Title: Low Phosphorus Tolerance Mechanisms: Phosphorus Recycling and Photosynthate Partitioning in the Tropical Forage Grass, *Brachiaria* Hybrid cultivar Mulato Compared with Rice

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Abbreviations: APase, acid phosphatase; P, phosphorus; PEPC, phospho*enol*pyruvate carboxylase; PEPP, phospho*enol*pyruvate phosphatase; Pi, inorganic phosphate; PK, pyruvate kinase; PPT, phospho*enol*pyruvate transporterl RNase, ribonuclease;

The *Brachiaria* hybrid cv. Mulato is well adapted to low-fertility acid soils deficient in phosphorus (P). To study the grassy forage's mechanisms for tolerating low P supply, we compared it with rice (*Oryza sativa* L. cv. Kitaake). We tested by using nutrient solution cultures, and quantified the effects of P deficiency on the enzymatic activities of phosphohydrolases and on carbon metabolism in P-deficient leaves. While P deficiency markedly induced activity of phosphohydrolases in both crops, the ratio of inorganic phosphorus to total P in leaves was greater in *Brachiaria* hybrid. Phosphorus deficiency in leaves also markedly influenced the partitioning of carbon in both crops. In the *Brachiaria* hybrid, compared with rice, the smaller proportion of ^{14}C partitioned into sugars and the larger proportion into amino acids and organic acids in leaves coincided with decreased levels of sucrose and starch. Hence, in P-deficient leaves of the *Brachiaria* hybrid, triose-P was metabolized into amino acids or organic acids. Results thus indicate that the *Brachiaria* hybrid, compared with rice, tolerates low P supply to leaves by enhancing sugar catabolism and by inducing the activity of several phosphohydrolases. This apparently causes rapid P turnover and enables the *Brachiaria* hybrid to use P more efficiently.

Keywords: *Brachiaria* hybrid - Low P tolerance - Phosphohydrolase - Photosynthate distribution - Rice (*Oryza sativa* L.)

Introduction

A major constraint to agriculture on tropical and subtropical soils is phosphorus (P) deficiency (Fairhust et al., 1999), affecting more than 2 billion hectares. Applying large amounts of fertilizer to correct P deficiency is not feasible for most resource-poor farmers in developing countries. Agricultural productivity thus becomes limited to extensive livestock raising on native or introduced pastures. For sustainable P management in agriculture, we need more information on the mechanisms involved in making plants more P-use efficient (Vance et al., 2003).

To overcome P deficiency, plants develop several strategies, including the well-known one of secreting acid phosphatase (APase), ribonuclease (RNase), and organic acids into the rhizosphere to improve P availability in the soil (Tadano et al., 1993; Duff et al., 1994; Green 1994; Jones 1998; Rao et al., 1999). Another strategy of P-deficient plants is to use P efficiently within the cell. Phosphohydrolases may function as a P-recycling mechanism in plants. RNase is probably involved in the overall turnover of RNA (Löffler et al., 1992). Induction of RNase under P deficiency occurs in cultured tomato cells and *Arabidopsis* spp. (Nürnberger et al., 1990; Löffler et al., 1992; Bariola et al., 1994) and the role of APase in efficient use of P in the cell has also been studied by Yan et al. (2001) and Yun and Kaepeller (2001). Although the role of inducing phosphohydrolase during P deficiency has been widely discussed, no specific view has yet been accepted.

Phosphorus deficiency also affects carbon metabolism, and the short- and long-term effects of P deprivation on photosynthesis are well documented (Rao, 1996). Effects of P deficiency were similar in C_3 (sunflower and wheat) and C_4 (maize) species (Jacob and Lawlor, 1991). In Pi withdrawal experiments of the range of C_3, C_{3-4} intermediate, C_4 annual and perennial monocotyledons and dicotyledons species, it was shown that C_3 and C_4 species had similar photosynthetic P use efficiency but the growth of C_3 species was more affected by Pi supply than C_4 species, moreover leaf photosynthetic rates were not correlated with growth response (Halsted and Lynch, 1996). These results indicated that relative growth rate decreased before any significant effect on photosynthesis. It is possible that the P recycling mechanism might be operating differently between C_3 and C_4 crops. P deficiency affects the partitioning and transport of photosynthates, although these differ among species as, for example, the partitioning of carbon between starch and sucrose. P deficiency causes accumulation of starch and decrease in sucrose in soybean leaves, whereas sucrose, glucose, and starch increase in sugarbeet leaves (Qiu and Israel, 1994; Rao and Terry, 1995).

Pieters et al. (2001) showed that, under P deficiency, the source:sink ratio also has a great effect on carbon export from source leaves. That is, P deficiency restricts carbon export from source leaves by more than four times, compared with P-sufficient plants. Decreasing the source:sink ratio by reducing light for sink leaves prevented a decrease in carbon export, and light-reduced P-deficient plants maintained carbon export at a value of more than double that in fully illuminated P-deficient plants. Increased carbon allocation to roots under P deficiency was observed in monocots, especially C_4 monocots such as *Brachiaria* (Halsted and Lynch, 1996).

From the study of the effect of low P on carbohydrate distribution into chemical compounds, Theodorou and Plaxton (1993) showed that P deficiency induces some glycolytic enzymes, such as phospho*enol*pyruvate carboxylase (PEPC) and phospho*enol*pyruvate phosphatase (PEPP). These catalyze the bypass reaction of pyruvate kinase (PK), which is responsible for regulating carbon flow from glycolysis to the TCA cycle. This induction may play an important role in carbon metabolism under P-deficient conditions. PEPC replenishes intermediates of the TCA cycle, and may help regulate both carbon-nitrogen metabolism, and P recycling under P deficiency.

Kondracka and Rychter (1997) observed that, in P-deficient bean leaves, the rate of malate synthesis increases, and the accumulation of aspartate and alanine (products of PEP metabolism) is enhanced. In early stages of P deficiency, the increased activity of PEPC and use of PEP in amino acid synthesis are probably the most important reactions for P recycling in bean leaves during photosynthesis. Thus, PEP metabolism by PEPC and PEPP, or PEP transport via PPT (phospho*enol*pyruvate transporter; which transports PEP from the cytosol into chloroplasts in leaves) may affect carbon distribution under P deficiency.

