Chapter 4

Acid Phosphatase Kinetics as a Physiological Tool for Assessing Crop Adaptability to Phosphorus Deficiency

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Additional information is available at the end of the chapter

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Abstract

Acid phosphatases (APase) exuded from the roots is important in mobilizing organic phosphate in the soil . Enzyme kinetics can provide reliable physiological markers to detect the potential for superior plant performance under low P . Kinetic constants for the secreted APase could be used as an early physiological indicator for P stress tolerance in legumes, Desmodium tortuosum , Phaseolus vulgaris,Vigna unguiculata and Crotalaria juncea were grown from seed in +P and -P nutrient solutions and plants were harvested during the early vegetative phase in order to collect the root exudates in vivo and for dry biomass, leaves soluble Pi, and total P in the dry biomass. Root surface Na-soluble APase was extracted from +P and -P grown plants by incubating three intact plants in beakers with their roots immersed in a 0.1 M NaCl solution. Secreted APase was obtained with the roots of three plants individually immersed in a dialysis tube (12 kD) containing NaCl 100 mM and then transferred to a recipient containing 3L of the same solution. Kinetic constants Km and Vmax were determined using a range substrate (p-NPP)concentration (S). Activity (v) was expressed as μmoles PNP/h per g root fresh (FWr) or dry weight DWr. Graphical representations were used for the determination of the Km and Vmax: Linewaver-Burk double reciprocal plot 1/v vs. 1/S plot; Hanes-Wolf plot S/v vs. S and Woolf-Augustinsson-Hofstee plot v vs. v/S. The first visual indication of P deficiency was a reduction in leaf area and dry biomass and a higher soluble Pi in the leaves of +P plants. Activity was higher in -P plants at the beginning of the growth period and the proper timing for the onset of the P-stress was apparently crucial for the induction of APase. For Phaseolus vulgaris Km values apparently indicate the lack of phosphate starvation-inducible APase and a higher Vmax in -P plants; however, with the combination of a high Km with a high Vmax plant behaviour could be improved under P deficiency. In Vigna unguiculata the low Vmax in -P plants may be compensated for by its lower

Km. Crotalaria juncea showed considerably greater kinetic diversity, but Km was lower in -P plants. The practical implications of Km and Vmax are explained in terms of the potential for P-liberation under limiting Pi ; to be efficient an increase in Pi uptake is likely to occur if the APase released has a low Km (in the neighborhood of the soil P concentration) and a high Vmax as found for Desmodium, Phaseoulus and Vigna. The Km provided a means of comparing the enzyme from high or low-P plants indicating that Km is a reliable physiological tool for assessing plant adaptability to P-deficiency and it is suggested that Km, Vmax with total leaf area and relative growth rate (RGR).

Keywords: plant enzymes, root secretion, abiotic stress

1. Introduction

Phosphorus (P) is an essential element in the mineral nutrition of plants and under P starvation a reduction in biomass and leaf area among other physiological traits, is observed. Phosphorus occurs in soils not only as mineral phosphates but also as organic compounds that in order to be available to the roots they must be first hydrolyzed and several studies now indicate that acid phosphatase may have a role in mobilizing organic phosphate in the soil that in infertile tropical soils may contribute significantly to ameliorate P bioavailability from sparingly soluble P forms. Acid phosphatase (APase) from several plant species and genotypes has been shown to liberate P from soil thus decreasing organic phosphorus in the rhizosphere [8, 26, 27] and a large body of evidence now exists showing that P deficiency can trigger an increase in activity of root secreted APase in a variety of wild and cultivated plants and that the activity of the enzyme can vary among species along with the severity of P starvation [4, 24, 25]; however [28] showed that some tropical forage such as *Brachiaria* hybrids and *Arachis pintoi* have the ability to tolerate low-phosphorus stress without showing any increase in APase activity in root exudates. According to [29], the role of secreted APase in plant adaptation to low phosphorus availability is unclear. Enzyme kinetics can used to identify simple and reliable physiological markers, for screening purposes to detect the potential for superior plant performance under low P concentrations. Ascencio [4, 5] calculated the kinetics constants (Km and Vmax) for the exuded APase from different plants species grown under P- sufficient and P-deficient conditions. The numerical values of the Km for the substrate p-nitrophenyl-Phosphate (p-NPP) provided a means of comparing the enzyme from high or low P plants, so it is suggested that Km and enzyme activity (Vmax) may be used as physiological indicators to differentiate plants grown under P deficiency or sufficiency. Km values are related to the substrate concentration at which enzyme velocity is half of the maximum (Vmax), they are not strictly constants as those that are found with the purified enzyme as the activity is assayed using the crude root exudates as they might be released to the soil. Acid phosphatase enzymes are released to the soil as part of the pool of extracellular enzymes along with other products of root exudation; however, under laboratory conditions different forms of the enzyme are present depending on the procedure that is used to collect the enzyme. Collection of the

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enzyme *in vivo* can be performed directly from the intact plant by immersing their roots in the extracting solution which is assayed without purification (root surface Na-soluble APase) or by separating the enzyme from other exuded compounds by enclosing the roots of the intact plant inside a tube or a dialysis membrane (secreted APase). Collection of the enzyme *in vitro* from the root and other plant tissues (mostly leaves or seedlings) is achieved by grinding and collecting the resulting solution (extractable APase). Further purification of the enzyme in order to assess whether new APase isoenzymes are induced by P deficiency can be achieved using any of the extraction methods described above. The properties of the purified enzyme secreted in vivo by plant roots was first reported by Tadano [23], for the secreted enzyme from Lupinus roots under P-deficient conditions. Under P-deficient conditions new APase isoforms, which are different forms for the enzyme, are released. It has been shown [1,27] that APase isoforms were inducible under P deficient conditions but that for Lupinus, the major activity in the rhizosphere soil and in roots grown under hydroponic conditions corresponded to a previously purified APase secreted by the roots; thus in order to compare the potential of APase in the root exudates released by different plant species and genotypes that may increase plant performance under phosphorus deficient conditions, root secretions can be obtained *in vivo* and acid phosphatase activity measured in the secreted or exuded solution without further purification.

The objective of the present chapter is to give a broader picture, from an agronomical point of view, as to how kinetic constants for the APase secreted "in vivo" by the roots of leguminous plants grown under P-deficiency or sufficiency, could be used as an early physiological indicator for P stress tolerance.

1.1. Research methods

The leguminous plants species (*Desmodium tortuosum* (Sw.) DC, *Phaseolus vulgaris* L var Manuare, *Vigna unguiculata* (L) Walp cv Tuy and *Crotalaria juncea* L, were used in this study. *Desmodium tortuosum* (beggarweed) Fabaceae, is a slow growing tropical non-grain legume that grows wild, and on small farms, in association with other crop species as a nitrogen source, as a forage legume and green manure. *Phaseolus vulgaris* (common bean) and *Vigna unguiculata* (cowpea), -Fabaceae are very important grain crops used as a protein source worldwide; *Crotalaria juncea* (sunn hemp) Fabaceae is widely grown throughout the tropics and subtropics as a source of green manure, fodder and lignified fiber and has been recently looked at as a possible bio-fuel. Plants were grown from sterilized seeds and water culture experiments were performed in a highly ventilated greenhouse in plastic 950 ml pots with aerated Hoagland solutions containing either sufficient (+P) or deficient (-P) (mM P as KH_2PO_4 depending of the species). After establishment, plants were harvested during the early vegetative phase for each species and separated into groups in order to perform kinetic studies for the APase in the root exudates collected in vivo, for dry biomass determinations and to measure soluble Pi content in fresh leaves and total P in the dry biomass. The Soluble Pi content in leaves was measured using leaf discs (0.5-10 g fresh weight) macerated in cold 2% acetic acid a the extract diluted and centrifuged at 4 ^{o}C and the clear supernatant used for Pi concentration determinations using the colorimetric phosphomolybdate reaction. Total P in the dry biomass was measured using previously digested material with H_2SO_4:H_2O_2. Phosphorus efficiencies were calculated

(when indicated) as mg total P in plant in dry root biomass per mg P in the nutrient solution (phosphorus absorption efficiency PAE), and as g the total dry biomass per mg total P in the plant respectively (phosphorus use efficiency PUE).

