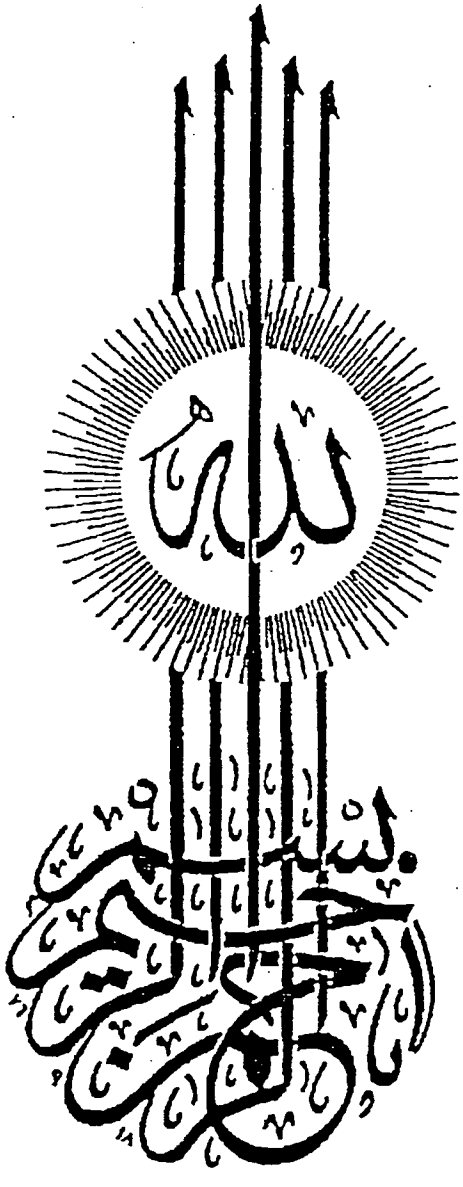
Surface phosphatase activities on roots of Pakistani wheat cultivars

G. Mustafa Chandio



IN THE NAME OF GOD, MOST GRACIOUS, MOST MERCIFUL

Surface Phosphatase Activities on Roots of Pakistani Wheat Cultivars

by

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in the University of Durham, England

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This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

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January 1994

DEDICATED TO THE POOR MAN BEHIND THE PLOUGH

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ABSTRACT

A study of the surface phosphatase activity of different wheat cultivar roots was carried out, using two substrates, p-nitrophenyl phosphate (pNPP) and 4-methylumbelliferyl phosphate (4-MUP), and different pH values ranging from 3 to 10.0. Seedlings of the wheat cultivars, Indus-66, Jauhar-78 and Pavan, were grown in Hoagland solution under P-sufficient and P-limited conditions, each for 5, 10 and 15-days. The optimum pH for phosphatase activity differed in the different cultivars, but was always acidic, ranging from pH 4.0 to 6.0.

P-limited plants showed greater phosphatase activity than P-sufficient ones with both substrates. Phosphatase activity increased within the first 5-day interval of the plants being deprived of phosphorus, and reached a peak by the 10-day age.

In 5-day old plants, cv Pavan showed highest PMEase activity (0.06 μmol mg d.wt $^{-1}$ h $^{-1}$ at pH 5.0); however in cv Indus-66 (0.08 μmol mg d. wt $^{-1}$ h^{-1}) and cv Jauhar-78 (0.07 μmol mg d. wt $^{-1}$ h $^{-1}$) the highest activity was in 10-day old plants at pH 4.5 and 6.0, respectively. This occurred with both substrates. The pH optima of P-sufficient and P-limited plants were similar.

As root surface phosphatase activity is an adaptive response, this could be used to distinguish cultivars suitable for growing in low inorganic phosphate fertility situations.

ABBREVIATIONS

^{o}C	degree Celsius
mg	milligramme
µg	microgramme
l	litre
cv	cultivar
d. wt	dry weight
h	hour
mM	millimolar
µM	micromolar
µmol	micromoles
P	phosphorus
Pi	inorganic phosphorus
PMEase	phosphomonoesterase
PDEase	phosphodiesterase
pNPP	para-nitrophenyl phosphate
pNP	para-nitrophenol
4-MUP	(4-methylumbelliferyl phosphate
CAPS	3 (cyclohexamino)-1-propanesulphonic acid
DMG	3, 3-dimethyl glutaric acid
EDTA	ethylenediaminetetra-acetic acid
HEPES	N-2-hydroxymethylpiperazine-N′-2-ethanesulphonic acid
pKa	dissociation constant

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INTRODUCTION

1.1 General Introduction

Wheat (*Triticum aestivum* L.) is the most important cereal crop in the world. It is mentioned in the Bible first in Genesis, Chapter 30, verse 14 (Wolfe & Kipps, 1959). The evolution of wheat as a crop plant occurred almost 10,000 years ago in the fertile crescent of the Middle East (Harlan & Zohary, 1966). The main wheat-producing regions are Southern Russia, the central plains of the United States and adjacent areas in Canada, the Mediterranean basin, north central China, India, Argentina and south-western Australia (Riley, 1965). Wheat is not a major food crop in the tropics, but it is grown at higher altitudes and in certain lowland areas in sub-tropical areas. In Pakistan wheat is grown during the cooler season of the year and usually under irrigation; it can also be grown in the dry season.

Triticum aestivum is the crop widely adopted throughout the world. It is grown over a wide range of precipitation and temperature conditions, mostly in the 25° to 50° latitude range. Wheat production has expanded into the lower latitudes of less than 15°N as a cool season crop (Khalifa *et al.*, 1977).

Essential nutrients contained in wheat are carbohydrates (60-80 %, mainly as starch), protein 8-15% (which containing adequate amounts of all essential amino acids except lysine, trytophan and methionine), fats (1.5-2.0 %), minerals (0.5-2.0%), vitamin B complex and vitamin E. (Values are expressed as % dry weight).

The great popularity of wheat as a crop is based on several facts:

(i) Its carbohydrates and proteins are well balanced;

(ii) It is produced economically with nearly complete mechanization;

(iii) It can be kept in storage for a long time as whole wheat or as flour;

(iv) It can be grown in a wide variety of climates throughout the world (Shaw, 1955).

1.1.1 SEED GERMINATION

Seed germination occurs between 4 and 37°C, with 20-25°C being optimal. The minimum moisture content for germination is 35-45% of grain dry weight, and germination is more rapid as moisture increases above this level. Germination can occur at a relative humidity of 97.7%, which is below the permanent wilting point for the plant (Owen, 1952). As the seedling develops, it becomes more susceptible to water deficiency (Milthorpe, 1950).

Light is not of great importance in controlling germination of wheat (Grahl, 1965). The minimum temperature for the growth is 3-4°C; the optimum temperature is 25°C, and the maximum is approximately 30-32°C.

1.1.2 WHEAT ROOTS

The fibrous root system of wheat consists of two distinct components, the seminal (seedling) and nodal (adventitious, secondary, crown) roots. These differ in the point and time of their origin, in features of their anatomy, and to some extent in their function (Troughton, 1962).

The seminal roots arise directly from, or below, the seed. Nodal roots develop from the nodes in the crown in sequence as the main culm and tillers develop with the earlier developed roots being longer and more branched than later ones. The main culm roots first tend to dominate the nodal root system. Seminal roots are deeper, finer, have a higher order of branching than nodal roots and tend to have a near linear distribution with depth. They tend to have a direct downward orientation (Belford *et al.*, 1987). Nodal roots remain active throughout the season, and provide a greater fraction of other roots in the lower profile.

Nodal roots are distributed exponentially with depth (negatively); after midseason they constitute the major weight of the root system in the upper profile. Nodal roots have an oblique outward development pattern that reorients in the downward direction, and this increases the extent of lateral rooting. Mackey (1973) indicated that the early developed nodal roots increase effectiveness in water and nutrient absorption compared with the laterally developing ones which are thicker, shorter and less branched. Nodal growth normally begins about 25-30 days after plant emergence unless growth is prevented by low temperatures. Dry surface soil at the crown can prevent the initial extension of nodal roots, and major surface soil drying during tillering can slow or prevent new nodal root growth from tiller nodes.

Winter cereals tend to produce a larger weight of roots than spring sown ones (Troughton, 1962). Presumably this is due to the longer period of growth at lower temperatures. Cultivars differ in the distribution and size of their root system (Pinthus & Eshel, 1962; Asana & Singh; 1967; Derera *et al.*, 1969).

Optimum rooting strategies depend on the climate and soil type. The depth of root penetration depends on the age of the plant, the fertility and physical condition of the soil and soil moisture. Wheat cultivars differ in the aerial extent and depth of penetration of their root systems. Cultivars which are most resistant to lodging have a large number of coarse and thick, strong roots extending horizontally in the soil. Non-resistant cultivars have smaller and more flexible roots or roots that penetrate almost vertically downwards (Troughton, 1962).

1.2 PHOSPHORUS

P is the eleventh most abundant element in nature. Its concentration is estimated as 0.1% by weight in the lithosphere, and is thus classed as a trace element. 80% of the

reserves are contained in phosphorite deposits in ocean sediments and 15% in igneous and metamorphic rocks (Holtan *et al.*, 1988). P is a vital structural component of nucleic acids, nucleoproteins, phytin phospholipids, adenosine triphosphate (ATP) and numerous phosphorylated compound.

As a component of nucleic acids, P is built into the DNA of the chromosome, and the RNA of the nucleus and ribosome, where it is vital to nuclear and cell division and regulation of every cellular process. P is a component in the phosphoplipids of cell membranes, regulating movement of materials in and out of the cells and organelles.

P is necessary for the growth, development and maintenance of plants and animals. Therefore, it is essential that the supply of P in agricultural soils be maintained. If crops are removed from the land, the supply of P in the soil may become the limiting factor in crop production. Large quantities of P compounds are used as fertilizers.

1.2.1 PHOSPHORUS IN SOIL

P in agricultural soils has been investigated more than other plant nutrients; it is a major nutrient element because of its complex reaction with soil constituents. Most soils contain between 0.022 and 0.083 % P. In soil profiles on seemingly uniform parent materials, the minimum % P usually occurs in the lower A or upper B horizon (Winters & Simonson, 1951). This minimum presumably results from absorption of P by plants and also losses due to leaching. The higher P content found in the surface soil may be due to the return of some of the P absorbed by plants and also retention of this P by the surface soil against the rapid downward movement by leaching.

P in the soil solution is important in areas where the roots are actively absorbing P. The required concentration of P in the soil solution depends primarily on the crop species being grown and the level of production (Fox, 1981-1982).

1.2.2 FORMS OF PHOSPHORUS

The phosphates in soils are derivatives of phosphoric acid, H_3PO_4. They can be divided into two broad categories: inorganic and organic phosphorus.

1.2.3 INORGANIC PHOSPHORUS

Inorganic phosphorus (P_i) may be classified according to its physical, mineralogical or chemical nature or a combination of these. Only a small fraction of the total P in the environment is concentrated in deposits consisting of mainly phosphate minerals (Brink, 1978).

Inorganic P is often present as apatite (calcium phosphate) of which the commonest form is fluorapatite [Ca10 $F_2(PO_4)$ 6] and is a common stable, accessory mineral in rock types. The P content of apatite in igneous and metamorphic rock can be, as high as 18% but normally it is lower than 12%. Phosphate minerals of various kinds have been found in soils. P readily forms insoluble compounds in the soil which precipitate out. Complexes are formed with other ions such as iron and aluminium.

P is present in water bodies in the form of inorganic phosphate ions (PO_4^{3-}, HPO_4^{-}, H_2PO_4), inorganic polymers, organic compounds and living organisms, and in living organisms and detritus. Many forms of phosphorus are not readily available for plant growth, but may become available through microbial action in the water. Much of the earth's P resource is in the inorganic form, but the continual cycling of P inorganic systems means that the size of the organic P pool is constantly changing, as the biota respond to changes in their physical environment (Hooper, 1973).

1.2.4 ORGANIC PHOSPHORUS

Soil contains various forms of organic P that are found in plants and micro organisms. P may be added to the soil in the form of chemical fertilizer, or may be incorporated as leaf litter, plant residues or animal remains. P occupies a critical position both in plant growth and in the biology of soil. Organic P compounds enter the soil in vegetation that undergoes decay. Anderson (1961) reported that nucleic acids account for 0.6 to 2.4% of the soil organic P.

Agricultural crops commonly contain 0.05 to 0.5% P in their tissues. In soil a large reservoir of organic P cannot be utilized directly by plants which emphasizes the role of microorganisms in converting the organic P to inorganic forms. By their actions, the bacteria, fungi and actinomycetes make the bound element in remains of vegetation and in soil organic matter available to succeeding generations of plants.

1.3 FUNCTION OF PHOSPHORUS IN PLANTS

The organic forms of P can be classified into two groups:

(i) storage and structural compounds;

(ii) compounds of intermediate metabolism.

(i) includes phytin, phospholipids and nucleoproteins. P in seed is stored as phytin, the calcium magnesium salt of inositol hexaphosphoric acid. During germination the compound is hydrolyzed to release inorganic phosphate.

P in the enzymes is present in the coenzyme protein, but it does not appear to be involved directly in the catalysis. In the metabolites, P plays a direct role as a carrier of energy. Phosphate in several organic linkages may be split off by hydrolysis with a relatively high yield of energy. This type of phosphate group is called high energy

phosphate. The most important carrier of high energy phosphate is ATP which is the sourse of energy that powers every energy-requiring biochemical process in plants. Phosphate groups in phytin, phospholipids, and nucleoprotein do not possess high energy bonds and yield less energy on hydrolysis. P high energy bonds have the property of being stable in water.

The concentration of (Pi) in plants may be expected to be influenced by the supply in the soil, and measurements of (Pi) in plant tissues are sometimes made to provide an index of the supply of P in the soil.

It has long been known that adequate levels of P are associated with early maturity of the crops, particularly in grain crops. P is associated with greater strength of cereal straw. The quality of fruits, forage vegetable and grain crop is said to be improved and disease resistance increased when crops have satisfactory P-nutrition. Phosphorylated compounds are important in photosynthesis.

1.3.1 ROOT GROWTH

P sometimes is said to stimulate root growth, the implication being that P has some special effect on the growth of roots that it does not have on the above-ground portion of the plant. If root designates the subterranean storage tissue of the root crops then the P supply does have a special effect. When a crop is deficient in P fertilization increases the yield of roots more than the above-ground parts. Plants deficient in P tend to have high carbohydrate content; when the supply of P is increased, the proportion of the carbohydrate translocated to the roots decreases. If P availability is high, young plants absorb P rapidly; rapid absorption of P early in the life of the plant is conductive to rapid development. Williams (1948) found the rate of P absorption required for the plants to attain maximum growth decreases as concentration of P increases.

