ROLE OF PHOSPHO*ENOL*PYRUVATE CARBOXYLASE IN THE ADAPTATION OF A TROPICAL FORAGE GRASS TO LOW-PHOSPHORUS ACID SOILS

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ABSTRACT

As *Brachiaria* hybrid cv. Mulato was adapted to acid soils with extremely low phosphorus (P) contents, we investigated its low P tolerance mechanisms comparing with wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L. cv. Kitaake). Among the three plant species, the highest P use efficiency (PUE) in low P soil was recorded in

Brachiaria hybrid, which remarkably increased in P-deficiency and soil acidity, while P-deficiency had less effect on PUE of wheat and rice. As exudation of organic acid anions from roots is considered as one of the important mechanisms of adaptation to low P soil, we investigated the role of phospho*enol*pyruvate carboxylase (PEPC), which is closely related to organic acid metabolism and Pi recycling in C_3 plants. As expected the PEPC activity of *Brachiaria* hybrid (C_4 plant) leaves was 51 to 129-fold higher than those estimated for wheat and rice (both C_3 plants). PEPC activity in leaves and roots of *Brachiaria* hybrid increased up to 2 and 3 folds respectively, and decreased malate inhibition ratio in leaves in response to P-deficiency. However, PEPC activity and malate inhibition ratio was less affected in wheat and rice under P deficiency. *Brachiaria* hybrid synthesized higher amounts of organic acids (*e.g.,* oxalate and fumarate) in leaves, especially under P-deficiency. Results from these experiments indicated that PEPC activated in *Brachiaria* hybrid under low P and pH conditions may contribute to its greater adaptation to tropical acid soils with low P availability.

Key Words: *Brachiaria*, Phosphorus acquisition; P use efficiency; Organic acids; PEPC activity; Al tolerance.

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INTRODUCTION

Phosphorus (P) deficiency is an important factor limiting crop production in tropical and sub-tropical soils.[1-5] In these soils, P forms insoluble compounds with a number of di- and tri-valent cations (*e.g.*, Al^{3+}, Fe^{3+}) and it is the least readily available nutrient in the rhizosphere.[6,7] Crop yield on 30-40% of arable land in the world is limited by P availability.[8] Concerns over depletion of high-grade phosphate rock, the necessary fertilizer raw material, and cost of P fertilization in developing countries have stimulated the search for means of saving and utilizing P more efficiently.[2,5, 9,10]

Correcting P deficiency with application of P fertilizer is not a viable approach for resource-poor farmers in the tropics and sub-tropics, especially on soils with high P-fixing capacity.[4,5] Under such conditions, the integration of field crops with forage cultivars that can make most efficient use of the P supplied as maintenance fertilizer application represents a key element of sustainable crop-livestock systems in the tropics.[11,12,13] Genetic variations in P uptake efficiencies have been widely reported in many crops[13], such as clover[14] and maize.[15] Plant traits responsible for P uptake efficiency include rhizosphere acidification, root exudation of organic acid anions and phosphate mobilizing enzymes, root morphology, uptake kinetics and symbiotic association with mycorrhizal fungi.[6,13,16-18]

Plants have evolved two broad strategies for P acquisition and use in nutrient-limiting environments: (1) those aimed at efficient of use; and (2) those directed toward enhanced acquisition or uptake.[5,19] Processes that use efficiently the acquired P involve decreased growth rate, increased growth per unit of P uptake, remobilization of internal Pi, modification in internal carbon metabolism that bypass P-requiring steps, and alternative respiratory pathways.[5, 20, 21] By comparison, processes that lead to increased P uptake include enhanced secretion of phosphatases and exudation of organic acids, changes in root morphology and enhanced expression of Pi transporters.[5, 6]

To enhance P uptake, because of their high affinity for divalent and trivalent cations, organic acid anions released from the roots is thought to displace P from insoluble complexes, making it more available for uptake by plants.[22, 23] Organic acid anions also play an important role in detoxification of Al both externally and internally.[16,17,24-26]

Enhanced expression and activity of phospho*enol*pyruvate carboxylase (PEPC) has been linked with P deficiency-induced biosynthesis and root exudation of carboxylic acids.[27-32, 21]

Phospho*enol*pyruvate carboxylase (PEPC) is a cytosolic enzyme widely distributed in most plant tissues, green algae and microorganisms but not in animal cells.[33] PEPC is a homotetrameric enzyme that catalyzes the β-carboxylation of phospho*enol*pyruvate by HCO_3^- in the presence of a divalent cation to yield Pi and oxaloacetate (OA), which is readily converted to malate by NAD(P)-malate dehydrogenase.[33] It is an important enzyme for the carbon economy of the cell, playing a central role in CO_2 fixation of C_4 and crassulacean acid metabolism (CAM) plants.[33] PEPC activity produces OA and malate that replenish the citric acid cycle, the so-called anaplerotic function, providing carbon skeletons for nitrogen assimilation.[35] In maize leaves, the addition of nitrate, ammonium and glutamine promotes the activation of PEPC expression,[36,37] lending support to the proposal that PEPC activity plays an important role connecting carbon and nitrogen metabolism. Recently, several abiotic stresses (monovalent cations, drought, cold and hypoxia) induced expression of PEPC in roots of wheat seedlings.[38] These findings suggest that PEPC may play an important role in the adaptation of plants to environmental stress factors.