1.2. Acid phosphatase activity and kinetic constants

The extraction experiment for the root surface Na-soluble acid phosphatase from +P and -P grown plants was performed by incubating three intact plants from each P treatment in 250 mL beakers wrapped in aluminum foil with their roots immersed in 100 mL of a 0.1 M NaCl solution inside a well lighted refrigerator at 4 C. After 6 h the solutions in the three flasks per P treatment were separated and filtered using Whatman paper # 1, and as the crystal-clear filtrate was obtained, used for the kinetic studies [5].The solution with the enzyme obtained in this experiment is referred to as extracted APase. The secretion experiment for the acid phosphatase enzyme was performed with the roots of +P and -P intact plants individually immersed in a dialysis tube (12 kD) containing NaCl 100 mM and then transferred to a recipient containing 3L of the same solution following the procedures reported [24].. After 24 h the solution inside the dialysis tubes of three plants was collected for kinetic studies when plants in the low P treatment showed moderate P deficiency symptoms as shown by growth inhibition. The solution with the enzyme obtained in this experiment is referred to as secreted APase. Root surface Na-soluble acid phosphatase (APase) activity (reaction velocity v) was assayed using aliquots of the extracted or secreted enzyme with the substrate p-nitrophenyl-1-phosphate, buffer Na-acetate 50 mM pH 5.0 in a water bath at 34C. Reaction was stopped after 30 min with a saturated Na_2CO_3 solution and the yellow p-nitrophenol (PNP) read at 410 nm in an spectrophotometer Varian DMS-90. Kinetic constants Km and Vmax were determined using a range substrate concentration (S) based on the Km value for the purified enzyme [1, 16]. Two different ranges depending on the species (0.2; 0.33; 0.50; 1,00;1,43; 2,00; 2,50; 3,33 and 5,0 or 1.0 ; 1.25; 1.43; 1.67; 2.0; 2.50; 3.30 and 5.50 mM p-NPP) were assayed as indicated in the enzyme activity plots using the root exudation or the secretion from different plants. APase activity (v) was expressed as μmoles PNP/h per g root fresh (FWr) or dry weight DWr. The first step was to examine the v vs. S curve that reflects the hyperbolic Michaelis- Menten to determine the degree of substrate saturation; in theory as the velocity of the enzyme reaction (v) responds in a characteristic way to increasing substrate concentration, but when crude extracts clear substrate saturation is not always observed, and in order to determine the affinity constant Km and maximal velocity of the enzyme reaction Vmax different graphical representations based on linear transformations of the data are used for the determination of the affinity constant (Km) and the maximal velocity of the enzyme reaction (Vmax). The most widely used graphical representations are: Linewaver-Burk double reciprocal plot 1/v *vs*. 1/S plot; Hanes-Wolf plot S/v *vs*. S and Woolf-Augustinsson-Hofstee plot v *vs*. v/S, where v represents initial enzyme velocity at any given substrate concentration (S). Under the conditions of this study, the Km refers to the apparent Km for the enzyme activity, or the concentration of substrate at which activity is one half the maximal velocity and Vmax refers to the apparent maximal velocity for enzyme activity, which is the maximum rate of P- hydrolysis. Enzyme kinetics data were analyzed using the Hyper32 free software.

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2. Results

The first visual indication of P deficiency as observed for the plants of this study, was a reduction in leaf area to different degrees in different species, which was reflected in lower biomass values. The large differences among -P and +P plants are explained in part due to soluble Pi concentration in leaves which remained higher in +P plants during the entire growth period. In spite of the large differences in growth and Pi content, P efficiencies were much higher in the low P plants where larger differences were found for PUE (indicating a superior ability of -P plants to convert phosphorus into biomass). The superior acquisition efficiency (PAE) for some plant species has been attributed to a more efficient mycorrhizae and/or root acid phosphatase activity in addition to other factors as superior uptake kinetic. For the plants of this study we focused the strategy on the kinetic constants for the secreted APase under P deficient conditions. Enzyme activity is induced under P deficient conditions which depends on the proper timing for the onset of the P stress; the low Pi content in leaves of -P plants on the other hand was compensated for by a 10 times higher phosphorus use efficiency (PUE) for the plants of this study, which is in agreement with the hypothesis that the efficient recycling of Pi inside the plant is an important mechanism for survival under conditions of P starvation.

2.1. Acid Phosphatase Kinetics of *Desmodium tortuosum* (beggar weed)

After 28 days of planting the roots of four plants grown under either P sufficiency (+P 1.0 mM P) or deficiency (-P 0.01 mM P) were immersed in the collection flasks or in the dialysis membrane and after the extraction period aliquots were taken to perform kinetic studies for (a) the extracted enzyme and (b) the secreted enzyme. For the secreted enzyme saturation kinetics was observed for the enzyme from both high and low grown plants (+P and -P) as substrate concentration was increased from 0.2 to 5.0 mM, as seen from the hyperbolic Michaelis-Menten plot in Figure 1a and Figure 2a Figure +P (a) and -P (a).

The apparent Km and Vmax values determined from Lineweaver-Burk, Hanes and Hofstee plots; Figures +P and -P. For the high P plant enzyme, Km values of 2.81, 1.54, 2.63 and 1.07 mM p-NPP and Vmax of 80.5, 57,3 80.85 and 51.6 μmol PNP/h/g FW roots were calculated from the plots depicted in Figure 1 (b, c and d) in Figure +P For the low-P plant enzyme values were 0.92, 0.56, 1.58 and 0.73 mM p-NPP and 105.1, 87.6, 138.4 and 101.3 μmol PNP/h/g FW roots (Figure 2 (b, c and d) (Figure -P). As seen from the results obtained in this investigation, lower Km a higher Vmax values were found for the enzyme from -P Desmodium plants with any of the plots used to transform the data. For the extracted enzyme Vmax values were similar for the +P and -P plant enzymes but a lower Km was found for -P as compared to +P grown plants: 2.00, 1.48, 1.78,1.61 mM p-NPP *vs* 3.23, 3.27, 2.96, and 2.69 mM p-NPP for +P, as calculated from the plots seen in Figures 3 and 4, for +P and –P treatments using flasks for the extraction of the enzyme. Besides enzyme velocity (Vmax) was lower for the extracted enzyme between 8.85 and 11.63 μmol PNP/h/g FW roots for +P and -P plants) as compared to the secreted enzyme.

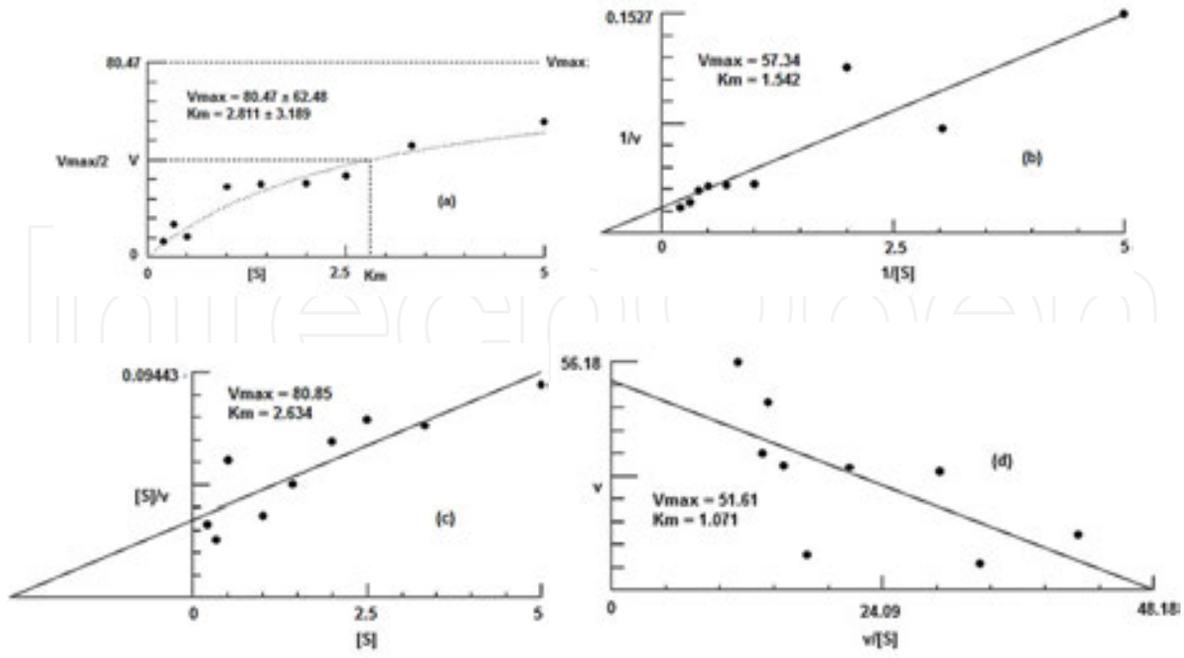

Figure 1. Enzyme kinetics plots of the acid phosphatase activity from *Desmodium tortuosum* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

