African Journal of Biotechnology Vol. 7 (20), pp. 3620-3627, 20 October, 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB08.438
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Organic acids in the rhizosphere and root characteristics of soybean (*Glycine max*) and cowpea (*Vigna unguiculata*) in relation to phosphorus uptake in poor savanna soils

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Accepted 2 September, 2008

Root characteristics associated with phosphorus (P) uptake under limiting soil-P conditions were examined in two sets of greenhouse experiments. Average diameter and length of soybean, cowpea, maize and sorghum roots were assessed after 7 weeks in three low-P soils amended with P fertilizer at 0, 3, 6, 11 and 23 mg P/kg. Organic acids in the rhizosphere of soybean, cowpea and pigeon pea were separately evaluated in one soil amended with or without rock phosphate, iron phosphate, aluminium phosphate, calcium phosphate, or triple super phosphate. Unplanted soil served as the control. The growth of soybean, cowpea, maize, and sorghum was significantly improved with P application in all the soils and the amount of P applied played an important role. The shoot dry matter yield and P accumulation correlated significantly with the root length of cowpea and the average diameter of sorghum roots. Citric acid was the only organic acid detected in measurable quantities in the rhizosphere of all plants tested; on average, it varied from 4 (pigeon pea) to 17 (soybean) µmol/g soil. For soybean, the secretion of citric acid appeared important for P acquisition in P-limiting environments whereas for cowpea, the size of the roots may be more important.

Key words: Legumes, low phosphorus availability, rhizosphere, sparingly soluble P.

INTRODUCTION

Plant-available phosphorus (P) is inherently low in most soils of the West African moist savanna, where it is the second most limiting nutrient. Since P is a non-renewable resource, external sources are needed to replenish soil P pools. Socioeconomic constraints hamper the use of inorganic fertilizers by resource-poor farmers. Therefore, the development of management strategies that enhance P availability is vital to improve crop production in the area.

Differences among plant species or genotypes in P acquisition under limiting conditions provide opportunities that can be manipulated to improve P availability. Some crop species adapt to low-P environments through various processes, such as changes in root characteristics (root length, root diameter, root hairs), association with mycorrhiza, and modification of the rhizosphere chemistry. The extent of a root system (including the root hairs) is of the utmost importance in P acquisition, particularly in a limiting environment, since a larger root/ soil contact surface can be achieved (Gahoonia and Nielsen, 2004a, b). The volume of soil explored by the root system of barley genotypes has been shown to depend on the length of the root hairs; genotypes with long root hairs produced higher grain yields under low-P conditions than those with short root hairs (Gahoonia and Nielsen, 2004b). There is considerable evidence that organic acids can enhance P availability in soils (Bolan et

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Table 1. Selected properties of the savanna soils.

Parameter	Soil		
	Danayamaka	**Kasuwan Magani**	**Shika**
Olsen-P (mg/kg)	2.11	1.75	2.49
Resin-P (mg/kg)	0.628	0.931	1.954
K_D (L/kg)	21	28	25
Sand (g/kg)	510	330	470
Silt (g/kg)	300	240	300
Clay (g/kg)	190	430	230
pH $_{(1:2.5,\ water)}$	6.00	5.49	5.92

K_D represents P distribution coefficient (i.e., the ratio of adsorbed P over P in solution) measured at a soil solution concentration of 1 mg P/L.

al., 1994; Jones, 1998; Hocking, 2001; Gahoonia and Nielsen, 2004a). Root-released organic acids may influence P availability by (i) competing for P-binding sites (and thus reduce P adsorption), (ii) increasing the solubility of P compounds, (iii) altering the surface features of soil particles, and (iv) forming complexes with cations bound to P or responsible for holding together organic molecules containing P (Bolan et al., 1994; Hocking, 2001). However, the amount of P solubilized by low-molecular-weight organic acids (LMWOAs) in soils is variable and does not correlate well with soil characteristics that influence P availability (Jones et al., 2003). Therefore, it is necessary to identify and ascertain the probable concentrations of LMWOAs in the rhizosphere of crop species where strategies that focus on organic acid exudation for P mobilization are contemplated. This paper presents the results of studies conducted to evaluate root characteristics and LMWOAs in the rhizosphere of soybean (*Glycine max*) and cowpea (*Vigna unguiculata*) in low-P savanna soils. Soybean and cowpea are important grain legumes with immense potential to improve the nutritional status and welfare of resource-poor farmers. They are commonly intercropped in the area or grown in rotation with cereal crops, such as maize (*Zea mays*) and sorghum (*Sorghum bicolor*).