The suppression of root development in general, combined with the paucity of fibrous root system in P-deficient plants, is especially limiting to normal plant growth. The weak root systems further render P-deficient plants highly sensitive to damage by root-rotting fungi. Young plants are particulary vulnerable to destruction when their root growth is too slow to keep ahead of advancing fungi.

1.4 PHOSPHATASES

Phosphatases are enzymes which catalyze the hydrolysis of both esters and anhydrides of phosphoric acid and under some circumstances certain phosphatases act from one substrate to another. They are often divided into acid and alkaline phosphatase according to their pH optima.

Phosphates are defined by Van Wazer (1973) as chemical structures in which each P atom is more or less tetrahedrally surrounded by four oxygen atoms. In condensed phosphates, 1 2 or 3 corners of the phosphate tetrahedra are shared with other tetrahedra through P-O-P bonds. Orthophosphate, made up of a single PO_4 group, is the principal form of P used in plant nutrition, being the form which is biologically mobile, and the main state in which P is interchanged between the various biological compounds of an ecosystem.

1.4.1 CLASSIFICATIONS OF PHOSPHATASE

The Commission on enzymes of the International Union of Biochemistry has classified all of these enzymes into five major groups.

1. Phosphoric monoester hydrolases (EC. 3. 1. 3) - Phosphomonoesterases
2. Phosphoric diester hydrolases (EC. 3. 1. 4) - Phosphodiesterases

3. Tri-phosphoric monoester hydrolases (EC. 3. 1. 5)

4. Hydrolase splitting anhydride bonds in phosphoryl containing anhydrides (EC. 3. 6. 1)

5. Hydrolase splitting P-N bond (EC. 3. 9) - Phosphoamidases

1.4.2 PHOSPHOMONOESTERASES

Phosphoric monoester hydrolases (phosphomonoesterases) are the acid and alkaline phosphatases. The term "phosphatase" is often used synonymously with phosphomonoesterases, abbreviated to PMEase (Flynn *et al.*, 1986). They catalyse P-O bond cleavage and have a wide range of substrate specificities. Phosphoric diester hydrolases catalyse the hydrolysis of phosphoric diester to yield a phosphoric monoester and an alcohol. The group is further subdivided according to the specific substrates catalysed. The least substrate-specific are phosphodiesterases, abbreviated to PDEase, which include the nucleases.

1.4.3 PHOSPHODIESTERASES

Phosphodiesterases (PDEase) are distinguished into two categories by their ability to hydrolyze 3'and 5' nucleotides. Phosphodiesterase I hydrolyzes nucleic acids to nucleoside 5'-phosphates and phosphodiesterase II hydrolyzes nucleic acids to nucleoside 3'-phosphates (Kelly *et al.*, 1975). The ability of PDEase to hydrolyze nucleic acid is incorporated as a tool in molecular biology.

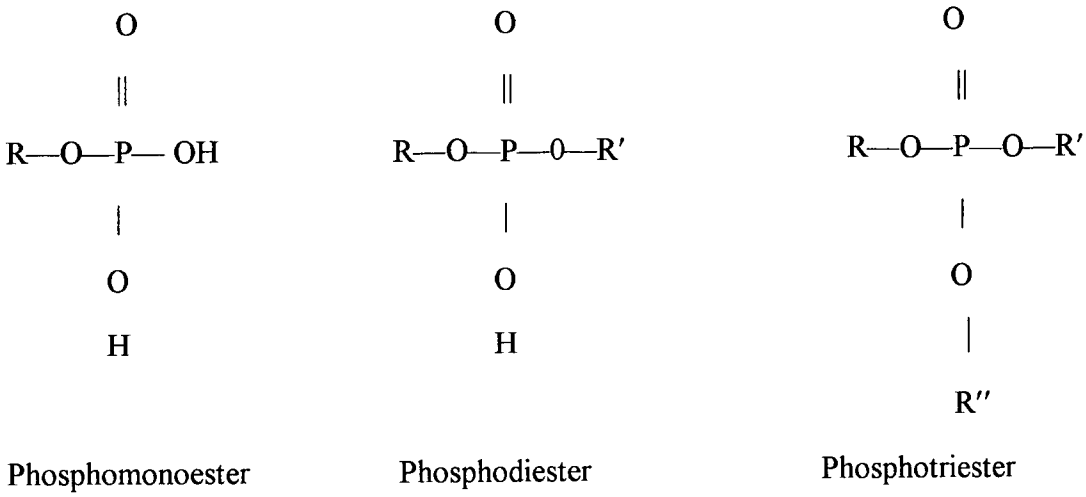
PDEase or nucleases have been isolated from a wide range of organisms e. g. cow (Kelly *et al.*, 1975), rat (Futai & Mizuno, 1967), carrot (Harvey *et al.*, 1970) and human urine (Ito *et al.*, 1987).

PDEase are typically alkaline and inhibited by EDTA. The activity of EDTA-treated

PDEase is completly restored by zinc and particularly by calcium and magnesium (Ito *et al.*, 1987), which suggested that PDEase are called zinc-metallo enzymes like PMEase.

PDEase in all cases are inhibited by Pi and in most cases PDEases are inhibited by ascorbic acid. It has been demonstrated that preparations of supposedly purified PMEase have diesterase activity. This activity was attributed to contaminating PDEase.

Fig. 1. 1. General formulae of phosphate esters.



Phosphomonoester Phosphodiester Phosphotriester

McComb *et al.*, (1979) suggested that phosphatases may be involved in metabolic procesess other than hydrolysis, such as transport of substances across membranes and synthesis of new organic phosphates.

Phosphatase activity will depend mainly on the type and concentration of the substrate and the enzyme. Other factors which affect phosphatase activity are temperature, ionic strength, pH and metal ions (McComb *et al*, 1979; Juma & Tabatabai, 1988).

1.5 PHOSPHATASES IN NATURE

Phosphatases have been observed in bacteria, algae and higher plants (Siuda, 1984; Press & Lee, 1983).

1.5.1 BACTERIAL PHOSPHATASE ACTIVITY

Phosphatases are adaptive enzymes and have been shown to be induced in bacteria (Torriani, 1960), when P concentrations become limiting. Bacteria are one of the most important contributers to environmental phosphatase activity. A large amount of work has concentrated on PMEase in heterotropic bacteria, with most research on PMEase structure and function in *E. coli* (McComb *et al.*, 1979). PMEase activity in freshwater heterotrophic bacteria has been demonstrated in lake waters (Jones, 1972a; Petterson, 1980; Chrost *et al.*, 1984; Hallemejko & Chrost, 1984). The majority of work on aquatic bacterial PMEase has been carried out with, marine forms which, in general, appear to have PMEases that are located in the periplasmic space.

1.5.2 ALGAL PHOSPHATASE ACTIVITY

Phosphatase activity has been found in all major groups of algae. Synthesis of phosphatase with external function has been frequently demonstrated in cultured algae (Kuenzler, 1965; Healey & Hendzel, 1973; Wynne, 1981). Phosphatases are located on the cell surface or in cell membranes and the release of extracellular phosphatase in culture has been reported by several authors (Healey & Hendzel, 1973; Aaronson & Patni, 1976). Phosphatase activities in aquatic environments have been attributed to algae (Petterson, 1980). The major work on algae in aquatic environments has concentrated on extracellular

phosphatase (Jansson *et al,* 1988) which can make up a substantial amount of the activity in the lake water.

The enzymes associated with phosphatase activity are located on the external cell membrane, where they are accessible to substrates outside the cell. As the cell membranes are relatively impervious to phosphate esters it is obviously advantageous to the algae to hydrolyse complex organic compounds of P outside the cell and then take up inorganic phosphate. Kuenzlar (1965) found that marine algae with the ability to hydrolyse glucose 6-phosphate in phosphate-deficient medium did not take up the whole molecule; they hydrolysed the ester extracellularly and took up the phosphate ion that resulted from hydrolysis.

Increased phosphatase activity in many species in the absence of orthophosphate has led to the suggestion that measurement at time of harvesting would be likely to respond to changes in nutrient status (Whitton, 1991).

1.5.3 PHOSPHATASE ACTIVITY IN HIGHER PLANTS

Phosphatase activity of plant roots could be important in the releasing phosphate from soil organic phosphatase, especially if the enzyme were liberated into the adjacent soil. Phosphatase activity appears in media in which cells of higher plants have been grown (Oleson *et al.*, 1974a, Yamaoka *et al.*, 1969).

Acid phosphatase PMEase activity has been reported in a number of plant tissues, including roots. Histochemical evidence shows that acid phosphatase activity of roots is localised in apical meristems and outer surface cells (Esterman & McLaren, 1961; McLean & Gahan; 1970; Shaykh and Roberts, 1974). In addition to acid phosphatase, the presence of other hydrolases such as ester, arylasulfatase , and glucuronidase has been reported on root surfaces of corn root tips. McLean and Gahan (1970) suggested that the hydrolase

activities present in the root cap may help in the breakdown of intracellular material, thereby loosening the cells.

Chang and Bandurski (1964) suggested that these hydrolases could solublize macromolecular constituents of organic matter in soil. This could yield low molecular weight organic and inorganic compounds, which could be utilized by plants. Plant root phosphatases may be involved in the mineralization of organic P present in the soil or in added organic matterials.

Several workers have reported phosphatase activity in higher plants in field and laboratory conditions. Juma and Tabatabai, (1988) reported that acid phosphatase activity in roots of maize and soyabean were inhibited by metal ions. In wheat, comparison of root and leaf phosphatase activity in plants grown in nutrition solution showed that leaf phosphatase was not necessarily a better measure of P status of the growing plants than root phosphatase (McLachlan & De Marco, 1982) .

1.6 PHOSPHATASES AS DEFICIENCY INDICATOR

After nitrogen-limitation, P-limitation in plants constitutes the second most important soil fertility problem throughout the world. Much work has been undertaken to characterize and understand the behaviour of P in the soils. In spite of this effort, the mineralogy of phosphates in the soil is still poorly understood.

Acid phosphatase, which is present in plant roots (Rogers *et al.*, 1942) and predominant in acid soils, is believed to be responsible for hydrolysis of organic P in soils. Therefore, the activity of this enzyme is significant in P cycling and plant nutrition.

Root surface (RS) phosphatases are one of the means by which a plant may hydrolyze immobilized phosphate groups and consequently make them available to its roots (Woolhouse, 1969; McLachlan, 1976); such non-specific phosphatases are mostly

phosphomonoesterses. Phosphatase activity in general may be influenced by P nutritional status of roots (Bieleski, 1973; McLachlan, 1980b; Boutin *et al.*, 1981).

Nutrient deficiencies are major factors limiting the productivity of agricultural plants. The commonly used methods for diagnosis of nutrient deficiencies consist of appraisal of visual symptoms, and leaf or soil analysis. However, these methods may be inaccurate or symptoms may appear too late for the cause to be remedied without significant loss of production (Epstein, 1972; Leece., 1976; Cox & Robson, 1980). There has therefore been interest in the use of enzymes as biochemical markers of nutrient deficiencies (Bar-Akiva, 1971; Leece, 1976).

The nutritional status of plants also has a great influence on the root secretion. When plants grown under nutrient stress conditions, such as P and iron deficiency, plant roots secrete functional organic compounds. Therefore, the root secretion ability of plants plays an important role in growth under nutrient stress conditions. It is considered that root secretion is one of the adaptive mechanisms in response to nutrient deficiencies.

Phosphatase activities in plants typically increase when plants become P-limited. The activities of ion transport systems in much of the plant root system seem to be regulated by the demand for mineral nutrients created by the growth of cells elsewhere in the plant.

It is well known that P-limited plants absorb phosphate more rapidly when the supply is restored than do P-sufficient controls (Bowen, 1970). Under P-sufficient conditions it has been found that tissue extracts from plants have an increased phosphatase activity. Besford (1979a).indicated that of 15 nutrient imbalances only P-limitation markedly increased acid phosphatase activity in leaves; he suggested that the enzyme might be used in a biochemical test to indicate P-limitation in tomato.

Phosphatase activity has been demonstrated in vivo under sterile and non-sterile conditions (Ridge & Rovira, 1971). Increases in the activity of external accessible phosphatases of roots of terrestrial plants growing with low levels of phosphate have been

demonstrated in vivo using wheat roots (McLachlan, 1980). The effect of P-limitation on phosphate bound to cell walls extracted from roots has been reported.

Woolhouse (1969) found that the phosphatase activity of cell walls from *Agrostis tenuis* was 2-fold higher when plants were grown with 1.0 μM phosphate than when plants were grown with 100 μM phosphate. Besford (1979 b) reported that soluble phosphatase activities also increased with P-limitation in leaves of wheat, however, phosphatase activities in wheat leaves were not a reliable index on P-limitation, as activities were influenced by water deficit and leaf age, as well as by P-limitation. Barrett-Lennard and Greenway (1982) further noted that in mature leaves of P-limited plants high phosphatase activities persisted for up to 12 days following the addition of adequate levels of P to the soil. Thus in these leaves high phosphatase activities are indicated.

Besford (1979b) found acid phosphatase activity in seven plant species, including wheat, in sand culture. Besford (1979b) further reported work, which suggested that it may well be possible to use this enzyme as an indicator of P-limitation in all the crops tested except dwarf rice.

Tarafdar and Jungk (1987) reported increased acid phosphatase in the rhizosphere of wheat and clover which was considered to induce the depletion of organic phosphates. Hayashi and Takijima (1953) observed that extracellular phosphatase activity of rice, wheat and barley roots under P-limited increased 2-5 fold compared with that of normal roots. They also reported that this activity was concentrated in the root cap cell, coating root tip of corn and degraded cells of barley and rice plants (Hayashi & Takijima, 1956). It has been reported that acid phosphatase, which was secreted by tomato roots and suspension cultured cells, increased under P-limitation (Goldstein *et al.*, 1988). It has been frequently reported that the activity of acid phosphatase in plant tissues increased under P-limited conditions (Silberbush *et al.*, 1981). P-limitation in *Spirodela* is very similar to that in other higher plants. It has already been shown that the pattern of neither phosphate nor

nitrogen metabolism is drastically altered by P-limitation in this plant (Bieleski, 1968). Reid and Bieleski (1970) reported that it might still be expected that changes in enzyme pattern could arise in other higher plants. Besford (1978 &1979a) suggested that soluble phosphatase activity may be useful as a biochemical index of P-limitation in leaves of tomatoes. Phosphatase activities increased 8-fold as the concentration of phosphate fell from 0. 30% to 0. 07% dry weight.

