studies, activity of PEPCase was between 0.02-0.1 units/mg protein in black mustard suspension cells (Duff *et al.*, 1989), 0.02-0.08 units/mg protein in tomato leaves, and 0.02-0.2 units/mg protein in tomato roots (Pilbeam *et al.*, 1993). Compared with these data, the results of the present study are a little higher: 0.05-0.3 units/mg protein in wheat leaves and 0.2-0.5 units/mg protein in roots. In addition, Pilbeam et al. (1993) observed that differences in PEPCase activity between phosphorus deficiency and sufficiency in tomato roots were higher than that in leaves, but the results for PEPCase in the present study in wheat (Figure 5 and 16) are opposite to that. These differences may result from differences between the species used.

The PEPCase from ripening banana fruits was reported by Law and Plaxton (1993) to have two subunits of about 103 (α-subunit) and 100 kD (β-subunit). In the Western blot analysis of PEPCase in this study (Figure 11), however, only one 116 kD band was observed in the lane containing banana fruit extract, and the two major bands which are probably the two subunits of PEPCase from wheat were of molecular weight 116 and 112 kD instead of 103 and 100 kD. It is possible to make

slight differences in molecular weight estimation for a protein because of different acrylamide concentration of gels, and different molecular weight standard used. However, the Western blot analysis agreed with the result of activity of PEPCase: there was greater increase of activity in leaves than that in roots under phosphorus deficiency (Figure 5). In addition to the major bands, some high molecular weight (>200 kD) proteins which can be detected by anti-(banana fruit PEPC) IgG in phosphorus-deficient leaf tissue seem to be good indicators of phosphorus deficiency, because they begin to appear in seedlings grown in 100 μM Pi, which was just below the critical Pi concentration. These proteins significantly increased as phosphate concentration decreased. The appearance of these proteins suggested that there may be a common antigenic epitope among several proteins involved in the mechanism of adaptation for phosphorus deficiency.

In the case of PFP, Theodorou and Plaxton (1994) showed that PFP activities increased in phosphorus deficient black mustard seeding in leaves, stems and roots. Only the 66 kD subunit was detected by anti-(potato PFP) immune serum in leaves, while both the 66 kD and 60 kD subunits were detected in stems and roots. In the present study, PFP activity in roots did not increase in phosphorus deficiency (Figure 6), and the 66 kD subunit was not be detected in roots except in seedlings grown in 500 μM Pi. Both subunits of PFP were detected in wheat seedlings leaves (Figure 11). Moreover, the lower molecular weight subunit of PFP in wheat tissue was about 63 kD instead of 60 kD, while the two bands shown in the lane for potato tuber extract were 66 and 60 kD (Figure 11), the same as reported in previous study

(Theodorou, 1992). This result suggests that the protein structure of PFP in wheat is slightly different from potato and black mustard, but has the same antibody recognition site. In addition, PFP in wheat, especially in the roots, may have different physiological functions from dicots such as black mustard.

In summary, all three of the enzymes examined in this study have potential for use as indicators of phosphorus status. Future research should focus on three areas identified here: (a) purification and characterization of individual isozymes of acid phosphatase including development of antibodies specific for wheat leaf APase; (b) purification and characterization of the high molecular weight proteins that were detected by the antibody to PEPCase; (c) the physiological function of PFP in wheat roots.

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In summary, all three of the enzymes examined in this study have potential for use as indicators of phosphorus status. Future research should focus on three areas identified here: (a) purification and characterization of individual isozymes of acid phosphatase including development of antibodies specific for wheat leaf APase; (b) purification and characterization of the high molecular weight proteins that were detected by the antibody to PEPCase; (c) the physiological function of PFP in wheat roots.

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