MATERIALS AND METHODS

Soil

Top soil (0 - 15 cm) was collected from farmers' fields in three villages (Shika, Kasuwan Magani, and Danayamaka) in the northern Guinea savanna of Nigeria, air dried, and sieved (4 mm) to remove large debris. Selected properties of the soils are shown in Table 1. They had low levels of available P and also relatively low P sorption capacity, as seen from their P distribution coefficients. The soils were used for two sets of greenhouse experiments at the International Institute of Tropical Agriculture, Ibadan, Nigeria. The following legume and cereal crops were used: *Glycine max* (soybean, var. TGx 1448-2E), *Vigna unguiculata* (cowpea, var. Dan-ila), *Cajanus cajan* (pigeon pea, var. MO92 ICP 8807), *Zea mays* (maize, var. Oba Super II), and *Sorghum bicolor* (sorghum, var. Kaura, local).

Experiment one: assessment of root characteristics

Soil was weighed (1.5 kg) into plastic pots and mixed thoroughly with powdered triple super phosphate (TSP) at different rates (0, 3, 6, 11 and 23 mg P/kg) to provide varying levels of P in soil solution. A nutrient solution was added to the soil in all pots to provide (mg/kg): 64 N, 28 K, 28 Ca, 14 Mg, 19 S, 0.7 Fe, 0.7 Mn, 0.7 Zn, 0.7 Cu, 0.07 Bo, 0.14 Mo, and 0.07 Co. The soil was brought to 65% field capacity with distilled water. Soil samples were taken from each treatment after 24 h to estimate the quantity of P in soil solution, determined as resin-P (data not shown) after extraction with anion exchange resin in the chloride form (Tiessen and Moir, 1993). Soybean, cowpea, hybrid maize, and sorghum were planted at the rate of four seeds of a crop in each pot (24 h after P addition), and thinned to two after emergence. The cereal crops were included because they are integral components of legume cropping systems in the area. The plants were watered with distilled water as required and allowed to grow for 7 weeks. All treatments had three replications and the pots were arranged on greenhouse benches in a randomized complete block design. The plants were harvested by cutting the shoot at soil level. The roots were separated from the soil by sieving and later washed with tap water. Subsamples were taken for the estimation of arbuscular mycorrhizal fungi (AMF) infection by the method of Giovannetti and Mosse (1980), after the roots had been cleaned and stained as described by Phillips and Hayman (1970). Another set of subsamples was taken to establish other root characteristics by scanning on a flatbed scanner. The acquired images were analyzed for root length and average diameter, using DT-SCAN image analysis software (Delta-T Devices, Cambridge, UK). The plant samples were dried (65°C), weighed, and ground for the determination of total N and P contents (IITA, 1982).

Experiment two: assessment of organic acids in the rhizosphere

Root study containers (RSCs), adapted from Kuchenbuch and Jungk (1982) and Zoysa et al. (1997), were constructed with PVC pipes (internal diameter ≈ 40 mm) (Figure 1) to allow a well defined compartmentalization of the plant/soil interface. Each RSC had two compartments: (i) about 30 mm deep and covered with 25 µm nylon mesh at one end, and (ii) about 50 mm deep, was fitted with a 100 µm nylon mesh at one end. Soil was mixed thoroughly with different P sources of varying solubility at a rate of 21 mg P kg^{-1} soil. These were rock phosphate (RP), iron phosphate (Fe-P), aluminium phosphate (Al-P), calcium phosphate (Ca-P), and TSP. A soil without P addition (zero P) was included. Each RSC was filled with 180 g of dry soil (60 g in the upper compartment and 120 g in the lower