Increased yields of acid enzymes in P-limited medium have been reported with tomato leaves (Hewitt & Tatham 1960), germinated barley seed (Bianchetti & Sartirana, 1967) and tobacco callus tissue (Ueki & Sato, 1970). It is well known that phosphatase activity appears in media in which cells of higher plants have been grown. Oleson *et al.* (1974) studied the effect of extracellular Pi on acid phosphatase activity in the medium of cultured tobacco cells, and found evidence that new synthesis of enzymes was induced by Pi depletion. Lipton *et al.* (1987) reported that secretion of citric acid and succinic acid by alfalfa roots increased when the plants were limited in P.

Bieleski and Johnson (1972) found in duckweed (*Lemna minor*) that a large activity of phosphatase, which increased 10-20 fold following P-limited growth was located in the cell wall or the external membranes of roots, where it was assumed to be the source of an increase in inorganic P esters in the medium.

Phosphatase activity as a P-deficiency indicator has been studied and tested in several cyanobacteria species and it has been found that alkaline phosphatases were increased when phosphorus was limiting (Islam & Whitton, 1992). Acid phosphatase activity may, like alkaline phosphatase activity, increase at low phosphate concentrations (Olsson, 1990). Aaronson and Patni (1976) found that both acid and alkaline phosphatases were repressed or inhibited by extracellular inorganic phosphate. Kuenzler and Perras (1965) stated that only one of a sample of marine algal species showed increase in the acid phosphatase activity after increased P-limitation. Price (1961) reported that the synthesis

of acid phosphatases by *Euglena gracilis* was repressed by phosphate. Acid phosphatase may thus have some role as an indicator, as does the activity of alkaline phosphatases.

1.7 ACID PHOSPHATASE AND EFFECT OF IONS

Acid phosphatases are enzymes of wide specificity which cleave phosphate-ester bonds and thus play an important role in the mineralization of organic phosphate in the environment, where their activity may be correlated with low levels of free inorganic ions Spiers & McGill, 1979; Appiah & Thomas, 1982).

Phosphatases differ in their optimum pH hence the common separation into acid and alkaline phosphatases. Acid phosphatases have their optimum activity below pH 7.0, generally between pH 4.0 and 6.0; this does not mean that they are totally inactive at, or do not tolerate, other pH values.

Acid phosphatases are non-metallic enzymes, which are not activated by divalent cations, and are specially inhibited by fluoride (Cembella *et al.*, 1984). In higher plants an investigation carried out by Juma and Tabatabai (1988) into inhibition of acid phosphatase by metal ions revealed that Zn (II) and W (VI) had little effect on acid phosphatase activity in maize roots , but these ions inhibited activity in soybean roots by 25% and 62 %, respectively. Hasegawa *et al.* (1976) reported that Hg (II), Cu (II), Fe (III), Zn (II) and Co (II) inhibited acid phosphatase activity of wheat roots by 100, 82, 82 ,46 , and 31 %, respectively.

The present work suggested that root phosphatase activity could be a significant indicator of the current P status of the plant cultivars.

(i) The root phosphatase activity provides a comparative measurement of its ability to obtain P from a given situation.

(ii) Phosphatase activity is an important measure of the current P status of the plant. It is

possible that the root morphology, and plant growth rate may be important where P supply is limited by the diffusion rate.

The efficiency of plant species to absorb nutrients from the soil has great importance, particularly in underdeveloped countries, in the light of increasing demand for fertilizers and decreasing fertilizer resources (Blair, 1977).

1.8 AIMS

Wheat root phosphatase activity may be an important indicator for the identification of cultivars with the potential to grow in soils with low P availability.

The aims of the project were:

1. To investigate the influence of plant P-status on phosphatase activity the roots of different wheat cultivars taken from Pakistan.
2. To study changes in root phosphatase activity during growth of the cultivars.
3. To examine the influence of environmental factors on the phosphatase activity of the roots.

Chapter 2

MATERIALS AND METHODS

2.1 COMPUTING

Two computing systems were used for the study: Microsoft word 5.5 using the IBM PS/2 model 30 and VIG IV/33. Graphic output was carried out using the SIGMA PLOT (4.1) suite of software.

2.2 pH

pH measurements were carried out using an Ingold combination WTW E50 electrode and EIL meter (model 7050). The probe was calibrated with BDH standard buffer solutions, prepared in MilliQ water, immediately before a measurement was taken; the pH of the buffers was arranged so that one was higher and one lower than the pH of the solution under investigation.

2.3 MASS DETERMINATION

Mass was measured on an Oertling R51 balance to five decimal places. All references to weight refer to mass.

2.4 FLUOROMETRIC ANALYSES

A Baird-Atomic Fluoripoint Spectrofluorometer was used for fluorescence measurement for phosphatase activity when using 4-MUP as a substrate. Polystyrene cuvettes with a pathlength of 1.0 cm were used for measurements at wavelengths of 440 nm emission, and 356 nm excitation.

2.5 CLEANING OF GLASSWARE

All the glassware was washed in tap water with 2% Decon (Decon Laboratories Ltd, England) detergent, then rinsed with distilled water six times prior to drying at 105°C. All volumetric glassware was dried at room temperature; plastic was dried at 40°C.

2.6 MEDIA

All media were prepared from BDH Analar grade chemicals in MilliQ water and then kept in the refrigerator at 4°C until required. The stock solutions were renewed every 3 months.

2.7 SUBSTRATES

Substrates used for assaying phosphatase activity

REAGENTS	SUPPLIER
(p-nitrophenyl phosphate) pNPP	Sigma Chemical Co., USA
(4-methylumbelliferyl phosphate) MUP	Sigma Chemical Co., USA

2.8 BUFFER SOLUTIONS

Buffers used to investigate the effect of pH on phosphatase activity (each at 50 mM) were as follows. For abbreviations, see page 6.

Table 2.1 Composition of buffer solutions.

pH	buffer	buffering capacity	pKa at 20 °C
3.0	DMG -NaOH	3.2-7.6	3.66 and 6.20
3.5	Glycine -HCL	2.2-3.6	2.35
4.0	DMG -NaOH	3.2-7.6	3.66 and 6.20
4.5	DMG -NaOH	3.2-7.6	3.66 and 6.20
5.0	DMG -NaOH	3.2-7.6	3.66 and 6.20
5.5	DMG -NaOH	3.2-7.6	3.66 and 6.20
6.0	DMG -NaOH	3.2-7.6	3.66 and 6.20
6.5	DMG -NaOH	3.2-7.6	3.66 and 6.20
7.0	HEPES -NaOH	6.8-8.2	3.66 and 6.20
7.5	HEPES -NaOH	6.8-8.2	7.50
8.0	HEPES -NaOH	6.8-8.2	7.50
8.5	HEPPS -NaOH	7.3-8.7	7.50
9.0	Glycine-NaOH	8.6-10.6	8.0
	(AMeP-NaOH)	9.0-10.5	9.60
9.5	Glycine-NaOH	8.6-10.6	9.69
	(AMeP-NaOH)	9.0-10.5	9.60
10	Glycine-NaOH	8.6-10.6	9.69
	(AMeP-NaOH)	9.0-10.5	9.60
10.3	Glycine-NaOH	8.6-10.6	9.60
	(AMeP-NaOH)	9.0-10.5	9.69
	Na_2CO_3. $NaHCO_3$	9.2-10.8	10.33
11.0	CAPS-NaOH	9.8-11.1	10.40

All the chemicals used in the buffers were obtained from British Drug House Ltd. (BDH), except HEPES, which was supplied by Sigma Chemical Co., USA.
Buffers were prepared in the Chu 10-D assay medium to a final concentration of 50 mM and were stored in the refrigerator at 2°C until required.

2.9 ASSAY MEDIUM CHU 10-D

Table 2.2 Composition of Chu 10-D medium.

Major elements	mg l^{-1}	µM
$NaHCO_3$	15.85	188.6
KCl	4.28	57.38
$MgSO_4.7H_2O$	25.00	101.4
$CaCl_2.2H_2O$	35.83	243.7
Added as Fe chelate		
Na_2-EDTA.2HO	1.67	4.17
Trace elements		
$MnCl_2.4H_2O$	0.04	2.28
$CuSO_4.5H_2O$	0.02	0.078
$CoSO_4$ $7H_2O$	0.01	0.035
$NiSO_4.7H_2O$	0.038	0.03
$ZnSO_4.7H_2O$	0.056	0.19
$Na_2MoO_4.2H_2O$	0.007	0.028
H_3BO_3	0.72	11.56

(Gerloff *et al.*, 1950).

Assay media were prepared from stock solutions which were prepared and stored in a refrigerator; dilution of the stock solutions was made using Milli-Q water. The media were used for up to 1 week before being discarded.

To prepare one litre of assay medium 1ml each of $NaHCO_3$, $MgSO_4$ and $CaCl_2 \cdot 7H_2O$, 0.50 ml KCl, 0.25 ml Fe EDTA plus 0.125 ml from the micronutrient mixture were added to a volumetric flask, then the final volume was made up to 1L with Milli-Q water. Trace elements were added as microelements. EDTA was used as a chelating agent in order to prevent the precipitation of iron in the medium.

2.10 DRY WEIGHT

Glass vials were labelled, put in the 105 °C oven overnight and then weighed. After the assay was terminated, roots were transferred from the assay vials to the weighed bottles and they were returned to the oven overnight. The following day the bottles were removed from the oven and placed in a desiccator. The bottles were then each reweighed. The dry weight of each root was obtained by subtracting the initial weight of the bottle from the final weight (of the bottle and root).

2.11 PLANT MATERIAL

Three different wheat cultivars were used, their seeds were obtained from the Wheat Section of Agricultural Research Institute, Tandojam, Sindh, Pakistan.

2.12 PEDIGREE OF WHEAT CULTIVARS

The naming and origins of the cultivars is as follows:

i) Indus-66 = Kalyan sona's II 8156

ii) Jauhar-78= Nayab NF 600 RAD

iii) Pavon = Pavon/s/ vicam s - 271 x CIANO 'S - site cerros/ Blue Bird s -78/ 4

2.13 SEED TREATMENT

The seeds of wheat cultivars were surface sterilized by soaking in a 5% sodium hypochlorite solution with up to 10 minutes shaking, followed by washing with tap and distilled water.

2.14 GERMINATION

Wheat seeds were allowed to germinate on Whatman filter paper in sterilized plastic Petri dishes in a growth room maintained at 25°C and a 16-hour photoperiod.

2.15 TRANSFER OF SEEDLINGS

After emergence the seedlings were transplanted from Petri dishes to honey jars containing Hoagland solution (-P, +P). Holes were drilled in the lids of the honey jars and the seedlings were supported with cottonwool so that the roots were in the nutrient solution. Nine seedlings were transfered to each honey jar. Cultivars and (-P, +P) conditions were marked separately on the jar to indentify the difference.

2.16 PLANT AGE

The plants were harvested at 5, 10 and 15-days after germination.

2.17 HOAGLAND'S SOLUTION

Table 2.3 Composition of Hoagland's solution

KNO_3	1.02	$g\ L^{-1}$
$Ca(NO_3)2$	0.492	
$NH_4H_2PO_4$	0.230	
$MgSO_4.7H_2O$	0.049	
$MnCl_2.4H_2O$	1.81	
H_3BO_3	2.86	
$ZnSO_4.7H_2O$	0.22	
$CuSO_4.5H_2O$	0.08	
$H_2MoO_4.H_2O$	0.09	
$FeSO_4.7H_2O$	0.5%	
$C_4H_4O6\ Na_2$	0.4%	

(Tartaric acid).

(Arnon & Hoagland, 1940).

The solution was prepared (-P,+P) separately. In (-P) the salt NH_4Cl_2 was added instead of the salt NH_4. H_2PO_4. The other salts were the same for both conditions. The quantity of solution depended on the plant material and number of honey jars. The final solution was prepared from stock solutions, which were kept in a refrigerator except for KNO_3 which was prepared freshly when needed. One ml from each stock solution was taken to prepare 1 litre, the final volume was made up with distilled water. Each honey jar contained 500 ml. 0.3 ml of a mixture of iron sulphate and tartaric acid was added three times a week. The solution was changed weekly, i.e. twice for 10-day and 15-day plants.

2.18 PHOSPHATASE ASSAY PROCEDURE

The wheat plants were taken from the growth room, young roots were cut from the plants and washed three times in the assay medium. All the universal bottles were marked as replicates accordingly along with their pH control bottles. The substrates pNPP and 4-MUP were prepared in assay medium with a final concentration 100μM. The pH values of the buffers were checked before pipetting the required quantity; 1.6 ml was dropped into each universal bottles. Each pH buffer was added to the 4 replicate bottles along with their control. Assay medium (1.25 ml) was used uniformly in all replicates and control bottles (total 2.85 ml). Roots were excised to approximately 1.5-cm length and randomised in water in a Petri-dish before being washed with assay.

The chopped roots were placed in the replicate bottles (except for controls). Following the addition of the roots, substrate (0.1ml pNPP/ 4-MUP) was pipetted into each bottles, including the control (without root) bottles. Before running a particular experiment the maximum assay time was decided based on a time course experiment. For the assay, a 25°C controlled temperature water tank was used. All the bottles were placed in a tray in the shaking tank and the material was then left to shake for up to 30 min, depending on the plant material. For pNPP the terminator 5 M NaOH was added to each bottle, while K_2HPO_4 + NaOH + EDTA terminator was used for 4-MUP. A different quantity was used according to the buffer (acid or alkaline). For 4-MUP at pH's from 3.0 to 5.5 the concentrations of terminator used were 100 mM NaOH plus 100 mM K_2HPO_4 and 2.5 mM EDTA. Above pH 6.0 the concentrations of terminator were 50 mM NaOH, K_2HPO_4 and 2.5 mM EDTA.

For pNPP, substrate measurements were made on a spectrophotometer at 405 nm using glass cuvettes with a path length of 1.0 cm, while for 4-MUP reading a Baird Atomic Fluoripoint Spectrofluorometer was used for fluorescence measurement.

2.19 ASSAYING FOR PHOSPHATASE ACTIVITIES

Wheat root samples were assayed for phosphatase activity from pH 3 to 10.0. These values were chosen for identifying the acid and alkaline activities (see page 31).