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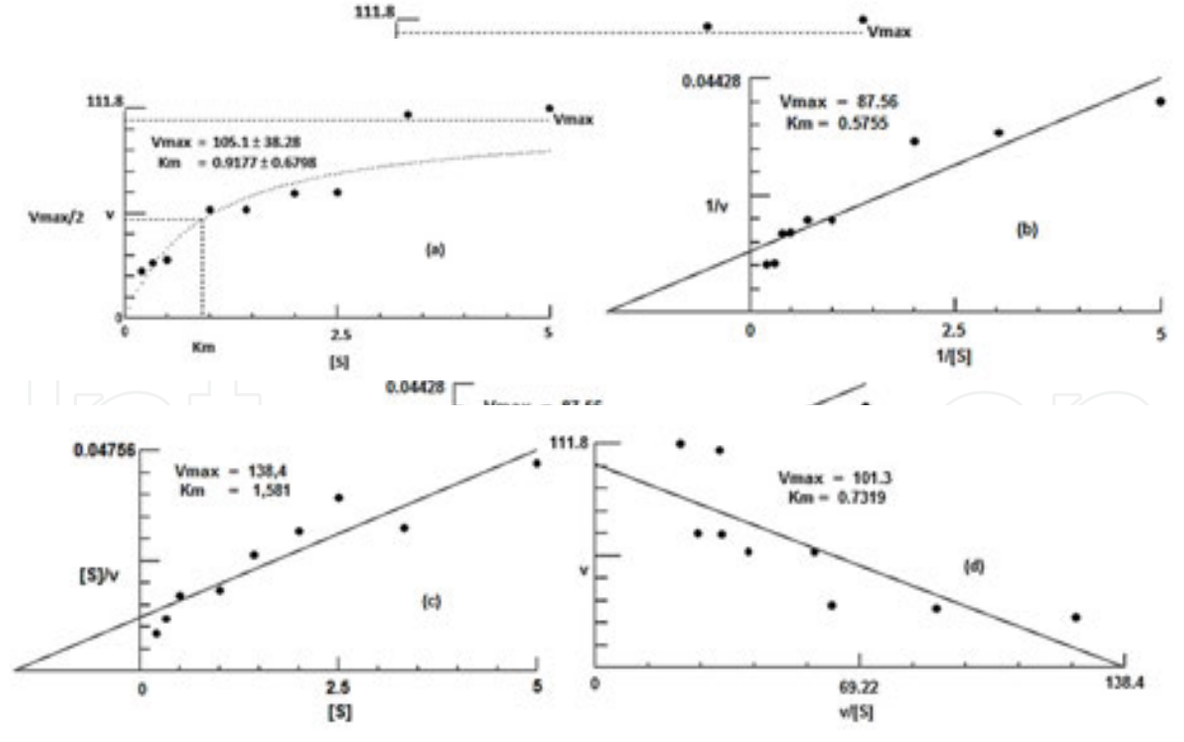

Figure 2. Enzyme kinetics plots of the acid phosphatase activity from *Desmodium tortuosum* roots with the substrate p-nitrophenylphosphae (p-NPP). The secreted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

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2.2. Acid Phosphatase Kinetics in *Phaseoulus vulgaris* (common bean)

After 14 days of planting the roots of four plants grown under either P sufficiency (+P 1.0 mM P) or deficiency (-P 0.005 mM P) were immersed in the dialysis membrane

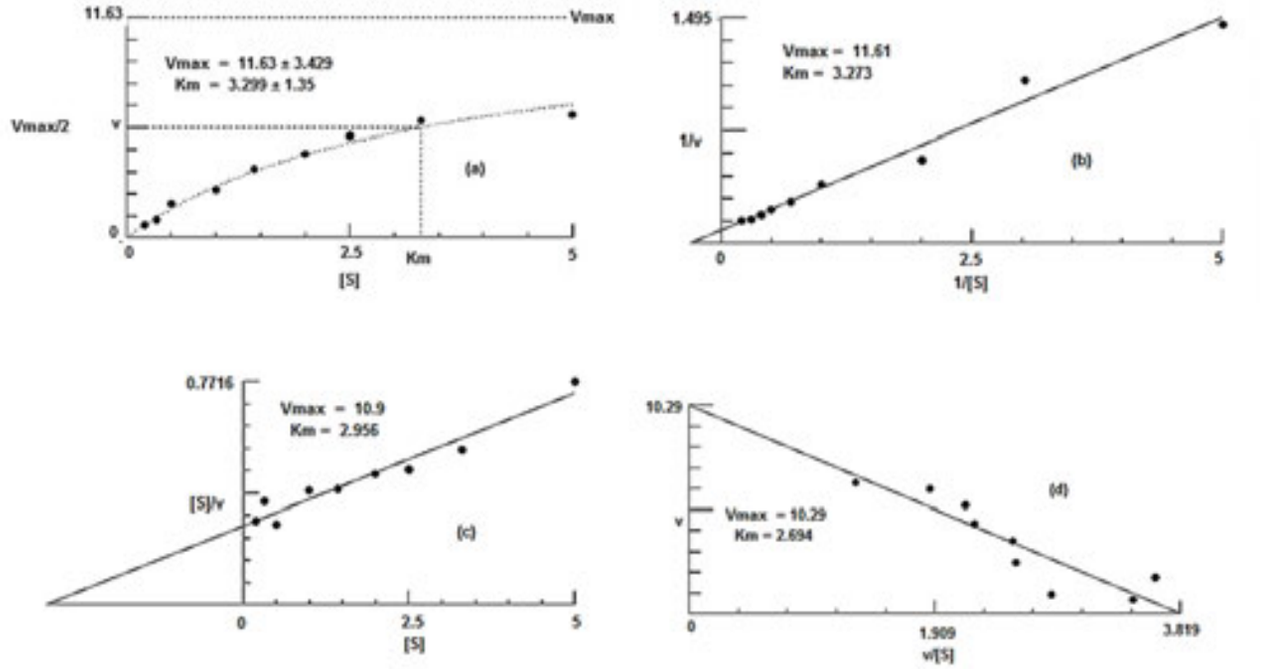

Figure 3.Enzyme kinetics plots of the acid phosphatase activity from *Desmodium tortuosum* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

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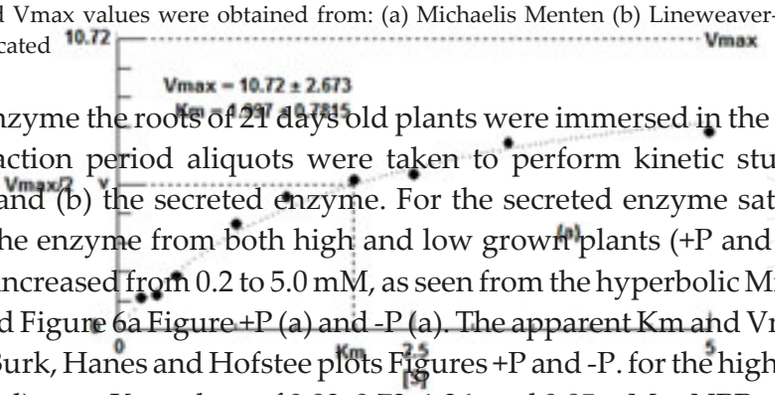

For the extracted enzyme the roots of 21 days old plants were immersed in the collection flasks and after the extraction period aliquots were taken to perform kinetic studies for (a) the extracted enzyme and (b) the secreted enzyme. For the secreted enzyme saturation kinetics was observed for the enzyme from both high and low grown plants (+P and -P) as substrate concentration was increased from 0.2 to 5.0 mM, as seen from the hyperbolic Michaelis-Menten plot in Figure 5a and Figure 6a Figure +P (a) and -P (a). The apparent Km and Vmax determined from Lineweaver-Burk, Hanes and Hofstee plots Figures +P and -P. for the high P plant enzyme Figure 5 (a, b, c and d) were Km values of 0.93, 0.73, 1.36, and 0.85 mM p-NPP and Vmax values of 8.5, 7.66, 10.13, and 8.34 µmol PNP/h/ g FW roots. For the low P grown plants Km values were similar to those found for the +P plant enzyme as calculated from plots in Figure 6 (a, b, c, and d) while the Vmax values were higher for the low P plants between 13.36 and 14.28 as calculated from Figure 6 (a, b c and d). For the extracted enzyme Km values were inconsistent and no clear differences were found between +P and -P plants (calculated from plots in Figure 7 and 8 respectively).