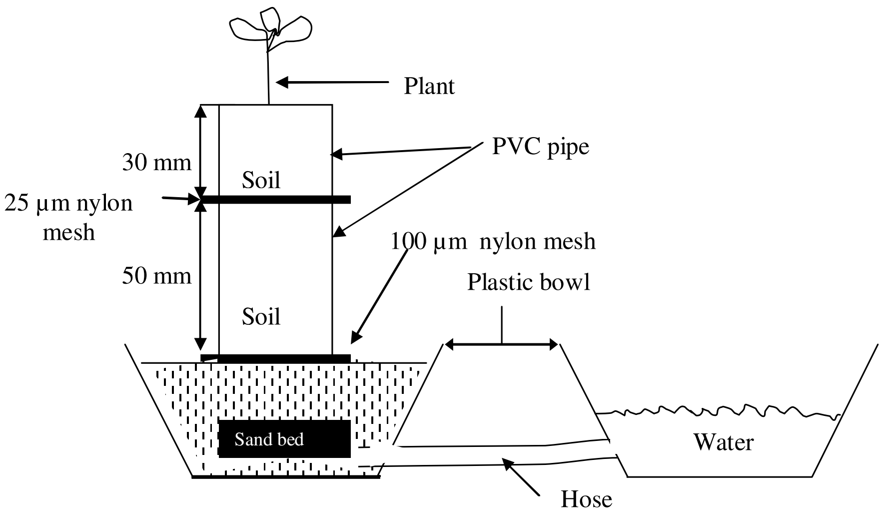

Figure 1. Schematic representation of the root study container.

part). A solution of other nutrient elements (as in experiment one) was applied to the upper compartment. The RSCs were placed on a sand bed connected to a water reservoir (containing distilled water) to keep them moist. Pre-germinated seeds of soybean, cowpea, and pigeon pea were planted in the upper compartments of the RSCs; the control treatments (with and without P addition) were left unplanted. All treatments were replicated three times and arranged in a completely randomized design. The plants received water from the sand bed and were grown for 42 days.

The 25 µm nylon mesh forced the roots to grow horizontally along the mesh, thereby forming a root mat at the interface. The soil below this mesh (i.e., in the lower compartment of the RSCs) is regarded as the rhizosphere. At 42 days after planting, the rhizosphere soil was gradually pushed out of the PVC pipe and sliced successively at 1 mm from the root mat down to 5 mm, and then at 20 mm for the determination of organic acids, pH, and resin-P. The soil slices were stored in a freezer (< –10°C) pending analysis. The organic acids were determined in only the first 1 mm slice of soil by high performance liquid chromato-graphy (HPLC); pH was determined in all slices at a soil: water ratio of 1:2.5, and resin-P was determined as described by Tiessen and Moir (1993).

Organic acid determination

Three millilitres of MQ H_2O were added to 3 g of wet soil in a 15 mL falcon centrifuge tube and shaken for 2 h on a mechanical shaker. The soil slurry was centrifuged (7500 rpm, 20 min) and the supernatant carefully decanted and stored at 5°C in sealed vials pending analysis. For the HPLC analysis, 1mL of the extract was acidified with 100 µL of H_3PO_4 (85% HPLC grade) to remove humic substances and improve peak shape, and filtered through a 0.45 µm membrane. The clear extract (20 µL) was injected into the chromatograph. The mobile phase was 0.025 M KH_2PO_4 (spiked with 85% HPLC-grade H_3PO_4) at pH 2.5; the flow rate was 1.5 mL min^{-1}. A reverse-phase column (Alltima Prevail C18, 5 µm, length 250 mm, and internal diameter 4.6 mm) was used for the separation of the peaks and detection was by UV–VIS spectrophotometer at a wavelength of 210 nm. Standards and calibration curves were prepared for the following organic acids: oxalic ($C_2H_2O_4$), citric ($C_6H_8O_7$), fumaric ($C_4H_4O_4$), malic ($C_4H_6O_5$), formic (CH_2O_2), acetic ($C_2H_4O_2$), lactic ($C_3H_3O_3$), tartaric ($C_4H_6O_6$), succinic ($C_4H_6O_4$), and trans-aconitic ($C_6H_6O_6$). The retention times of observed peaks were compared with those of the standards and the identified organic acids were quantified with the calibration curves. The results were expressed on a dry soil basis.