Inherent differences in efficiencies of P uptake and utilization exist between tropical forage grass and legume species.[39] The International Center for Tropical Agriculture (CIAT) has identified several tropical forages that are adapted to infertile acid soils.[40-41] Among the selected forages, *Brachiaria* grasses are very important species which showed

high adaptation to low P supplying acidic soils. Differential adaptation efficiencies among forage species to P deficiency and excess Al (in high soil acidity) have been observed by other researchers.[1, 5] These differential responses to P deficiency and/or Al are of interest to plant breeders because this variability can be used to improve acid-soil adaptation in high-yielding cultivars.[40] Rao and co-workers investigated growth, P uptake and utilization efficiencies of a number of forage species under various tropical soils.[39,42,43] They found that phosphorus use efficiency (PUE) in grass (*e.g.*, *Brachiaria* spp.) is much higher than that of the legumes (*e.g.*, *Stylosanthes* spp.) regardless of P supply. Because of their higher adaptation to low-fertility acid soils, *Brachiaria* species have been introduced as commercial pastures in 50-70 million hectares of native savannas in tropical America since the 1960s.[44] Widespread adoption of forage cultivars depends on efficiently acquiring P from the soil and using them for growth. Plant attributes appear to be linked to different strategies to acquire and use phosphorus. Understanding these linkages is fundamental in integrating plant attributes in a selection index. It is essential to elucidate mechanism of plant species conferring superior adaptation to P deficient acidic soils.[5]

Although knowledge about the principal mechanisms involved in efficient P acquisition by plants has evolved substantially during recent years,[5,13,45] the detailed mechanisms of internal P requirements among different genotypes of crop plants are still not fully understood. In this paper, we have evaluated the role of phospho*enol*pyruvate carboxylase in improving PUE under P deficiency and soil acidity in *Brachiaria* hybrid cv. Mulato comparing with wheat (low P-sensetive) and rice (low P-tolerant).

MATERIALS AND METHODS

Experiment 1. Effect of low pH and low P in soil

Plant materials and cultivation of plants

Experiment was conducted in a glasshouse (43˚3' N, 141˚2' E, altitude 17 m; maximum temperature 32˚C; minimum temperature 16˚C; average photoperiod during experiment = 14.8 h light and 9.2 h darkness; maximum photon flux density = 1550 µmol m^{-2} s^{-1}) of Graduate School of Agriculture of Hokkaido University, Sapporo, Japan from June to August 2000. Seeds of *Brachiaria* hybrid cv. Mulato CIAT 36061 (developed from parents of *Brachiaria ruziziensis* clone 44-06 and *Brachiaria brizantha* cv. Marandú and identified as FM9201/1873), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L. cv. Kitaake) were surface sterilized with 1% of sodium hypochlorite for 10 min. Rice seeds were germinated on a petri dish for 3 days. Seeds (germinated in case of rice) (2-6) were sown in small plastic pots (160 ml) containing soils with two levels of P (+P and -P) and three levels of pH (4.0, 4.5 and 5.0), of which soil were collected from a long-term (25 years-old) experimental field without P fertilizer input, with pH (H_2O) 4.0. Initially, soil was fertilized with 1.87 g N kg^{-1} and 0.8 g K kg^{-1} soils as $(NH_4)_2SO_4$ and K_2SO_4, respectively. In case of +P treatments, $Ca(H_2PO_4)_2$ was added at the rate of 2.22 g kg^{-1} of soil while no P was added

in -P treatments. Available phosphorus concentration (Bray II, mg/100g soil) was 5.5-5.9 in –P treatment, and 14.0-15.6 in +P treatment, which was higher in lower pH soil. The pH levels of soil were adjusted by adding appropriate amounts of calcium carbonate or 0.1 N H_2SO_4 to the soil. Water was conventionally supplied by deionized water. Each treatment was replicated for 6 times arranged in complete randomized block design.

Sampling methods

Plants were harvested at 28-50 days of cultivation. Their roots were washed with tap water and then gently washed with deionized water, and plants were separated into root, stem and leaf. Half of each sample was dried in air-forced oven at 80°C for 72 h, then weighed, and ground for nutrient analysis. Another half of each sample was chilled by liquid nitrogen and then stored at -80°C for PEPC and organic acid analyses.
Leaves of *Brachiaria* hybrid, wheat and rice were analyzed for PEPC and organic acid analysis.

Nitrogen and phosphorus concentration

Dried sample was digested with sulfuric acid and hydrogen peroxide (Mizuno and Minami 1980). Total nitrogen and P were measured by semi-micro Kjeldahl-method and vanado-molybdate yellow method, respectively as described previously[46]. This analytical method was applied also in experiment 2.