Chapter 3

RESULTS

3.1 PRELIMINARY EXPERIMENTS ON RICE

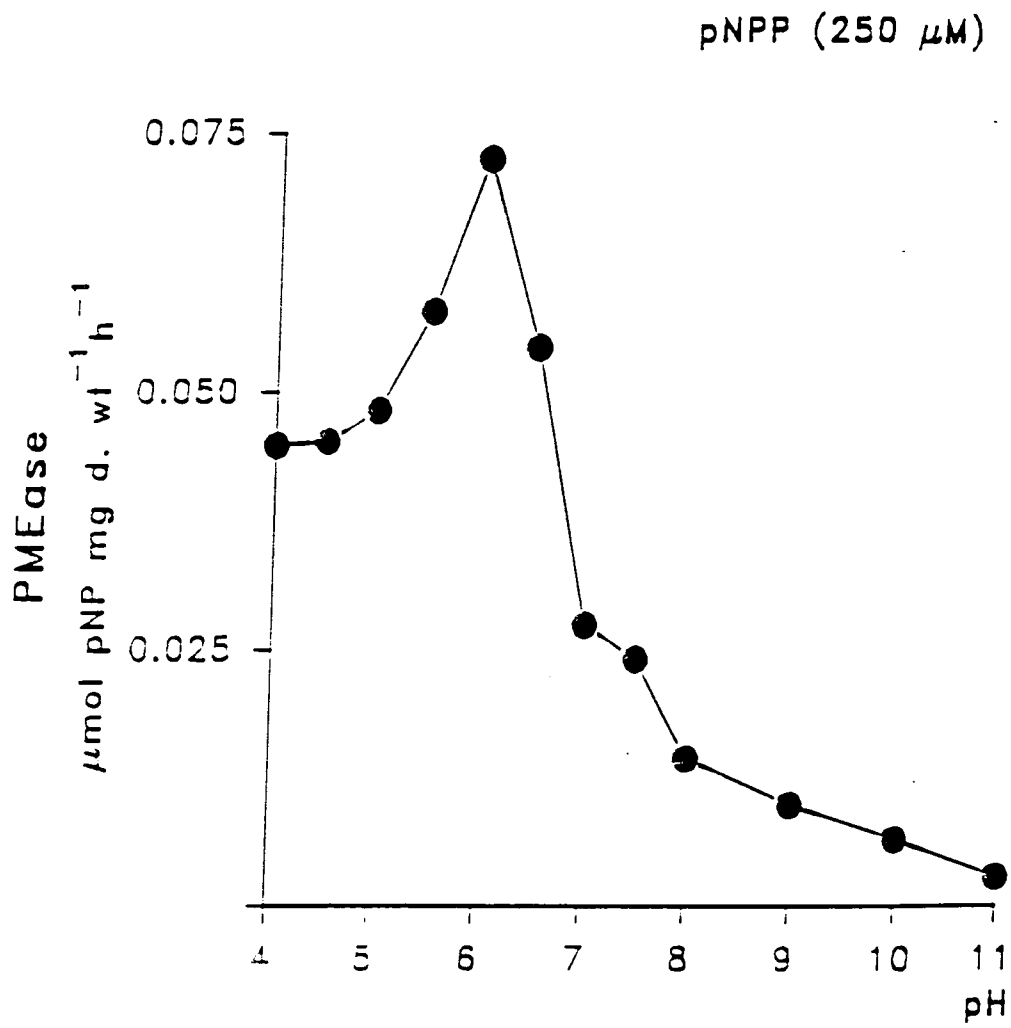
Before detailed experiments were carried out on wheat , preliminary experiments were performed on deepwater rice (which had been tested previously at Durham) in order to establish the methodology. The aim was to investigate the root phosphatase activities of deepwater rice cv Chota Bawalia using buffers from pH 4 to 11.0, and the substrate 250 μM pNPP.

Root sections were (approximately 1.5-cm long) cut and washed three times in assay medium prior to carrying out the assays. At each pH value four replicate samples were used. All assays were carried out in universal bottles incubated in a growth tank at 25°C.

Assays were carried out with buffers at a final concentration of 50 mM, which was chosen as a suitable concentration for buffering physiological media. The substrate, pNPP, was used with a final concentration of 250 μM. Incubations were run for 1 h and the reaction was stopped using a terminator (5M NaOH).

Fig.3.1 shows the phosphatase activity of the rice roots. The highest phosphatase activity occurred at pH 6.0. Once the methodology for assaying phosphatase activity of rice roots was established, this was then applied to wheat and was modified where necessary.

Fig. 3. 1 Influence of pH on PMEase activity of deepwater rice roots (cv Chota Bawalia)



3.2 WHEAT

Preliminary experiments were performed in order to finalise the methodology for the project. In various experiments different methods were used to select the best combination of assay terminator and its quantity. Cultivars Indus-66, Jauhar-78 and Pavon were selected on the basis of their difference in pedigree (see Methods). Given the origins of these cultivars it was necessary to grow the seedlings in a controlled environment growth room. Each cultivar was grown under identical conditions to minimise physiological variations.

Different variables were used in the phosphatase assay experiments e.g. two substrates pNPP and 4-MUP, three cultivars and two P-conditions (sufficient versus limited). Their components were combined with plants of different ages: 5, 10 and 15-days after germination. The results for each individual experiment are shown in Figures 3.2 to 3.10 and are discussed briefly. The overall results are shown in (Tables. 3.1- 3.3).

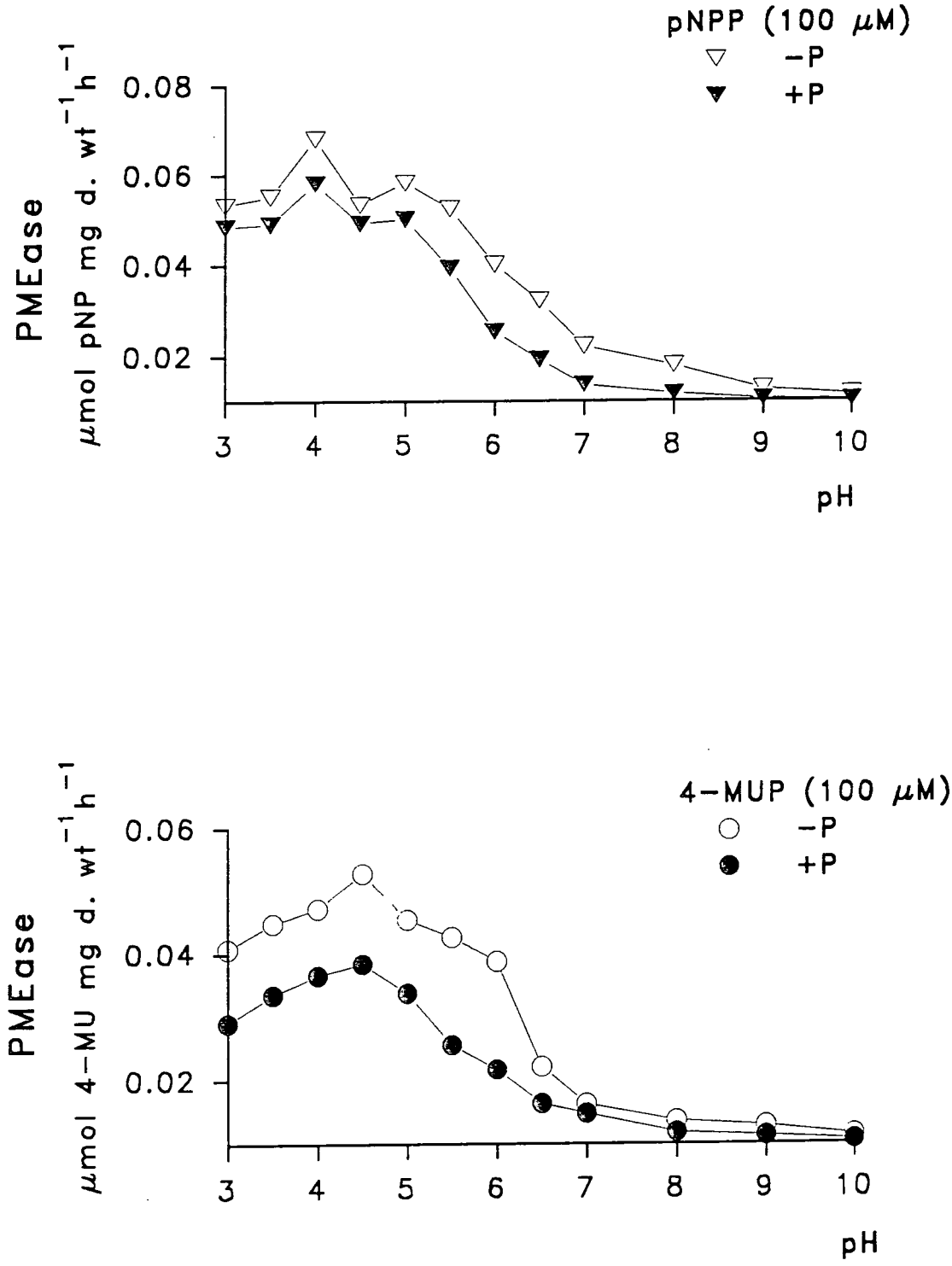
3.2.1 Five-day old plants

All the above cultivars were grown in medium for 5-days. Equal amounts of +P or -P versions of nutrient solution were applied as the growth medium. On the final day of treatment the seedlings were harvested and the level of phosphatase activity in the roots was determined.

3.2.1.1 Cultivar Indus-66

The influence of pH on PMEase activity measured using the two substrates pNPP and 4-MUP is shown in (Fig.3. 2). The maximum rate of activity was observed between pH

Fig. 3. 2 Influence of pH on PMEase activity of 5-day old wheat roots (cv Indus-66) grown with and without phosphorus



4.0 and 4.5 with both substrates. A possible shoulder also occurred at pH 5.0, above which activity gradually declined to pH 7.0. Above this PMEase activity was negligible at all pH values.

The substrate 4-MUP showed an apparently slightly different response to pNPP with a relatively lower value at pH 3.0. The overall activity was lower at all pH values.

The phosphatase activity of roots of P-sufficient plants was less than that of P-limited plants for both substrates. However, the optimum pH for activity peaks of P-sufficient and P-limited plants was similar with both substrates.

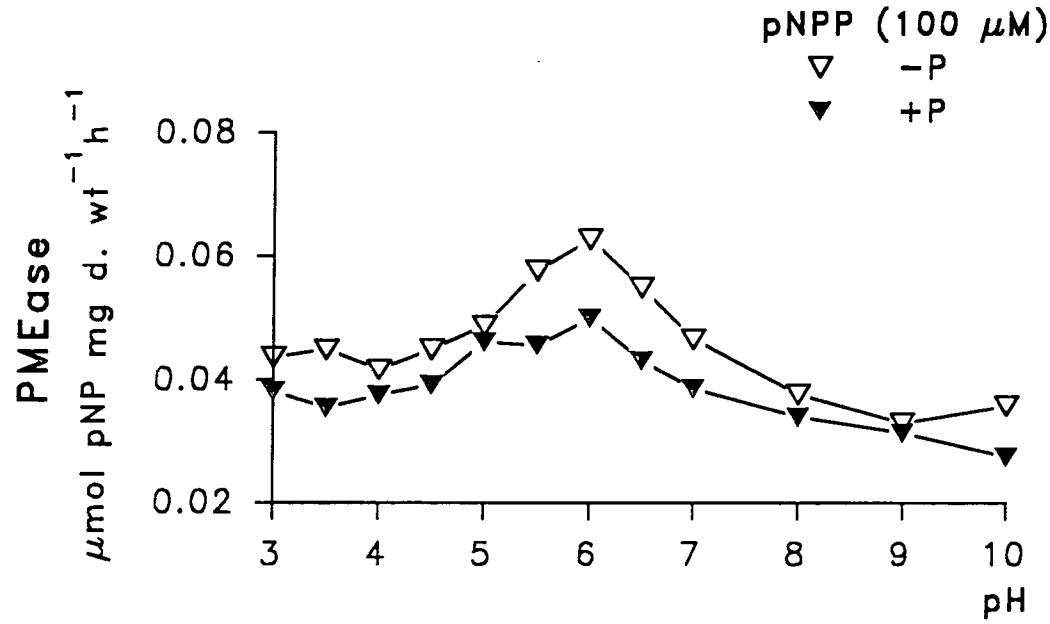
3.2.1.2 Cultivar Jauhar-78

Use of the substrates pNPP and 4-MUP showed different values for optimum pH (pH 6.0 and 4.5, respectively). Above pH 6.0, PMEase activity decreased as the pH values increased, proportionally more with 4-MUP than pNPP. Again, P-limited plants showed more activity at all pH values with both substrates (Fig. 3. 3).

3.2.1.3 Cultivar Pavon

Data show that PMEase activity using the substrate 100 μM pNPP was detectable between pH 3-7 with an optimum at pH 5.0. However, 4-MUP showed maximum activity at a slightly lower pH (4.5) value than pNPP. With increasing pH,there was a decline in activity under both conditions. The phosphatase activity of the roots of P-sufficient plants was less than that of P-limited plants with both substrates at all pH values (Fig. 3. 4).

Fig. 3. 3 Influence of pH on PMEase activity of 5-day old wheat roots (cv Jauhar-78) grown with and without phosphorus