Statistical analyses

Treatment and interaction effects were analyzed with the GLM procedure of the SAS software (SAS, 1999). The separation of means was done with the PDIFF option. Correlation coefficients were computed with the CORR procedure of SAS to assess the relationships among selected parameters.

RESULTS

Experiment one: plant growth, root characteristics, and AMF infection

Application of P increased significantly the shoot dry matter yield (DMY) of both legumes and cereals in all soils but the magnitude depended on the amount of P applied (Figure 2). A significant soil × P rate interaction ($P < 0.05$) was observed only with soybean and sorghum. Nevertheless, the largest response to P application by all the crops was observed in Shika soil. The amount of P accumulated in the shoot biomass of all the crops also depended on the rate of P application (Figure 3) but no significant interaction between soil and P rate was recorded for cowpea, maize, and sorghum. Hence only treatment means are presented. The shoot DMY and P accumulation correlated significantly with the root length of cowpea but not for soybean or the cereal crops (Table 2). However, a significant relationship existed between shoot DMY and P accumulation of sorghum and the average diameter of the roots. The rate of AMF infection of the roots of both the legume and cereal crops in all the soils was significantly higher at low P supply than at high P (Figure 4), and the relationship between AMF infection

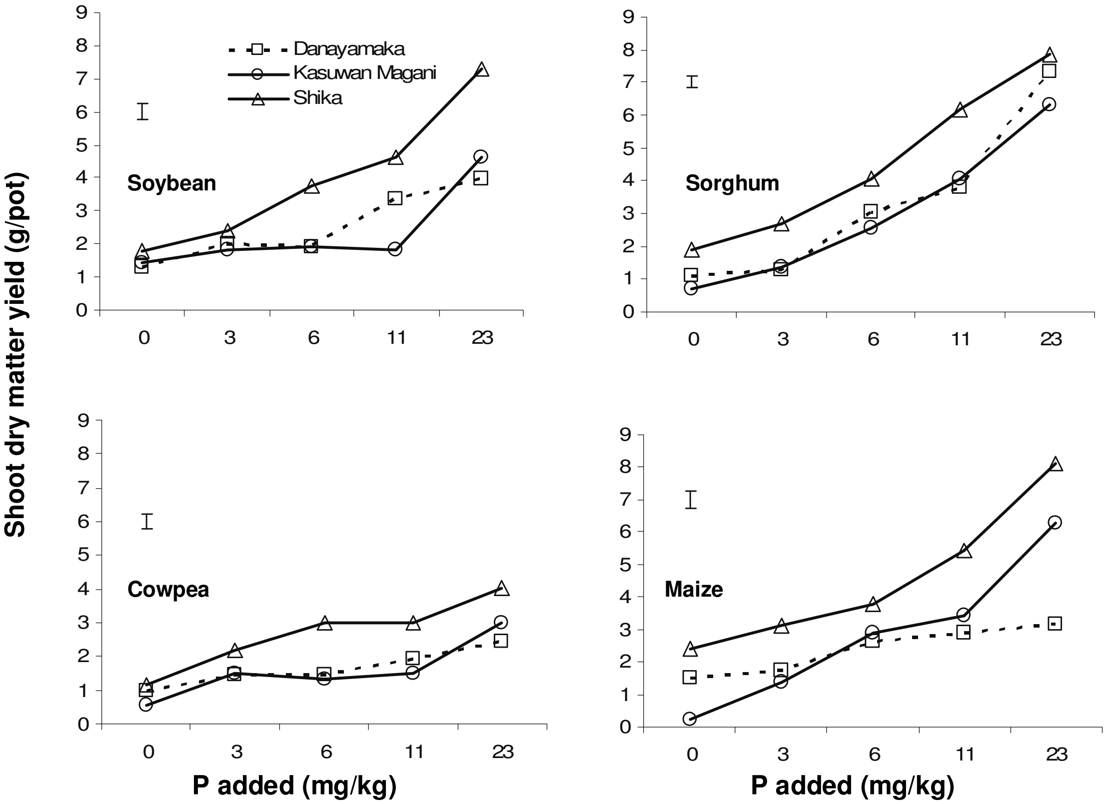

Figure 2. The shoot dry matter yield of soybean, cowpea, sorghum, and maize grown in three soils under greenhouse conditions expressed as a function of the amount of P added. Bars indicate standard error of means.