Organic acid analysis

Lyophilized leaves were homogenized in cold 0.01N HCl (sample:HCl=1:10 in leaves) to determine the organic acid concentrations in the plant. The extract was filtered with a membrane filter (pore size = 0.45 μm). The organic acid anions (oxalate, malate, citrate, fumarate, and α-ketogluterate, which was dominant) were analyzed by a Capillary Ion Analyzer (CIA, Waters) under the following measurement conditions: electrolyte, 2.5% CIA-PAK^{TM} OFM Anion BT in 120 mM $Na_2B_4O_7$; capillary fused silica (50 μm x 60 cm); and detection, 185 nm. Identification and detection of organic acid anions were done by comparing their retention time and absorption spectra with those of known standards. This method was applied also in experiment 2.

Phospho*enol*pyruvate carboxylase (PEPC) activity assay

For the measurement of PEPC activity under malate inhibition conditions, 200μl of the supernatant of the protein extraction solution was mixed with 300μl of saturated Na_2CO_3, and set on ice for 10 min. The precipitate was obtained by centrifugation at 17,000 g for 10 min at 4^0C, then resuspended with 20μl of 50 mM Hepes-NaOH (pH 7.5), 5%

(v/v) glycerol, 5 mM $MgCl_2$, 1 mM EDTA, 14 mM 2-mercaptoethanol, 1 mM PMSF, and 10 μg mL^{-1} chymostatin. After centrifugation at 17,000 g for 5 min at 4^0C, 20 μl of the supernatant was mixed with 913 μl of pre-reaction mixture containing 100 mM Hepes-NaOH (pH 7.3), 10 mM $MgCl_2$, 1 mM Na_2CO_3, and 0.2 mM NADH (dissolved in 50 mM Tris-HCl (pH 7.4), and 20 μl of 50 mM malate and 2 units of MDH. The change in the amount of NADH was monitored after the addition of 40 μl of 50 mM PEP.

To measure the maximum activity of PEPC, 0.2 g of leaf was homogenized with 50 mM Hepes-KOH (pH 7.4), 10 % (v/v) glycerol, 1 mM EDTA, 10 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 10 μM leupeptin, and 5 % (w/v) polyvinyl-polypyrrolidone. 25 μl of the supernatant was mixed with 908 μl of pre-mixture, containing 100 mM Hepes-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM $NAHCO_3$, 0.2 mM NADH (in 50 mM Tris-HCl), 2 μl of MDH (diluted to 2 units with 50 % (v/v) glycerol). The amount of NADH was monitored after the addition of 40 μl of 100 mM PEP. The amount of soluble protein was determined by the Bradford method, using BSA as a standard.[47] This method was applied also in experiment 2.

Statistical analysis

All the statistical analyses were done using the SPSS (Windows 10.0) computer program.

Experiment 2. PEPC response to low phosphorus in hydroponic solution

Plant culture

Brachiaria hybrid cv. Mulato and rice (*Oryza sativa* L. cv. Kitaake) were grown hydroponically under greenhouse conditions. Seedlings were pre-cultured in a 56 L vessel containing 2.12 mM N (NH_4NO_3), 0.77 mM K (K_2SO_4:KCl=1:1), 1.25 mM Ca ($CaCl_2 \cdot 2H_2O$), 0.82 mM Mg ($MgSO_4 \cdot 7H_2O$), 35.8 µM Fe ($FeSO_4 \cdot 7H_2O$), 9.1 µM Mn ($MnSO_4 \cdot 4H_2O$), 46.3 µM B (H_3BO_3), 3.1 µM Zn ($ZnSO_4 \cdot 7H_2O$), 0.16 µM Cu ($CuSO_4 \cdot 5H_2O$), 0.05 µM Mo ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$), with 6 µM P ($NaH_2PO_4 \cdot 2H_2O$). After one week pre-culture, plants were transplanted to 56 L vessels with three levels of P concentration (0 µM, 6 µM and 32 µM, respectively) for two weeks. Phosphorus concentration was measured and adjusted to the respective levels of treatment everyday. The pH of the nutrient solution was adjusted to 5.2 ± 0.1 everyday. The nutrient solution was completely renewed once a week. Three plants were pooled for one replication and each experiment was conducted with three replications. A total of three experiments were conducted. Half of the collected plants was dried in 80˚C oven for 3 days and weighed. The remaining half was frozen in liquid nitrogen and stored at –80 ˚C until the analysis of Pi and enzyme activities.