Brachiaria species are the most widely planted tropical forage grasses in the world. For example, in Brazil alone, more than 70 million hectares are planted to *Brachiaria* pastures. *Brachiaria* species are well adapted to low-fertility acid soils of the tropics because they are highly tolerant of high aluminum, low P, and low calcium (Rao et al., 1995, 1996; Wenzl et al., 2002). Under P-deficient conditions, the grasses improve their P acquisition by enhancing root growth, uptake efficiency, and ability to use poorly available plant P (Rao, 2001). Although they have much lower internal requirements for P than do other grasses, they show interspecific differences (Rao et al., 1996).

The ongoing *Brachiaria* breeding program at the Centro Internacional de Agricultura Tropical (CIAT), conducted in collaboration with the Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), has generated several promising genetic

recombinants (genetically recombined apomictic or sexual hybrids) (Miles and do Valle, 1996). Field evaluation of these hybrids identified one apomictic hybrid, FM9201/1873 (from *Brachiaria ruziziensis* clone 44-06 and *Brachiaria brizantha* cv. Marandú), as productive when grown on P-deficient, low-fertility, acid soils in both wet and dry seasons (Rao et al., 1998). This hybrid, the world's first commercial *Brachiaria* hybrid, was recently released in Mexico as cv. Mulato, and is being adopted by farmers in the tropics.

Before this study, we had previously evaluated the *Brachiaria* hybrid's tolerance of low P supply, and found that it had very high P-use efficiency (g of biomass produced per mg of P uptake). Our current study aims to determine the hybrid's mechanisms for tolerating low P supply, that is, the physiological basis of its high P-use efficiency. We quantified the effects of P deficiency on phosphohydrolases and carbon metabolism in its leaves, and compared them against the values for the rice (*Oryza sativa* L.) cultivar Kitaake, which is also relatively tolerant of low P and low pH conditions.

Results

Plant growth and nutrient status

Results on changes in total P concentrations in the nutrient solution in each container within 24 h showed that the 3 levels of P treatment 0 μM P, 6 μM P and 32 μM P provided conditions of complete P deprivation, low P supply and adequate P supply, respectively, during the treatment period of 2 weeks. Maximum amount of P depletion of up to 85% to 100% was observed at 2 weeks after treatment with 6 μM P while it was only up to 40% with 32 μM P treatment.

Although plant growth decreased with P deficiency, the difference between the treatments 6 μM P and 32 μM P was not significant (Table 1). The total dry weight (DW) of the *Brachiaria* hybrid decreased by 33% in the 0 μM P treatment, and lower P supply had greater effect on leaf and stem growth than on root growth. Rice also decreased its growth with P deficiency, but its growth in the 0 μM P treatment was greater than that of the *Brachiaria* hybrid. Root to shoot ratio increased with P deficiency in both species (Table 1).

However, P-use efficiency (the inverse of P concentration) was greater in the *Brachiaria* hybrid (1.85 g DW per 1 mg P) than in rice (1.61 g DW per 1 mg P) (Table 2). Thus, in the 0 μM P treatment, the *Brachiaria* hybrid showed greater tolerance of low concentrations of tissue P, compared with rice. In contrast, P-use efficiency in the 32 μM P treatment was greater in rice than in the *Brachiaria* hybrid. Apparently, the

mechanisms of P-use efficiency differ between the two test crops. Although P-use efficiency was different, total dry weight in the 32 μM P treatment was similar for the two test crops, indicating that both crop plants would serve as good models for understanding how different plant mechanisms function in relation to P nutrition.

Nitrogen concentration was stable in the *Brachiaria* hybrid, regardless of P treatment, whereas it decreased markedly in rice with P deficiency (Table 2). Phosphorus concentration decreased markedly in both test crops with P deficiency (Table 2), which was lower at 0 μM P treatment, especially in leaves of the *Brachiaria* hybrid.

Inorganic P (P_i) concentration, expressed on the basis of dry weight also decreased markedly with P deficiency in both test crops (Table 3), with P_i concentration being higher in leaves than in roots. P_i concentration in stems was higher in rice than in the *Brachiaria* hybrid, although when P was supplied at 6 μM P or 32 μM P, P-use efficiency was similar for both the *Brachiaria* hybrid and rice.

Phosphohydrolase activity

We measured APase and RNase activities as P-recycling enzymes to discover whether they have any role in increasing the efficiency of acquired P in the plant. APase and RNase activities were both induced by P deficiency. The *Brachiaria* hybrid had higher APase activity, especially in shoots, than did rice. Specific activity of APase in leaves was 5.2 to 7.7 times higher in the *Brachiaria* hybrid (Fig. 1A) than in rice (Fig. 1B) and, in stems, 2.7 to 3.5 times higher. APase activity in roots was noticeably induced in both test crops when they were grown without P.

RNase activity in leaves and stem was also higher in the *Brachiaria* hybrid than in rice (Figs 1C and 1D), while being slightly lower in roots. RNase activities in *Brachiaria* hybrid leaves were 4.4 to 5.8 times higher than in rice leaves and 1.7 to 5.4 times higher in stems.

Organic acid concentration in leaves

Total organic acid concentration was higher in the *Brachiaria* hybrid than in rice (Fig. 2). With P deficiency, organic acid concentration dropped by 37% in the *Brachiaria* hybrid and by 55% in rice. Results on organic acid composition showed that oxalate and fumarate were the main organic acids in the *Brachiaria* hybrid, whereas oxalate concentration was high in rice only in the 32 μM P treatment. In rice, the marked decrease in oxalate was accompanied by increased malate and citrate concentrations in leaves.

Carbohydrates in leaves

The concentrations of glucose, fructose, and sucrose dropped with P deficiency in the *Brachiaria* hybrid (Figs. 3A, 3B, 3C, respectively). The concentration of glucose was also dropped with P deficiency in rice, while it was more prominent in *Brachiaria* hybrid. Starch (Fig. 3D), glucose, and fructose concentrations were higher in the *Brachiaria* hybrid than in rice, with sucrose concentration being higher in rice (Fig. 3C). The level of starch decreased in the *Brachiaria* hybrid with P deficiency while it increased in rice.

Enzyme activities catalyzing the PEP-consuming reaction

The three enzymes that catalyze the PEP-consuming reaction in glycolysis are PEPC, PEPP, and PK. Phospho*enol*pyruvate carboxylase (PEPC) activity was greater in the *Brachiaria* hybrid than in rice (Table 4). In rice, it decreased by a factor of 2.4 with P deficiency, whereas it did not change in the *Brachiaria* hybrid.