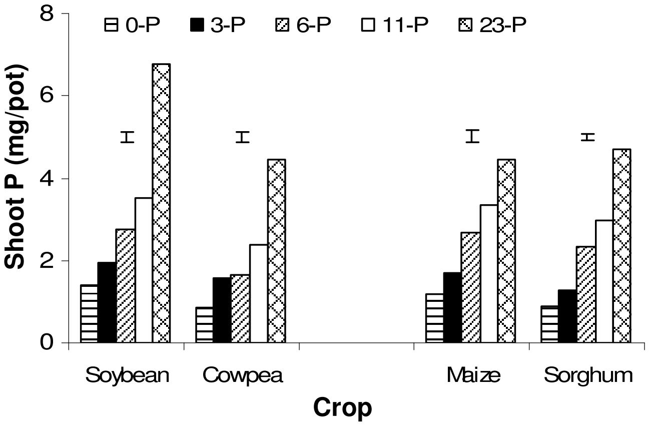

Figure 3. Accumulation of P in the shoot biomass of soybean, cowpea, maize, and sorghum as affected by different rates of P application. Figures in legend refer to the amount of P applied (e.g., 3-P = 3 mg P/kg). Plotted values are means of three soils. Bars indicate standard error of means.

and shoot P accumulation was negative (Table 2). For the legumes, a higher rate of infection occurred in Shika and Kasuwan Magani soils (more obvious for soybean) than in Danayamaka soil.

Experiment two: accumulation of organic acids in the rhizosphere

Citric acid was the major organic acid detected in the rhizosphere of the legume crops. The largest amount (17.48 µmol/g soil, equivalent to a concentration of 44 µM in solution) was obtained in the rhizosphere of soybean while the lowest value (4.06 µmol/g soil) was observed in the rhizosphere of pigeon pea (Figure 5). The amount of citric acid measured in the rhizosphere of cowpea was significantly lower than in soybean and significantly higher than in pigeon pea. In addition, citric acid could not be detected in the control treatments (that is, soil with or without P but without a crop). No significant crop × P source interaction ($P > 0.05$) was observed, hence only average values across P sources and across crop species are shown; a relatively larger amount was obtained with Al-P than with the other sparingly soluble P sources and TSP (Figure 6). Other organic acids were not detected except for traces of malic, acetic, and lactic acids that were observed in some treatments.

A significant interaction occurred between crop and P source for shoot P accumulation ($P = 0.001$). Soybean accumulated different amounts of P from the P sources (Figure 7). Cowpea accumulated more from TSP and lower but similar amounts from all other sources. Shoot P

Table 2. Pearson correlation coefficients (n = 45) for the relationships between selected root-related parameters and shoot P accumulation and dry matter yields of the legume and cereal crops in savanna soils.

Root-related parameter	Shoot P accumulation (mg/pot)	Shoot dry matter (g/pot)	Shoot P accumulation (mg/pot)	Shoot dry matter (g/pot)
	Cowpea		Soybean	
Arbuscular mycorrhizal fungi (%)	-0.467**	-0.481***	-0.606***	-0.639***
Root length (m/pot)	0.688***	0.650***	-0.101	-0.079
Average root diameter (mm/pot)	0.317*	0.452**	-0.025	0.042
	Sorghum		Maize	
Arbuscular mycorrhizal fungi (%)	-0.811***	-0.819***	-0.642***	-0.638***
Root length (m/pot)	0.264	0.289	0.071	0.152
Average root diameter (mm/pot)	0.459**	0.448**	0.243	0.256

'*' Significant at $P < 0.05$; '**' Significant at $P < 0.01$; '***' Significant at $P < 0.001$.