RESULTS

Experiment 1. Effect of low pH and low P in soil

Dry matter production and amount of phosphorus and nitrogen absorbed

Dry weights of three crops were lower at -P treatment than those at +P treatment (Table 1). Dry weights of wheat and rice decreased with decrease of soil pH regardless of P treatments. However, dry weight of *Brachiaria* hybrid at -P treatment were higher at pH 4.5 than those of pH 4.0 and 5.0, adapting well to low pH (4.0) even at low levels of soil P, which was more adaptable than the higher pH (5.0). At +P treatment, dry weight of *Brachiaria* hybrid was not much affected by soil pH. Root-to-shoot ratio of *Brachiaria* hybrid increased by P-deficiency, whereas that of wheat and rice was stable or decreased by P-deficiency (Table 1). This ratio remained constant in *Brachiaria* hybrid and rice regardless of decreasing soil pH, however decreased in wheat by a decrease of soil pH. Thus, relative root growth was vigorous in *Brachiaria* hybrid and rice under low pH, also vigorous in rice under low pH, and weak in wheat under low P and low pH. Consequently, root function of *Brachiaria* hybrid was maintained or stimulated under low pH and low P soils.

Amount of P absorbed by plants was quite similar to dry matter production, indicating that P accumulation and tolerance to low pH were key factors that contributed to plant production in this experiment (Table 2). When relative P absorption ability under acidic low P soil was estimated as (amount of P at -P. pH 4.0/ amount of P at +P. pH 5.0), this ratio

was 0.52 in *Brachiaria,* 0.33 in wheat, 0.47 in rice, suggesting that *Brachiaria* hybrid had greater ability to absorb P from acidic low P soil than other crops.

Amount of nitrogen absorbed by *Brachiaria* hybrid was less affected by P and pH treatments, whereas response of amount of N absorbed to P and pH treatments was almost similar to those of dry matter production and absorption of P by rice and wheat (Table 3). It appeared that root activity of *Brachiaria* hybrid was less affected because of constant ability of nitrogen uptake. However, available P in soil was affected by P treatment and pH, it was assumed that plant growth is affected by P availability. Root activity was severely depressed in wheat by low P and low soil pH and in rice by low pH.

Phosphorus concentration

In *Brachiaria* hybrid P concentration in leaf (mg/g) was 1.1 to 1.3 at –P treatment and 1.6 to 1.9 at +P treatment and in root was 1.0 to 1.1 at –P treatment and 1.0 to 1.4 at +P treatment (Table 4). However in rice and wheat, P concentration was higher, especially in leaf compared with that of the *Brachiaria* hybrid.

Phosphorus use efficiency (PUE)

Phosphorus use efficiency (PUE) was higher in *Brachiaria* hybrid than that of wheat and rice (Table 5). PUE of *Brachiaria* hybrid increased tremendously by P deficient treatment, however, that of rice and wheat was remained constant or slightly increased. In

Brachiaria hybrid, P use efficiencies were remained constant, or decrease slightly with a decrease of soil pH. Thus *Brachiaria* hybrid seems to utilize absorbed P more efficiently compared to wheat and rice especially under low pH condition.

Organic acids concentration in leaves and roots

Fumarate was found as a major organic acid in leaves of *Brachiaria* hybrid regardless of P and pH levels followed by oxalate (Table 6). Oxalate concentrations in leaves of *Brachiaria* hybrid were decreased under P deficient condition. Fumarate was also a major organic acid in wheat leaves followed by malate. A trace amount of oxalate was detected in leaves of wheat. On the other hand, oxalate, α-ketogluterate, malate and citrate were detected in leaves of rice plant. It appeared that phosphorus and pH treatments had less effect on amount of total organic acid anions in rice plants. Total organic acid level was higher in *Brachiaria* hybrid than in rice and wheat, indicating that in *Brachiaria* hybrid organic acid metabolism and its pool was active and large.

PEPC activity in leaves

PEPC maximum activity in leaves was extremely high in *Brachiaria* hybrid because of its C_4 photosynthetic pathway (Table 7). PEPC maximum activity was higher in –P treatment than in +P treatment, and did not respond to soil pH. PEPC maximum activity of rice and wheat was slightly higher in -P treatment than in +P treatment.

Malate inhibition ratio of PEPC in *Brachiaria* hybrid was lower in –P treatment than in +P treatment, especially at lower soil pH (Table 7). This ratio in wheat did not respond to P and pH treatment, and in rice did not respond to P treatment, but increased with decrease of soil pH.

Experiment 2. PEPC response to low phosphorus hydroponic medium

Phosphorus concentration

Phosphorus concentration in leaf (mg/g) and root of both *Brachiaria* hybrid and rice was extremely low with -P treatment (Table 8). Phosphorus concentration in *Brachiaria* hybrid leaf was 0.67 at –P treatment and 8.33 at +P treatment. Thus in nutrient culture, P treatment was extremely low at –P treatment and high at +P treatment, comparing with soil culture experiment (Table 4).

Organic acid anion concentration in leaf

In *Brachiaria* hybrid, oxalate and fumarate were dominant organic acids, and these two decreased by –P treatment (Table 9). In rice, oxalate, α-ketogluterate, malate, and citrate were main organic acids. However, P response was different between oxalate, α-ketogluterate (decreasing by -P treatment) and malate, citrate (increasing by –P treatment).

PEPC activity and malate inhibition ratio

By -P treatment, PEPC activity of *Brachiaria* hybrid decreased in leaf, and increased in roots (Table 10). Also by –P treatment, PEPC activity of rice remained almost constant in both leaf and roots.