In both test crops, phospho*enol*pyruvate phosphatase (PEPP) activity was induced by P deficiency: 5.6 times in the *Brachiaria* hybrid and 6.0 times in rice, although in leaves, it was higher in the *Brachiaria* hybrid than in rice.

Pyruvate kinase (PK) activity in rice was 1.6 times higher in the 0 μM P than in the 32 μM P treatment. Although PK activity in the *Brachiaria* hybrid was also higher in the 0 μM P treatment, it was not significant.

^{14}C partitioning

In both test crops, photosynthetically fixed carbon pools were mainly distributed to sugars, and the ^{14}C distribution ratio to sugars increased with P deficiency (Fig. 4). The effect of P deficiency on ^{14}C partitioning to sugars was larger in rice than in the *Brachiaria* hybrid. The distribution ratio to sugars was 68% in rice and 50% in the *Brachiaria* hybid in the 0 μM P treatment. The ^{14}C distribution ratio to the amino acid and organic acid pools was greater in the *Brachiaria* hybrid than in rice, and slightly increased with P deficiency in the *Brachiaria* hybrid. The ^{14}C distribution ratio to phosphate esters markedly decreased with P deficiency in both test crops. The ^{14}C distribution ratio to the residue fraction, which is supposed to contain protein, starch, and nonstructural carbohydrate pools, increased with P deficiency in both test crops.

Discussion

Brachiaria species are well adapted to the low-fertility acid soils of the tropics because they are highly tolerant of high aluminum and low supplies of P and calcium (Rao et al., 1995, 1996; Wenzl et al., 2001). They have much lower internal requirements for P than other grasses because they are able not only to acquire P with their extensive root systems but also to use the acquired P more efficiently for growth and metabolism. However, mechanisms of P-use efficiency are relatively less known in plants, especially in the *Brachiaria* hybrid that we have examined. Since carbon metabolism is known to be affected by the P status in plant tissue (Rao, 1996), we studied low-P-tolerance mechanisms, in terms of P recycling and carbon metabolism, in the *Brachiaria* hybrid, comparing them with those of rice.

When P supply in the culture was low, root:shoot ratio increased, especially in the *Brachiaria* hybrid. We found that, for the *Brachiaria* hybrid, vigorous root growth is a mechanism for acquiring larger amounts of P from low P supplies. Lower P concentration in *Brachiaria* hybrid leaves may indicate that the hybrid uses P more efficiently to sustain active metabolism for dry matter production.

In our study, the higher P_i proportion to total P was observed especially in the leaf of the *Brachiaria* hybrid (Table 3), compared with that of rice. This coincided with lower total P concentrations. We speculate that recycling of internal organic P compounds could be an important mechanism of contributing to the greater P-use efficiency in the *Brachiaria* hybrid.

Though the nitrogen concentration of each organ in rice decreased with lower P supply, it was rather stable or even increased (in the case of stem) in *Brachiaria* hybrid (Table 2). Since nitrogen supply is known to improve plant productivity, it is suspected that maintaining higher nitrogen level in plant organ is also one of the important features of the *Brachiaria* hybrid to tolerate low P condition.

The function of phosphohydrolases in phosphorus recycling

Bosse and Köck (1998) have shown APase and RNase to be induced during P deficiency, and that this induction is associated with P turnover in plants. In our study, APase and RNase activities were both strongly induced in both test crops by P deficiency (Fig. 1). Induction of APase activity was markedly higher in roots under P-deficient conditions.

Duff et al. (1994) reported the existence of extracellular APase in roots, where it is localized mainly in apical meristems and outer and surface cells. It is involved in hydrolyzing and mobilizing P_i from organic phosphates in the soil for plant nutrition. The induction of APase in roots may also be associated with excretion. Bosse and Köck

(1998) suggested that the increase in activity of phosphohydrolases was a specific response to the decline of cellular available P_i in P-starved tomato seedlings. Although P_i in roots was lower than in leaves and stem of both test crops (Table 3), it was impossible to account for the difference of APase induction between roots, and leaves and stems only by the difference of intracellular P_i concentration.

APase activity in shoots was greater in the *Brachiaria* hybrid than in rice, indicating the possibility of rapid P turnover in the *Brachiaria* hybrid. While this may enable the *Brachiaria* hybrid to survive under low P conditions, it is possible that APase may not be a major mechanism for scavenging or acquiring P because differences in APase induction could not sufficiently account for the diverse growth response of genotypes of bean plants and maize under P deficiency (Yan et al., 2001; Yun and Kaepfler., 2001). However, we observed in our study that APase activity was induced by P deficiency and that it correlated with P-use efficiency, as indicated by the lower value of total P concentration, so that the function of APase in adaptation to low-P conditions should not be underestimated at least in *Brachiaria* hybrid and rice.

RNase activity was also high in roots under P-deficient conditions (Fig. 1). Nürnberger et al. (1990) and Löffler et al. (1992) showed that extracellular and intracellular RNase were induced in tomato-cell culture under P deficiency. Extracellular RNase could help degrade the RNA from senescing cells that have been either damaged or lysed, and also help degrade any RNA that might be present in the rhizosphere. Thus, the high RNase activity in roots may be associated with secretion similar to APase.

RNase activity in shoots was also greater in the *Brachiaria* hybrid than in rice, indicating that RNase also contributes to rapid P turnover. Glund et al. (1990) showed that, in the relationship between P_i concentration and RNase activity, induction under phosphate starvation occurs while the intracellular content of phosphate is very high.

In contrast, Bosse and Köck (1998) and Köck et al. (1998) suggested that a sensing mechanism exists within the cell, which induces activation of transcriptional processes and thus unlocks the system that is normally blocked by the presence of P_i. RNase induction in both the *Brachiaria* hybrid and rice was associated with a decrease in intracellular P_i concentration (Fig. 1 and Table 3), indicating the existence of a sensing mechanism in the cell. Further research work is needed to determine the dynamics of levels of Pi and other P compounds together with changes in enzyme activities and gene expression under low P deficiency. RNA turnover can occur in the vacuole because of the localization of RNase, and that the uptake of RNA into the vacuole may occur through autophagy (Matile, 1978). If such a mechanism works under

P deficiency, RNase induction can contribute to rapid RNA degradation in the vacuole.