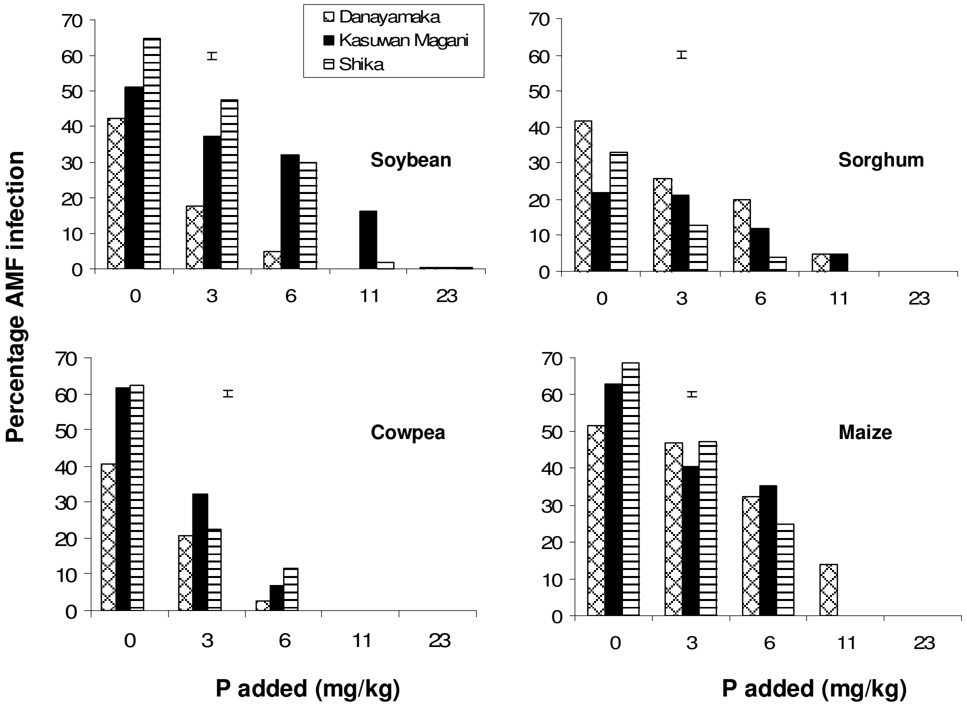

Figure 4. The percentage AMF infection rate of soybean, cowpea, sorghum, and maize roots as influenced by different rates of P addition. Bars indicate standard error of means.

accumulation in soybean increased significantly with the addition of Fe-P, Ca-P, and TSP, but in cowpea only with added TSP. No significant relationship was observed between P accumulation in the shoot biomass of soybean and citric acid measured in its rhizosphere. There was no significant change in soil pH with respect to the distance from the root mat of either soybean or cowpea, irrespective of the P source, but on average, the addition of TSP lowered the pH slightly (data not shown). In general, the concentration of resin-P tended to increase slightly with the distance from the root mat of cowpea or soybean (data not shown). Thus, lower values were measured at 1 mm from the root mat than at 20 mm, indicating that the plants were able to obtain P beyond the root mat, and the rate of solution P depletion at the interface was probably lower than the rate of its replenishment.

DISCUSSION

Root characteristics (such as hair length, diameter and

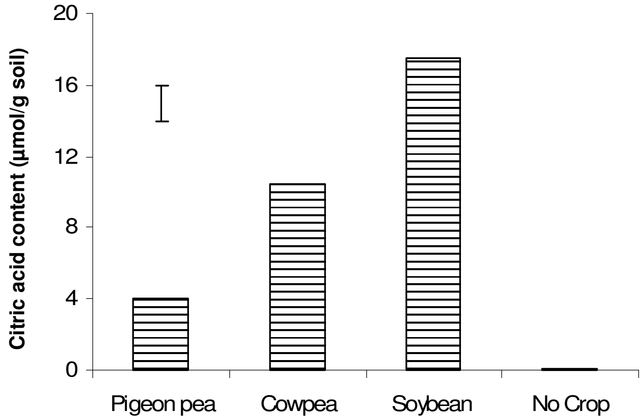

Figure 5. The concentration of citric acid in the rhizosphere of crops grown with different P sources in root study containers in the greenhouse. Plotted values are means across P sources. 'No crop' refers to the control treatment. Bar indicates standard error of means.