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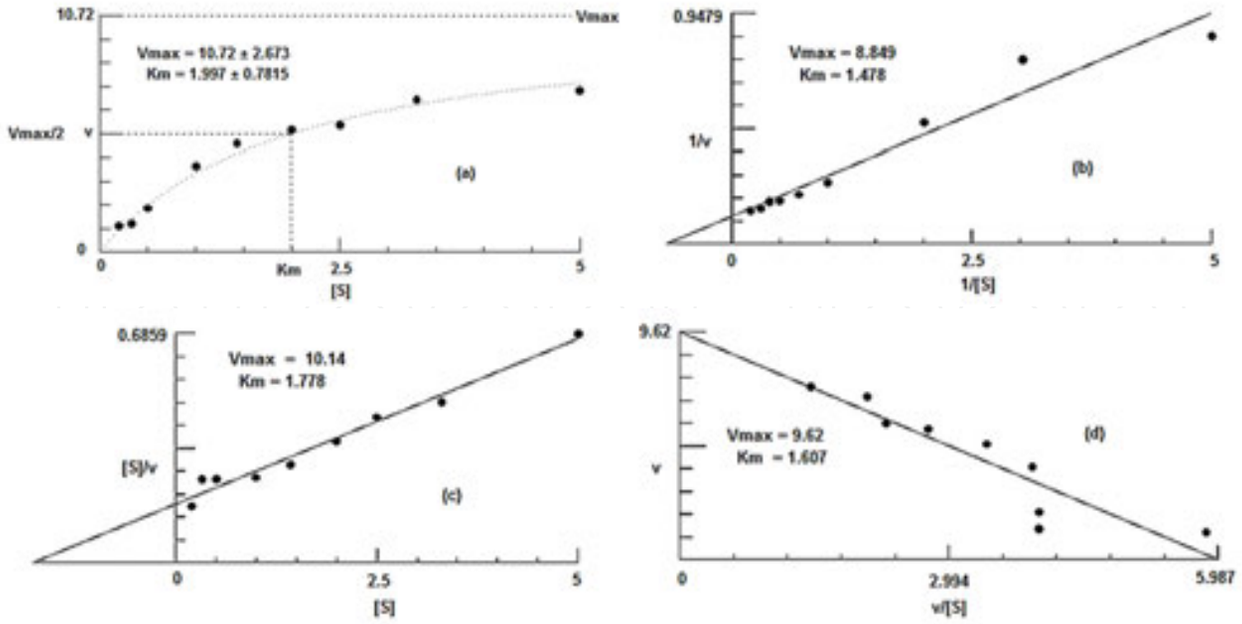

Figure 4. Enzyme kinetics plots of the acid phosphatase activity from *Desmodium tortuosum* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

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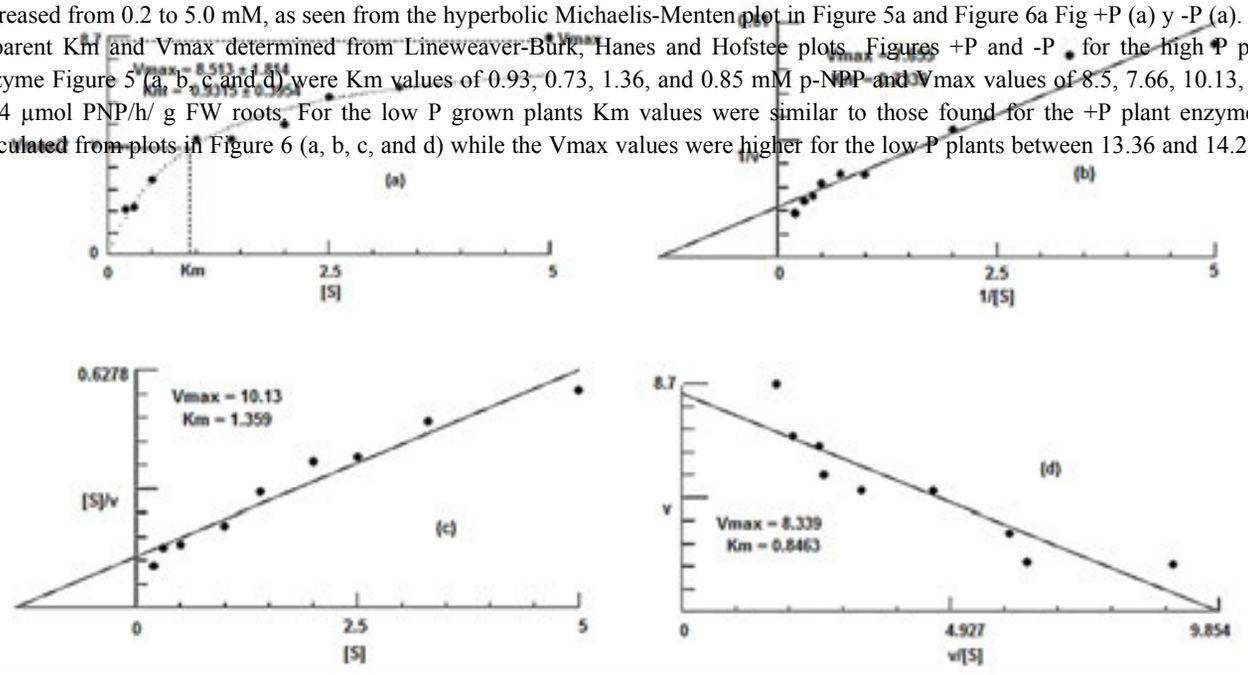

Figure 5. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

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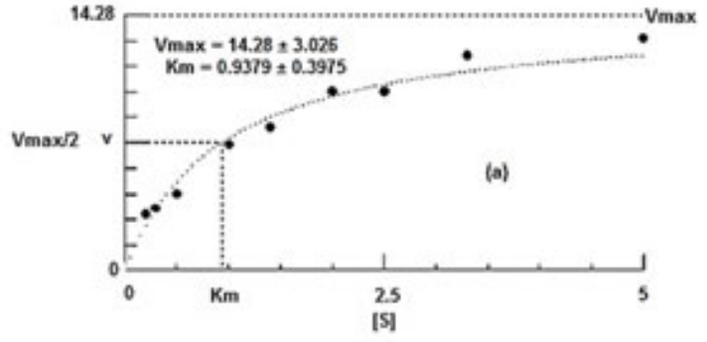