Carbon partitioning in phosphorus-deficient plants

Phosophohydrolases were induced by P deficiency as a P-recycling system. Coinciding with such a mechanism, carbon metabolism could also be altered under P deficiency. We therefore studied photosynthate partitioning under P deficiency, tracing photosynthetically fixed ^{14}C in leaves. In rice, photosynthates mainly distributed to sugars, which consist of sucrose, indicating that rice enhanced the sucrose synthesis pathway (Fig. 4). Hence, sucrose concentration in rice leaves was remarkably high (Fig. 3). The ^{14}C distribution ratio to sugars increased with P deficiency. However, as the ^{14}C distribution ratio to amino acids and organic acids decreased (Figs. 3, 4), it is assumed that sucrose catabolism was restricted with P deficiency. Thus Pi releasing pathway during sucrose synthesis from primary photosynthate (triose phosphate) may, therefore, not contribute efficiently to P recycling. However, the ^{14}C distribution ratio to sugars in the *Brachiaria* hybrid was not as marked as in rice (Fig. 4), and the effect of P deficiency was smaller.

The decrease of total organic acids and carbohydrates in *Brachiaria* hybrid leaves under P deficiency suggests that the *Brachiaria* hybrid can sustain active amino acid and organic acid pathways with enhanced sugar catabolism, using P efficiently under P deficiency.

PK and its bypassing enzymes catalyze the PEP-consuming reaction in leaves, with PEPP activity increasing by a factor of 5.6 to 6.0 with P deficiency (Table 4). This induction of PEPP is likely to be associated with P recycling, as Duff et al. (1989) suggest. Since the value of PEPP was 3 to 4 times higher in *Brachiaria* hybrid than in rice, we speculated that more active role for PEPP is expected in *Brachiaria* hybrid under P deficiency. PK was also induced by P deficiency, but not significantly so in the *Brachiaria* hybrid (Table 4). PEPC activity was slightly induced by P deficiency in rice but not in the *Brachiaria* hybrid (Table 4). Kondracka and Rychter (1997) suggest that facilitating the PEP metabolism may be important in P recycling process. PEPC and PEPP are considered to function in P recycling as PK-bypass pathways by reducing the usage of P molecule and maintain the carbon flow to TCA cycle. If these enzyme activities are induced in P recycling, then the carbon flow to the TCA cycle is expected to increase.

The ^{14}C distribution ratio to amino acids and organic acids increased slightly in the *Brachiaria* hybrid with P deficiency (Fig. 4), indicating that these bypassing enzymes may function to facilitate carbon flow to the TCA cycle. However, in rice, the

^{14}C distribution ratio to amino acids and organic acids decreased with P deficiency. Therefore, the PK bypassing mechanism under P deficiency may not contribute to facilitating the carbon flow to the TCA cycle in rice.

In addition to the PK-bypassing mechanism, carbon export from chloroplast to cytosol via the triose-phosphate translocator (TPT) may be a process that significantly affects carbon partitioning under P deficiency. When plants are starved for P, triose-P exports from chloroplast to cytosol via TPT, and subsequent sucrose synthesis in the cytosol is likely to be restricted (Rao, 1996). The ^{14}C distribution ratio to sugars, which mainly consists of sucrose, increased with P deficiency in both crops (Fig. 4), indicating that restriction of triose-P exports from chloroplast to cytosol via TPT may not occur. Other forms of carbon export system from chloroplast to cytosol such as glucose transport (Häusler et al., 1998) and glycolate transport (Kondracka and Rychter, 1997) may also be related to carbon partitioning. These aspects and the importance of cellular differentiation and communication (bundle sheath vs mesophyll cells) in C_4 plants in P recycling processes under P deficiency need further research.

Conclusions

Our study shows that tolerance of low P in the *Brachiaria* hybrid and rice involved marked differences in P recycling and carbon metabolism. For the *Brachiaria* hybrid, low-P tolerance involved two major strategies: (1) increasing the ability to use P efficiently by inducing APase and RNase in shoots with P deficiency; and (2) enhancing sugar catabolism and subsequent synthesis of amino acids and organic acids in leaves under P deficiency.

For rice, strategies for low-P tolerance differed by involving (1) decreased carbon flow to amino acids and organic acids, and decreased N concentration; and (2) improved partitioning of photosynthates to sucrose, combined with restricted sugar catabolism.

Materials and Methods

Plant culture

Brachiaria hybrid cv. Mulato and rice (*Oryza sativa* L. cv. Kitaake) were grown hydroponically under greenhouse conditions (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}).

Seedlings were pre-cultured in a 56-L vessel, containing a nutrient solution made up of 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 1 week in pre-culture, 5 plants were transplanted to 56-L vessels containing nutrient solutions, each with one of three levels of P concentration (0 μM, 6 μM, and 32 μM) for 2 weeks. The experimental design was a randomized complete block with 3 replicates. *Brachiaria* hybrid and rice were cultured in different containers. Phosphorus concentration was adjusted every day, as was the pH (5.2 ± 0.1). P depletion in each treatment was monitored daily to make sure that transient P depletion is not a major problem during plant growth. The P levels selected represent P deprived, low P and adequate P conditions for plant growth. The nutrient solution was completely renewed once a week. Three plants were pooled for one replicate, and each experiment conducted with three replicates (obtained from three different containers). Half of the collected plants were dried in an oven at 105˚C for 3 days and weighed. The other half was frozen in liquid nitrogen and stored at -80˚C until analysis of P_i and enzyme activities.

Nitrogen and phosphorus concentration

Dried samples were digested with sulfuric acid and hydrogen peroxide (Mizuno and Minami, 1980). Total N was measured by the semi-micro Kjeldahl method, and P by the vanado-molybdate yellow method, as described by Watanabe et al. (1998a).

Inorganic phosphate

A frozen sample (about 0.5 g) was homogenized in 1 mL of 10% (w/v) PCA, using an ice-cold mortar and pestle. The homogenate was then diluted 10 times with 5% (w/v) PCA and placed on ice for 30 min. After centrifugation at 10,000 *g* for 10 min at 4˚C, the supernatant was used to measure the P_i, using the molybdate-blue method described above.

Enzyme extraction

For the APase and RNase analyses, a frozen leaf sample (about 0.5 g) was homogenized in 2 mL of 100 mm sodium acetate buffer (pH 5.6), using a cold mortar and pestle. After centrifuging at 17,000 g for 20 min at 4˚C, the supernatant was collected as a crude extract and stored at -80˚C until analysis.