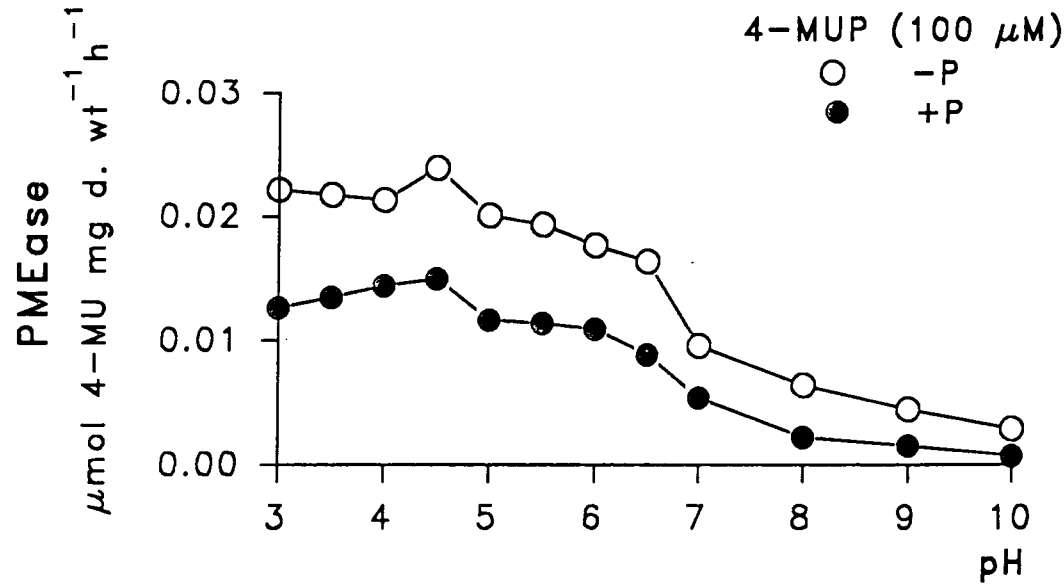
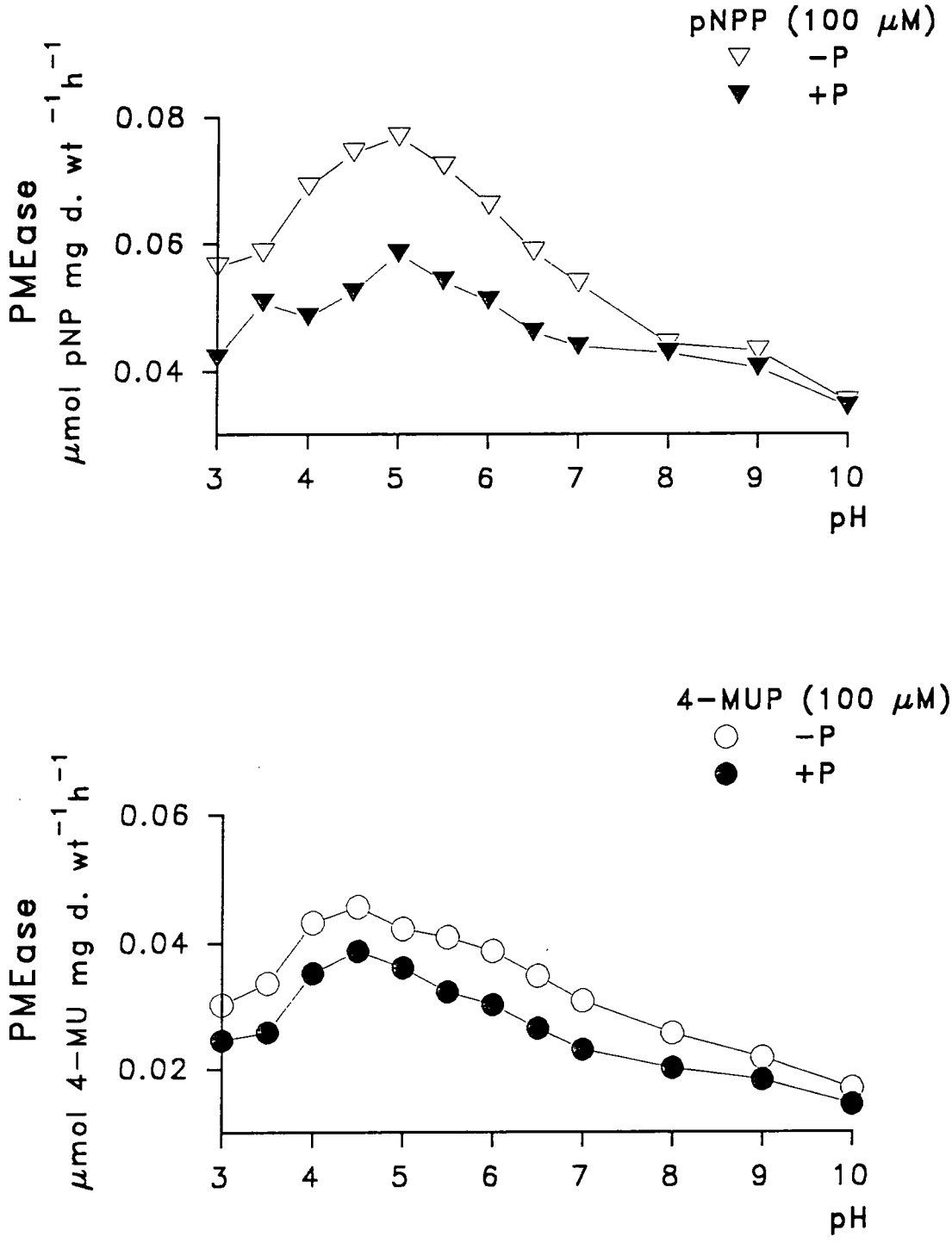


Fig. 3. 4 Influence of pH on PMEase activity of 5-day old wheat roots (cv Pavon) grown with and without phosphorus



3.2.2 Ten-day-old plants

Comparison of cultivars for 10-day old plants for phosphatase PMEase activity was carried out using two substrates (pNPP and 4-MUP). This experiment was carried out using all cultivars.

3.2.2.1 Cultivar Indus-66

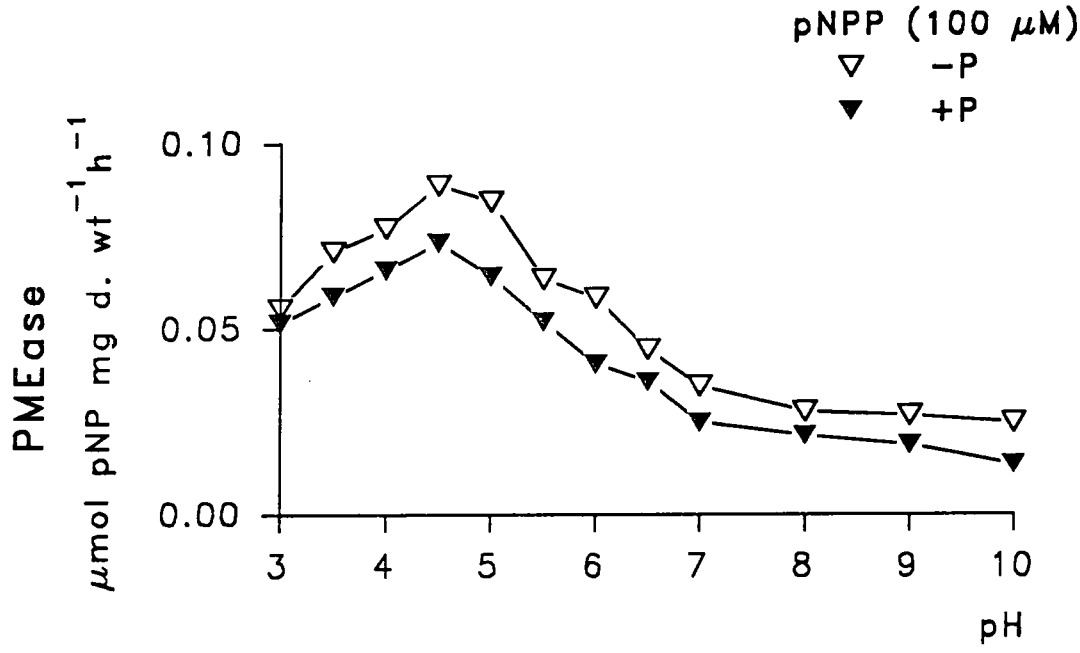
PMEase was assayed using the 100 µM pNPP and 4-MUP substrates. Phosphatase activity with pNPP substrate showed that roots of P-limited plants had more activity than those of P-sufficient plants (Fig.3. 5). The optimum pH (pH 4.5) for P-sufficient and P-limited plants was, however, similar.Above pH 4.5 the activity declined gradually to pH 10.0. The maximum activity of roots of P-limited plants was 0.08 µmol mg d. wt $^{-1}$ h $^{-1}$ compared with 0.07 µmol mg d. wt $^{-1}$ h $^{-1}$ for P-sufficient plants.

The use of 4-MUP gave the same response for optimum pH (4.5). Above this value the PMEase activity decreased to pH 6.5. The activity dropped sharply to pH 7.0. The maximum activity under the P-limited condition was 0.06 µmol mg d. wt $^{-1}$ h $^{-1}$.

3.2.2.2 Cultivar Jauhar-78

Cv Jauhar-78 showed two peaks of PMEase activity, at pH 4.5 and pH 6.0, with the maximum (0.07 µmol mg d. wt $^{-1}$ h $^{-1}$) at pH 6.0. The PMEase activity then dropped sharply to pH 7.0. A decline was observed proportionally from pH 8.0-10.0. Roots of P-limited plants showed more overall activity than P-sufficient plants.

Fig. 3. 5 Influence of pH on PMEase activity of 10-day old wheat roots (cv Indus-6) grown with and without phosphorus



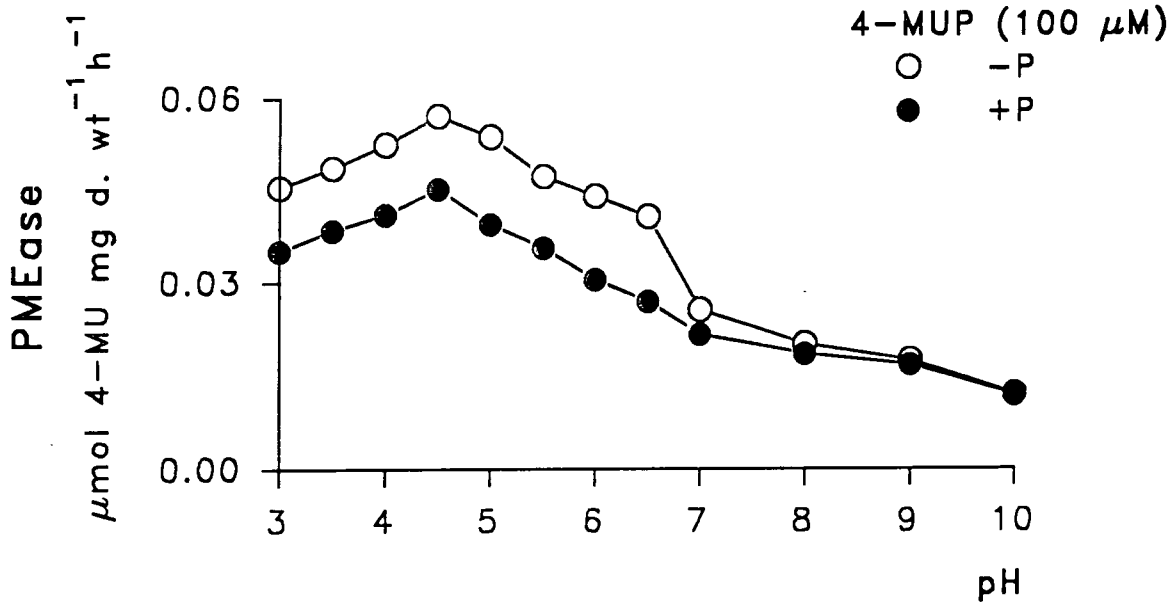
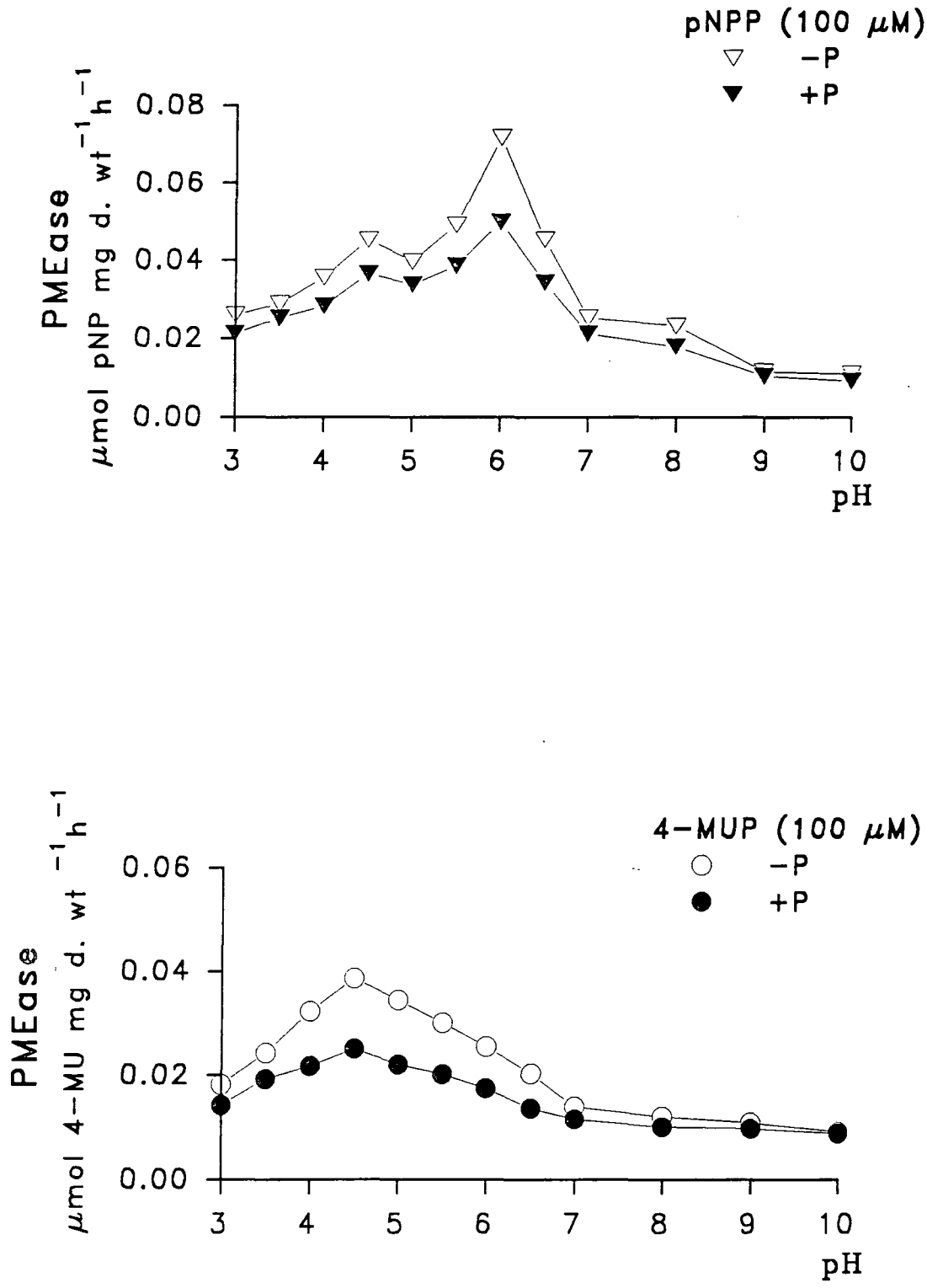


Fig. 3. 6 Influence of pH on PMEase activity of 10-day old wheat roots (cv Jauhar-78) grown with and without phosphorus



Use of the substrate 4-MUP showed different responses for pH optimum. The maximum phosphatase activity (0.04 µmol mg d. wt $^{-1}$ h $^{-1}$) was observed at pH 4.5. Over the range of pH values used, the activity of roots of P-sufficient plants was less than that of P-limited plants (Fig.3. 6). An increase in activity of 30-35% was noted for P-sufficient plants compared to the P-limited plants.