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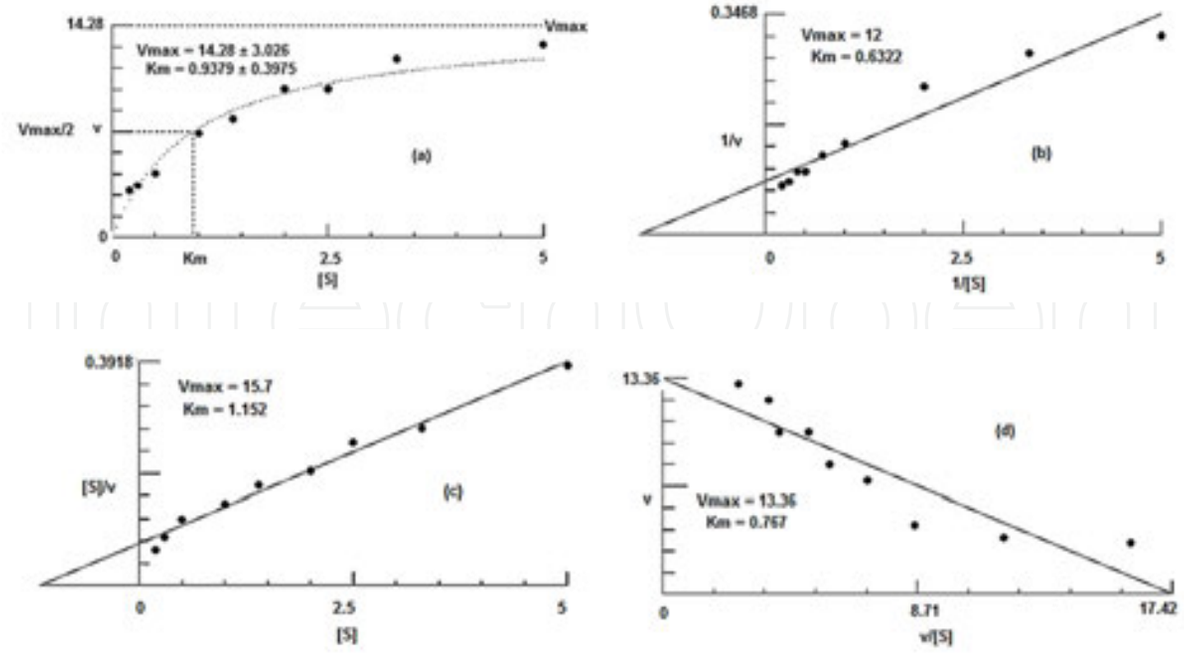

Figure 6. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

Figure 6. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

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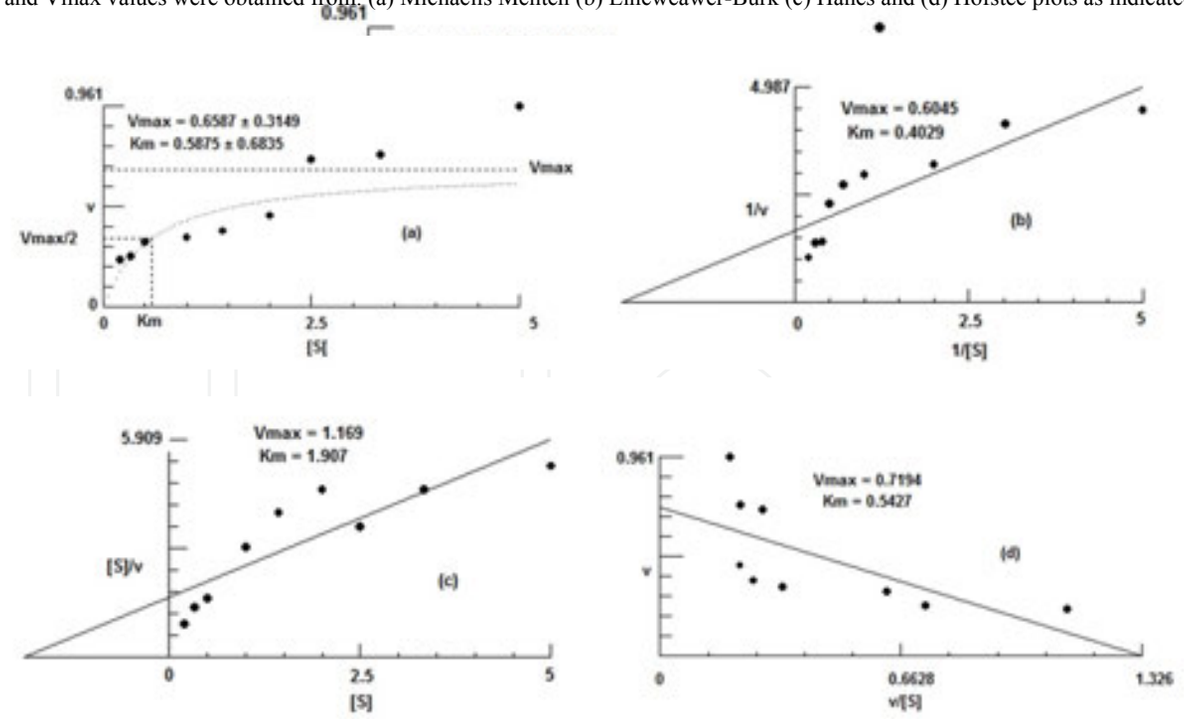

Figure 7. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

Figure 7. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

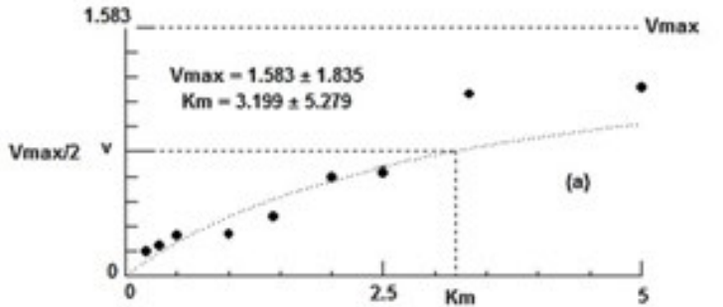

Figure 7. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

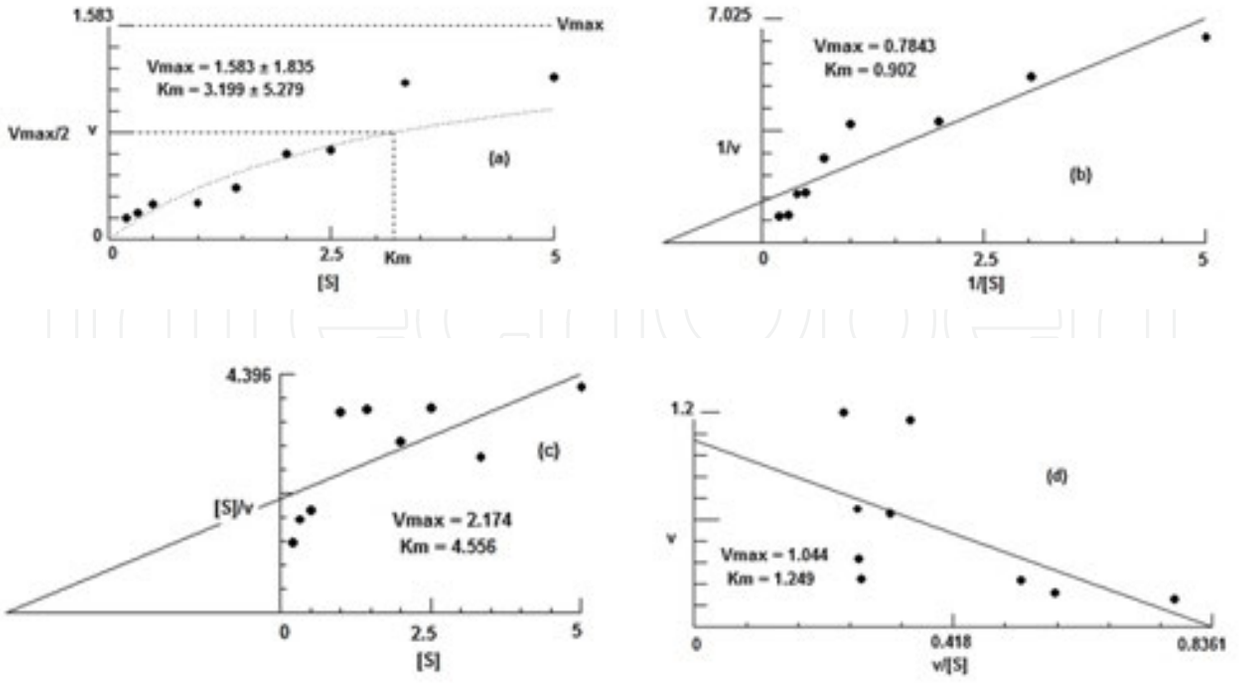

Figure 8. Enzyme kinetics plots of the acid phosphatase activity from *Phaseolus vulgaris* roots with the substrate p-nitrophenylphosphate (p-NPP). The extracted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