For the PEPC, PEPP, and PK analyses, a frozen leaf sample (about 0.5 g) was homogenized in 2 mL of extraction buffer containing 100 mm Tris-HCl pH 7.5, 5% (w/v) ethyleneglycol, 5 mm sodium phosphate, 50 mm NaF, 10 mm EDTA, 14 mm 2-mercaptoethanol, 2 mm benzamidine-HCl, 1 mm PMSF, 10 μm leupeptin, 16 μm chymostatin, 1 μm microcystin-LR, and 5% (w/v) PVPP. After centrifuging at 17,000 g for 5 min at 4 ˚C, the supernatant was collected as a crude extract and stored at -80˚C until analysis.

Enzyme activity

APase activity was measured according to Ozawa et al. (1995) and RNase activity to Bosse and Köck (1998). To measure PEPC, the crude extract was centrifuged at 17,000 *g* for 10 min at 4˚C, and the supernatant was used. PEPC activity was assayed in a solution containing 100 mM Hepes-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.2 mM NADH, 12 units of MDH, 5 mM G6P, 4 mM PEP, and crude extract. The reaction was started by adding PEP, and absorbance at 340 nm monitored.

PEPP and PK activity was measured in the same way as for PEPC activity, except for the composition of the reaction buffers, which was as follows: (1) buffer for PEPP = 50 mM sodium acetate (pH 5.6), 5 mM $MgCl_2$, 0.2 mM NADH, 2 mM PEP; and (2) buffer for PK = 100 mM Mes-Tris (pH 6.2), 10 mM $MgSO_4$, 40 mM KCl, 0.2 mM NADH, 2 mM ADP, 5 mM DTT, 0.2 mM ammonium molybdate, and 2 mM PEP.

Soluble protein was measured by the Bradford method (Bradford, 1976), using BSA as a standard.

Organic acids in leaves

A lyophilized leaf sample (about 100 mg) was used to extract organic acids, after agitating with 5 mL of 0.01 N HCl for 1 h. After centrifuging at 3000 *g* for 10 min, the supernatant was collected and then filtered, using a 45-μm membrane filter. The organic acids were then analyzed, using capillary electrophoresis (Quanta 4000 CE, Waters, USA; Watanabe et al., 1998b).

Carbohydrates in leaves

A lyophilized leaf sample (about 50 mg) was incubated with 80% (v/v) ethanol

at 40˚C for 17.5 h to extract glucose, fructose, and sucrose. The ethanol extract was filtered through filter paper and concentrated to dryness, using a rotary-evaporator. It was then solubilized with water, and the following were added: 1.25 mL of 85 mm $K_4[Fe(CN)_6]$, 1.25 mL of 250 mm $ZnSO_4$, and 2.5 mL of 0.1 m NaOH. The sample was filtered through filter paper to remove precipitated protein. The solution was made up to its final volume of 25 mL by water.

For starch analysis, a lyophilized leaf sample (about 50 mg) was incubated with 5 mL of DMSO and 1.25 mL of 8 N HCl at 60˚C for 30 min. After cooling to room temperature, 10 mL of water was added and adjusted to pH 4.5 with NaOH. The solution was made up to its final volume of 25 mL by water.

Carbohydrate contents were measured with an F-kit purchased from Roche Diagnostics Corporation.

^{14}C labeling experiment

For the experiment, we used plants grown under either the 0 μm P or the 32 μm P treatment for 2 weeks after pre-culture. $^{14}CO_2$ was generated by adding 30% PCA into $NaH^{14}CO_2$ (18.5 kBq). $^{14}CO_2$ was fed to the plants for 5 min in a vinyl package under natural lighting (photon flux density was between 1000 and 1200 μmol m^{-2} s^{-1}). Only shoot tissue was collected and frozen immediately in liquid nitrogen. The sample was lyophilized and stored at -20˚C until analysis. A lyophilized sample (about 100 mg) was ground in 10 mL of a solution (methanol:chloroform:water:formic acid = 12:5:5:1) with a cold mortar and pestle to remove chlorophyll and lipids. It was centrifuged at 16,000 *g* for 15 min at 4˚C, the supernatant collected, and 2.5 mL of chloroform and 3.5 mL of water added. The whole supernatant was agitated for 1 min, followed by centrifuging for 15 min. The upper layer was collected as extract A. Then 4 mL of water was added to lower layer, and again agitated for 1 min, and centrifuged again. The upper layer was collected as extract B.

Solution B (99% formic acid:methanol = 1:10) was then added to the residue collected from the first centrifuging, and the whole centrifuged again for 10 min. After centrifuging, the supernatant was collected as extract C and the residue as residue A. Extracts A, B, and C were mixed and concentrated, using a rotary-evaporator at 40˚C, and further concentrated to dryness with a centrifugal concentrator. The lyophilized sample was resuspended in 1 mL of water and centrifuged to remove insoluble deposits. The supernatant was collected, and the procedure repeated. Both supernatants were used for the column fractionation experiment, and the residue collected as residue B.

The sample solution was embedded into a column of SP Sephadex C-25 and 50

mL of water was applied to the column. The column was previously equilibrated, using 0.5 M ammonium sulfate and 7% formate. The eluate was embedded into a column of QAE Sephadex A-25, which was equilibrated with 0.5 M sodium formate, and 50 mL of water subsequently applied to the column. The eluate was then collected as a sugar fraction.

To collect an amino acid fraction, we applied 50 mL of 0.2 N ammonia water to the SP Sephadex C-25 column and collected the eluate. Likewise, for an organic acid fraction, we applied 40 mL of 4% formic acid to the QAE Sephadex A-25 column and collected the eluate. By applying 30 mL of pyridine-formic acid buffer we collected the eluate as a phosphate ester fraction.

All of the fractions were concentrated, using a rotary-evaporator at 40˚C, then making up their volumes to 10 mL by water. From each sample, 1 mL was mixed with toluene scintillator (DPO 4 g, POPOP 200 mg, and nonion 300 mL, made up to 1 liter with toluene) and placed in darkness overnight. Radioactivity was measured, using a liquid scintillation counter (Aloka, Liquid Scintillation Counter, LSC-5100).

Residues A and B were mixed and dried in an oven at 105˚C, and their total weight measured. After adding 0.5 mL of NCS-II to 10 mg of the residues, the solution was incubated at 50˚C for 30 min. Then, 0.5 mL of benzoyl peroxide (0.2 mg mL^{-1} toluene) was added, and incubated at 50˚C for 30 min, followed by adding 0.5 mL of hydrogen peroxide, and the whole incubated at 50˚C for 30 min. After mixing with toluene scintillator (DPO 4 g, POPOP 200 mg, cabosyl 15 g, and nonion 300 mL, and the whole made up to 1 liter with toluene), it was placed in darkness for the night and radioactivity measured.