Malate inhibition ratio of PEPC in leaf decreased in *Brachiaria* hybrid, but remained constant in rice under –P treatment (Table 10). This ratio was higher in rice than in *Brachiaria* hybrid, indicating that PEPC of rice was mostly inactive.

DISCUSSION

Brachiaria species are adapted to low-fertility acid soil of the tropics because they are highly tolerant to high aluminum and low phosphorus, low calcium.[43] In P deficient condition, they may improve P acquisition by enhancing its root growth, uptake efficiency and ability to utilize poorly available P to plants. It was expected that *Brachiaria* species adapted to low P medium by two strategies; 1) efficient P-uptake, and 2) efficient P utilization in tissue. From acidic low P soil, *Brachiaria* hybrid had relatively high ability to absorb P from soil compared to rice and wheat (estimated from Table 2). This was owing to high root activity in *Brachiaria* than the other two crops (estimated from nitrogen uptake in Table 3). It appeared that in *Brachiaria* hybrid under low P conditions (both soil and hydroponics culture), 1) PEPC activity increased in both leaves and roots, 2) malate

inhibition ratio in leaves decreased, and 3) organic acid levels decreased. Therefore, it is highly probable that high P uptake depends on high PEPC activity and high rate in organic acid metabolism resulting in exudation of organic acids from roots to solubilize relatively less available P in soil.

On the other hand, P use efficiency (PUE) of *Brachiaria* hybrid was extremely higher in -P treatment than +P treatment, comparing with wheat and rice (Table 5). The PUE of *Brachiaria* hybrid was significantly augmented in response to P-deficiency, which was maximum (0.88) at pH 4.5. Therefore, *Brachiaria* hybrid has an excellent P utilization mechanism in tissue once P is absorbed. Higher P use efficiency in *Brachiaria* under low P supplying and high acidic soils was found by earlier investigators.[39,42-43] A high P use efficiency is advantageous in low P acid soils, because the plant can then maximize the amount of biomass produced per unit P and thus dominate use of the resources available.[5,42]

To explain one of the mechanisms of high P use efficiency, it has been suggested that P deficiency induces some glycolytic enzymes.[48] Phospho*enol*pyruvate carboxylase (PEPC) and phospho*enol*pyruvate phosphatase (PEPP) that catalyze bypass reaction of pyruvate kinase (PK), responsible for the regulation of carbon flow from glycolysis to TCA cycle,[21] are induced under P deficiency. This induction is supposed to play an important role in organic acid metabolism and Pi recycling under P deficient condition.[49] In P deficient bean leaves, the increased rate of malate synthesis and enhanced accumulation of aspartate and alanine, the products of PEP metabolism, were observed.[49] It was suggested that the increased activity of PEPC and the utilization of PEP to amino acids synthesis might be the

most important response for phosphate recycling in bean leaves at the early stage of P deficiency. Similar increases in PEPC activity in response to P-deficiency have been noted in the cluster roots of white lupin [20] in chickpea (*Cicer arietinum*), oilseed rape, and *Sesbania rostrata*.[5,27,50] Also, enhanced expression and activity PEPC has been linked with P-deficiency-induced biosynthesis and root exudation of carboxylic acids.[27-30]

Under P-deficient conditions, the PEPC reaction, which liberates oxaloacetate and Pi, may have a function for Pi recycling in PEP catabolism as a bypass for the ADP- and Pi-depended pyruvate kinase.[48] In general, however, C_4-PEPC in C_4 cycle should not contribute to Pi recycling and large amounts of organic acids production because C_4 cycle substrates are recycling in C_4 cycle. In the present experiment, PEPC activity in leaves of *Brachiaria* hybrid, C_4 plant, was increased up to 3 folds in response to P-deficiency (Table 7). It appeared that higher PEPC activity might be related to higher PUE in *Brachiaria* hybrid. In current study, we could not estimate whether C_4- or C_3-PEPC of *Brachiaria* hybrid had a function in responding to P deficiency, suggesting the need for further research to define the precise role of PEPC of *Brachiaria* in adaptation to low P acid soils.

In summary, in *Brachiaria* under acidic low P soil 1) P uptake remained high because of high relative P absorption ability and high root activity (estimated from high nitrogen absorption rate), and 2) PUE was significantly high which was appeared to associate with higher PEPC activity and lower malate inhibition ratio in leaves. Thus, PEPC activity contributed greatly to P uptake/and PUE of *Brachiaria* hybrid and less contributed to rice and wheat (low P use efficient crops) under low P and pH conditions. Consequently, total organic acid level in *Brachiaria* leaf was lower in -P treatment than in +P treatment

indicating that organic acids were metabolized actively by increased PEPC activity and decreased malate inhibition ratio in leaves. Taken together, these results suggest that PEPC activated in *Brachiaria* hybrid under low P and pH conditions may contribute to its greater adaptation to tropical acid soils with low phosphorus availability.