Vmax values were lower than those for the secreted enzyme although higher for -P plants, as found for the secreted enzyme . However, no clear tendency was found in the Km values for the extracted APase collected from root extracts. Acid Phosphatase Kinetics in *Vigna unguiculata* (cowpea) After 14 days of planting the roots of four plants grown under either P sufficiency (+P 1.0 mM P) or deficiency (-P 0.005 mM P) were immersed in the dialysis membrane and after the extraction period aliquots were taken to perform kinetic studies for the secreted enzyme. Data for the extracted enzyme are not presented as they were very low and similar for +P and -P plants and in some cases not detectable. For the secreted enzyme saturation kinetics was observed for the enzyme from both high and low grown plants (+P and -P) as substrate concentration was increased from 1.0 to 5.0 mM, as seen from the hyperbolic Michaelis-Menten plot in Figure 9a and Figure 10a Fig +P (a) -P (a). The apparent Km and Vmax determined from Lineweaver-Burk, Hanes and Hofstee plots Figures +P and -P . for the high P plant enzyme Figure 9 (a, b, c and d) were Km values of 1.02, 1.31, 1.04 and 0.96 mM p-NPP and Vmax values of 4.11, 4.46, 4.12 and 4.03 µmol/h/ g FW roots. For the low P grown plants Km values were lower (0.72, 0.68, 0.98 and 0.71) to those found for the +P plant enzyme as calculated from plots in Figure 10 (a, b, c, and d) while the Vmax values were higher for the high P plants (4.11, 4.46, 4.12, and 4.03 as compared to 3.23, 3.19, 3,52 and 3.23 µmol PNP/h/g FW roots for -P enzyme as calculated from Figures 9 and 10. Acid Phosphatase Kinetics in *Crotalaria juncea* (sunn hemp) After 20 days of planting the roots from +P (0.86 mM P) and -P (.004 mM P) intact plants were individually immersed in a dialysis membrane and after an extraction period of 24 h, aliquots from the solution inside the dialysis membrane were taken to perform the kinetics studies of root secreted acid phosphatase. Two separate experiments were performed using different plants. The following concentration range for the substrate was used: 1.0, 1.25, 1.43, 1.67, 2.0, 2.50, 3.30 and 5.50 mM p-NPP and as no clear substrate saturation was observed for either +P or -P enzymes using the hyperbolic Michaelis-Menten plot, the apparent Km

Vmax values were lower than those for the secreted enzyme although higher for -P plants, as found for the secreted enzyme . However, no clear tendency was found in the Km values for the extracted APase collected from root extracts.

2.3. Acid Phosphatase Kinetics in *Vigna unguiculata* (cowpea)

After 14 days of planting the roots of four plants grown under either P sufficiency (+P 1.0 mM P) or deficiency (-P 0.005 mM P) were immersed in the dialysis membrane and after the extraction period aliquots were taken to perform kinetic studies for the secreted enzyme. Data for the extracted enzyme are not presented as they were very low and similar for +P and -P plants and in some cases not detectable. For the secreted enzyme saturation kinetics was observed for the enzyme from both high and low grown plants (+P and -P) as substrate concentration was increased from 1.0 to 5.0 mM, as seen from the hyperbolic Michaelis-Menten plot in Figure 9a and Figure 10a Fig +P (a) -P (a). The apparent Km and Vmax determined from Lineweaver-Burk, Hanes and Hofstee plots Figures +P and -P. for the high P plant enzyme Figure 9 (a, b, c and d) were Km values of 1.02, 1.31, 1.04 and 0.96 mM p-NPP and Vmax values of 4.11, 4.46, 4.12 and 4.03 µmol/h/ g FW roots. For the low P grown plants Km values were lower (0.72, 0.68, 0.98 and 0.71) to those found for the +P plant enzyme as calculated from plots in Figure 10 (a, b, c, and d) while the Vmax values were higher for the high P plants (4.11, 4.46, 4.12, and 4.03 as compared to 3.23, 3.19, 3,52 and 3.23 µmol PNP/h/g FW roots for -P enzyme as calculated from Figures 9 and 10.

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2.4. Acid Phosphatase Kinetics in *Crotalaria juncea* (sunn hemp)

After 20 days of planting the roots from +P (0.86 mM P) and -P (.004 mM P) intact plants were individually immersed in a dialysis membrane and after an extraction period of 24 h, aliquots from the solution inside the dialysis membrane were taken to perform the kinetics studies of root secreted acid phosphatase. Two separate experiments were performed using different plants. The following concentration range for the substrate was used: 1.0, 1.25, 1.43, 1.67, 2.0, 2.50, 3.30 and 5.50 mM p-NPP and as no clear substrate saturation was observed for either +P or -P enzymes using the hyperbolic Michaelis-Menten plot, the apparent Km and Vmax values for the two experiments were determined from Lineweaver-Burk, Hanes and Hofstee plots and linear transformations were adjusted to the best fit line.

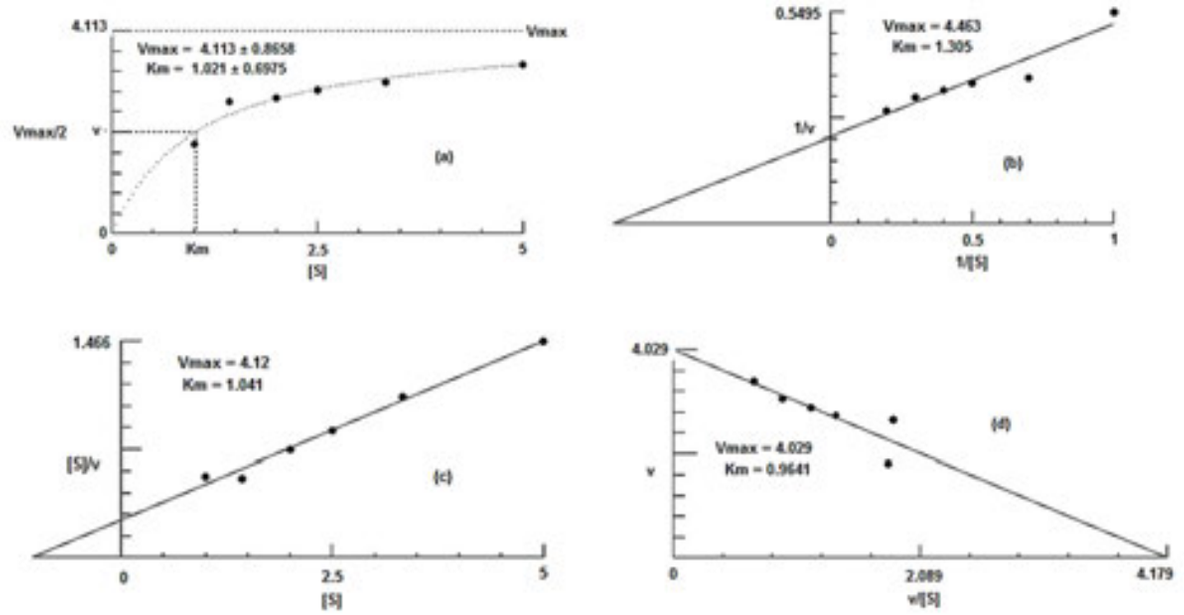
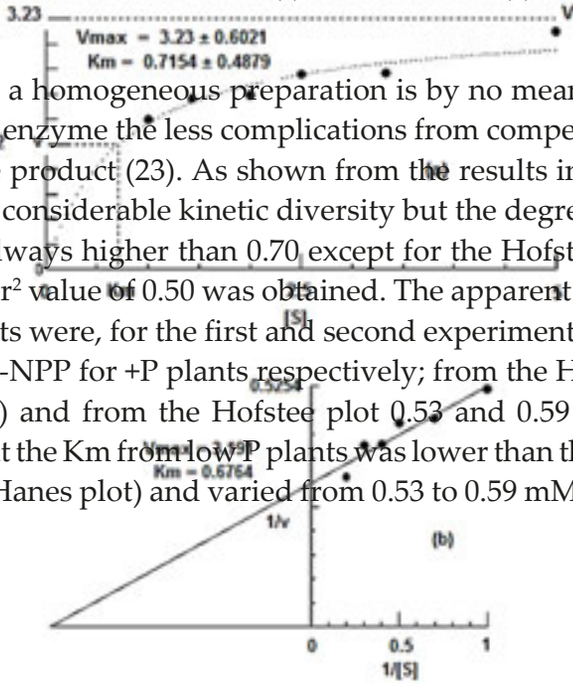