3.2.2.3 Cultivar Pavon

When pNPP substrate was used, PMEase activity was detected between pH 3-7. When the pH was increased, a decline in activity was observed. The maximum PMEase activity (0.06 µmol mg d. wt $^{-1}$ h $^{-1}$) was recorded at pH 5.0.when using 4-MUP (Fig.3.7); differences in PMEase activity and optimum pH were observed. Roots of P-limited plants had maximum rate of activity of (0.04 µmol mg d. wt $^{-1}$ h $^{-1}$), while a lower rate of activity (0.03 µmol mg d. wt $^{-1}$ h $^{-1}$) was observed in the P-sufficient conditions. The P-limited plants gave an increase in activity of 9.3%- 25.4% compared with the P-sufficient plants for pNPP & 4-MUP respectively. Optimum pH for activity of P-sufficient and P-limited plants was similar using both conditions.

3.2.3 Fifteen-day old plants

3.2.3.1 Cultivar Indus-66

PMEase activity was detected between pH 3-7 (Fig.3. 8) with optimal activity at pH 4.5, after which the activity declined gradually. The maximum rate of activity (0.06 µmol mg d. wt $^{-1}$ h $^{-1}$) was observed in plants grown under P- limited conditions. However, when the activity was tested using the substrate 4-MUP different results were observed.

Fig. 3. 7 Influence of pH on PMEase activity of 10-day old wheat roots (cv Pavon) grown with and without phosphorus

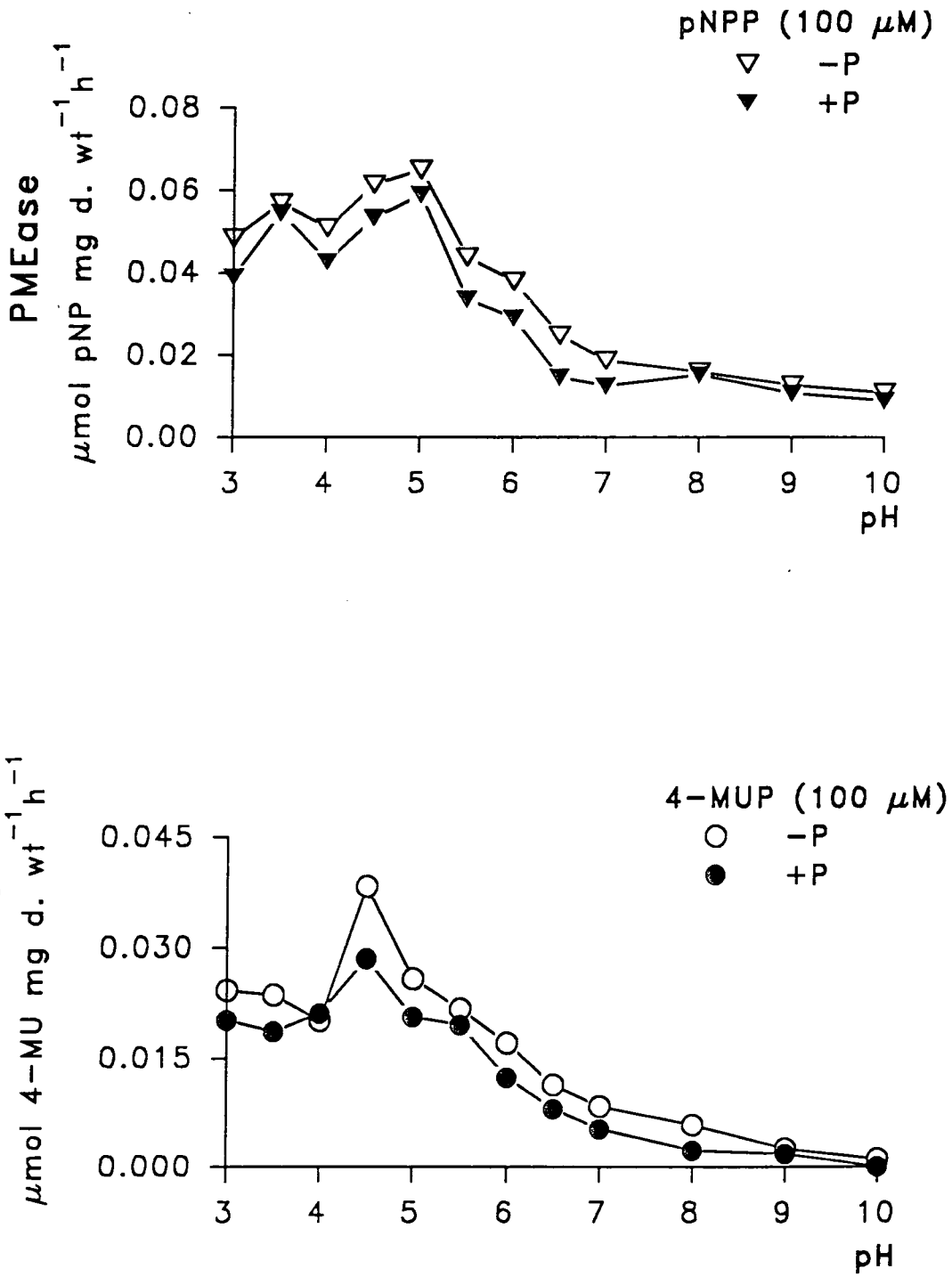
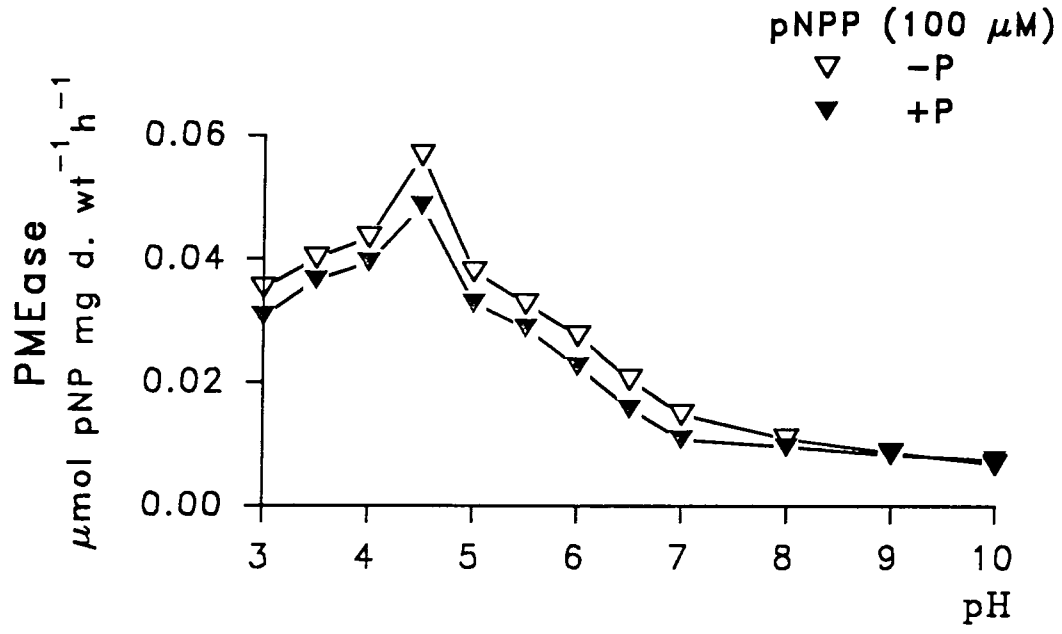
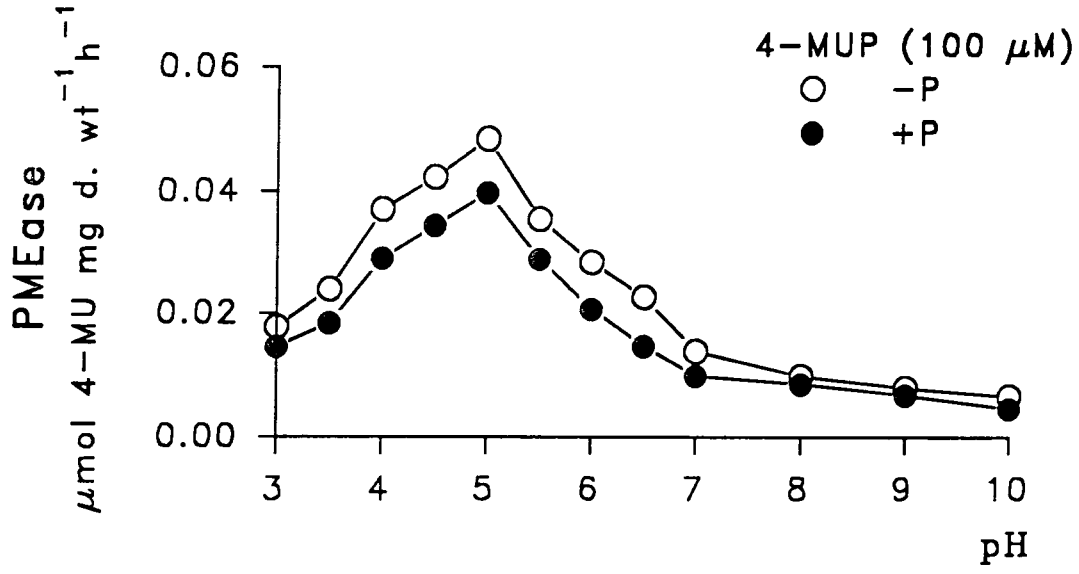


Fig. 3. 8 Influence of pH on PMEase activity of 15-day old wheat roots (cv Indus-66) grown with and without phosphorus





over the range of pH values tested the maximum rate of activity (0.05 µmol mg d. wt $^{-1}$ h $^{-1}$) occurred at pH 5.0. Roots of P-limited plants showed more activity than P-sufficient plants.

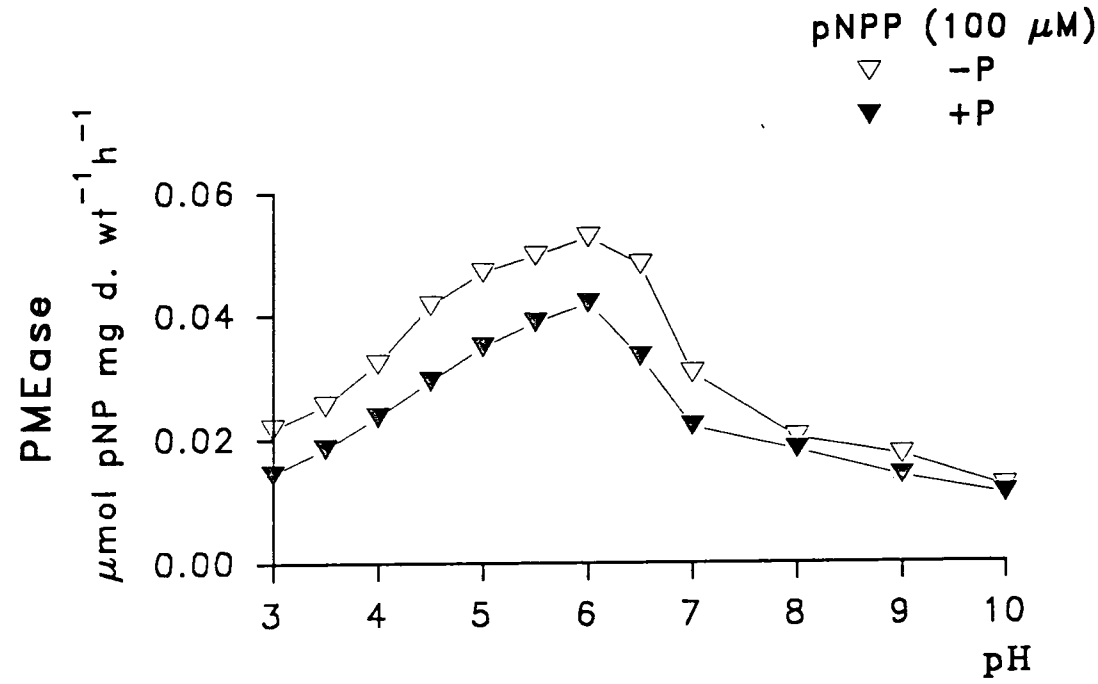
3.2.3.2 Cultivar Jauhar-78

The results show (Fig.3. 9) different pH optima and rates of activity with the two substrates. The maximum rate of activity (0.05 µmol mg d. wt $^{-1}$ h $^{-1}$) occurred at pH 6.0 using substrate pNPP; A lower maximum rate of activity of (0.02 µmol mg d. wt $^{-1}$ h $^{-1}$) was observed at pH 5.0 using 4-MUP. Roots of P-limited plants showed higher activity using both substrates. Use of pNPP shows that after pH 7.0 the activity declined sharply up to pH 10.0, while the trend of decline was observed from pH 6.0 using the substrate 4-MUP.

3.2.3.3 Cultivar Pavon

Over the range of pH values used, the maximum rate of PMEase activity was observed at pH 5.0 with both substrates (Fig.3. 10). However, a rate of 0.06 µmol mg d. wt $^{-1}$ h $^{-1}$ was observed using pNPP as substrate but only 0.04 µmol mg d. wt $^{-1}$ h $^{-1}$ with the substrate 4-MUP. The roots of P-limited plants showed highest PMEase activity than P-sufficient plants using both substrates. The results further show that above the optimum at pH 5.0 activity declined up to pH 7.0 and there was no clear difference in activity up to pH 10.0 with substrate 4-MUP.