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Table 1. Effect of phosphorus and pH treatments on dry matter production (g/plant).

Plant	Organ	-P			+P		
		pH 4.0	pH 4.5	pH 5.0	pH 4.0	pH 4.5	pH 5.0
Brachiaria hybrid	Shoot	0.19±0.03	0.27±0.02	0.20±0.02	0.27±0.03	0.33±0.02	0.32±0.02
	Root	0.06±0.02	0.09±0.01	0.06±0.01	0.08±0.02	0.10±0.01	0.09±0.02
	Total	0.25±0.07c	0.36±0.03b	0.26±0.04c	0.35±0.04b	0.43±0.04a	0.41±0.04a
	Root-to shoot ratio	0.32	0.33	0.30	0.30	0.30	0.28
Wheat	Shoot	0.03±0.01	0.03±0.00	0.04±0.01	0.02±0.00	0.04±0.00	0.07±0.01
	Root	0.01±0.00	0.02±0.01	0.03±0.01	0.01±0.00	0.03±0.01	0.05±0.02
	Total	0.04±0.01d	0.05±0.01c	0.07±0.02b	0.03±0.01e	0.07±0.01b	0.12±0.03a
	Root-to shoot ratio	0.33	0.66	0.75	0.50	0.75	0.71
Rice							
	Shoot	2.14±0.25	2.11±0.24	2.44±0.28	2.39±0.27	2.25±0.26	2.81±0.32
	Root	0.41±0.09	0.41±0.09	0.45±0.10	0.40±0.09	0.47±0.11	0.49±0.11
	Total	2.55±0.12c	2.52±0.05c	2.89±0.13b	2.79±0.05bc	2.72±0.14bc	3.30±0.11a
	Root-to shoot ratio	0.19	0.19	0.20	0.17	0.21	0.17

Note: Values are the mean of three replicates. Treatment means are different ($P<0.05$) if denoted by different letters (Duncan's multiple range test).

Table 2. Effect of phosphorus and pH treatments on total amount of phosphorus (mg P/plant) accumulated.

Name of plant		-P			+P		
	Organ	pH 4.0	pH 4.5	pH 5.0	pH 4.0	pH 4.5	pH 5.0
Brachiaria hibrid	Shoot	0.27±0.02	0.32±0.01	0.27±0.01	0.58±0.01	0.69±0.02	0.52±0.02
	Root	0.06±0.01	0.08±0.00	0.06±0.01	0.11±0.01	0.15±0.01	0.11±0.04
	Total	0.33±0.05c	0.38±0.01c	0.33±0.04c	0.69±0.02b	0.84±0.03a	0.63±0.07b
Wheat	Shoot	0.05±0.01	0.06±0.01	0.09±0.01	0.06±0.01	0.11±0.01	0.13±0.01
	Root	0.03±0.01	0.04±0.00	0.04±0.03	0.03±0.01	0.06±0.01	0.11±0.01
	Total	0.08±0.02c	0.11±0.00c	0.13±0.02bc	0.09±0.02c	0.17±0.01b	0.24±0.01a
Rice	Shoot	4.68±0.16	4.94±0.10	5.58±0.17	8.21±0.09	8.45±0.27	10.32±0.24
	Root	0.58±0.06	0.45±0.03	0.67±0.04	0.78±0.01	1.10±0.05	0.96±0.08
	Total	5.26±0.36c	5.39±0.16c	6.25±0.29c	8.99±0.09b	9.55±0.55b	11.28±0.32a

Note:Values are the mean of three replicates. Treatment means are different ($P<0.05$) if denoted by different letters (Duncan's multiple range test).

Table 3. Effect of phosphorus and pH treatments on total amount of nitrogen (mg N/plant) accumulated.

Name of plant		-P			+P		
	Organ	pH 4.0	pH 4.5	pH 5.0	pH 4.0	pH 4.5	pH 5.0
Brachiaria hibrid	Shoot	4.73±0.28	3.86±0.06	4.46±0.25	4.46±0.14	4.36±0.10	4.37±0.19
	Root	0.78±0.16	0.82±0.06	0.62±0.14	0.74±0.06	1.05±0.01	0.66±0.04
	Total	5.50±0.42a	4.68±0.04a	4.62±0.32a	5.20±0.18a	5.41±0.08a	5.04±0.29a
Wheat	Shoot	0.78±0.07	1.53±0.04	1.90±0.07	0.86±0.04	2.19±0.09	2.76±0.09
	Root	0.32±0.11	0.73±0.01	0.85±0.08	0.39±0.03	0.95±0.09	1.31±0.12
	Total	1.10±0.14d	2.26±0.03c	2.75±0.09bc	1.25±0.17d	3.14±0.13b	4.07±0.18a
Rice	Shoot	73.70±2.70	83.81±1.51	98.91±1.78	90.73±0.63	85.80±2.48	107.44±0.54
	Root	8.72±1.14	8.13±0.01	9.74±0.22	8.51±0.25	9.91±0.57	9.17±0.21
	Total	82.42±6.22d	91.94±3.69cd	108.65±3.64ab	99.24±0.82bc	95.71±5.09c	116.61±1.52a

Note:Values are the mean of three replicates. Treatment means are different ($P<0.05$) if denoted by different letters (Duncan's multiple range test).