Figure 9. Enzyme kinetics plots of the acid phosphatase activity from *Vigna unguiculata* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

Figure 9. Enzyme kinetics plots of the acid phosphatase activity from *Vigna unguiculata* roots with the substrate p-nitrophenylphosphate (p-NPP). The secreted enzyme was obtained from plants previously grown in P sufficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated

It is important to note that a homogeneous preparation is by no means necessary for kinetic analyses, but the purer the enzyme the less complications from competing reactions that may use up the substrate or the product (23). As shown from the results in Table 1, apparent Km and Vmax values, showed considerable kinetic diversity but the degree of adjustment for the linear equations (r^2) was always higher than 0.70 except for the Hofstee plot for +P plants in experiment 1, where a low r^2 value of 0.50 was obtained. The apparent Km values determined from Lineweaver-Burk plots were, for the first and second experiments, 0.53 and 0.57 mM for -P and 0.82 and 0.76 mM p-NPP for +P plants respectively; from the Hanes plot 1.75 and 0.79 (-P) and 0.62 and 1.13 (+P) and from the Hofstee plot 0.53 and 0.59 (-P) and 0.86 and 0.78 (+P).These results show that the Km from low P plants was lower than that for +P plants (except when calculated from the Hanes plot) and varied from 0.53 to 0.59 mM in -P and 0.76 and 1.13



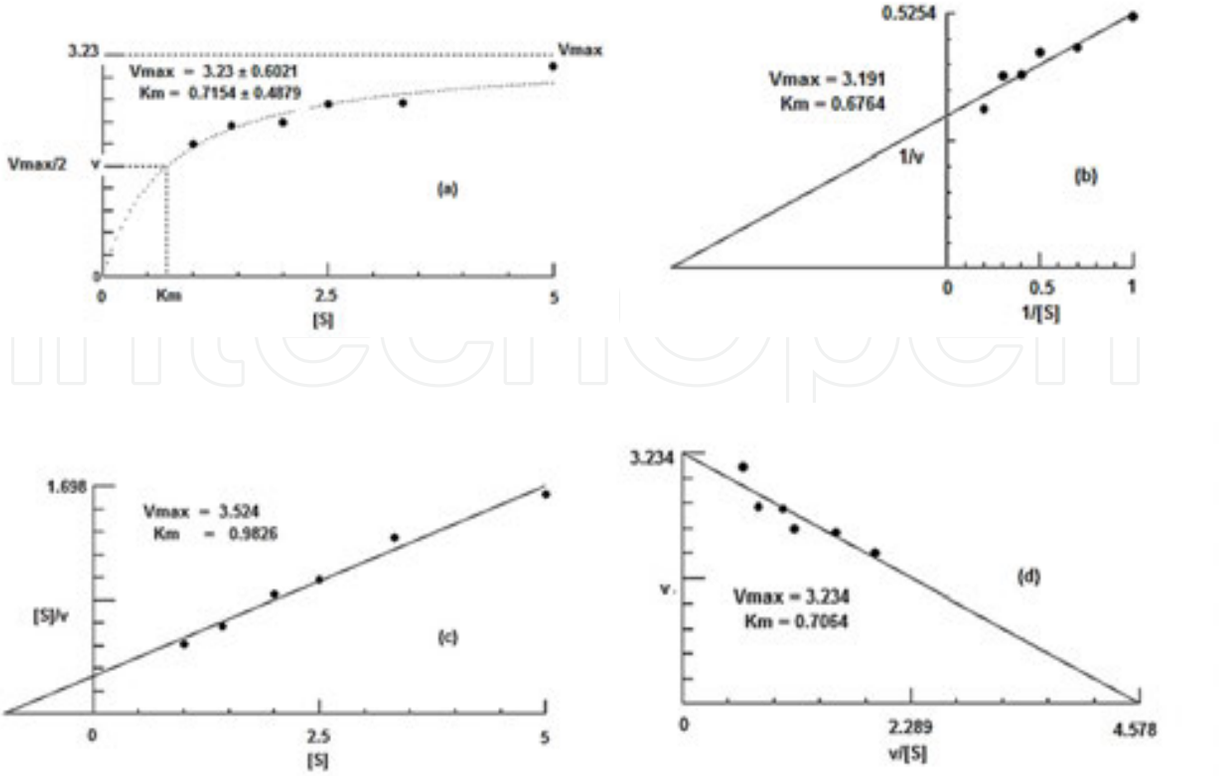


Figure 10. Enzyme kinetics plots of the acid phosphatase activity from *Vigna unguiculata* roots with the substrate p-nitrophenylphosphae (p-NPP). The secreted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

Figure 10. Enzyme kinetics plots of the acid phosphatase activity from *Vigna unguiculata* roots with the substrate p-nitrophenylphosphae (p-NPP). The secreted enzyme was obtained from plants previously grown in P deficient solutions. Apparent Km and Vmax values were obtained from: (a) Michaelis Menten (b) Lineweaver-Burk (c) Hanes and (d) Hofstee plots as indicated.

It is important to note that a homogeneous prearatin is by no means necessary for kinetic analyses, but the purer the enzyme the less complications from competing reactions that may use up the substrate or the product (23). As shown from the results in Table 1, apparent Km and Vmax values showed considerable kinetic diversity but the degree of adjustment for the linear equations (r^2) was always higher than 0.70 except for the Hofstee plot for +P plants in experiment 1, where a low r value of 0.50 was obtained. The aim of our study was to find out the differences in Km between -P and +P plant enzymes The apparent Vmax values determined from Lineweaver-Burk double reciprocal plot were 39.90 and 25.97 for -P and 25.44 and 25.38 μmol PNP/g root DW/h for experiments 1 and 2 respectively; from the Hanes plot these values were 33.78 and 28.08 (-P) and 38.04 and 28.73 (+P) and from the Hofstee plot 36.94 and 26.12 (-P) and 26.13 and 25.64 (+P).These results show that Vmax values were similar for the secreted enzyme from roots of low P and high P plants. Thus for practical purposes and according to the results presented in this study, the Lineweaver-Burk and Hofstee plots are the best options to fit the data, if the objective of the study is to find out the differences in Km between -P and +P plant enzymes

mM in +P plants. Thus for practical purposes and according to the results presented in this study, the Lineweaver-Burk and Hofstee plots are the best options to fit the data, if the objective of the study is to find out the differences in Km between -P and +P plant enzymes The apparent Vmax values determined from Lineweaver-Burk double reciprocal plot were 39.90 and 25.97 for -P and 25.44 and 25.38 μmol PNP/g root DW/h for experiments 1 and 2 respectively; from the Hanes plot these values were 33.78 and 28.08 (-P) and 38.04 and 28.73 (+P) and from the Hofstee plot 36.94 and 26.12 (-P) and 26.13 and 25.64 (+P).These results show that Vmax values were similar for the secreted enzyme from roots of low P and high P plants.

Plot	Experiment	Treatment	Equation	r^2	Km (mM p-NPP)	Vmax (μmol PNP/h/g DW r)
Lineweaver	I	-P	Y =0,0271+0,0144x	0,9209	0,53	36,90
		+P	Y= 0,0393+0,0321x	0,7144	0,82	25,44
	II	-P	Y=0,0385+0,0221x	0,8869	0,57	25,97
		+P	Y=0,0394+0,0299x	0,8542	0,76	25,38
Hanes	I	-P	Y=0,0520+ 0,0296x	0,9075	1,75	33,78
		+P	Y=0,0163+ 0,0262x	0,9980	0,62	38,04
	II	-P	Y=0,0282+0,0356x	0,9948	0,79	28,08
		+P	Y=0,0393+0,0348x	0,9888	1,13	28,73
Hoffstee	I	-P	Y=36,9396-0,5331x	0,8929	0,53	36,94

Plot	Experiment	Treatment	Equation	r^2	Km (mM p-NPP)	Vmax (μmol PNP/h/g DW r)
Lineweaver	I	-P	Y =0,0271+0,0144x	0,9209	0,53	36,90
		+P	Y= 0,0393+0,0321x	0,7144	0,82	25,44
	II	-P	Y=0,0385+0,0221x	0,8869	0,57	25,97
		+P	Y=0,0394+0,0299x	0,8542	0,76	25,38
Hanes	I	-P	Y=0,0520+ 0,0296x	0,9075	1,75	33,78
		+P	Y=0,0163+ 0,0262x	0,9980	0,62	38,04
	II	-P	Y=0,0282+0,0356x	0,9948	0,79	28,08
		+P	Y=0,0393+0,0348x	0,9888	1,13	28,73
Hoffstee	I	-P	Y=36,9396-0,5331x	0,8929	0,53	36,94