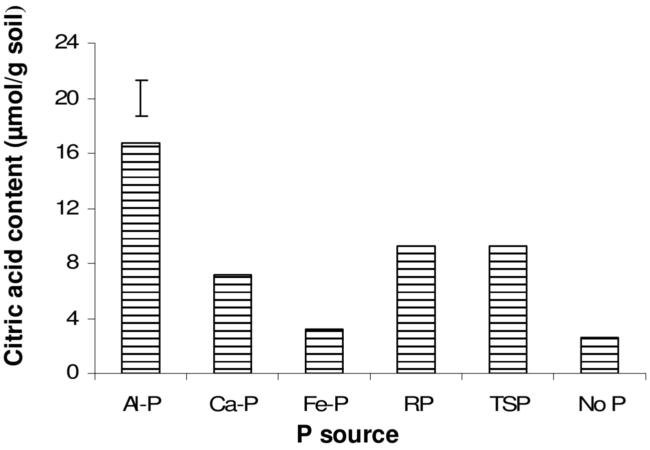

Figure 6. The effect of different P sources on the concentration of citric acid in the rhizosphere of soybean, cowpea, and pigeon pea grown in root study containers in the greenhouse. Plotted values are means across crop species. 'No P' refers to soil without P addition. Bar indicates standard error of means.

length) have been reported to play significant roles in the uptake of P by plants growing in low-P environments (Krasilnikoff et al., 2003; Gahoonia and Nielsen, 2004b). The length of roots of cowpea (but not of soybean and cereal crops) played an important role in P uptake in our first experiment, given the significant relationships that occurred among shoot DMY and P accumulation and root length. Krasilnikoff et al. (2003) reported that the cowpea variety Dan-ila adapts to low P availability by expanding its root system (root and root hairs) to explore a relatively larger volume of soil. The results of our study indicate that root length (and by implication, the extent of the root system) is a critical feature for P acquisition by this genotype, irrespective of the level of P supply in soil. This assertion is supported by the non-occurrence of a significant soil × P rate interaction ($P > 0.05$) for both shoot DMY and P accumulation. Unlike cowpea, the relative importance of the size of the roots of soybean for soil P acquisition (even under low P supply) was not evident. However, this needs to be verified under field conditions where root growth is unrestricted.

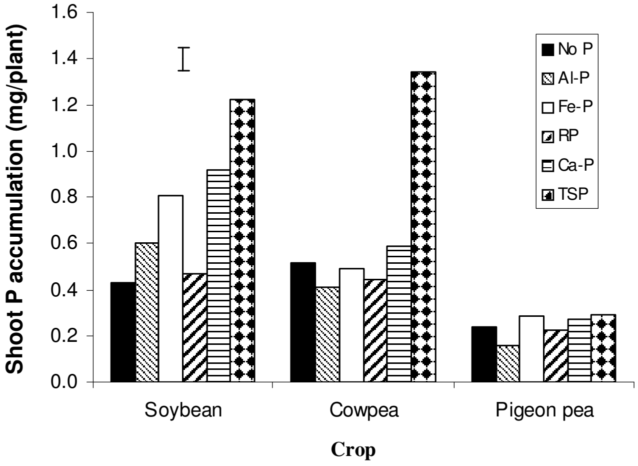

Figure 7. The accumulation of P in the shoot biomass of soybean, cowpea, and pigeon pea grown in root study containers with different sources of P. Bar indicates standard error of means.

The role of AMF in the uptake of P by the crops under low-P conditions was not visible as shoot P accumulation still remained very low without added P, even though the highest rate of AMF infection of the roots in all the soils was recorded in this treatment (Figure 4). Nevertheless, the importance of the mycorrhizal symbiotic association cannot be ruled out. The decreasing rate of AMF infection observed as P supply increased and the absence of infection when P addition was increased to 23 mg kg^{-1} could be attributed to the concentrations of P in the soil solutions achieved with these rates that probably exceeded the optimum for the symbiotic interaction between the host plants and the (indigenous) mycorrhizal fungi species. Aziz and Habte (1987) emphasized the existence of threshold, optimum, and inhibitory amounts of soil solution P for mycorrhizal symbiotic activity. These authors showed that the colonization of cowpea roots by mycorrhizal fungi (*Glomus aggregatum*) was considerably retarded when the concentration of P in the soil solution was increased to 0.087 mg/L. This value is far below the level in solution with the addition of 23 mg P/kg to any of the soils used in the present study.