***Table 4*.** Effect of phosphorus and pH treatments on the phosphorus concentration (mg g^{-1}) in plant tissues..

		-P			+P		
Organ	Plant	pH 4.0	pH 4.5	pH 5.0	pH 4.(	pH 4.5	pH 5.0
Leaf	*Brachiaria* hybrid	1.28±0.05c	1.14±0.01d	1.21±0.04cd	1.89±0.03a	1.93±0.01a	1.58±0.03b
	Wheat	2.11±0.01b	1.55±0.01d	2.28±0.73b	2.74±0.37a	2.26±0.02b	1.79±0.24cd
	Rice (*Oryza sativa*)	2.24±0.03c	2.19±0.04cd	2.14±0.02d	2.92±0.03b	3.10±0.07a	3.10±0.06a
Stem	*Brachiaria* hybrid	1.44±0.01b	1.28±0.03b	1.56±0.00b	2.29±0.05a	2.33±0.02a	1.52±0.13b
	Wheat	3.75±0.06b	3.11±0.12c	2.51±0.08d	5.27±0.03a	3.62±0.03b	3.21±0.31c
	Rice (*Oryza sativa*)	2.18±0.02d	2.38±0.03c	2.22±0.13d	3.76±0.12b	4.08±0.08a	3.94±0.01a
Root	*Brachiaria* hybrid	1.06±0.01a	0.98±0.02a	1.06±0.05a	1.35±0.06a	1.39±0.02a	1.03±0.43a
	Wheat	3.23±0.02ab	2.08±0.01d	1.67±0.01e	3.33±0.06a	2.25±0.01c	3.10±0.01b
	Rice (*Oryza sativa*)	1.42±0.03c	1.09±0.05d	1.43±0.12c	2.02±0.03b	2.29±0.01a	1.93±0.05b

Note: Values are the mean of three replicates. Different letters in each organ in a species under various P and pH treatments differ significantly ($P<0.05$) if denoted by different letters (Duncan's multiple range test). .

Table 5 . Phosphorus use efficiency*of whole plants grown in soil with phosphorus and pH treatments.

Plant	-P			+P		
	pH 4.0	pH 4.5	pH 5.0	pH 4.0	pH 4.5	pH 5.0
Brachiaria hybrid	0.79±0.04ab	0.88±0.05a	0.79±0.01ab	0.53±0.01c	0.52±0.01c	0.72±0.07b
Wheat	0.36±0.01b	0.49±0.01 a	0.49±0.05a	0.30±0.02c	0.40±0.01b	0.39±0.01b
Rice	0.48±0.01a	0.47±0.01a	0.48±0.01a	0.31±0.01b	0.29±0.01c	0.30±0.01bc

Note: *Phosphorus use efficiency = Total dry weight (g/plant) / Total amount of phosphorus uptake (mg/plant).
Values are the means of three replications ±SE. Different letters in each species indicate statistically significant ($P<0.05$) by Duncan`s multiple range test .

Table 6. Organic acid concentration (μ mol/g leaf dry weight) in leaves of plants grown in soil with phosphorus and pH treatments.

Plant	Treatments							
	P	pH	oxalate	fumarate	α-ketoglutarate	malate	citrate	total
Brachiaria hybrid	-P	4.0	12±0.1b	159±7.2b	n.d.	n.d.	n.d.	171±3.7
		4.5	9±0.1d	106±0.7d	n.d.	n.d.	n.d.	115±0.4
		5.0	10±0.3cd	121±1.9c	n.d.	n.d.	n.d.	131±1.1
	+P	4.0	20±1.2a	156±2.1b	n.d.	n.d.	n.d.	176±1.6
		4.5	11±0.2bc	146±0.5b	n.d.	n.d.	n.d.	157±0.4
		5.0	11±0.1bc	248±2.a	n.d.	n.d.	n.d.	259±1.4
Wheat	-P	4.0	2±0.0c	35±0.2e	n.d.	3±0.1e	n.d.	40±0.2
		4.5	3±0.1b	69±0.1c	n.d.	8±0.7ab	n.d.	80±0.3
		5.0	4±0.2a	109±2.1a	n.d.	8±0.3ab	n.d.	121±0.9
	+P	4.0	0.9±0.1d	28±0.9f	n.d.	5±0.9d	n.d.	34±0.6
		4.5	3±0.1b	48±0.2d	n.d.	9±0.2a	n.d.	60±0.2
		5.0	3±0.1b	94±0.7b	n.d.	6±0.3cd	n.d.	103±0.4
Rice	-P	4.0	38±1.2c	n.d.	25±0.3a	23±0.3ab	15±3.6ab	101±3.7
		4.5	39±2.9bc	n.d.	6±0.1c	24±5.3ab	7±1.3b	76±0.4
		5.0	42±1.5ab	n.d.	5±0.3c	28±5.6a	13±1.1ab	88±1.1
	+P	4.0	42±1.0ab	n.d.	5±1.3c	17±2.1bc	13±4.0ab	77±1.6
		4.5	44±1.3a	n.d.	12±0.5b	11±0.4c	16±0.1a	83±0.4
		5.0	39±0.6bc	n.d.	4±0.1c	20±1.3abc	7±0.4b	70±1.4

Note : n.d. = not detected. Values are the means of three replications ±SE. Different letters in each organic acid in each crop species under various P and pH treatments differ significantly.Treatment means are different ($P<0.05$) if denoted by different letters (Duncan's multiple range test).