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Figure Legends

Figure 1. APase (A, B) and RNase (C, D) activities of a *Brachiaria* hybrid (A, C) and rice (B, D). Plants were grown at three different levels of P for 2 weeks. APase and RNase activities were measured in leaves (black bars), stem (hatched bars), and roots (white bars). APase activities were assayed in sodium acetate buffer (pH 5.6), using *p*-nitrophenylphosphate as substrate. One unit of APase activity was defined as the activity that liberated 1 μmol of *p*-nitrophenylphosphate per minute. RNase activities were assayed in sodium acetate buffer (pH 5.6), using 250 μg of yeast RNA as the substrate. One unit of RNase activity was defined as the activity that liberated the amount of soluble nucleotide corresponding to one unit of nucleotide per minute. One unit is the amount of nucleotide that has an A_{260} of 1.0 in a volume of 1.0 mL, according to Wilson (1975). Values are the means ± SE of three replicates.

Figure 2. Organic acid concentration in *Brachiaria* hybrid and rice leaves. Plants were grown with or without P for 2 weeks. Organic acid content was measured, using capillary electrophoresis. We identified each peak of an organic acid by comparing the peaks with standards. Contents were determined by comparing the peak area of the sample with that of the corresponding standard. Values are the means ± SE of three replicates.

Figure 3. Carbohydrate concentration in *Brachiaria* hybrid and rice leaves: (A) glucose, (B) fructose, (C) sucrose, and (D) starch concentrations. Plants used for the assays were grown with P (white bars) or without P (hatched bars) for 2 weeks. Carbohydrate concentration was determined by enzymatic analyses. Values are the means ± SE of three replicates.

Figure 4. Distribution of photosynthetically assimilated ^{14}C in the *Brachiaria* hybrid and rice. $^{14}CO_2$ (18.5 kBq) was fed to P-deficient or P-sufficient plants under natural lighting. After $^{14}CO_2$ was fed for 5 min, only shoots were harvested and used for assays. ^{14}C assimilated in shoots was fractionated into amino acids, organic acids, phosphate esters, sugars, and residue, using the ion-exchange columns. The residual fraction is considered to consist of protein, starch, and constituents of cell walls such as cellulose, hemicellulose, and lignin. Values are the means ± SE of three replicates.

Table 1 Dry weight (DW) among plant parts of a *Brachiaria* hybrid and rice grown at three different levels of phosphorus (P) supply

Species	Treatment (μM P)	DW (g per plant)				Root/Shoot ratio
		Leaf	Stem	Root	Total plant	
Brachiaria hybrid	0	0.425 [a] ± 0.102	0.248 [a] ± 0.008	0.438 [a] ± 0.021	1.11 [a] ± 0.035	0.651 [a] ± 0.025
	6	0.707 [b] ± 0.054	0.395 [b] ± 0.027	0.539 [a] ± 0.082	1.64 [ab] ± 0.159	0.484 [b] ± 0.045
	32	0.799 [b] ± 0.102	0.439 [b] ± 0.062	0.449 [a] ± 0.059	1.69 [b] ± 0.223	0.363 [c] ± 0.005
Rice	0	0.400 [a] ± 0.017	0.583 [a] ± 0.049	0.365 [a] ± 0.009	1.35 [a] ± 0.072	0.373 [a] ± 0.019
	6	0.487 [b] ± 0.016	0.645 [a] ± 0.021	0.403 [a] ± 0.003	1.53 [a] ± 0.035	0.357 [a] ± 0.014
	32	0.542 [b] ± 0.030	0.700 [a] ± 0.048	0.363 [a] ± 0.027	1.61 [a] ± 0.104	0.292 [b] ± 0.004

Values are the means ± SE of three replicates. Means followed by the same letter within the column are not significantly different, according to Duncan's multiple range test ($P \leq 0.05$).

Table2 Nitrogen and P contents in different plant parts of a *Brachiaria* hybrid and rice grown at three different levels of P supply

Nutrient	Species	Treatment (μM P)	Concentration (mg per g DW)				P-use efficiency (g DW per mg P)
			Leaf	Stem	Root	Total plant	
Nitrogen	*Brachiaria* hybrid	0	44.7 ^{ab} ± 0.62	43.3 ^{b} ± 0.82	32.0 ^{ab} ± 0.57	39.6 ^{a} ± 2.13	
		6	42.0 ^{a} ± 1.19	37.3 ^{a} ± 0.59	29.2 ^{a} ± 0.72	38.6 ^{a} ± 3.84	
		32	45.2 ^{b} ± 0.69	38.3 ^{a} ± 0.63	33.1 ^{b} ± 1.07	40.2 ^{a} ± 0.29	
	Rice	0	33.4 ^{a} ± 0.79	15.1 ^{a} ± 0.58	18.0 ^{a} ± 0.57	21.3 ^{a} ± 0.66	
		6	44.9 ^{b} ± 0.55	24.6 ^{b} ± 0.26	23.9 ^{b} ± 0.42	30.8 ^{b} ± 0.39	
		32	50.5 ^{c} ± 0.26	30.7 ^{b} ± 0.41	29.6 ^{c} ± 0.40	37.1 ^{c} ± 0.14	
Phosphorus	*Brachiaria* hybrid	0	0.44 ^{a} ± 0.01	0.62 ^{a} ± 0.06	0.58 ^{a} ± 0.06	0.54 ^{a} ± 0.03	1.85 ^{a} ± 0.10
		6	1.69 ^{b} ± 0.07	1.94 ^{b} ± 0.01	1.78 ^{b} ± 0.08	1.78 ^{b} ± 0.02	0.56 ^{b} ± 0.01
		32	11.0 ^{c} ± 0.33	6.92 ^{c} ± 0.28	8.35 ^{c} ± 0.57	9.26 ^{c} ± 0.37	0.11 ^{c} ± 0.00
	Rice	0	0.74 ^{a} ± 0.01	0.57 ^{a} ± 0.03	0.56 ^{a} ± 0.05	0.62 ^{a} ± 0.03	1.61 ^{a} ± 0.09
		6	2.03 ^{b} ± 0.02	1.83 ^{b} ± 0.13	1.78 ^{b} ± 0.09	1.88 ^{b} ± 0.07	0.53 ^{b} ± 0.02
		32	6.29 ^{c} ± 0.08	7.00 ^{c} ± 0.16	5.40 ^{c} ± 0.09	6.40 ^{c} ± 0.12	0.16 ^{c} ± 0.00

Values are the means ± SE of three replicates. Means followed by the same letter within the column are not significantly different, according to Duncan's multiple range test ($P \leq 0.05$).