Citric acid was the only organic acid detected in measurable quantities in the rhizosphere of the plants studied and results from soybean were superior to those from cowpea and pigeon pea. Because this organic acid was not detected in the control treatments (with or without P, without plant) the measured amounts are unequivocally root-released. Root-released organic acids (such as citric acid) have been shown to increase the solubility of strongly bound soil P (Bolan et al., 1994; Ishikawa et al., 2002), and thus, to promote P uptake under limiting conditions. But no correlation was found between shoot P accumulation and the amount of citric acid detected in the rhizosphere of the plants in the present study. Although increased citric acid exudation during P starvation has been reported in some plants (Hoffland et al., 1992), larger amounts of citric acid were observed in the treatments amended with the various P sources (including the readily soluble source) than in those without added P (Figure 6). The level of organic acids in soil solution across many ecosystems varies from 1 to 50 µM, which is rather low (Jones et al., 2003) and other studies have also been reported (van Hees et al., 2005) where low P supply did not result in short-term increases. The concentration of citric acid (17 µmol/g soil) observed in the rhizosphere of soybean is equally low. Considering that Ishikawa et al. (2002) have shown that at least 100 µM of organic acid (e.g., citric, malic) is needed to liberate about 1.6 µM P from a strongly bound P pool in an Alfisol, the question arises whether the amount of citric acid (or other organic acids) estimated in the rhizosphere of the plants is adequate to solubilize sparingly soluble P and to raise availability sufficiently to eliminate symptoms of P-deficiency. However, it can be expected that the amount detected may be several times lower than the actual quantity exuded by the roots. Organic acids can undergo biodegradation or sorption processes after release (Jones et al., 2003), and these may reduce the concentration in soil solution. Although organic acids play diverse functions in the rhizosphere (Bolan et al., 1994; Hocking, 2001), the significant increase in shoot P accumulation of soybean following the addition of Fe-P and Ca-P, and the slight increase following the addition of Al-P (Figure 7) indicate that the contribution of citric acid observed in the rhizosphere of soybean to the release of P from the various sources cannot be ruled out. Moreover, it is known that citric acid can increase P availability by solubilizing Ca, Al, and Fe phosphates (Gahoonia et al., 2000), and the degree of this effect is dependent on soil type (Jones et al., 2003) and the form of P in the soil. For example, shoot P accumulation in soybean was lower with the addition of Al-P than with the addition of Fe-P, Ca-P, and TSP even though the average concentration of citric acid due to the addition of Al-P was higher than the concentrations due to the addition of Fe-P, Ca-P, and TSP (Figure 6). The relatively high amount of citric acid in the Al-P treatment may be attributed to the apparent slight increase in Al content (compared to the other treatments) since it has been reported that Al increases citric acid exudation by the roots of some leguminous species (Hocking, 2001). With pigeon pea, relatively small quantities of citric acid were produced compared to those from soybean and cowpea. This is expected, considering that pigeon pea has been shown to exude a different form of organic acid (piscidic acid) in response to P stress (Ae et al., 1990). Although no relationship could be established between the amounts of organic acids detected and resin-P measured in the rhizosphere, there was evidence of resin-P depletion near the root mat of soybean and cowpea due to uptake by the plants.

In conclusion, it is evident from the data presented that the cowpea genotype studied depended, to a large extent, on the morphology of its roots for soil P acquisition (even under limiting conditions). Nevertheless, the detection of citric acid in its rhizosphere signifies that other strategies may be involved. Soybean tended to modify its rhizosphere chemistry by exuding citric acid. However, further studies are necessary to elucidate genotypic variation in the exudation of citric acid by these grain legumes, and the possible relationships with crop growth under field conditions.

ACKNOWLEDGEMENTS

The Directorate General for Development Cooperation (DGDC) is gratefully acknowledged for sponsoring this work under the collaborative project between KU Leuven and IITA on “Achieving Development Impact and Environmental Enhancement through Adoption of Balanced Nutrient Management Systems by Farmers in the West African Savanna”. The authors thank Christine Peeters and Rik Deliever of the Didactic Laboratory, KU Leuven, and Susan Ikerra of Mlingano Agricultural Research Institute, Tanga, Tanzania, for help with organic acid analysis. The editorial assistance provided by Rose Umelo is acknowledged.

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