Fig. 3. 9 Influence of pH on PMEase activity of 15-day old wheat roots (cv Jauhar-78) grown with and without phosphorus



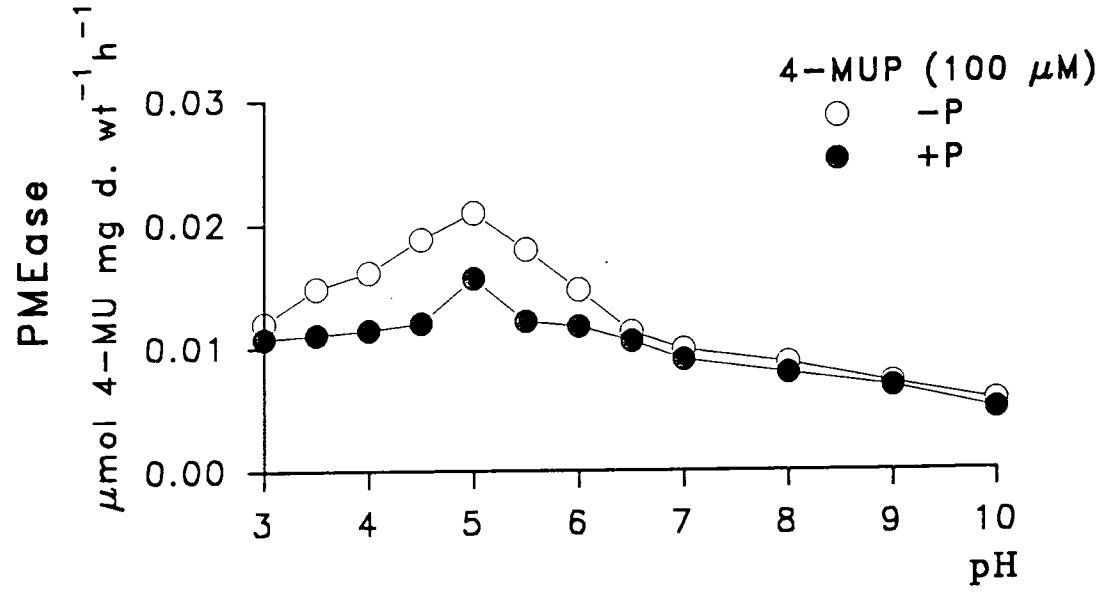
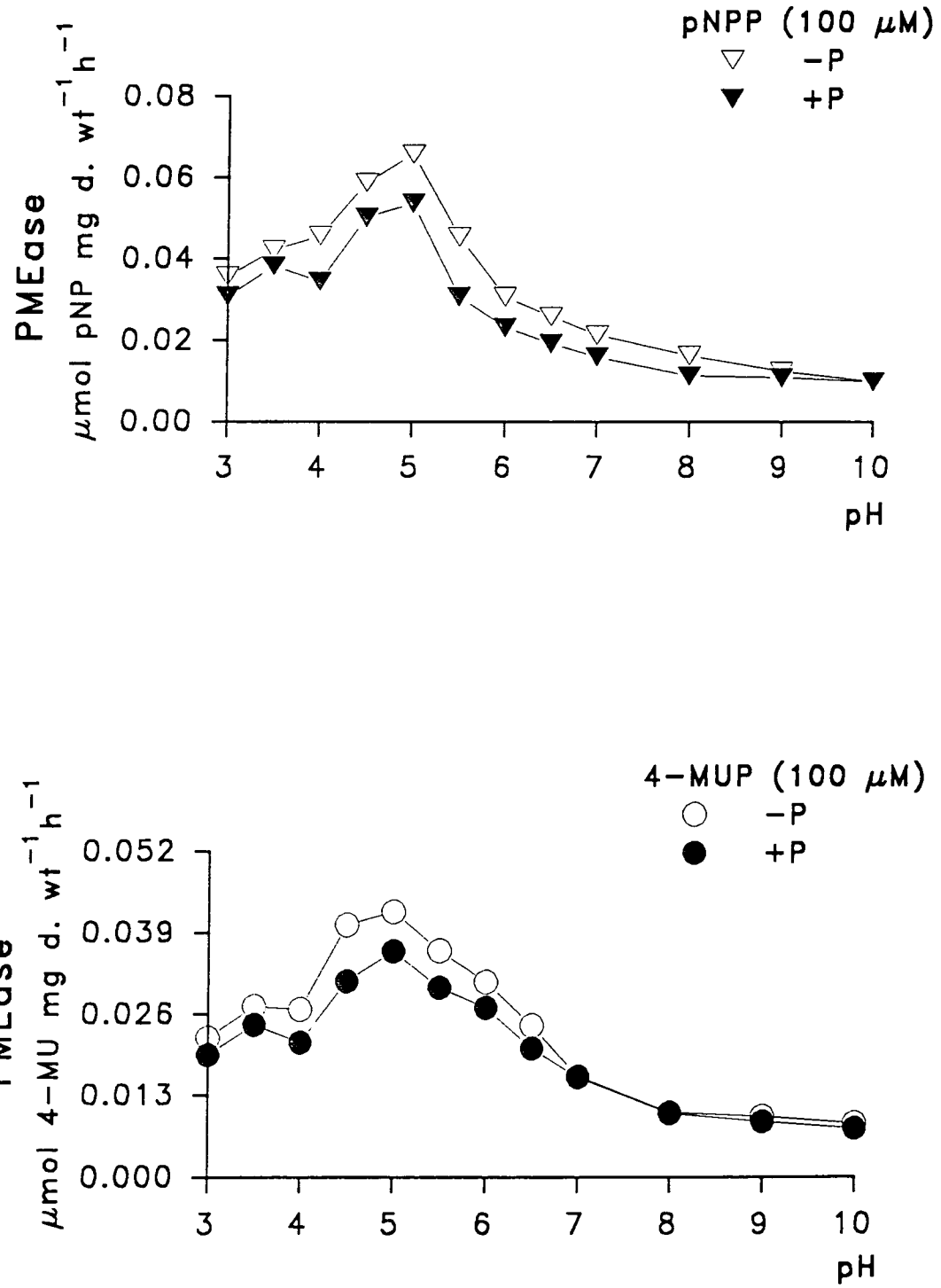


Fig. 3. 10 Influence of pH on PMEase activity of 15-day old wheat roots (cv Pavon) grown with and without phosphorus



3.3 OVERVIEW OF RESULTS

The results from the various experiments are brought together in (Tables 3. 1 to 3. 3). Statistical analyses of these values are shown in Appendices 1-12. From these the principal conclusions were:

(i) Roots of P-limited plants showed more phosphatase activity than P-sufficient plants.

2) Maximum phosphatase activity was observed at 10-day old plants. Cultivar Indus-66 and Jauhar-78 showed higher phosphatase activity than cv Pavon .

3) All the cultivars showed acid phosphatase activity at various pH values ranging from pH 4 to 6.0.

4) Both substrates showed different responses for PMEase activity within and between cultivars, but maximum phosphatase activity was observed in P-limited plants.

5) When the substrate pNPP was used more phosphatase activity was detected than when 4-MUP was used as the substrate.

Table 3.1 PMEase activity over a range of pH values in 5-day old seedlings of three wheat cultivars, Indus-66, Jauhar-78 and Pavon, grown with and without phosphorus

pH	pNPP						4-MUP					
	Indus-66		Jauhar-78		Pavon		Indus-66		Jauhar-78		Pavon	
	+ P	- P	+ P	- P	+ P	- P	+ P	- P	+ P	- P	+ P	- P
3.0	0.048	0.053	0.030	0.040	0.042	0.056	0.029	0.040	0.012	0.022	0.024	0.030
3.5	0.049	0.055	0.035	0.044	0.051	0.059	0.033	0.044	0.013	0.021	0.026	0.034
4.0	0.058	0.068	0.037	0.040	0.048	0.069	0.036	0.047	0.015	0.021	0.035	0.043
4.5	0.049	0.053	0.039	0.045	0.052	0.074	0.038	0.052	0.011	0.023	0.038	0.045
5.0	0.050	0.058	0.046	0.049	0.058	0.077	0.033	0.045	0.011	0.020	0.036	0.042
5.5	0.039	0.052	0.045	0.058	0.054	0.072	0.025	0.042	0.011	0.019	.0032	0.040
6.0	0.025	0.040	0.050	0.063	0.051	0.066	0.022	0.038	0.011	0.017	0.030	0.038
6.5	0.019	0.032	0.043	0.055	0.046	0.058	0.016	0.022	0.008	0.016	0.026	0.035
7.0	0.014	0.022	0.038	0.046	0.043	0.053	0.014	0.016	0.005	0.009	0.023	0.031
8.0	0.011	0.018	0.034	0.038	0.042	0.044	0.011	0.014	0.002	0.006	0.020	0.025
9.0	0.010	0.013	0.031	0.033	0.040	0.043	0.011	0.012	0.001	0.004	0.018	0.021
10.0.	0.010	0.011	0.027	0.035	0.034	0.035	0.010	0.011	0.000	0.003	0.014	0.016

Table 3. 2 PMEase activity over a range of pH values in 10-day old seedlings of three wheat cultivars, Indus-66, Jauhar-78 and Pavon, grown with and without phosphorus.

pH	pNPP						4-MUP					
	Indus-66		Jauhar-78		Pavon		Indus-66		Jauhar-78		Pavon	
	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P
3.0	0.051	0.055	0.021	0.025	0.039	0.048	0.035	0.045	0.014	0.018	0.020	0.024
3.5	0.058	0.071	0.025	0.029	0.054	0.057	0.038	0.049	0.019	0.024	0.018	0.023
4.0	0.066	0.077	0.028	0.036	0.042	0.051	0.041	0.052	0.022	0.033	0.021	0.020
4.5	0.072	0.088	0.036	0.045	0.053	0.061	0.045	0.057	0.025	0.038	0.028	0.038
5.0	0.064	0.084	0.033	0.040	0.058	0.065	0.039	0.053	0.022	0.034	0.020	0.025
5.5	0.052	0.063	0.038	0.049	0.033	0.043	0.036	0.047	0.020	0.030	.0019	0.021
6.0	0.040	0.058	0.049	0.071	0.029	0.037	0.030	0.044	0.017	0.025	0.012	0.017
6.5	0.035	0.044	0.034	0.045	0.014	0.024	0.026	0.041	0.013	0.020	0.008	0.011
7.0	0.024	0.034	0.021	0.025	0.012	0.018	0.021	0.026	0.011	0.014	0.005	0.008
8.0	0.021	0.027	0.017	0.023	0.015	0.015	0.018	0.020	0.010	0.012	0.002	0.006
9.0	0.018	0.026	0.010	0.011	0.010	0.013	0.016	0.017	0.009	0.010	0.002	0.002
10.0.	0.013	0.024	0.009	0.010	0.009	0.011	0.011	0.012	0.008	0.009	0.000	0.001

Table 3.3 PMEase activityover a range of pH values in 15-day old seedlings of three wheat cultivars, Indus-66, Jauhar-78 and Pavon, grown with and without phosphorus.

pH	pNPP						4-MUP					
	Indus-66		Jauhar-78		Pavon		Indus-66		Jauhar-78		Pavon	
	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P	+P	-P
3.0	0.030	0.035	0.014	0.022	0.031	0.035	0.014	0.018	0.010	0.012	0.019	0.022
3.5	0.036	0.040	0.018	0.025	0.038	0.042	0.018	0.023	0.011	0.014	0.024	0.027
4.0	0.039	0.043	0.023	0.032	0.034	0.045	0.029	0.037	0.011	0.016	0.021	0.026
4.5	0.048	0.066	0.029	0.041	0.050	0.059	0.034	0.042	0.012	0.018	0.031	0.040
5.0	0.032	0.038	0.034	0.047	0.053	0.066	0.040	0.048	0.015	0.021	0.036	0.042
5.5	0.028	0.033	0.038	0.049	0.031	0.045	0.029	0.035	0.012	0.018	0.030	0.036
6.0	0.022	0.027	0.049	0.052	0.023	0.031	0.021	0.028	0.011	0.014	0.027	0.031
6.5	0.015	0.020	0.033	0.048	0.019	0.025	0.014	0.022	0.010	0.011	0.020	0.024
7.0	0.011	0.015	0.022	0.030	0.016	0.021	0.009	0.014	0.009	0.009	0.015	0.016
8.0	0.009	0.011	0.018	0.020	0.011	0.016	0.008	0.009	0.008	0.008	0.010	0.010
9.0	0.008	0.008	0.014	0.017	0.010	0.012	0.007	0.007	0.006	0.007	0.008	0.009
10.0	0.007	0.007	0.010	0.012	0.009	0.009	0.004	0.006	0.004	0.005	0.007	0.008

Chapter 4

DISCUSSION

4.1 RICE

Phosphorus plays a key role in reproduction and energy transfer processes in plants. Therefore, P-status, development and growth are closely related phenomena. In order to play an effective role in inorganic phosphorus supply in phosphorus-depleted situations phosphatase should be capable of efficient operation at the pH of the environment in which the associated organisms commonly work. It is therefore important to ascertain phosphatase activity in relation to pH.

The results presented in the previous chapter show that roots of deepwater rice cv Chota Bawalia had " surface" phosphatase activity (Fig.3.1), with maximum activity, using 250 μM pNPP, at pH 6.0. Above pH 6.0 activity declined as the pH values increased.

The results agree with those of other workers who have found similar pH optima for phosphatase (s). These include phosphatases bound to cell walls extracted from the roots of *Agrostis tenuis* (Woolhouse, 1969) and phosphatase (s) derived from walls of cultured cell of tobacco (Suzuki & Sato, 1973) and rice (Igaue *et al.*, 1976). In rice, phosphatase activity from P-limited roots after 12-day was 5 times higher than that of the controls and was followed by a decline after 20-days (Takijima, 1953). Further,Yamaoka *et al* (1969) surveyed extracellular acid phosphatase enzymatic activities in tobacco cultures and found a rapid decrease in the inorganic (Pi) concentration in the culture fluid with age.

4.2 WHEAT

Comparison of root phosphatase activities between different wheat cultivars was made using the substrates pNPP and 4-MUP. Wheat proved to be similar to deepwater rice in

that differences in activity between P-sufficient and P-limited plants were demonstrated. The overall results are discussed below first by individual cultivar and then an overall comparison is made.

4.2.1 Cv Indus-66

PMEase activity measured using the two different substrates (pNPP & 4-MUP) indicated that the maximum rate of activity was obtained between pH 4.0 to 5.0. Activity was detected in 5-day plants and reached a peak in 10-day plants. No significantly greater activity was found in the 15-day old plants. Both substrates showed that there was effective phosphatase activity, but there was a slight difference in the measured optimum pH. When pNPP was used as substrate there was an optimum at pH 4.0 with all ages of plant. However, when 4-MUP was used the optimum for 15-days old plants was pH 5.0. The change in optimum pH appeared to be related to the substrate rather than the cultivar.

Comparison of root phosphatase activity between P-sufficient and P-limited plants showed that the latter always had higher activity. When plants became more deficient over the growing period the relative difference between P-sufficient and P-limited plants increased.

4.2.2 Cv Jauhar-78

Phosphatase activity of 5-day old plants was greater in P-limited than P-sufficient plants. The substrates pNPP and 4-MUP showed maximum activity at pH 6.0 and 4.5, respectively. Similar pH optima were recorded for plants which were 10-days old and these showed higher phosphatase activity than the 5-day old plants.

These plants showed two possible peaks for phosphatase activity with pNPP, one at pH 4.5 and other at pH 6.0 recorded for 10-day old plants. A more detailed study would be needed to establish this point. With 4-MUP the optimum changed to pH 5.0 for 15-day old plants compared with pH 4.5 for the 10-day old specimens. Although total activity was lower for 15-day than 10-day plants.

4.2.3 Cv Pavon

Both substrates gave the same pattern for phosphatase activity in this cultivar (3.4). With maximum activity at pH 4.5-5.0. However, the results suggested the possibility that there may be a slight shift in pH response with age of the plants in the case of 4-MUP. As with the other cultivars phosphatase activity was higher in P-limited than P-sufficient plants. Maximum phosphatase activity was observed when the plants were at 5-days old. With increase in plant age decreases in phosphatase activity were observed with both substrates. This contrasts with the behaviour of the other two cultivars (Table.3.2) where maximum activity occurred in 10-day-old plants.