Table 3 Contents of P_i and P_i:total P ratio in different plant parts of a *Brachiaria* hybrid and rice grown at three levels of P supply

P_i parameter	Species	Treatment (μM P)	Leaf	Stem	Root	Total plant
P_i concentration (mg per g DW)	*Brachiaria* hybrid	0	0.13 [a] ± 0.01	0.08 [a] ± 0.00	0.06 [a] ± 0.00	0.09[a] ± 0.00
		6	0.73 [a] ± 0.06	0.22 [a] ± 0.02	0.30 [b] ± 0.02	0.47 [a] ± 0.02
		32	7.43 [b] ± 0.63	3.48 [b] ± 0.18	4.63 [c] ± 0.05	5.66[b] ± 0.35
	Rice	0	0.13 [a] ± 0.01	0.08 [a] ± 0.01	0.05 [a] ± 0.01	0.08[a] ± 0.01
		6	0.57 [b] ± 0.06	0.24 [a] ± 0.02	0.31 [a] ± 0.03	0.36[b] ± 0.03
		32	3.61 [c] ± 0.12	4.05 [b] ± 0.16	3.04 [b] ± 0.39	3.67[c] ± 0.12
P_i:total P ratio (%)	*Brachiaria* hybrid	0	29.6 [a] ± 1.19	14.1 [a] ± 2.24	11.3 [a] ± 1.88	17.5[a] ± 0.85
		6	43.5 [b] ± 3.31	11.1 [a] ± 1.15	16.7 [a] ± 1.05	26.2[b] ± 0.81
		32	67.1 [c] ± 4.38	50.2 [b] ± 0.93	56.0 [b] ± 4.45	61.1[c] ± 2.96
	Rice	0	17.0 [a] ± 1.88	13.7 [a] ± 0.61	8.5 [a] ± 0.79	13.6[a] ± 0.66
		6	27.8 [b] ± 2.75	13.3 [a] ± 1.84	17.3 [a] ± 1.91	19.1[b] ± 1.05
		32	57.4 [c] ± 2.55	57.8 [b] ± 1.96	56.2 [b] ± 6.23	57.4[c] ± 1.35

Values are the means ± SE of three replicates. Means followed by the same letter within the column are not significantly different, according to Duncan's multiple range test ($P \leq 0.05$).

Table 4 Enzyme activities, which are functioning in PEP metabolism, in leaves of *Brachiaria* hybrid and rice

Species	Treatment	Activity (μmole min^{-1} mg^{-1} protein)		
	(μM P)	PEPC	PEPP	PK
Brachiaria hibrid	0	4.39^{a}±0.062	0.218^{a}±0.007	0.052^{a}±0.010
	32	3.79^{a}±0.145	0.039^{b}±0.002	0.038^{a}±0.005
Rice	0	0.04^{a}±0.006	0.060^{a}±0.001	0.087^{a}±0.006
	32	0.09^{b}±0.001	0.010^{b}±0.001	0.053^{b}±0.001

Plants used for the assays were grown with or without phosphorus for 2 weeks. The effect of phosphorus concentration in the medium was determined separately between *Brachiaria hybrid* and rice by t-test ($p \leq 0.05$).

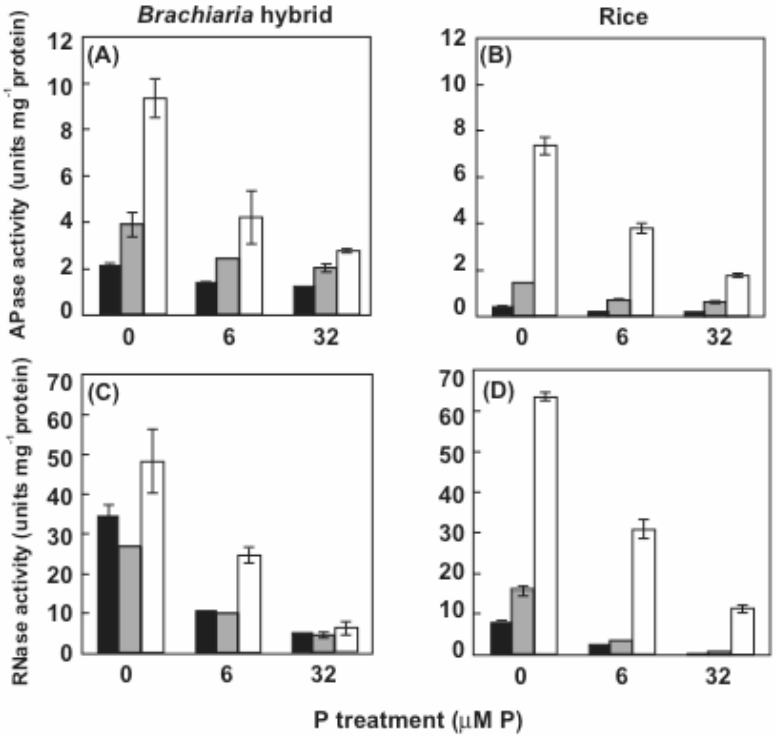
Brachiaria hybrid
Rice
(A)
(B)
(C)
(D)
APase activity (units mg-1 protein)
RNase activity (units mg-1 protein)
0
6
32
P treatment (μM P)