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Plot	Experiment	Treatment	Equation	r^2	Km (mM p-NPP)	Vmax (μmol PNP/h/g DW r)
Hanes	I	-P	Y=0,0520+ 0,0296x	0,9075	1,75	33,78
	I	+P	Y=0,0163+ 0,0262x	0,9980	0,62	38,04
	II	-P	Y=0,0282+0,0356x	0,9948	0,79	28,08
		+P	Y=0,0393+0,0348x	0,9888	1,13	28,73
Hoffstee	I	-P	Y=36,9396-0,5331x	0,8929	0,53	36,94
		+P	Y=26,1334-0,8593x	0,4997	0,86	26,13
	II	-P	Y=26,1232-0,5892x	0,8487	0,59	26,12
		+P	Y=25,6493-0,7819x	0,7837	0,78	25,64

Table 1. Apparent Km and Vmax values for the root secreted acid phosphatase in +P and -P grown plants of *Crotalaria juncea*. (Ascencio and Santana, unpublished) -P= 0,004 mM P; +P= 0,86 mM P

3. Discussion

Root acid phosphatase activity for the plants was higher in -P plants at the beginning of the growth period (depending on the species) and that the proper timing for the onset of the P-stress is apparently crucial for induction of APase in different species [2, 6]. It has been shown in this and many other reports in the literature, that P deficient conditions in the plant can trigger APase activity [5, 10, 23]; and that new isoenzymes could be activated under P deficiency in roots [12, 16], leaves [29] and seedlings [14].However for bean and cowpea APase activity appears not to be inducible when 0.02 mM P was used to grow the plants under P deficient conditions even though differences in P concentration in the dry matter were large enough to suspect that plants were suffering from mild P stress [9]. In the present study 0.005 mM P was used in the low P treatment and even though large differences were not found for enzyme activity between +P and -P plants larger differences in total dry matter, leaf area and soluble Pi concentration in leaves were found. However from an agronomic point of view and focusing on the APase role to dissolve organic-P in soils with low P bioavailability, the enzyme secreted with the root exudates to the soil is a major concern in plant adaptation, specially to phosphorus-limited tropical soils [21]. Like many other secreted proteins the APase is glycosylated, which protects the enzyme against proteolytic enzymes and contributes to its stability over a wide pH range. Secretory APase can liberate bound P from soil and have shown to deplete organic P in the rhizosphere of several plant species; however, mineral phosphorus is hardly available in tropical soils and for organic P to be used in agricultural soils, increased secretion of APase may be involved as part of the coordinated adaptive strategies to withstand P deficiency. However, under soil conditions where extracellular enzymes such as APase function are associated with soil colloids, a large fraction of the free enzyme may be immobi-

lized as extracellular enzymes such as APase primarily function associated with soil colloids [20]. Compared to the free enzyme, properties and kinetic behavior of such complexed enzymes had a different pH activity dependence and sensitivity to temperature and protein degradation [19]. According to reports in the literature, the kinetics of APase in synthetic enzyme complexes simulating those usually encountered in soil, showed Michaelis-Menten kinetics with a lower Vmax and higher Km values as compared to the free enzyme [13]. Many APase isozymes exist in the root and leaves but only one of them was secreted into the rhizosphere in a large amount [25]. When the enzyme was mixed with aqueous solutions extracted from a P-deficient soil its activity declined to 55% of its original activity after 14 days and to 9% after 21 days. We have performed experiments applying the secreted APase enzyme solution obtained from low P grown plants of *Centrosema rotundifolia* and *Crotalaria juncea* to low P soils [6]; according to the results APase activity in the soils showed significant differences depending on soil type and root secretion but was higher in soils with the secreted APase from *Crotalaria* plants. Under the conditions of a higher Km the enzyme will not efficiently function under P starvation as a higher substrate concentration is needed to achieve half the maximal velocity. Under these circumstances, in order to unbind APase to perform efficiently, besides having a lower Km value, the roots should have the ability to secrete larger amounts of the enzyme into the rhizosphere to compensate for the low Vmax. It has been shown that the APase secreted by white lupin roots is stable in soil solution and shows low substrate specificity which is important to improve their ability to use organic P [12]. According to our results, true saturation Michaelis-Menten kinetics was not observed for all the species, specially for the enzyme from +P plants; we have also found similar results with crude extracts from other wild and cultivated species, and as seen from the shape of the plots of enzyme velocity *versus* substrate concentration, the presence of several isoenzymes should not be discarded. In this context the Hofstee plot (v vs, v/S) is the best alternative in detecting the presence of multiple enzymes that catalyze the same reaction [23]. For agronomic purposes, it is better to assay the crude enzyme secretion or extract, without further purification, as it is the form that it is released from the roots to the environment. Differences in APase activity for *Phaseolus vulgaris* as seen from the Km values apparently indicate the lack of phosphate starvation-inducible APase, as it has been found in other crops, for example, see [14]; Vmax values on the other hand were higher in -P plants; however the combination of a high Km with a high Vmax could improve plant behaviour under P deficiency. The opposite was noted with *Vigna unguiculata* where a low Vmax in -P plants may be compensated by a lower Km. As compared to *Phaseoulus* and *Vigna,* the APase secreted from the roots of *Crotalaria juncea* showed considerably greater kinetic diversity depending on the methods of plotting enzyme kinetics data for the calculation of Km and Vmax values for -P and +P plants. For maximum efficiency it seems reasonable to expect that the enzyme from low P plants under the conditions of this study would have a low Km and a high Vmax; we have found for *Crotalaria* differences in the Km from -P and +P plants, but not for Vmax where the values were similar for the enzyme secreted from low P and high P plants, as found for *Phaseoulus and Vigna*. The less suited combination for the enzyme to perform efficiently under P deficient conditions is to have a high Km and a low Vmax (which means that the substrate concentration must be high and does not compensate for a low Vmax). From our results, it is seen that the enzyme from -P plants is better suited

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to cope with P deficiency, due to a consistent lower Km. In this context, favorable kinetic properties (low Km and highVmax) as well as the amount of secreted enzyme are important as one may compensate for each other; in this connection the best combination for the enzyme to perform efficiently under natural conditions, where a low P concentration exist in the soil, would be a low Km and a great amount of secreted enzyme to the environment. This might be the better strategy for plant species to perform efficiently under low P soils. We have shown from previous studies that leguminous plants have developed several growth strategies to withstand P limitations imposed by the soil; under P deficiency total leaf area, relative growth rate (RGR) and root length were reduced by 50% in severely stressed *Desmodium tortuosum* and other plant species [4,5], and that for tomato APAse activity was highly correlated to development and recovery from P stress and that total weight and average root diameter decreased under P stress while root surface area per unit dry weight increased, [10]. As large differences were found in Relative Growth Rate (RGR) between the high and low P plants and as these differences were consistent for all the species analyzed, RGR is an adequate physiological indicator of plant performance under P deficient conditions and a useful tool if used in screening purposes.

4. Conclusions

The practical implications of kinetic constants Km and Vmax for the enzyme exuded by the roots of several plant species were analyzed in this study in terms of the potential for P-liberation under limiting condition of Pi bioavailability in soil; an increased Pi uptake is likely to occur if the APase released by the roots has a low Km value (in the neighborhood of the soil P concentration) and a high Vmax, in order to be efficient in liberating Pi from the soil organic-P pool. It has been shown that one of the advantages of the APase secreted from the roots of some leguminous species such as lupinus, was a higher Vmax value as compared to the enzyme from other species. We have found similar results for enzyme from *Desmodium, Phaseoulus* and *Vigna*. The numerical value of the Km for the substrate p-nitrophenyl-P provided a means of comparing the enzyme from high or low-P plants.Our results showed that Km is a reliable physiological tool for assessing plant adaptability to P-deficiency and its is suggested that Km, enzyme activity (Vmax), total leaf area and relative growth rate (RGR) may be used as physiological indicators to differentiate plants grown under P deficiency or sufficiency.

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