Table 7 . PEPC activity and malate inhibition ratio of PEPC in leaves of plants grown in soil with phosphorus and pH treatment.

Plant	Treatments		Malate inhibition ratio*	PEPC activity
	Phosphorus	pH		(μ mol/mg protein/ min)
Brachiaria hybrid	-P	4.0	0.33±0.02	8.46±0.09b
		4.5	0.24±0.06	9.15±0.15a
		5.0	0.58±0.01	9.51±0.23a
	+P	4.0	0.54±0.07	4.40±0.07c
		4.5	0.49±0.04	5.32±0.11d
		5.0	0.43±0.06	5.80±0.11c
Wheat	-P	4.0	0.59±0.02	0.13±0.01b
		4.5	0.61±0.04	0.12±0.02c
		5.0	0.65±0.02	0.15±0.01a
	+P	4.0	0.48±0.05	0.09±0.01d
		4.5	0.68±0.01	0.04±0.00f
		5.0	0.54±0.02	0.05±0.00e
Rice	-P	4.0	0.72±0.09	0.11±0.01b
		4.5	0.69±0.01	0.16±0.02a
		5.0	0.49±0.06	0.10±0.01c
	+P	4.0	0.76±0.01	0.09±0.01d
		4.5	0.44±0.02	0.12±0.02b
		5.0	0.40±0.01	0.10±0.01c

Note: Values are the means of three replications ±SE. Different letters in each species indicate statistically significant ($P<0.05$) by Duncan's test.
*Malate inhibition ratio = [(- malate PEPC activity)-(+ malate PEPC activity)] / [(- malate PEPC activity)].

Table 8 . Phosphorus concentrations (mg g^{-1} DW) in plants grown in hydroponics with different phosphorus treatment.

Plant	Phosphorus	Phosphorus concentration (mg/g DW)		
	Treatment	Leaf	Root	Whole plant
Brachiaria hybrid	-P	0.67±0.01b	1.09±0.06b	0.84±0.03b
	+P	8.33±0.12a	7.77±0.06a	8.20±0.08a
Rice	-P	0.73±0.01b	0.70±0.01b	0.72±0.00b
	+P	6.95±0.15a	6.34±0.09a	6.83±0.14a

Note : Values are the means of three replications ±SE. Different letters in each organ in each crop species under P treatments differ significantly.Treatment means are different ($P<0.05$) if denoted by different letters (Duncan's multiple range test).

Table 9 . Concentrations of (μ mol/g DW) of organic acids in leaves of plants grown in hydroponics with different phosphorus treatments.

Plant	P treatment	oxalate	fumarate	α-ketogluterate	malate	citrate	Total
Brachiaria hybrid	-P	10.5±1.1b	90.4±8.0b	n.d.	n.d.	n.d.	100.9
	+P	27.4±5.6a	134.0±16.3a	n.d.	n.d.	n.d.	161.4
Rice	-P	14.1±0.30b	n.d.	9.5±1.20b	15.3±0.10a	3.98±0.40a	42.9
	+P	69.6±6.80a	n.d.	18.7±1.20a	3.97±0.9b	2.94±0.80b	95.2

Note : n.d. : not detected.Values are the means of three replications ±SE. Different letters in each organic acid in each crop species under P treatments differ significantly.Treatment means are different ($P<0.05$) if denoted by different letters (Duncan's multiple range test).

***Table 10*.** PEPC activity (μ mol/min/mg protein) and malate inhibition ratio in leaves of plants grown hydroponical in different phosphorus treatment

Plant	P treatment	Malate inhibition ratio* in lea	PEPC activity	
			Leaves	roots
Brachiaria hyb	-P	0.49±0.03	2.01±0.20b	0.31±0.02a
	+P	0.62±0.01	3.11±0.27a	0.18±0.02b
Rice	-P	0.84±0.02	0.06±0.01a	0.21±0.02a
	+P	0.89±0.01	0.05±0.00b	0.18±0.01b

Note: Values are the means of three replications ±SE. Different letters in each organ in each crop species under P treatments differ significantly. Treatment means are different ($P<0.05$) if denoted by different letters (Duncan`s multiple range test).

*Malate inhibition ratio = [(- malate PEPC activity)-(+ malate PEPC activity)] / [(- malate PEPC activity)]