Fig. 1

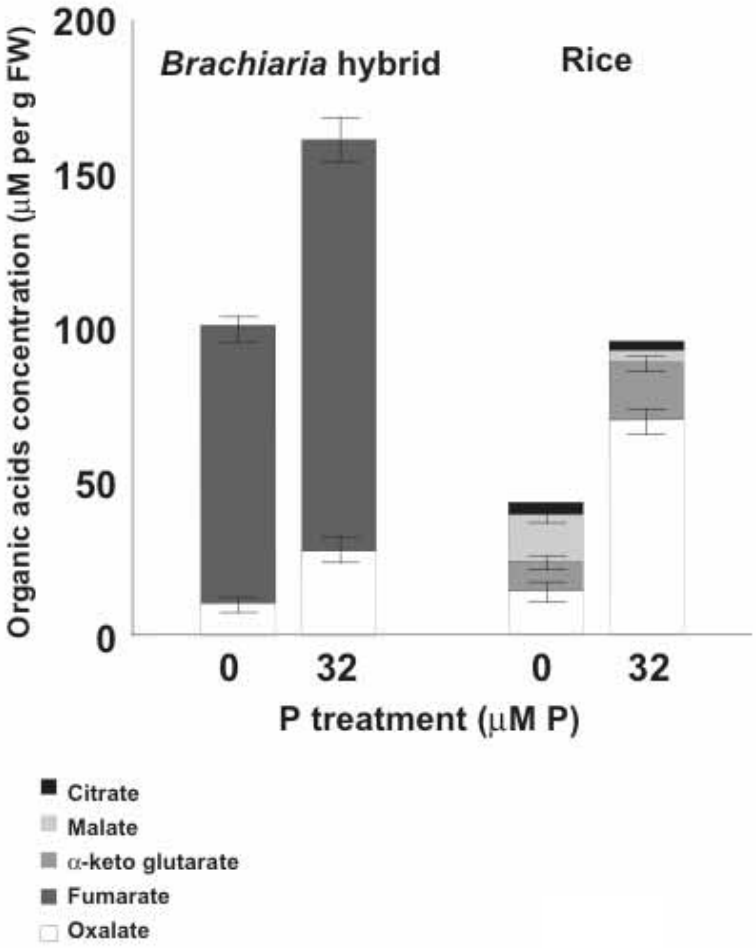
Organic acids concentration (μM per g FW)
200
150
100
50
0
Brachiaria hybrid
Rice
0
32
0
32
P treatment (μM P)
Citrate
Malate
α-keto glutarate
Fumarate
Oxalate

Fig. 2

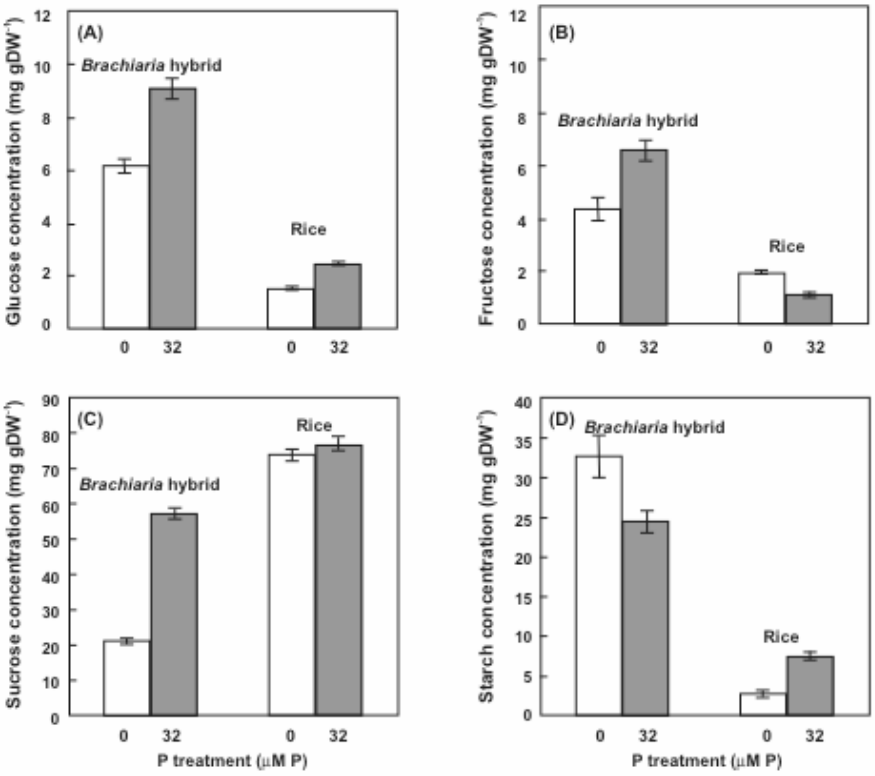
(A)
Brachiaria hybrid
Rice
Glucose concentration (mg gDW-1)
(B)
Brachiaria hybrid
Rice
Fructose concentration (mg gDW-1)
(C)
Brachiaria hybrid
Rice
Sucrose concentration (mg gDW-1)
(D)
Brachiaria hybrid
Rice
Starch concentration (mg gDW-1)
0
32
P treatment (μM P)

Fig. 3

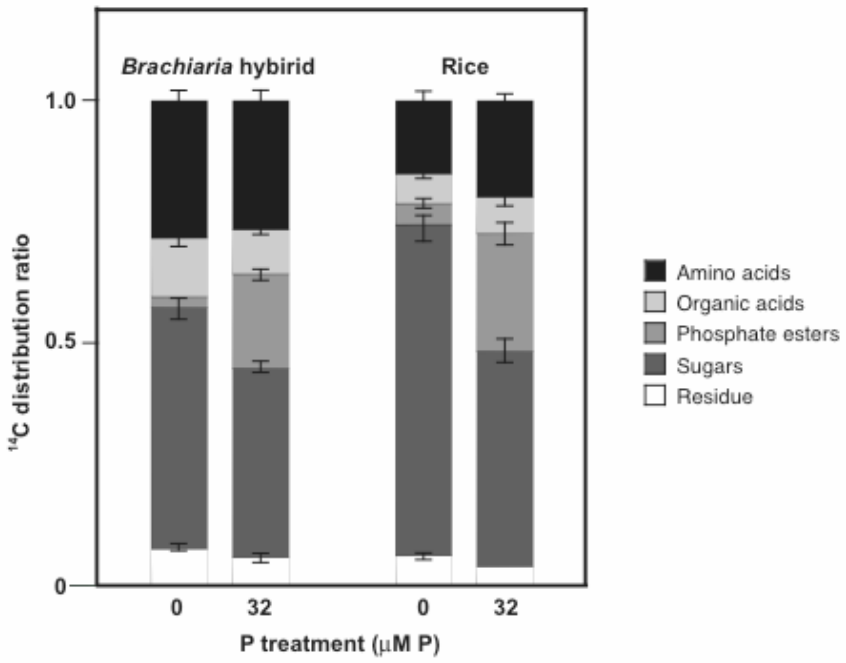
Brachiaria hybirid
Rice
1.0
0.5
0
^{14}C distribution ratio
0
32
0
32
P treatment (μM P)
Amino acids
Organic acids
Phosphate esters
Sugars
Residue

Fig. 4