4.3 Overall comparison

Comparison of P-sufficient and P-limited roots showed that the cultivars always had more phosphatase activity in P-limited plants. However, differences occurred between cultivars, suggesting that there may be differences in the ability of cultivars to grow in the less fertile soil situations. In the case of the cultivars tested, cv Indus-66, Jauhar-78 and Pavon may be the most useful for less fertile soil. However, when considering root phosphatase activity as a potential indicator of P-status of a plant in the field or laboratory,

it must be appreciated that this can be affected by several conditions: the physiological age of the plant at time of sampling, cultivar and level of available P in the substrate (soil or medium).

The results are in agreement with previous studies on other higher plants including wheat. Increases in soluble phosphatase activity with P-limitation have been found in roots of *Agrostis tenuis* (Woolhouse, 1969) and clover (Dracup, 1981). Plants respond to environmental conditions of low phosphate availability by increasing the activity of extracellular acid phosphatase (Goldstein *et al*., 1988; Lee, 1988). High leaf phosphatase activity has been reported for P-limited wheat plants (Besford, 1979b; McLachlan & De Marco, 1982). Phosphatase activity in leaves of wheat increased several fold with P-deficiency and water deficient (Barrett-Lennard & Greenway, 1982).

In wheat, phosphatase activity increased during the first 4 days of growth and reached the peak in 8 days (McLachlan, 1980). The optimum pH for activity lay in the range pH 5-6; P-limited plants had greater activity than P-sufficient ones.

Nutrient deficiencies are major factors limiting the productivity of agricultural plants. Phosphatase (s) on roots play an important role in the P-nutrition of higher plants allowing them to use P-esters as a source of P for growth. PMEase activity in crop plants typically increased when the plants became P-limited. Present results show that when plants were grown in P-sufficient and P-limited conditions the maximum response for phosphatase activity was observed under P-limited conditions as compared to P-sufficient conditions. Cultivars showed differences in that cv Indus-66 and Jauhar-78 had higher phosphatase activity than cv Pavon.

The results are in agreement with the findings of other workers, who detected acid phosphatase activity in wheat and other higher plants.

In isolated isozymes of acid phosphatase from wheat germ two activity peaks were obtained, at pH 4.5 and 5.5 (Verjee, 1969). He suggested the presence of more than one acid phosphatase while using the substrate p-nitrophenyl phosphate. The phosphatase of intact wheat roots is an acidic phosphatase, similar to that other higher plants, and characteristic of the plant root (McLachlan, 1980).

In wheat roots phosphatase activity increased only under P-limited conditions; it was unchanged in salt-stressed plants and inhibited under non-ionic osmotic stress (Szabo-Nagy *et al.*, 1992). When a comparison was made of P nutrition in seven plant species, including wheat, it was found that acid phosphatase activity was present with P-limited conditions in all crops (Besford, 1979b). After 3-days of P-limitation secretion of phosphatase into the medium by wheat roots was 20 times higher than that just before the treatment, and remained high for about 9 days, followed by a decline (Takijima, 1953).

Present results are in agreement with the above statement that when cultivars were grown for 10-days, the maximum phosphatase activity was observed as compared to the previous 5-day plants. Further work of McLachlan, (1980) supports this, in that in wheat the phosphatase activity increased within 4-days of plants being cultivated and reached a maximum at 8-days. P-limited plants had greater activity than P-sufficient ones. Wheat P-limited roots had approximately 1.6 times the phosphatase activity of non-deficient roots (Gilliam, 1970). Comparison of root and leaf phosphatase activities in wheat grown in nutrient solutions showed that leaf phosphatase activity was not a better measure of the P status than root phosphatase (McLachlan & De Marco, 1982). The present results are very similar to those for other higher plants with acid phosphatase activity under P-limitation. Roots of tomato plants under P-limited conditions showed higher acid phosphatase activity than sections of similar tissue from control plants. Phosphatase activity of rice, wheat and barley roots under P-limited conditions increased 2-5 fold

compared with that of normal roots (Besford & Syred, 1979; Szabo-Nagy *et al* , 1992). In cultures of tobacco the activity increased over the period of 8-days and reached a maximum on the 12th-day with the substrate pNPP (Ueki & Sato 1977). In *Zea mais* surface phosphatase activity increased, this enhancement was especially pronounced in new roots (Kummerova, 1986).

The present results differ from those of previous work done using the same substrate, which demonstrated the presence of both acid and alkaline phosphatase activity in *Spirodela* and a marked shift in favour of alkaline phosphatase activity with P-limited in plants (Bieleski, 1974). His test on the intact plant showed relatively greater alkaline than acid phosphatases activity in both control and deficient plants, with more of both forms present under P-limited conditions.

P-limitation is not the only condition which causes increased phosphatase activity. In roots of cucumbers, Ca-limitation caused a 10-fold increase in the specific activity of phosphatase using the substrate pNPP (Yamaya *et al.*, 1982).

Comparison of the data for wheat cultivars in this study showed that when plants were grown in P-sufficient and P-limited conditions, then significant differences were observed between treatments and cultivars.

When plants were grown for 10-day higher phosphatase activity was observed in two cultivars, Indus-66 and Jauhar-78, than in the third, cv Pavon. The activity was in favour of P-limited plants rather than P-sufficient ones. Variability in cultivars showed the effect of substrate and plant age. With both substrates it was shown that activity was present but always acidic rather than alkaline. The requirement for phosphate is most critical before emergence of the ear (Boatwright, 1966).

Acid phosphatase activity increased at the end of each of 4, 14 and 21-days respectively; under P-stress, these values were 2, 2.5 and 5-times higher than that of the control (Garcia and Ascencio, 1992). Sixteen days after planting of wheat the roots and

leaves of P-limited plants had only 20-30% the P content of sufficient plants. After 16-day of growth plants under P-stress had 41% more p-nitrophenol phosphate activity (Smyth & Chevalier, 1984). In wheat, using the substrate pNPP, it has been reported that mature leaves of P-limited plants had high phosphatase activities in the period for up to 12-days following the addition of adequate levels of P to the soil (Barrett-Lennard & Greenway, 1982).

Roots and above ground plant tissues of 10, 17, 24 and 28-day old plants of three wheat cultivars, grown in solution with different P concentrations have been examined (Kummerova *et al.*, 1989). A significant genotypic difference in acid phosphatase activity was found depending on organ, plant age and P concentration. Significant increase in acid phosphatase activity of root and leaves was observed in all cultivars.

The results presented here suggest that phosphatase activity of wheat roots increased linearly with the period of time spent in P-limited solution up to 10-days. No significant change in activity occurred in the remaining 5 days. There was a significant difference in the phosphatase activity of the roots associated with the P-treatment at each growth stage.

The comparison between wheat cultivars shows that significant differences in phosphatase activity can occur within them. This was a reflection of the differing ages of plants, phosphate availability, cultivar and substrates.

If the major part of the cultivars, variation in the phosphatase activity was due to genetic variability, then advantage could be taken of these differences by selecting cultivars to make the best use of less fertile soil situations.

Under similar growing conditions, plant species may differ in their ability to utilise soil phosphate or phosphate as fertilizer. Plant species which absorb nutrients efficiently from the soil are particularly important in underdeveloped countries, in the light of increasing fertilizer price and decreasing fertilizer resources (Blair, 1977).

The results reported here show that wheat cultivars are capable to mobilizing organic phosphatase through the root phosphatase. However, further work should be carried out to characterize these enzymes in full, in terms of surface phosphatase activities of wheat roots.

These results indicated that when plants become P-limited, the activity of acid phosphatase by the plant roots increased. Thus, this function of the roots is considered to be one of the widespread adaptive mechanisms of plants in order to grow in P-limited soils. It is considered that acid phosphatase from the wheat roots hydrolyzes organic phosphate compounds in the form of orthophosphate.

The ability of roots to secrete acid phosphatase differed among the cultivars. For instance, the activity of acid phosphatase by roots of cv Indus-66 and Jauhar-78 was remarkably high, while that of cv Pavon was comparatively low. In addition, the activity of acid phosphatase by the roots of cultivars varied with crop growth stages, in particular cv Pavon, where the activity of acid phosphatase by the roots under P-limited conditions was higher at an early growth stage. However, it is considered that the growth stages at which a large amount of acid phosphatase is found in the roots varied among wheat cultivars.

The contribution of acid phosphatase by the plant roots to the total amount of phosphorus absorbed by the plant grown on P-limited conditions in the soil is still unknown. Therefore, further investigations should be carried out to determine the contribution of the acid phosphatase by wheat roots in P-limited soils.

Whilst this study has shown that surface acid phosphatases are present on the roots of the wheat cultivars studied, the significance of their activities in an agricultural context is not clear since productive soils in Pakistan have pH values ranging from 7.0-8.5. It is possible, however, that the pH of the soil is not the same as that of the surface of the roots due to the activity of the plant itself.

SUMMARY

1) A brief study was made of deepwater rice cv Chota Bawalia from Bangladesh followed by a more detailed study of root surface phosphatase activity of wheat cultivars from Pakistan.

2) Deepwater rice showed highest phosphatase activity at pH 6.0.

3) The three wheat cultivars (Indus-66, Jauhar-78, Pavon) were brought from Pakistan and seedlings were grown at 25°C in Hoagland's solution under P-sufficient and P-limited conditions for 5, 10 and 15 days. Assays of phosphatase activity were carried out on detached roots in buffered assay medium using the substrates pNPP and 4-MUP; both were tested at 100 μM.

4) All cultivars showed maximum phosphatase activity between pH 4.0 and 6.0.

5) Roots of P-limited plants always showed more activity (per unit dry weight of root) than P-sufficient plants.

6) Maximum phosphatase activity of cv Pavon was noted in 5-day old plants and had a pH optimum of 5.0. In contract the highest activity for cv Indus-66 and Jauhar-78 was found in 10-day old plants with pH optima of 4.5 and 6.0 respectively.

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APPENDICES

Appendix 1: Use of ANOVA to test for influence of pH on the PMEase activity in 5-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using pNPP substrate.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.0035748	0.017874	306.59 **
pH	11	0.0076168	0.006924	118.76 **
Interaction	22	0.0039976	0.0001817	3.12 **
Error	36	0.0021005	0.0000583	
Total	71	0.0172897		

** $P < 0.001$

Appendix 2: Use of ANOVA to test for influnce of P on the PMEase activity in 5-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using substrate pNPP.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.003575	0.001787	9.71 **
Conditions	1	0.001522	0.001522	8.27 **
Interaction	2	0.000075	0.000038	0.20 NS
Error	66	0.012118	0.000184	
Total	71	0.017290		

** $P < 0.001$

NS Non-significant

Appendix 3: Use of ANOVA to test for influence of pH on the PMEase activity in 5-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using 4-MUP substrate.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.07197	0.003598	14.92 **
pH	11	0.003431	0.000312	1.29 NS
Interaction	22	0.003605	0.000164	0.68 NS
Error	36	0.008659	0.000241	
Total	71	0.022891		

** $P < 0.001$

NS Non-significant

Appendix 4: Use of ANOVA to test for influence of P on the PMEase activity in 5-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using 4-MUP substrate.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.007197	0.003598	15.92 **
Conditions	1	0.000113	0.000113	0.50 **
Interaction	2	0.000638	0.000319	1.41 NS
Error	66	0.014945	0.000226	
Total	71	0.022891		

** $P < 0.001$

NS Non-significant

Appendix 5: Use of ANOVA to test for influence of pH on the PMEase activity in 10-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using pNPP substrate.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.004597	0.002299	48.50 **
pH	11	0.0182452	0.0016587	3.50 *
Interaction	22	0.004886	0.0002222	4.69 **
Error	36	0.0017060	0.0000474	
Total	71	0.0292995		

** $P < 0.001$
* $P < 0.01$

Appendix 6: Use of ANOVA to test for influence of P on the PMEase activity in 10-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using pNPP substrate.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.004460	0.002230	6.26 *
Conditions	1	0.001250	0.001250	3.15 NS
Interaction	2	0.000089	0.000045	0.11 NS
Error	66	0.023501	0.000356	
Total	71	0.029299		

* $P < 0.01$
NS Non-significant

Appendix 7: Use of ANOVA to test for influence of pH on the PMEase activity in 10-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using substrate 4-MUP.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.0048614	0.0024307	75.72 **
pH	11	0.0065492	0.0005954	18.55 **
Interaction	22	0.0115445	0.0000299	0.93 NS
Error	36	0.0132240	0.0000321	
Total	71	0.0132240		

** $P < 0.001$
NS Non-significant

Appendix 8 : Use of ANOVA to test for influence of P on the PMEase activity in 10-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using substrate 4-MUP.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.004861	0.002431	21.32 **
Conditions	1	0.000767	0.000767	6.73 *
Interaction	2	0.000065	0.000033	0.29 NS
Error	66	0.007530	0.00114	
Total	71	0.13224		

** $P < 0.001$
* $P < 0.01$
NS Non-significant

Appendix 9: Use of ANOVA to test for influence of pH on the PMEase activity in 15-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using pNPP substrate.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.0002295	0.0001148	3.74 *
pH	11	0.0110254	0.0010023	32.65 **
Interaction	22	0.0038468	0.0001749	5.69 **
Error	36	0.0011040	0.0000307	
Total	71	0.0162058		

** $P < 0.001$
* $P < 0.01$

Appendix 10: Use of ANOVA to test for influence of P on the PMEase activity in 15-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using substrate pNPP.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.000230	0.000115	0.50 NS
Condition	1	0.000748	0.000748	3.25 NS
Interaction	2	0.000026	0.000013	0.06 NS
Error	66	0.015202	0.000230	
Total	71	0.016206		

NS Non-siginificant

Appendix 11: Use of ANOVA to test for influence of pH on the PMEase activity in 15-day old wheat cultivars Indus-66, Jauhar-78 Pavon using substrate 4-MUP.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.0018244	0.0009122	82.18 **
pH	11	0.0053355	0.0004850	43.69 **
Interaction	22	0.0011153	0.0000507	45.67 **
Error	36	0.0004005	0.0000111	
Total	71	0.0086757		

** $P < 0.001$

Appendix 12: Use of ANOVA to test for influence of P on the PMEase activity in 15-day old wheat cultivars Indus-66, Jauhar-78 and Pavon using substrate 4-MUP.

Source	DF	SS	MS	F. ratio
Cultivar	2	0.0018244	0.0009122	9.17 **
Condition	1	0.0002683	0.0002683	26.96 **
Interaction	2	0.0000170	0.0000085	0.08 NS
Error	66	0.0065659	0.0000995	
Total	71	0.0086757		

** $P < 0.001$

NS Non-significant